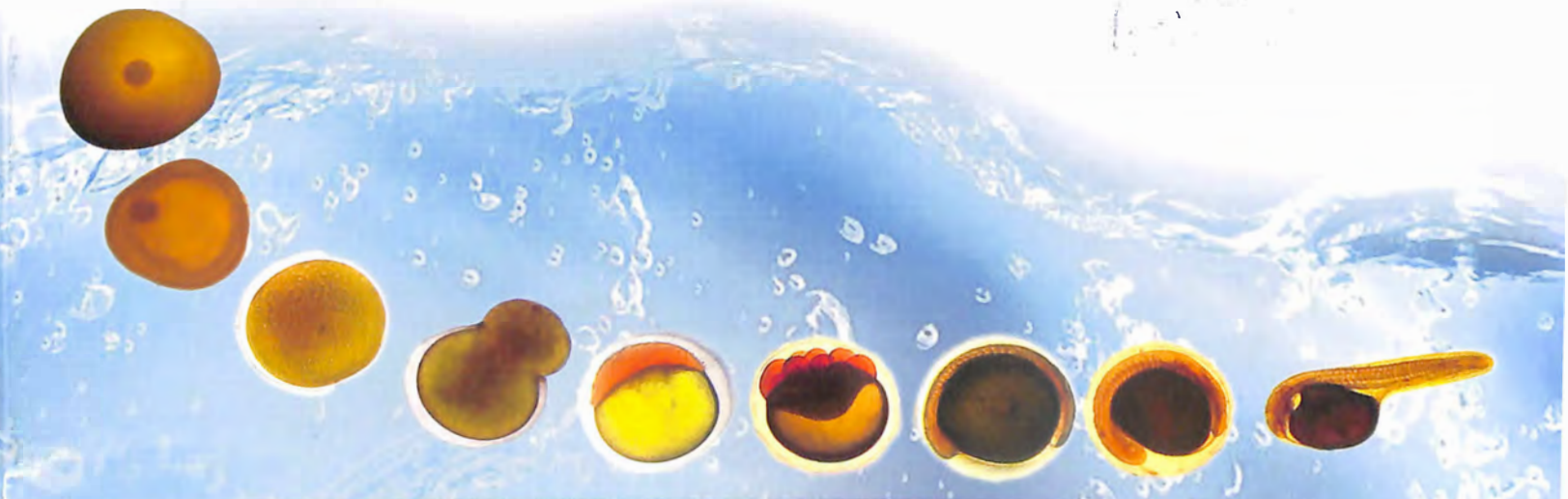
C2011/2
Vol:4 Issue:S8 August 2011 ISSN : 0974-6846

9th International Symposium on Reproductive Physiology of Fish

Cochin, India, August 9-14, 2011



Published by Indian Society for Education and Environment (iSee)
http://www.indjst.org

In memoriam

Prof. Dr. Pieter Gregorius Willem Johannes Van Oordt

Former Professor & Charman
Dept. of Experimental Zoology
University of Utrecht

Our colleague and friend, my teacher and supervisor, Professor Piet van Oordt passed away on 27 May 2011 at the age of 83. A few days before he has got a stroke, never regained conscious and died peacefully. Piet van Oordt started his study in Biology right after World War II at the University of Utrecht. He got his Ph.D. in 1956 at the same university and became Assistant professor at the Zoological Department. In 1968 he was appointed as full professor in Comparative Endocrinology and started his own research group in the Department of Zoology of the University of Utrecht, The Netherlands.

From the beginning of his scientific career, Piet's main interest was the endocrine regulation of amphibian reproduction. In the early seventies, however, he and his co-workers (Jan Lambert, Jan Peute, Rob van den Hurk, Goverdien van Ree and myself) decided to switch to the endocrine regulation of fish reproduction, not in the least because of the possibility for more strategic and applied research and, as a consequence of that, better funding. We never regretted this decision and we remember well Piet's active stimulation of his group into this new direction.For about five years the rainbow trout was the Group's experimental animal, but rather soon it was replaced by the African catfish. Apart from extensive laboratory studies in Utrecht on this species, it opened for Piet the opportunity to conduct a field project in his beloved country of Israel on the pheromonal induction of ovulation in the African catfish, which is native also in Israel.

Piet retired from his professorship in 1989 at the age of 62 and devoted himself for almost 20 years to many social activities. After his departure the research of his former group moved more and more into the direction of genetic and molecular endocrinology. Because of the limited genetic possibilities of the catfish this species was abandoned and is now replaced by the zebrafish. We will remember Piet van Oordt as a real personality, an excellent teacher and supervisor, but above all as a person characterised by honesty and integrity. It was a great honour for me having been appointed in 1990 as Piet's successor.

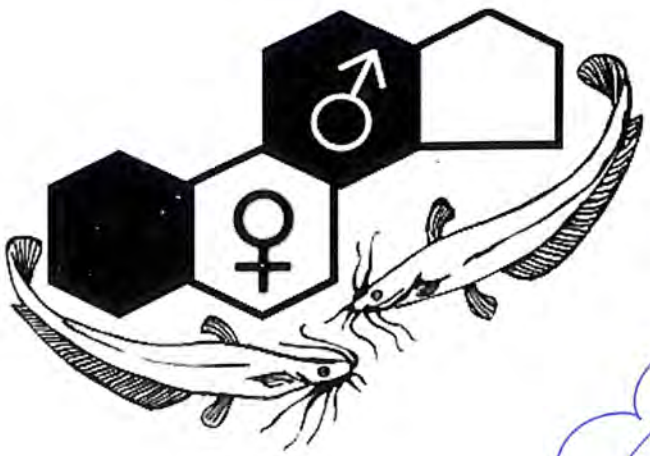
Henk Goos

Former Professor & Charman
Dept. of Experimental Zoology
University of Utrecht

9th International Symposium on Reproductive Physiology of Fish

Thrissur (Cochin), INDIA

August 9-14, 2011



Editors

K. P. Joy, R. M. Inbaraj, R. Kirubagaran and R. Chaube

Foreword

We are honoured and privileged to organize the 9th International Symposium on Reproductive Physiology of Fish (ISRPF) in Kochi, India from August 9-14, 2011.We thank the International Advisory Committee (IAC) of the 8th ISRPF, 2007, St. Malo, France for accepting our proposal and entrusting us with the responsibility of organizing the 9th ISRPF in Kochi. We also express our appreciation gratitude to the IAC members and other scientists for their active support, advice and suggestions in planning and preparing the scientific program. The program includes 8 symposium sessions, covering both platform and poster presentations. The scientific sessions cover areas of contemporary interests in fish reproductive physiology such as Reproductive Neuroendocrinology, Sex determination and gonad differentiation, Reproductive cycles, behavior and migration, Spermatogenesis, Folliculogenesis, Spawning, fertilization and early embryogenesis, Endocrine disruptors and reproductive toxicology, and Fish biotechnology and aquaculture. The detailed program is appended *inter alia*. The session on Spawning, fertilization and early Embryogenesis has been introduced with the object of focusing research in this direction. 190 abstracts were accepted for presentation, which include a keynote lecture, 8 plenary lectures, 49 platform presentations and 133 poster presentations. It is regretted that due to time constraints, the scientific committee could not include as many abstracts as it desired in the platform presentation category, without parallel sessions which has not been the convention in the ISRPF meetings. Consequent upon the decision of the National Organizing Committee (NOC) in consultation with the IAC, the abstracts are published as short papers in the form of the Symposium Proceedings. It is also intended to publish selected abstracts in a Special Issue of Fish Physiology and Biochemistry, Springer for which an agreement has been signed with the Publisher.

We extend a warm and hearty welcome to all the participants at the symposium and to India. The symposium venue Lulu International Convention Centre and Garden Hotels, Thrissur, in the erstwhile Kochi Kingdom and now a district of Kerala, is enchanting. We hope you will enjoy the stay and the excursion tour in and around Kochi.

We thank the members of the NOC and Local Organizing Committee for their untiring efforts for the smooth conduct of the symposium. We are grateful to The Principal and Board of Directors of the Madras Christian College for institutional facility and support of Head & faculty members of Zoology Department. We owe to the Government agencies of UGC, CSIR, MoEF, MoES, DBT, DST and ICAR and private agencies for financial support, which made us to subsidize the expenses. The timely printing and publishing of the Symposium Proceedings by the Indian Society for Education and Environment, Chennai is gratefully acknowledged. The enthusiasm and energetic service of the students and research scholars at Department of Zoology, Banaras Hindu University (Tapan Chourasia, Geeta Gautam, Rahul Singh, Arup Acharjee, Putul Banerji) and Department of Zoology, Madras Christian College (Nirmal Magdalenal, Gokulakrishnan, Madhu Magesh, Saravanan, Ramachandra Prabhu, Lynda Keren, Rose Gregoria, Prakash) are admirable and acknowledged.

Kochi
August 1, 2011

Editors

1600
+ 300
1900 - IRP

Catherine 05266

International Advisory Committee

Dr. Yoshitaka Nagahama, Japan
Dr. Wei Ge, HongKong
Dr. K.P.Joy, India (Chair of 9th ISRPF)
Dr. Yann Guiguen, France
Dr. Rüdiger Schulz, Netherlands
Dr. Peter Thomas, USA
Dr. Penny Swanson, USA
Dr. Hanna Rosenfeld, Israel
Dr. Norberg Birgitta, Norway
Dr. Hamid R. Habibi, Canada
Dr. Vance Trudeau, Canada
Dr. Gustavo Somoza, Argentina

Local Organizing Committee

Dr. R.W. Alexander Jesudasan
(Principal, Madras Christian College)
Mr. C. Sundaraj, Bursar, MCC
Dr. Arul Samraj, Head, Dept.Zoology, MCC
Dr. K. P.Joy,Varanasi (Chair of 9th ISRPF)
Dr. T.M. Jose, Kochi
Dr. C.S. Paulose, Kochi
Dr. E. Vijayan, Kochi
Dr. Oommen V Oommen, Kerala
Dr. Subash Peter, Kerala
Dr. R. Kirubagaran, NIOT, Chennai
Dr. Samuel Sukumar, MCC
Dr. R. Moses Inbaraj, MCC
(Organizing Secretary)
Dr. Manu Thomas Mathai, MCC
Dr. Ann Sudir, MCC
Dr. Logmanya Tilak,MCC
Dr. Lita Sunder, MCC
Dr. Sesh Serebiah, MCC
Dr. Mahesh Kumar, MCC
Dr. Nirmal Magdalenal, Chennai.

National Organizing Committee

Dr. S. Ayyappan, New Delhi
Dr. P.D. Prasada Rao, Nagpur
Dr. Samir Bhattacharya, Santiniketan
Dr. Shamim Haider, Varanasi
Dr. K.P. Joy, Varanasi (Chair of 9th ISRPF)
Dr. Panchanan Nath, Santiniketan
Dr. Bechan Lal, Varanasi
Dr. Saumen Kumar Maitra, Santiniketan
Dr. Radha Chaube, Varanasi
Dr. R. Kirubagaran, Chennai
Dr. R. Moses Inbaraj, Chennai
(Organizing Secretary)
Dr. B. Senthilkumaran, Hyderabad
Dr. Neeta Sehgal, New Delhi
Dr. N. Subhedar, Pune
Dr. J.K. Sundaray, Chennai

Organized by
MADRAS CHRISTIAN COLLEGE
Tambaram, Chennai 600059, India
Ph: +91 44 64531024 Mob: +91 9444442362 Fax: +91-44-22791024
www.9isrpf.org Email: os9isrpf@9isrpf.org College web: www.mcc.edu.in

Organizers thank the supporters:

- Ministry of Earth Sciences, Govt. of India (MoES)
- Department of Biotechnology (DBT)
- Indian council of Agricultural Research (ICAR)
- Council of Scientific and Industrial Research(CSIR)
- Central Institute of Brackish water Aquaculture(CIBA)
- Ministry of Environment & Forests (MoEF)
- University Grants Commission (UGC)
- Department of Science and Technology (DST)
- National Bureau of Fish Genetic Resources
- National Institute of Ocean Technology (NIOT), Chennai
- United Board for Christian Higher Education in Asia
- Participatory Learning Action Network and Training(PLANT), Chennai
- Banaras Hindu University
- Indian Journal of Science and Technology &
  Indian Society for Education and Environment (iSee), Chennai
- Jai Scientific Corporation, Varanasi
- The Educational Supplying Company, Varanasi
- Biotex Scientific Company, Varanasi
- Matri Shree Powertronics Pvt. Ltd., Varanasi
- Kiruba Associates, Chennai

Institutional support - Madras Christian College

9th International Symposium on Reproductive Physiology of Fish

August 9-14, 2011

Program schedule at a glance

August 9, 2011

Registration	-	3.00 - 6.00 PM
Inauguration	-	6.00 - 7.00 PM
Cultural program & Dinner	-	7.00 - 9.00 PM

August 10, 2011

Keynote Address	**Dr. Yonathan Zohar, USA**	09.00-10.00 AM
	Memoriam: P.G.W.J. van Oordt	10.00-10.10 AM

Session I: Reproductive Neuroendocrinology

Chair: Prasada Rao, India and Ishwar Parhar, Malaysia

Olivier Kah, France	-	Plenary Lecture	10.10 – 10.45 AM
Katsuhisa Uchida, Japan	-	Invited Lecture	10.45- 11.05 AM
Kataaki Okubo K, Japan	-	Invited Lecture	11.05 – 11.25 AM

Coffee break – 11.25 – 11.40 AM

Nagarajan Ganesan, Taiwan	-	Oral presentation	11.40 – 11.55 AM
Jon Hildahl, Norway	-	Oral presentation	11.55 – 12.10 AM
Nilli Zmora, USA	-	Oral presentation	12.10 – 12.25 PM
Levavi- Berta Sivan, Israel	-	Oral presentation	12.25 – 12.40 PM

Lunch – 12.40 – 01.40 PM

Ishwar Parhar, Malaysia	-	Special Lecture	01.40 – 02.05 PM
Bindhu Paul- Prasanth, India	-	Invited Lecture	02.05 – 02.25 PM
Matias Pandolfi, Argentina	-	Oral presentation	02.25 – 02.40 PM

Session II: Sex Determination and Gonad Differentiation

Chair: Yoshitaka Nagahama, Japan and Francesc Piferrer, Spain

Yann Guigen, France	-	Plenary Lecture	02.40 – 03.15 PM
Ching Fong Chang, Taiwan	-	Invited Lecture	03.15 – 03.35 PM
Deshou Wang, China	-	Invited Lecture	03.35 – 03.55 PM

Coffee break – 03.55 – 04.10 AM

Ricardo Hattori, Brazil	-	Oral presentation	04.10 – 04.25 PM
Ryu Nozu, Japan	-	Oral presentation	04.25 – 04.40 PM
Francesc Piferrer, Spain	-	Oral presentation	04.40 – 04.55 PM
Vizziano Cantonnet,Uruguay	-	Oral presentation	04.55 – 05.10 PM

Poster session 1 (Session 1 & II) - 05.10 – 07.30 PM

Entertainment/Cultural event, Dinner – 07.30 – 09.30 PM

August 11, 2011

Session III: Reproductive Cycles, Behaviour and Migration

Chair: Norberg Briggita, Norway and Alexander Scott, UK

Minoru Tanaka, Japan	-	Plenary Lecture	09.00 – 09.35 AM
Hiroshi Ueda, Japan	-	Invited Lecture	09.35 – 09.55 AM
Ignacio Carazo, Spain	-	Oral presentation	09.55 – 10.10 AM
Peter Hubbard, Portugal	-	Oral presentation	10.10 – 10.25 AM
Joao Saraiva, Portugal	-	Oral presentation	10.25 – 10.40 AM
Matthew Wylie, New Zealand	-	Oral presentation	10.40 – 10.55 AM
Eran Yanowski, Israel	-	Oral presentation	10.55 – 11.10 AM

Coffee break – 11.10 – 11.25 AM

Session IV: Gametogenesis - I (Spermatogenesis)

Chair:Matt Vijayan, Canada and Luiz Renato Franca, Brazil

Luiz Renato Franca, Brazil	-	Plenary Lecture	11.25 – 12.00 PM
M. Hulak, Czech Republic	-	Invited Lecture	12.00 – 12.20 PM
Sussane Trombley, Sweden	-	Oral presentation	12.20 – 12.35 PM

Lunch –12.40–01.40 PM

Bechan Lal, India	-	Invited Lecture	01.40 – 02.00 PM
Padmanav Routray, India	-	Invited Lecture	02.00 – 02.20 PM
Makoto Kusakabe, Japan	-	Oral presentation	02.20 – 02.35 PM
Florence Le Gac, France	-	Oral presentation	02.35 – 02.50 PM
Ana Gomez, Spain	-	Oral presentation	02.50 – 03.05 PM

Poster session 2 (Session III, IV & V) – 03.05 – 06.05 PM with coffee session

Travel and Dinner with entertainment at Vishnu Garden – 07.00 PM – 08.30 PM

August 12, 2011
(Excursion – Cochin)

Leaving to Cochin at 8.30 AM from Lulu by Bus
Boat Cruise with Lunch, Fort Kochi
Banquet Dinner at Bolgatty Palace, Cochin

August 13, 2011

Session V: Gametogenesis - II (Folliculogenesis)

Chair: Hirohiko Kagawa, Japan and Adelino Canario, Portugal

Joan Cerda, Spain	-	Plenary Lecture	09.00 – 09.35 AM
Wei Ge, HongKong	-	Invited Lecture	09.35 – 09.55 AM
Naoshi Hiramatsu, Japan	-	Invited Lecture	09.55 – 10.15 AM
Neeta Sehgal, India	-	Invited Lecture	10.15 – 10.35 AM
Sudipta Maitra, India	-	Oral presentation	10.35 – 10.50 AM
Mark Lokman, New Zealand	-	Oral presentation	10.50 – 11.05 AM
Suraj Unniappan, Canada	-	Oral presentation	11.05 – 11.20 AM

Coffee break – 11.20 – 11.40 AM

Session VI: Folliculogenesis (continued..), Spawning, Fertilization and Early Embryogenesis

Chair: Penny Swanson, USA and Graham Young, USA

Adam Luckenbach, USA	-	Plenary Lecture	11.40 – 12.15 PM
Graham Young, USA	-	Invited lecture	12.15 – 12.35 PM

Lunch –12.40–01.40 PM

Thomas Desvignes, France	-	Oral presentation	01.40 – 01.55 PM
Palacios-Salgado, Mexico	-	Oral presentation	01.55 – 02.10 PM
Kjetil Hodne,Japan	-	Oral presentation	02.10 – 02.25 PM
Labbe Catherine, France	-	Oral presentation	02.25 – 02.40 PM

Session VII: Reproductive Toxicology and Endocrine Disruptors

Chair: Charles Tyler, UK and Neeta Sehgal, India

Charles Tyler, UK	-	Plenary Lecture	02.50 – 03.25 PM
Vijayan M., Canada	-	Invited Lecture	03.25 – 03.45 PM
Amy Filby, UK	-	Oral presentation	03.45 – 04.00 PM
In Joon Hwang, Korea	-	Oral presentation	04.00 – 04.15 PM

Poster Session 3 (Session VI, VII & VIII) – 04.15 – 07.15 PM with coffee session

Meeting of International Advisory Committee- 6.00 PM

Entertainment program, Dinner – 07.15 PM – 9.30 PM

August 14, 2011

Session VIII: Fish Biotechnology and Aquaculture (Reproductive Manipulations)

Chair: Goro Yoshizaki, Japan and Hanna Rosenfeld, Israel

Hanna Rosenfeld, Israel	-	Plenary Lecture	09.00 – 09.35 AM
Abigail Elizur, Australia	-	Invited Lecture	09.35 – 09.55 AM
Jena K, India	-	Invited Lecture	09.55 – 10.15 PM
Goro Yoshizaki, Japan	-	Invited Lecture	10.15 – 10.35 PM

Coffee break – 10.35 – 11.00 AM

Hirohiko Kagawa, Japan	-	Invited Lecture	11.00 –11.20 AM
Ping Li, Czech Republic	-	Oral presentation	11.20 – 11.35 AM
Tapas Chakraborty, Japan	-	Oral presentation	11.35 – 11.50 AM
Samyra Lacerda, Brazil	-	Oral presentation	11.50 – 12.05 PM

General Assembly - 12.10-12.40 PM

Lunch – 12.40 – 01.40 PM

Valedictory Address	**Dr. Zvi Yaron, Israel**	02.00 – 3.30 PM

DETAILS OF SESSIONS AND PRESENTATIONS

Contents

Session II: Sex Determination and Gonad Differentiation

Chair: Yoshitaka Nagahama, Japan and Francesc Piferrer, Spain

Platform Presentation

Session III: Reproductive cycles, Behaviour and Migration

Chair: Norberg Briggita, Norway and Alexander Scott, UK

Platform Presentation

Poster Presentation

Session IV: Gametogenesis-I (Spermatogenesis)

Chair: Matt Vijayan, Canada and Luiz Renato Franca, Brazil

Platform Presentation

Poster Presentation

Session V: Gametogenesis-II (Folliculogenesis)

Chair: Hirohiko Kagawa, Japan and Adelino Canario, Portugal

Platform Presentation

Poster Presentation

Session VI: Folliculogenesis (continued..), Spawning, Fertilization and Early Embryogenesis

Chair: Penny Swanson, USA and Graham Young, USA

Platform Presentation

Poster Presentation

Session VII: Reproductive Toxicology and Endocrine Disruptors

Chair: Charles Tyler, UK and Neeta Sehgal, India

Platform Presentation

Poster Presentation

Session VIII: Fish Biotechnology and Aquaculture (Reproductive Manipulations)

Chair: Goro Yoshizaki, Japan and Hanna Rosenfeld, Israel

Platform Presentation

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

KEYNOTE ADDRESS

FISH AND REPRODUCTIVE PHYSIOLOGY - A JOURNEY FROM BASIC AND TRANSLATIONAL SCIENCE TO A RECOGNIZED VERTEBRATE MODEL

Yonathan Zohar

Department of Marine Biotechnology, University of Maryland, Baltimore County, Baltimore, MD 21202, USA

In September 1977, Dr. Roland Billard hosted the inaugural International Symposium on Reproductive Physiology of Fish (ISRPF) in Paimpont, France. As a young graduate student entering the field, I was fascinated by its depth and breadth - a scientific spectrum spanning from very fundamental research to innovative translational work. Myself and many others were drawn to the field with the hope of taking scientific ideas from the bench to their end-use. Indeed, our field has always addressed real-world challenges that could be overcome through basic research and understanding of gametogenesis, the hormones that control it, their mechanisms of action and interactions with the environment, and their complex regulation from the whole animal to the gene level. Over the past 9 ISRPFs, we have witnessed the growth of our field, the publication of a wealth of basic information in fish reproductive physiology, and its translation into practical uses and technologies for aquaculture and fisheries. Collectively, our basic research enabled the industry to produce good quality gametes, fertilized eggs and viable juveniles on demand, develop science-based approaches to induce reproductive sterility, control the timing of puberty and determine gender and sex ratio. Intensive research, driven by the applied challenges, transformed fish into a primary model in the field of vertebrate and animal reproductive physiology. Novel discoveries in environmental control of the brain-pituitary-gonadal (BPG) axis in fish, sex determination and differentiation, GnRH multiplicity and its functional significance, structure/function of fish gonadotropins, gonadal steroids and their unique receptors, cross-communications between the components of the BPG axis, endocrine disruptors, and many others have led to new paradigms in animal reproductive biology that have had direct impacts far beyond just fish, in the fields of animal agriculture, environmental health and biomedicine. As the life sciences entered into the era of genomics, and beyond, the field of fish reproductive physiology has taken full advantage of the relevant platforms to further its pioneering role in twenty-first century science. With zebrafish and medaka becoming recognized vertebrate models, fish reproductive physiologists were among the first to capitalize on the benefits of these species and associated technologies, such as mutagenesis, knockdown, gene-transfer and fluorescence-based imaging, to identify and study genes involved in reproduction and their regulation by environmental and endocrine factors. Since 1977, our field has seen rapid and very exciting scientific progress, its successful application, and the recognition of fish as an important vertebrate model in reproductive physiology. This presentation will provide a historical overview of some of the above concepts focusing on the GnRH-reproductive axis in several fish models.

Reproductive Neuroendocrinology

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

40 YEARS OF RESEARCH ON THE "REPRODUCTIVE BRAIN" OF TELEOST FISHES

Olivier Kah

Neurogenesis and Estrogens, University of Rennes 1, Campus de BeaulieuRennes, France.
olivier.kah@univ-rennes1.fr

This year marks the 40th anniversary of the first article ever published on the effects of a brain hypothalamic factor on pituitary gonadotrophin in a teleost fish, the carp [1]. Since this pioneer work, one generation of researchers has written more than 1250 papers dedicated to various aspects of the brain control over the reproductive axis of fish. What we have learnt is that, overall, the neuroendocrine circuits controlling the pituitary gonadotropic activity and the main molecular actors involved in this control are highly conserved in vertebrates, indicating that they are at least 450 million years old and, probably, that many of these actors, if not all, were inherited from invertebrates.

A first issue is to understand how the reproductive brain develops and is sexualized in accordance with the gonadal sex. While it is admitted in birds and mammals that the brain of males and females are different, this issue is poorly addressed in fish. It is probably due partly to the fact that there are important species differences. In general, it is admitted that the brain of fish exhibits a high sexual plasticity as exemplified by the fact that many species can change sex naturally or after hormonal treatment. This indicates that the brain of fish is not permanently sexualized as documented in other vertebrate models. How this can be achieved is likely linked to the fact that the brain of fish conserves throughout life properties of the embryonic brain of mammals, such as persistence of radial glia progenitors, high neurogenic activity and elevated aromatase activity. This probably allows sexual steroids to shape the brain in a region and sex-specific manner through a modulation of neurogenesis [2].

In terms of neuroendocrine control, the key player is GnRH that is the most potent neurohormone on LH release. Three GnRH genes are found in fish, the history of which is still matter of active debates. GnRH1 neurons in the preoptic area express a decapeptide, which varies from species to species and stimulates the synthesis and release of FSH and LH. In some families, GnRH1 seems to be replaced by GnRH3. However, there is some evidence to suggest that some GnRH genes could remain undiscovered in certain species, such as salmon or zebrafish. Recently, GnRH2, a universal ancestral GnRH variant was shown to act as a melatonin-stimulating factor in the European sea bass through direct projection in the pineal [3]. In sea bass, this new role involves one of 5 GnRH-Rs, and this pineal receptor is different from the one present in the pituitary. The other GnRH-Rs are still in search of functions and precise sites of expression. The GnRH system develops during early stages of embryogenesis. In the sea bass and other species, GnRH neurons and fibres reach their respective final destination in adult much earlier than the time of the first sexual maturation. This suggests that, as in mammals, this system develops early and needs to be activated at the time of puberty, which takes us to kisspeptins.

With the discovery of kisspeptin, year 2003 has seen a real revolution in the field of reproductive neuroendocrinology and puberty control. In mammals, kiss neurons are now acknowledged as essential activators of the GnRH systems and considered as integrators of metabolic, seasonal and hormonal signals. Most fish species have conserved 2 copies of an ancestral kiss gene, kiss1 and kiss2. However, the expression of these two genes and their relative importance in reproduction certainly varies from species to species. The zebrafish is so far the only species in which kiss expression has been deciphered at both the messenger and protein levels [4]. However, data in medaka [5] and sea bass (Escobar *et al.*, this meeting) suggest that the use of kiss1 and kiss2 genes might vary from species to species. The pituitary could also be a source of kisspeptins as indicated in zebrafish and sea bass. In any case, the role of kisspeptin on fish puberty has not been firmly established so far and we need more data regarding the roles of kisspeptins on GnRH neuron activity.

Another key question concerns the brain targets of hormones such as sex steroids, but also metabolic hormones or melatonin, and their effects on the circuits controlling reproduction. Although outstanding progress has been made, notably in the localization of receptors, we are far from understanding the respective roles of these hormones. It seems now clear that estrogens do not influence GnRH neurons directly. The possibility exists that, similar to rodents, kiss neurons are targets for estradiol as documented in zebrafish, medaka and sea bass. Thus as in mammals, kiss neurones could forward estrogen information to GnRH neurons. The functional significance of the high aromatase expression found in fish brain is still unclear and this makes it difficult to understand the roles of aromatizable vs. non aromatizable androgens. In addition, recent data point to

PR>AR>ER exp

the fact that neurosteroids are produced *de novo* in the brain of developing and adult fishes [6]. The roles of these neurosteroids vs. peripheral steroids are still open to speculation. Importantly, it was shown that testosterone can be actively transformed, through 5α-reductase and 3ß-hydroxysteroid-deshydrogenase, into the potent estrogenic androgen, 5 alpha-androstan-3 alpha,17 beta-diol [7]. This makes the situation very complicated to fully understand the roles of androgens vs. estrogens. Androgen receptors are widely distributed in the brain of fish as well as nuclear and membrane progesterone receptors (nPR). These nPR are up-regulated by estradiol in both larvae and adults. To add further complexity, we have evidence that radial glial cells express membrane estrogen receptors. Whether these correspond to the GPR30-expressing cells that we have identified in zebrafish is under investigation.

Leptin and melatonin receptors are widely expressed in the brain of fish. How exactly those signals are processed and transformed into reproductively relevant information is not known. Preliminary data indicate that kiss neurons are tightly related to leptin receptors and that their expression varies according to energy status. Kiss neurons are thus likely to be direct targets for leptin. On another hand, there is increasing indication that melatonin influences kiss neuron activities.

This lecture intends to provide a comprehensive and integrated view of the current situation and to raise a number of unsolved questions and bottlenecks in our understanding and mastering of fish reproductive physiology.

Unpublished data were obtained with the financial support of the CNRS, the University of Rennes 1, the EU Project LIFECYCLE (FP7-222719-1), the NEMO project, the ANR NEED to O. Kah.

References

[1]Breton *et al.* 1971. C R Acad Sci 273:2591-2594
[2]Le Page *et al.*, 2011. Eur. J. Neurosci. 32:2105-15
[3]Servili *et al.*, 2010. Endocrinology 151: 2265-2275.
[4]Servili *et al.*, 2011. Endocrinology 152:1527-1534.
[5]Kanda *et al.*, 2008. Endocrinology. 149:2467-2476.
[6]Diotel *et al.*, 2010. Frontiers Neuroendocrinol. 31 172–192.
[7]Mouriec *et al.*, 2009. Biol. Reprod. 80:889-896

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

EVOLUTIONARY ORIGIN OF A FUNCTIONAL GONADOTROPIN IN THE PITUITARY OF THE MOST PRIMITIVE VERTEBRATE, HAGFISH

Uchida K.[a], Moriyama S.[b], Sower S. A.[c], Nozaki M.[d]

[a] Department of Marine Biology and Environmental Sciences, Faculty of Agriculture, University of Miyazaki, Miyazaki 889-2192, Japan. Fax: +81-985-58-7224, e-mail: k-uchida@cc.miyazaki-u.ac.jp
[b] School of Marine Biosciences, Kitasato University, Sanriku, Iwate 022-0101, Japan
[c] Center for Molecular and Comparative Endocrinology and Biochemistry Program, University of New Hampshire, Durham, NH 03824, USA
[d] Sado Marine Biological Station, Niigata University, Sado, Niigata 952-2135, Japan

Introduction:

Reproduction in jawed vertebrates (gnathostomes) is controlled by a hierarchically organized endocrine system called the hypothalamic-pituitary-gonadal (HPG) axis. The HPG axis, which is specific to vertebrates, is considered to be an evolutionary innovation and seminal event that emerged prior to or during the differentiation of the ancestral jawless vertebrates (agnathans). In gnathostomes, two functional gonadotropins (GTHs), luteinizing hormone (LH) and follicle-stimulating hormone (FSH), are secreted from the pituitary and stimulate the gonads inducing the synthesis and release of sex steroid hormones, which in turn elicit growth and maturation of the gonads. The extant representative of the agnathans, hagfish, represent the most basal and primitive vertebrate that diverged over 550 million years ago. They are of particular importance in understanding the evolution of the HPG axis related to vertebrate reproduction. Nevertheless, our knowledge of the endocrine regulation of reproduction in the hagfish has been poorly understood. The objective of this study was to identify a pituitary glycoprotein hormone (GPH) and to examine its possible functions for gonadal activities in the most primitive vertebrate, the hagfish.

Methods:

The brown hagfish, *Paramyxine atami*, is sold in local markets in Niigata district, Japan. Pituitary cDNA library was prepared and the cDNA clones were

Fig.1. Correlation between pituitary GPH activities and gonadal development in hagfish. (A, B) Cellular activities of GPHβ cells in the hagfish pituitary. Note that intense immunoreactions are observed in mature female (B), while faint reactions presented in juvenile (A). (C, D) Relative GPHα and GPHβ gene expressions in the pituitary of female (C) and male (D) hagfish. Open bars represent GPHα gene expressions and filled bars represent GPHβ gene expressions. Note that two GPH transcripts in both sexes increase in well accordance with the developmental stage of the gonad.

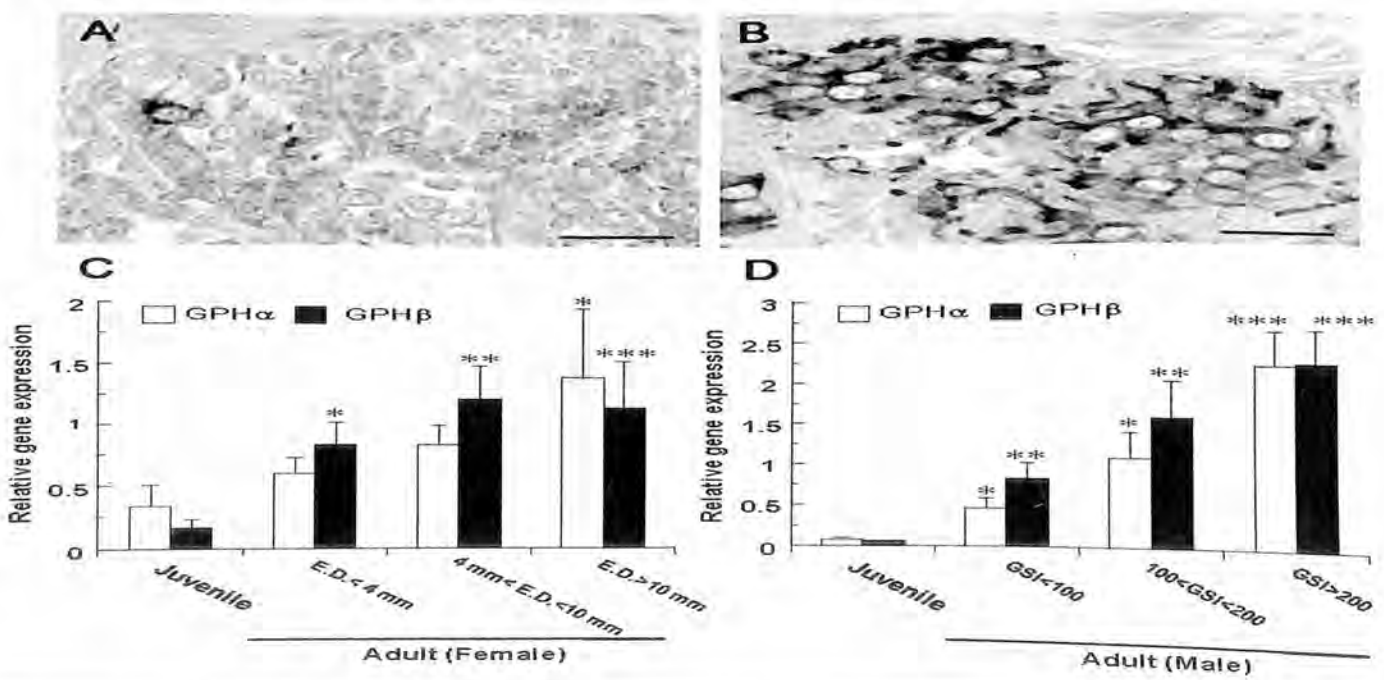
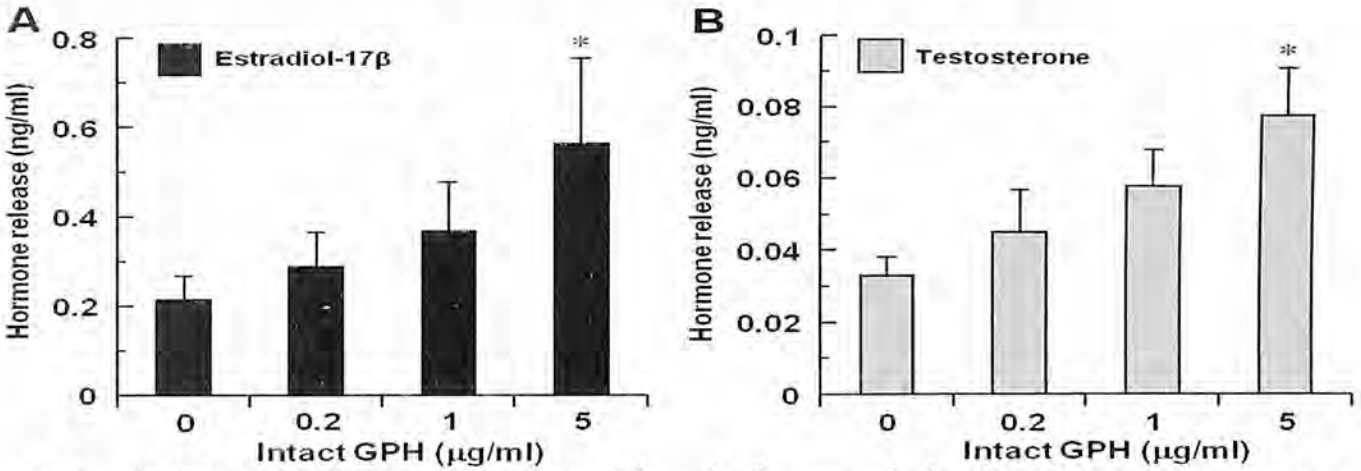


Fig. 2. Biological activity of native GPH from hagfish pituitary with *in vitro* bioassay. *In vitro* effects of native GPH on the releases of estradiol-17β (A) and testosterone (B) from organ cultured testis. It is notable that intact GPH stimulates the release of sex steroids from cultured testis *in vitro*, indicating "gonadotropic action" of hagfish GPH.



randomly selected. A total of 2304 clones were used for the sequence analysis. The molecular phylogenetic analysis of vertebrate GPHs was constructed using Maximum likelihood method. Cellular characterization of GPH-producing cells in hagfish pituitary was demonstrated by *in situ* hybridization and immunohistochemistry. We also examined correlation between pituitary GPH gene expression and gonadal development by quantitative real-time PCR assay. Purification and characterization of native GPH from hagfish pituitaries was performed, and the maturing testis was then cultured in the medium with or without native hagfish GPH at the same conditions. The concentrations of estradiol-17β (E2) and testosterone (T) in the medium were measured by Time-Resolved Fluoroimmunoassay (TR-FIA).

Results and Discussion:

The hagfish GPH consists of two subunits, α and β, which are synthesized and colocalized in the same cells of the adenohypophysis. The cellular and transcriptional activities of hagfish GPHα and β were significantly correlated with the developmental stages of the gonad (Fig. 1). The purified native GPH induced the release of gonadal sex steroids *in vitro* (Fig. 2). These results provide evidence that the hagfish GPH identified here has stimulatory effects on steroidogenesis and is thus considered a "GTH-like hormone" that can regulate gonadal functions. From our phylogenetic analysis, we propose that ancestral glycoprotein alpha subunit 2 (GPA2) and beta subunit 5 (GPB5) gave rise to GPHα and GPHβ of the vertebrate glycoprotein hormone family respectively. Based on the sequence and phylogenetic analyses, the identified hagfish GPHα- and β-subunits appear to be the typical pituitary GPHα and GPHβ subunits of gnathostomes. We hypothesize that the identity of a single functional GPH of the hagfish, hagfish GTH, provides critical evidence for the existence of a pituitary-gonadal system in the earliest divergent vertebrate that likely evolved from an ancestral, pre-vertebrate exclusively neuroendocrine mechanism by gradual emergence of a new control level, the pituitary, that is not found in the Protochordates.

Hagfish GTH, provides critical evidence for the existence of a pituitary-gonadal system in the earliest divergent vertebrate that likely evolved from an ancestral, pre-vertebrate exclusively neuroendocrine mechanism by gradual emergence of a new control level, the pituitary, that is not found in the Protochordates.

Conclusion:

We report the first identification of a glycoprotein hormone (GPH) in the pituitary of hagfish, the earliest divergent extant lineage and most primitive vertebrate. It seems most likely that an ancestral GPH gave rise to only one GTH in hagfish and that multiplicity of GPHs arose later during the early evolution of gnathostomes. We hypothesize that this functional pituitary GPH found in hagfish helps to delineate the evolution of the complex neuro/endocrine axis of reproduction in vertebrates. Furthermore, we propose that this HPG system likely evolved from an ancestral, pre-vertebpituitary gland.

SEX DIFFERENCES IN ESTROGEN AND ANDROGEN SIGNALING IN THE MEDAKA BRAIN

Okubo K.

Department of Aquatic Bioscience, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo, Tokyo 113-8657, Japan
Fax: +81-3-5841-5289, e-mail: okubo@marine.fs.a.u-tokyo.ac.jp.

Introduction:

Sex differences in the mammalian and avian brain are organized early during development as a result of a combination of hormonal and genetic events [1-3, 9]. This is an irreversible process, and thus the sex of the brain is permanently fixed. In contrast, the phenotypic sex of teleosts, including sex-specific reproductive behavior, can be manipulated by treatment with exogenous steroid hormones, even after reaching sexual maturity [10]. Furthermore, quite a few teleost species can spontaneously change their phenotypic sex in response to social and physiological events, even in adulthood [7]. These phenomena suggest that teleosts have an unknown, unique mechanism of brain sexual differentiation, which enables them to hold remarkable sexual plasticity throughout their lifetime. To dissect the molecular basis of sexual differentiation of the teleost brain, we examined sex differences in estrogen and androgen signaling in the brain of medaka *Oryzias latipes*.

Methods:

We searched for genes differentially expressed between the sexes in the medaka brain. One of the genes identified in the screen was that encoding the brain-predominant form of aromatase, the rate-limiting enzyme in the conversion of androgen to estrogen [4-6, 8]. Since estrogen and androgen are known to exert profound influences on sexual differentiation of the mammalian and avian brain, the aromatase gene was selected for further analysis of expression in the medaka brain. We also examined sex differences in the expression of nuclear receptors for estrogen and androgen in the medaka brain to assess the possible involvement of these receptors in sexual differentiation of the teleost brain.

Results:

The medaka aromatase gene was found to be expressed at higher levels in the female than male brain. Estrogen and androgen receptors also exhibited sexual dimorphism in the medaka brain, with each receptor having a discrete pattern of sex-biased expression. Detailed expression studies led to the identification of sex-specific action sites of aromatase, estrogen, and androgen in the medaka brain. We also found that the expression of aromatase and estrogen and androgen receptors in the medaka brain was not under the direct control of sex chromosome genes but relies mostly, if not solely, on steroid hormones.

Conclusion:

Sex differences in the expression of aromatase, estrogen receptors, and androgen receptors in the brain and their steroid-dependent regulatory system supposedly contribute substantially to the mechanisms underlying sexual differentiation and plasticity of the teleost brain.

References:

[1]ARNOLD, A.P., CHEN., X. 2009. What does the "four core genotypes" mouse model tell us about sex differences in the brain and other tissues? Front. Neuroendocrinol., 30: 1-9.

[2] ARNOLD, A.P., 2009. The organizational-activational hypothesis as the foundation for a unified theory of sexual differentiation of all mammalian tissues. Horm. Behav., 55: 570-578.

[3]BALTHAZART, J., CORNIL, C.A., CHARLIER, T.D., TAZIAUX M., BALL, G.F. 2009. Estradiol, a key endocrine signal in the sexual differentiation and activation of reproductive behavior in quail. J. Exp. Zool. A Ecol. Genet. Physiol., 311: 323-345.

[4]CALLARD, G.V., TCHOUDAKOVA,A.V., KISHIDA, M., WOOD., E. 2001. Differential tissue distribution, developmental programming, estrogen regulation and promoter characteristics of cyp19 genes in teleost fish. J. Steroid Biochem. Mol. Biol., 79: 305-314.

[5]DIOTEL, N. L.E., PAGE, Y., MOURIEC, K., TONG, S.K., PELLEGRINI, E., VAILLANT, C., ANGLADE, I., BRION, F., PAKDEL, F., CHUNG, B.C., KAH, O. 2010. Aromatase in the brain of teleost fish: expression, regulation and putative functions. Front. Neuroendocrinol., 31: 172-192.

[6]FORLANO, P.M., SCHLINGER, B.A., BASS, A.H. 2006. Brain aromatase: new lessons from non-mammalian model systems. Front. Neuroendocrinol., 27: 247-274.

[7]GODWIN, J. 2010. Neuroendocrinology of sexual plasticity in teleost fishes. Front. Neuroendocrinol, 31: 203-216.

[8]LE PAGE, Y., DIOTEL, N., VAILLANT, C., PELLEGRINI, E., ANGLADE, I., MÉROT, Y., KAH, O. 2010. Aromatase, brain sexualization and plasticity: the fish paradigm. Eur. J. Neurosci., 32: 2105-2115.

[9]MCCARTHY, M.M., WRIGHT, C.L., SCHWARZ, J.M. 2009. New tricks by an old dogma: mechanisms of the organizational/activational hypothesis of steroid-mediated sexual differentiation of brain and behavior. Horm. Behav ., 55: 655-665.

[10]MUNAKATA, A., KOBAYASHI, M. 2010. Endocrine control of sexual behavior in teleost fish. Gen. Comp. Endocrinol.165:456-468.

A NOVEL PEAK OF FUNCTIONAL NEUROSTEROIDOGENESIS AND ESTROGEN SIGNALLING IN THE EARLY BRAIN DEVELOPMENT OF ORANGE-SPOTTED GROUPER *EPINEPHELUS COIOIDES*

Nagarajan G. **Aruna A.** and **Chang C.F.**

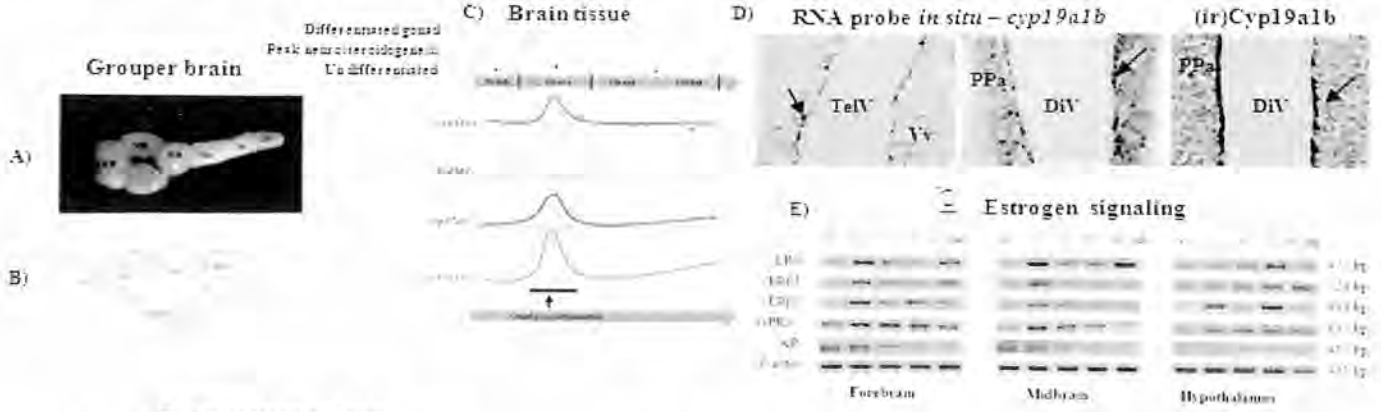
Department of Aquaculture, National Taiwan Ocean University, Keelung, 20224, Taiwan
Fax: +886-2-2462-1579 e-mail: nagarajangi@yahoo.com

Introduction:

Despite neurosteroidogenic enzymes are playing important roles in the regulation of brain development and function, the potential link between brain and gonad by the action of steroid hormones during gondal sex differentiation is still matter of debate in teleosts [1]. In recent years, we focused on the early brain development in response to the synthesis of neurosteroids and receptor activation during gonadal sex differentiation in orange-spotted grouper *Epinephelus coioides* (a mono-female sex development at the juvenile stage) and black porgy *Acanthopagrus schlegeli* (a mono-male sex development at the juvenile stage) [2,3]. The mechanism of brain sex differentiation and/or brain activation during early ages of female grouper fish is not fully understood yet. Moreover, there is an important lack of information regarding the expression of four key steroidogenic enzymes *cyp11a1* (P450scc), *hsd3b1*, *cyp17a1* and *cyp19a1b* in the early brain development and their potential links with gonadal sex differentiation. Hence, these phenomena support the selection of orange-spotted grouper as a model for neuroendocrine research, principally to understand the molecular mechanism of sex differentiation in female fish. Therefore, in the present study we aimed to test the hypothesis that 1) brain of orange-spotted grouper fish has the capability for *de novo* steroidogenesis and the production of these neurosteroids may have a correlation to the occurrence of gonadal sex differentiation and 2) we compared the expression of key genes and signalling in the protogynous grouper and protandrous black porgy in order to know whether this expression pattern has gender related event or not during gonadal sex differentiation. Here, we investigated the temporal expression pattern of the genes related to the neurosteroidogenesis and estrogen signaling in different days after hatching ages (dah) in the teleosts brain.

Methods:

In order to compare the gender effect on steroidogenic ability in the early brain, the dissection of grouper brain (Fig. A and B) was followed to our previous studies published in the male black porgy [3].



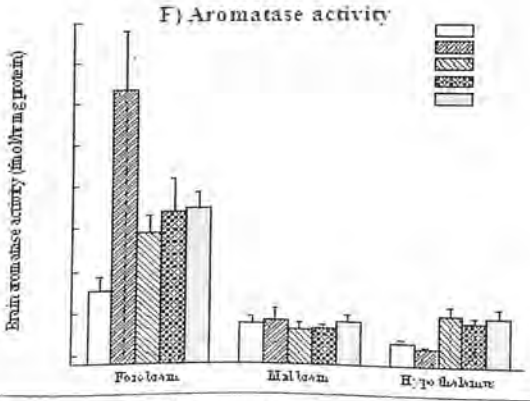


Fig. A. Dorsal view and B. lateral view of an adult orange-spotted grouper brain showing different parts in the brain.

Fig. C. Variation in the relative amount of mRNA encoding for *cyp11a1*, *hsd3b1*, *cyp17a1* and *cyp19a1b* in grouper brain at different days after hatching ages

Fig. D. Transfer section of grouper forebrain showing expression of cyp19a1b in the radial glial cells by ISH and IHC

Fig. E. RT-PCR analysis of the expression of the grouper estrogen signaling genes at different dah and F. aromatase activity in the grouper brain at different dah.

Neurosteroidogenic enzymes and estrogen related gene expressions were quantified by quantitative real-time PCR (qPCR) and RT-PCR analyses. We also measured brain aromatase activity by radiometric method and brain steroid hormone levels (brain estradiol 17β, E2 and testosterone, T) at different dah by enzyme immunoassay (EIA). Further, we demonstrated the anatomical localization of four key genes by *in situ* hybridization (ISH), and immunohistochemistry (IHC) for Cyp19a1b using our grouper brain Cyp19a1b specific antibody. Effects of exogenous E2 on the mRNA expression of steroidogenic genes were also reported.

Results and Discussion:

The brain is considered to be an important sex steroid producing tissue in teleosts [2, 3]. RT-PCR and qPCR analyses indicated that the mRNA expressions of *cyp11a1*, *hsd3b1* and *cyp17a1* were similarly increased in the brain around the period of gonadal differentiation (Fig. C). *cyp19a1b* mRNA expression and aromatase activity showed significant increases in the forebrain at 110 dah (Fig. C and F). mRNAs for ERs (*ERα*, *ERβ1*, *ERβ2*) and *GPR30* but not *AR*, were significantly increased at 110 dah (a time close to gonadal sex differentiation) in the forebrain and midbrain (Fig. E). Brain E2 levels, but not T, were increased in the forebrain at 120 dah. Similarly, a synchronous peaked expression of *StAR*, the key enzyme genes (*cyp11a1*, *hsd3b1*, *cyp17a1* and *cyp19a1b*) and estrogen receptors were reported at 4 months of black porgy [3]. Using ISH, *cyp11a1*, *hsd3b1* and *cyp17a1* genes were highly expressed in several discrete brain regions with overall similar expression pattern, and most of the hybridization signal seems to correspond to the neuronal cells. Whereas, expression of *cyp19a1b* studied by ISH and IHC showed that most prominent expressions were correspond to the radial glial cells (Fig. D). These data well matched with that of zebrafish brain aromatase studied by ISH and IHC. Exogenous E2 (1 μg/g BW) induced significant effects on the steroidogenic enzymes in the grouper brain.

Conclusion:

Therefore, we identified a peak of functional steroidogenic activity and estrogen signaling in the early teleost brain. Moreover, *pcna* transcripts (a marker for cell proliferation activity) were higher in the early brain at 110–150 dah. The early expression of these genes (including *cyp19a1b*) and aromatase enzyme, concurrent to gonadal sex differentiation, provide that these genes are functional during early postnatal development and gonadal sex differentiation, and have distinct contribution to different aspects of sex hormone, but not gender-related brain differention and/ or development.

References:

[1]WU, G.C., TOMY, S., LEE, M.F., LEE, Y.H., YUEH, W.S., LIN, C.J., LAU, E.L., CHANG, C.F. 2010. Sex differentiation and sex change in the protandrous black porgy, *Acanthopagrus schlegeli*. Gen. Comp. Endocrinol., 167: 417–421.

[2]NAGARAJAN, G., TSAI, Y.J., CHEN, C.Y., CHANG, C.F. 2011. Developmental expression of genes involved in neural estrogen biosynthesis and signaling in the brain of the orange-spotted grouper *Epinephelus coioides* during gonadal sex differentiation. J. Steroid Biochem. Mol. Biol., (accepted).

[3]TOMY, S, WU, G.C., HUANG, H.R., DUFOUR, S., CHANG, C.F. 2007. Developmental expression of key steroidogenic enzymes in the brain of protandrous black porgy, *Acanthopagrus schlegeli*. J. Neuroendocrinol., 19: 643–655.

DEVELOPMENTAL TRACING OF LH BETA GENE EXPRESSION USING A STABLE LINE OF TRANSGENIC MEDAKA REVEALS PUTATIVE DEVELOPMENTAL FUNCTION AND PITUITARY DISTRIBUTION

Hildahl J.[1], Sandvik G.K.[2], Lifjeld R.[1,2], Nagahama Y.[3], Haug T.M.[2], Okubo K.[3,4], Weltzien F.A.[1,2]

[1]Norwegian School of Veterinary Science, Department of Basic Science and Aquatic Medicine, Weltzien Lab, PO Box 8146 Dep, 0033, Oslo, Norway, e-mail: jon.hildahl@nvh.no, fax: +47 22597310
[2]University of Oslo, Department of Molecular Biosciences, Oslo, Norway
[3]National Institute of Basic Biology, Division of Reproductive Biology, Okazaki, Japan
[4]University of Tokyo, Department of Aquatic Bioscience, Tokyo, Japan

Background:

Luteinizing hormone (LH) and follicle stimulating hormone (FSH) are key regulators of vertebrate reproduction. The differential regulation of these hormones by pituitary gonadotrope cells, however, is still poorly understood. Fish, in contrast to mammals, have two distinct gonadotrope cell types, producing LH and FSH, respectively, making teleosts a good model to study the differential regulation of these hormones. Japanese medaka (*Oryzias latipes*) is a powerful model for fish reproductive physiology research, combining a wealth of advanced biotechnological tools, genomic resources, short generation time, a close phylogenetic relationship to many important aquaculture species and a well-defined sexual development. We have thus chosen to use the medaka model for our research to characterize the pituitary gonadotrope cells that produce and secrete LH and FSH.

Methods:

To this aim we have developed a stable transgenic line of medaka with the endogenous LH beta gene (*lhb*) promotor driving green fluorescent protein (*gfp*) expression by microinjection of a BAC construct with hrGFP inserted downstream of the *lhb* promotor. This line has been used to trace the developmental expression of LH beta using fluorescent light and confocal microscopy in whole larva and histological sections. Additionally, qPCR and *in situ* hybridization have been used to measure developmental and tissue specific gene expression.

Results and Discussion:

We demonstrate by *in situ* hybridization that *lhb* mRNA and Gfp protein are co-localized in pituitary cells of sexually mature medaka, and we have characterized the three dimensional distribution of LH gonadotrope cells in the pituitary. qPCR analysis reveals that the *lhb* gene is expressed early during development, already after 24 hours post-fertilization (hpf), and Gfp protein can first be detected by 32 hpf, approximately stage 20. Gfp expression starts posterior of the eyes as paired lateral clusters which then extend to the midline and posteriorly. By 48 hpf, Gfp producing cells are dispersed along the ventral surface of the larva, extending from behind the otic vesicle to the metencephalon – myelencephalon boundary in the developing hindbrain. By 72 hpf, dispersed Gfp producing cells extend to the mesencephalon – metencephalon boundary and a few distinct cells are located at the posterior margin of the eye. *In situ* hybridization of pituitary marker genes show that Gfp producing cells are initially localized outside the primordial pituitary and Gfp is first detected in the developing pituitary by 2 weeks post-fertilization. Confirmation of embryonic *lhb* expression in extra-pituitary Gfp-producing cells is currently underway to validate a potentially novel developmental function for LH.

Conclusion:

We have established a powerful model for characterizing the developmental and reproductive physiological regulation of LH gonadotropes. These data lay the framework for future research into the function of LH during early pituitary developmental studies. This transgenic line also provides the technological basis for further characterization of gonadotropes by cell specific gene expression analysis, electrophysiological and calcium imaging experiments (see abstract by Stransdabo et al, this meeting).

KISSPEPTIN – PHOTOPERIOD/GONADAL STEROID RELATIONSHIPS IN THE BRAIN OF TWO PERCIFORMS, THE STRIPED AND HYBRID BASSES

Zmora N., Stubblefield J., Zulperi Z., Klenke U., Zohar Y.

Department of Marine Biotechnology, University of Maryland Baltimore County, Baltimore, Maryland, USA, nzmora@umbc.edu, Fax: 1-410-2348896.

Introduction:

Reproduction in vertebrates is governed by the hypothalamic-pituitary-gonadal (HPG) axis, which is regulated by a variety of inputs from the environment and peripheral tissues. Environmental cues, such as day length (photoperiod) and temperature dictate the patterns of reproduction in seasonal breeders. Internal cues are predominantly conveyed by the gonads through gonadal steroids and peptides that feed back negatively and positively on the HPG axis. Kisspeptin has recently been implicated as a major player in the control of reproduction in vertebrates [1]. More importantly, it is emerging as the long-sought, yet elusive, mediator between the above cues and the HPG axis. A few studies in fish have demonstrated that estradiol (E2) has an effect on kisspeptin levels [2, 3]. However, the kisspeptin system shows some diversity between classes, especially with regard to the roles of the two kisspeptin systems (kiss1 and kiss2), found in most fish species. In addition, no report yet has shown the impact of photoperiod on kisspeptin as in mammals.

In order to determine the role of the two kisspeptin systems in mediating photoperiod and gonadal cues to the perciform HPG axis, the study investigated how each system's expression and neurons respond to photoperiodic and gonadal steroid signals in the two representative *Morone* models, the striped bass and the striped bass/white bass hybrid.

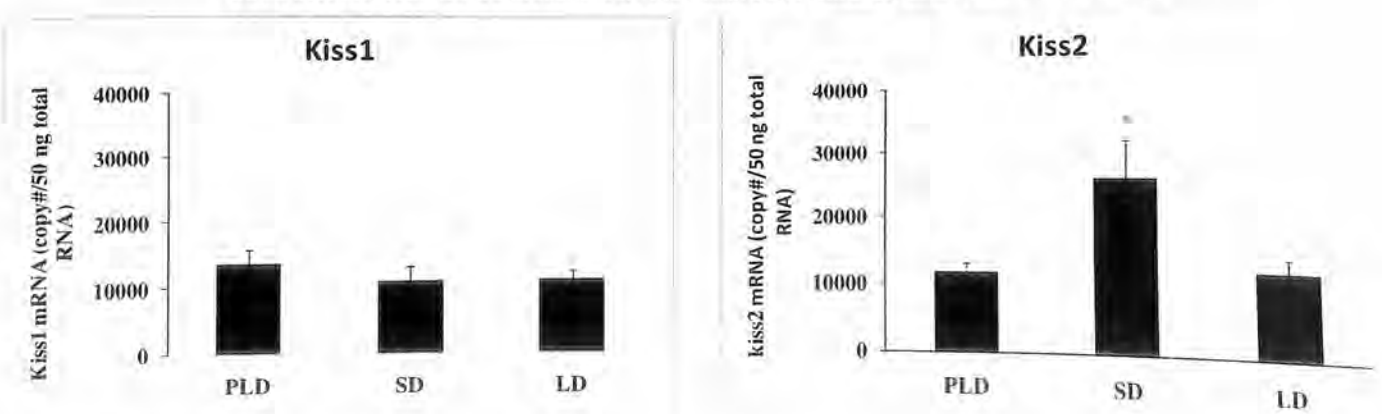
Methods:

Female hybrid bass were exposed to either a long day regime (LD, 16L:8D) or a short day regime (SD, 7L:17D) for 4 weeks. The experiment was conducted twice, in May when fish undergo the onset of puberty and in October when gonads are developing (recrudescent). Striped bass males at different reproductive stages were sham operated, gonadectomized (GDX), or GDX with subsequent steroid replacement via implants. All fish were held under ambient conditions for 10 days post-treatment. mRNA levels of *kiss1, kiss2* and their cognate receptors (*kiss1R* and *kiss2R*) in the brain were measured using Quantitative RT-PCR . Primers were designed based on the corresponding cDNA sequences (Genbank accession #: GU351864, GU351865, GU351869) and procedures were conducted as previously described [4]. *In-situ* hybridization (ISH) to detect neurons expressing *kiss1*, *kiss2* and *kissRs* was performed on 12 micron brain cryo-sections using biotin- or Dig-labeled anti-sense riboprobe. Signal was obtained using anti-Dig-AP (Roche) and NBT-BCIP as a substrate or the Tyramide Signal Amplification kit with Cy3 dye (Perkin Elmer).

Results and discussion:

The mRNAs of *kiss1* and *kiss2*, *kiss1R* and *kiss2R* were identified and cloned from the brain of adult striped bass. Our initial studies focused on verifying the regulatory potency of kiss1 and kiss2 on the reproductive axis. Both kisspeptin peptides (kiss1 - QDVSSYNLNSFGLRY-NH_2, kiss2 - SKFNFNPFGLRF-NH_2) induced the release of LH and upregulated *GnRH1* expression 24 hr post-injection. However, the response was more pronounced in May, when kiss2 also showed higher potency than kiss1.

Figure 1: Brain *kiss1* and *kiss2* mRNA levels following exposure to ambient (PLD), long (LD) and short (SD) photoperiods. Results are presented as the mean copy number ±SE. Statisticasignifican



Expression profiles revealed a different pattern for *kiss1R* than those of *kiss1*, *kiss2* and *kiss2R*.

In situ hybridization detected kiss1 and kiss2 neurons predominantly in the nucleus recessus lateralis and the preoptic area. Our studies also showed that the two kiss receptors are expressed in the same, or in close contact to, positively stained GnRH1 neurons in the POA, thus indicating a direct effect of kisspeptin on GnRH neurons. Significantly lower expression was observed for *kiss1/kiss1R* compared to *kiss2/kiss2R* using ISH and QRT-PCR.

Following exposure of hybrid females to short day or long day regimes, only *kiss2* transcript levels in the brains increased, by 2.5 fold, in the short photoperiod compared to the long photoperiod (Fig. 1). Simultaneously, *GnRH1* mRNA levels increased in the brains of the SD compared to LD exposed fish in pubertal (May trial) but not in recrudescent females (October trial). These results indicate that *kiss2* expression is positively regulated by short photoperiod. *GnRH1* expression, however, might be affected by additional factors like gonadal steroids, which are at higher levels during recrudescence.

Kiss1, *kiss2*, *kiss2R* and *GnRH1* transcript levels were reduced in GDX mid-gonadal developmental male striped bass treated with testosterone compared to GDX males. In contrast, pubertal males responded to the steroid replacement by upregulation of *kiss1* and *kiss2*. No change was observed in juvenile and recrudescent males. These results indicate that gonadal steroids affect *kiss1* and *kiss2* expression in a reproductive stage-dependent manner. These effects are in agreement with that of T on the HP axis, where steroids have a negative effect at early to mid-gonadal developmental stages and a positive effect in the pubertal stage.

Conclusions:

Our study shows that both kisspeptin systems in basses are involved in the regulation of the HPG axis. Additionally, by showing that kisspeptin expression responds to changes in light regime and in gonadal steroids, this study supports the emerging hypothesis that the kisspeptin system plays a mediating role in the gonadal- and environmental-HPG axes. The effect of these cues, however, is reproductive stage-dependent, which supports the notion that the kisspeptin system is a central processing system for a variety of signals.

References:

[1]KAUFFMAN, A.S., CLIFTON, D.K., STEINER, R.A. 2007. Emerging ideas about kisspeptin-GPR54 signaling in the neuroendocrine regulation of reproduction. Trends Neurosci., 30: 504-511.

[2]SERVILI, A., LE PAGE, Y., LEPRINCE, J., CARATY, A., ESCOBAR, S., PARHAR, I.S., SEONG, J.Y., VAUDRY, H., KAH, O. 2011. Organization of two independent kisspeptin systems derived from evolutionary-ancient kiss genes in the brain of zebrafish. Endocrinology, In press.

[3]KANDA, S., AKAZOME, Y., MATSUNAGA, T., YAMAMOTO, N., YAMADA, S., TSUKAMURA, H., MAEDA, K., OKA, Y. 2008. Identification of KiSS-1 product kisspeptin and steroid-sensitive sexually dimorphic kisspeptin neurons in medaka (*Oryzias latipes*). Endocrinology, 149(5): 2467-2476.

[4] WONG, T.T., GOTHILF, Y., ZMORA, N., KIGHT, K.E., MEIRI, I., ELIZUR, A., ZOHAR, Y. 2004. Developmental expression of three forms of GnRH and ontogeny of the hypothalamus-pituitary-gonadal axis in gilthead seabream (*Sparus aurata*). Biol. Reprod., 71: 1026-1035.

NOVEL NEUROPEPTIDES INVOLVED IN THE CONTROL OF PUBERTY AND REPRODUCTION IN FISH

Levavi-Sivan B., Biran J., Ben-Dor S., Palevitch O.

The Robert H. Smith Faculty of Agriculture, Food and Environment, Department of Animal Sciences, The Hebrew University of Jerusalem, Rehovot 76100, Israel.
e-mail: sivan@agri.huji.ac.il Fax: +972-89489307.

Introduction:

The findings that inactivation of kisspeptin signaling, because of mutations in the kisspeptin receptor, is associated with hypogonadotropic hypogonadism and absent or delayed puberty in man [1] stimulated the many subsequent studies that together have led to a new view of the neural control of GnRH release [2]. With this in mind, the recent observations [3] that mutations of the genes encoding neurokinin B (NKB) (*TAC3*) and its receptor (*TAC3R; NKBR*) were also associated with hypogonadotropic hypogonadism, is therefore of considerable interest. In order to study the physiological function(s) and evolutionary conservation of NKB, we cloned *tac3* and *tac3 receptor* cDNAs from several fish species, including zebrafish.

Methods:

Zebrafish tac3a, tac3b, tac3Ra and tac3Rb were cloned by synteny, according to Biran *et al.* [4]. A phylogenetic tree of all the currently known vertebrate neurokinin genes was generated with the neighbor-joining method. Real-time PCR and receptor transactivation assays were performed as previously described [4].

Results and Discussion:

A comparison of the zebrafish protein coding sequences of *tac3* cDNAs with those of the human and mouse TAC3 proteins showed identities of 23% and 24%, respectively. The phylogenetic tree showed that the vertebrate neurokinin genes fall into two distinct lineage groups. One lineage includes the mammalian and rodent TAC3, and all the piscine Tac3 that were cloned in the present study. The second lineage includes the mammalian and fish *tac1*. Nonetheless, high identity was found between different fish species, in the region encoding the NKB; all shared the common C-terminal sequence.

Many genes encoding the tachykinins have been found to encode a precursor that produces more than one tachykinin. However, in mammals TAC3 is unusual in that it encodes only a single tachykinin – the NKB. Interestingly, we have found that in zebrafish, exon 3 of both tac3s encodes a well conserved additional tachykinin. Furthermore, *in silico* analysis revealed that this new tachykinin exists in all other fish species whose genomes are known, and it was termed NKF. NKF also possesses the common C-terminal sequence FVGLM, known as the tachykinin signature.

zftac3a-expressing neurons were localized in specific brain nuclei that are known to be implicated in reproduction, whereas *zftac3b*-expressing neurons were more dispersed throughout the brain. Zebrafish *tac3a* – but not *tac3b* – mRNA levels gradually increased during the first few weeks of life, and peaked in fish with ovaries containing mature oocytes or with testes containing mature spermatozoa. Estrogen treatment of mature fish causes increase in *tac3a*, *kiss2* and *kiss1rb* expression in males, with no significant change in females.

Tac3Ra and tac3Rb transduce their activity via both PKC/Ca^{2+} and PKA/cAMP pathways. Both tac3 receptor types were very sensitive to amidation of their cognate ligands.

Conclusion:

These results indicate that the NKB/NKBR system may participate in puberty initiation in fish. Moreover, this novel system may be involved, in parallel with the kisspeptin system, in neuroendocrine regulation of GnRH secretion.

References:

[1]SEMINARA, S. B., MESSAGER, S., CHATZIDAKI, E. E., THRESHER, R. R., ACIERNO, J. S., SHAGOURY, J. K., BO-ABBAS, Y., KUOHUNG, W., SCHWINOF, K. M., HENDRICK, A. G., ZAHN, D., DIXON, J., KAISER, U. B., SLAUGENHAUPT, S. A., GUSELLA, J. F., O'RAHILLY, S., CARLTON, M. B. L., CROWLEY, W. F., APARICIO, S., COLLEDGE, W. H. 2003. The GPR54 gene as a regulator of puberty. New Eng. J. Med., 349: 1614-U1618.

[2]OAKLEY AE, CLIFTON DK, STEINER RA. 2009. Kisspeptin Signaling in the Brain Endocr. Rev., 30: 713-743.

[3]TOPALOGLU, A. K., REIMANN, F., GUCLU, M., YALIN, A. S., KOTAN, L. D., PORTER, K. M., SERIN, A., MUNGAN, N. O., COOK, J. R., OZBEK, M. N., IMAMOGLU, S., AKALIN, N. S., YUKSEL, B., O'RAHILLY, S., SEMPLE, R. K. 2009. TAC3 and TACR3 mutations in familial hypogonadotropic hypogonadism reveal a key role for Neurokinin B in the central control of reproduction. Nat. Genet., 41: 354-358.

[4]BIRAN J, BEN-DOR S, LEVAVI-SIVAN B. 2008. Molecular identification and functional characterization of the kisspeptin/kisspeptin receptor system in lower vertebrates. Biol. Reprod. 79: 776-786.

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

KISSPEPTINS REGULATE FISH REPRODUCTION

Ishwar S. Parhar

Brain Research Institute, Jeffrey Cheah School of Medicine & Health Sciences, Monash University Sunway Campus, PJ 46150, Malaysia, ishwar@med.monash.edu.my

Gonadotropin-releasing hormone (GnRH) is the primary regulator of vertebrate reproductive functions. We have cloned novel genes that are emerging as important regulators of GnRH and the reproductive axis, *kiss1* (encoding Kisspeptin-1), *kiss2* and their G-protein coupled Kisspeptin receptors (*kissr1* and *kissr2*), in non-mammalian vertebrates, the teleost. In the zebrafish, *kiss1* mRNA expression was observed in the ventral habenula, and *kiss2* mRNA expression was observed in the posterior tuberal nucleus and the periventricular hypothalamic nucleus. Laser capture microdissection coupled with real-time PCR showed *kissr1* mRNA expression in the ventral habenula, while *kissr2* mRNA expression was seen throughout the brain. Luciferase-reporter assay showed higher response of zebrafish Kiss-R1 to Kiss1 and that of zebrafish Kiss-R2 to Kiss2. The expression pattern of *kiss1*, *kiss2*, *gnrh2* and *gnrh3* mRNA levels were significantly increased at the start of the pubertal phase, suggesting regulatory roles of kisspeptins on GnRH neuron types during sexual maturation, which is supported by the expression of Kiss-R2 in the three GnRH neuron types (GnRH1, GnRH2 and GnRH3) in the tilapia. Using double-label *in situ* hybridization, we showed co-expression of *kissr1* and *kiss1* mRNAs in the ventral habenula. Further, central administration of Kiss1 significantly decreased the amount of *kiss1* mRNA, while increased *kissr1* and *c-fos* mRNA in the ventral habenula. These observations suggest that Kiss1 is autoregulated through Kiss-R1 in the habenula. In sexually mature female zebrafish, Kiss2 but not Kiss1 administration significantly increased β-subunit of gonadotropins mRNA levels in the pituitary, which suggests that hypothalamic Kiss2 is the predominant regulator of gonadotropin synthesis in teleosts. Hence the regulation of reproduction by the hypothalamic Kisspeptin-Kiss receptor-GnRH system is well conserved in vertebrates.

GPR54

SEXUAL BEHAVIOR IN TELEOSTS: ROLE OF GONADAL HORMONES

Paul-Prasanth B*, and Nagahama Y^O

*Amrita Centre for Nanosciences and Molecular Medicine, Amrita Institute of Medical Sciences, Kochi-41, India.
bindhuprash@gmail.com
^ONational Institute for Basic Biology, Okazaki, Japan

In general among vertebrates, sexual behavior is governed by the sex-steroids produced by the central nervous system and the peripheral organs, mainly gonads. The general consensus among mammals is that the estradiol-17β (E_2) synthesized by CNS is essential for masculinization of the brains in male embryos, while E_2 has not been shown to have any such effect on the feminization process in the female brains. However, recent reports have started to reveal that E_2 has a role in the feminization of the female brain also, though postnatal unlike the male embryos. A recent study from mice has shown that prepubertal E_2 is critical for the display of the normal lordosis behavior in females. Unlike mammals, no reports have given any evidence for the organizational roles of E_2 in masculinization of the male brain of fishes in general. There have been reports on the role of E_2 in the feminization of the female brain among fishes. Studies done using various fish models have shown that the brains of fishes are highly plastic. Administration of sex steroids can alter the sex-specific behavioral patterns in fishes even after sexual maturation. Using Japanese medaka as a model organism we have investigated whether E_2 has a role in controlling the female sexual behavior during adulthood. Japanese medaka (*Oryzias latipes*) is a small teleost fish that exhibits sexually dimorphic behavioral patterns during mating in adulthood. Adult males perform the mating dance in a stepwise manner in order to attract the females and the steps include following, dancing, crossing etc. On the contrary, females do not display any particular mating behavior other than receptiveness. Recently, we have shown that sexual differences become established in medaka brain only at around 1-2 months post hatching under the influence of gonadal hormones. Therefore, we exposed the breeding females of medaka to exemestane (EM), a steroidal inhibitor of aromatase to block the production of E_2 in order to examine the role of E_2 in feminization of the female brain in medaka. EM-treated females began to exhibit male-specific sexual behavior from the 5th day of treatment (dot) when they were paired with sexually mature normal females. By 10 dot, a significant decrease was found in the levels of estrogens like estrone (E_1) and E_2 in the brain. Further, we examined whether the changes in sexual behavior was accompanied with local modulations in the brain transcriptome by microarray analysis. A large number of sex-specific genes were found to be modulated region-specifically in the brain in response to the inhibition of aromatase at 10 dot. One of these genes was the gene coding for the brain form of aromatase in teleosts namely, *cyp19a2*. Further analysis of *cyp19a2* by real-time PCR and in situ hybridization revealed that its transcription was positively regulated by levels of E_2 in the brain and intriguingly, unlike mammals, transcripts of *cyp19a2* was more in the female brain than that of the male brain. More interestingly, expression of *cyp19a2* was found to be specific to the periventricular gray zone of the optic tectum in females and the presence or absence of these transcripts in this area was under the direct control of E_2

levels in the brain. Further investigation to find whether ovarian estrogens had any role in the up regulation of *cyp19a2* in the female brain by ovariectomy revealed that it was the levels of estrogens in the ovary that was responsible for the increased levels of *cyp19a2* transcripts and thus, the estrogens in the brain of the females in medaka. Further, the ovariectomized females failed to display any male-specific sexual behavior, suggesting that androgens produced by the ovaries in the absence of E_2 should have been responsible for the induction of male-specific sexual behavior in EM-treated females. Our results demonstrated that estrogens from the ovaries suppressed male sexual behavior in the females of medaka through direct up regulation of the transcription of *cyp19a2*.

EFFECTS OF ARGININE VASOTOCIN ON THE HYPOTHALAMIC-PITUITARY-GONADS AXIS: A BEHAVIOURAL APROACH

Ramallo M.[1], Grober M.[2], Pandolfi M.[1]

[1]Departamento de Biodiversidad y Biología Experimental, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Argentina. Ciudad Universitaria. Pabellón II. CABA (C1428EHA). pandolfi@bg.fcen.uba.ar.
[2]Department of Biology, Georgia State University, USA.

Introduction:

The neuropeptide arginine vasotocin (AVT) has been linked to reproductive plasticity in a variety of teleosts with distinctive types of alternative phenotypes. The manipulation of AVT levels affects behaviours that are exhibited predominantly by animals of one phenotype, such as courtship and aggression [1, 2]. AVT is differentially expressed as a function of alternative phenotypes modulating social behaviour in many fish species [3].*Cichlasoma dimerus* is a cichlid species in which males are found in one of two basic alternative phenotypes that are linked to both social and reproductive status. Non territorial (NT) males have dark ground coloration and lack immediate access to reproduction when compared with territorial (T) brightly colored males, that actively defend a territory. The aim of this study was to characterize the AVT system in males of *C. dimerus* in relation to social status and aggressive behaviour, with particular emphasis on the various putative sites of action of AVT across the hypothalamic-pituitary-gonads (HPG) axis, and its effects on reproductive and social physiology.

Methods:

The location and distribution of AVT neurons within *C. dimerus'* brain was studied by immunohistochemistry. Each AVT-ir soma was assigned to either the parvocellular (pPOA), magnocellular (mPOA) or gigantocellular (gPOA) preoptic area (POA) subpopulation based on neuroanatomical position, somata morphology and size. To assess differences in neural phenotype associated to social status, the size and number of AVT-ir somata, and the size of AVT-ir nucleus were measured in coronal sections. Cell numbers from each cell group were determined by duplicate by naked-eye observation. Cell and nucleus profile area were computed from digital images by tracing the cell somata's/nuclei's profile with a digitizing pen.

To study the effect of AVT on pituitary LH and FSH secretion, pituitaries were incubated for 24 h with AVT (control, 0.1, 1 and 10 μM). Beta-FSH and beta-LH presence in the culture media was semiquantified by immunoblotting. To investigate the effect of AVT on testicular androgen release testicular fragments were incubated with or without different AVT doses (control, 0.1, 1 and 50 mM). Androgen levels were measured by RIA.So as to study AVT gene expression in different organs, total RNA was isolated from the POA, head kidney, trunk kidney, intestine, muscle, testes and liver. RT-PCR was performed using specific primers for *C. dimerus* AVT preprohormone.

Results and Discussion:

AVT-ir neurons were localized exclusively within the POA. AVT-ir cells extended from behind the anterior commissure to the posterior POA, above the optic chiasm. Immunoreactivity was observed in all 3 POA cell subpopulations: pPOA, mPOA and gPOA, lining the border of the 3rd ventricle. Axons from pPOA cells, targeted ventrally and away from the ventricle. The greatest concentration of AVT-ir axons occurred within the POA where fibers from mPOA and gPOA cells, formed a dense preoptic-hypophyseal (PO-H) tract that entered the pituitary trough the anterior and posterior infundibular stalks. Within the pituitary, immunostaining was observed mainly in the *pars nervosa*, proximal *pars distalis* and *pars intermedia*. Some AVT-ir axons from mPOA and gPOA cells targeted other brain regions. AVT-ir fibers were observed in the dorsal sac of the pineal complex, where AVT is believed to play a role in the coordination between photoperiodic cues and reproductive events. Thus, in *C. dimerus* mPOA cells might be involved in the synthesis of AVT which mainly acts as a neurohormone. In turn, the association between AVT-ir nerve terminals with the adenohypophysis, suggests a possible role as a moderating factor for hormone release. Nonetheless, not all AVT-ir fibers from the mPOA incorporated to the PO-H tract which suggests that AVT may act as a neuromodulator, possibly affecting sensory association pathways with social relevance. T individuals had mPOA neurons 11% larger compared with NT males. There were no differences in cells/section number and nuclear area between T and NT males for any of the 3 subpopulations analyzed.In *C. dimerus*, AVT significantly increased pituitary's gonadotropin release and testicular androgen synthesis *in vitro*. Beta-LH showed a triphasic response to AVT increasing doses, while beta-FSH and androgen responses were directly proportional. Androgens would modify, reinforce and/or stabilize the neural circuits

associated with behaviour typical of T males. Expression and peptide synthesis was found in small cells from the interstitial compartment of the testes, presumed to be Leydig cells. Though many studies have informed AVT expression on the testes of various vertebrates [4, 5], to our knowledge this is the first report of AVT detection at a cellular level in a teleost.

Conclusion:

In *C. dimerus*, the AVT system is highly complex with multiple sites of action along the HPG axis. AVT expression levels in mPOA cells are differentially associated to the different social phenotypes in which increased AVT expression, triggers behavioural and physiological changes typical of T males, ensuring access to territory and thus the possibility to reproduce.

References:

[1]SEMSAR K., KANDEL F. L., GODWIN J., 2001. Manipulations of the AVT system shift social status and related courtship and aggressive behaviour in the bluehead wrasse. Horm. Behav., 40(1):21-31.

[2]SANTANGELO N., BASS A. H., 2006. New insights into neuropeptide modulation of aggression: field studies of AVT in a territorial tropical damselfish. Proc. R. Soc., B 273, 3085–3092.

[3]GREENWOOD A. K., WARK A. R., FERNALD R. D., HOFMANN H. A., 2008. Expression of AVT in distinct preoptic regions is associated with dominant and subordinate behaviour in an African cichlid fish. Proc . Biol. Sci., 275(1649):2393-402.

[4]FILLION C., MALASSINE A., TAHRI-JOUTEI A., ALLEVARD A. M., BEDIN M., GHARIB C., HUGUES J. N., POINTIS G., 1993. Immunoreactive AVP in the testis: immunocytochemical localization and testicular content in normal and in experimental cryptorchid mouse. Biol. Reprod., 48(4):786-792.

[5]SINGH AND JOY K.P., 2008. Immunocytochemical localization, HPLC characterization, and seasonal dynamics of vasotocin in the brain, blood plasma and gonads of the catfish *Heteropneustes fossilis*, Gen. Comp. Endocrinol., 159: 214–225.

NEGATIVE IMPACT OF STRESS ON REPRODUCTION: ROLE OF BRAIN AND GILL DURING SALINITY RESPONSE IN TILAPIA

Aruna A., Nagarajan G. and Chang C.F.

°Department of Aquaculture, National Taiwan Ocean University, Keelung, 20224, Taiwan, Fax: + 886-2-2462-1579
n.aruna@yahoo.com

Introduction:

Negative impact Stress is considered to be the physiological resultant of demands that exceed an organism's regulatory capacities. Response to stressors is vitally important for normal response, allowing the organism to avoid or cope with challenges to homeostasis. Sex steroids actually have profound influence on stress perception in the brain. Stress manifests centrally in the nervous system, converging at the hypothalamus and the final product of stress hormone, cortisol is believed to contribute to the stress-induced reproductive suppression during stress by central actions on the pituitary or hypothalamus. Suppression of reproductive hormones reduced gamete viability and gonadal growth retardation. Therefore, the aim of the present study was to test the hypothesis that stress has a negative impact on reproduction. To address this, we examined the differential expression pattern of stress hormones (corticotrophin releasing hormone, CRH; CRH-receptor, CRH-R; adrenocorticotropic hormone, ACTH and beta-Na+/K+ ATPase, β-NKA) and reproductive hormones (luteinizing hormone, LH; follicle stimulating hormone, FSH; gonadotropin releasing hormone, GnRH1, 2 and 3) in freshwater (FW) and seawater (SW) acclimated tilapia in the forebrain, midbrain, hypothalamus, pituitary and gill during salinity stress.

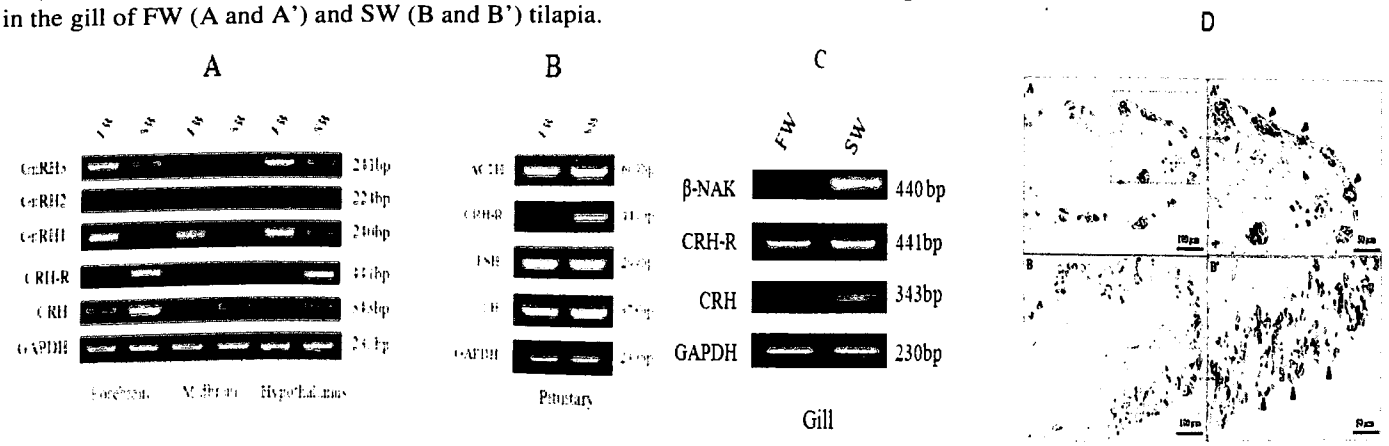
Methods:

RT-PCR analysis was performed to find out the mRNA expression of CRH, CRH-R, ACTH, β-NKA, GnRH, LH, and FSH in the brain, pituitary and gill of tilapia during salinity stress. *In situ* hybridization study was also performed to localize the transcripts of hypothalamic neuropeptide (CRH, GnRH1 and GnRH3) in the tilapia brain, and immunolocalization also carried out by using α-Na+/K+-ATPase (α-NKA) antibody to differentiate the mitochondria rich cells in the gill at FW and SW.

Results and Discussion:

The present results demonstrated that the intensity of the band of CRH and CRH-R was strongly detected in the forebrain, midbrain and hypothalamus of SW fish. However, the mRNA expression of GnRH1 and GnRH3 was weak in the forebrain, midbrain and hypothalamus of SW fish compared to their respective FW fish (Fig.1A). In addition, there was no significant difference observed in LH, FSH and ACTH in the pituitary, whereas increased expression of CRH-R was found in the SW fish (Fig. 1B). Stress increased the expression of CRH, which led to a suppression of GnRH1 and GnRH3 in the hypothalamus. Similarly, the transcripts of gill CRH [1], CRH-R, β-NKA [2] (Fig. 1C) and α-NKA protein (Fig. 1D) were increased in the SW fish to maintain the homeostasis and balance the acid-base regulation. Furthermore, the CRH, GnRH1 and GnRH3 transcripts hybridization signals were detected in the tilapia brain especially in the telencephalic ventricle (TelV) (Fig. 2B, E and H) and preoptic area (POA) (Fig. 2C, F and I), which implied that the stress induce CRH neuron and this may activate GnRH, which then

Fig.1. RT-PCR analysis of (A) CRH, CRH-R, GnRH1, GnRH2 and GnRH3 in the forebrain, midbrain and hypothalamus, (B) LH, FSH, CRH-R and ACTH in the pituitary, (C) CRH, CRH-R and β-NKA in the gill and (D) immunolocalization of α-NKA in the gill of FW (A and A') and SW (B and B') tilapia.



suppress reproduction. Stress exerts profound inhibitory effects on reproductive function by suppressing the pulsatile release of GnRH.

Conclusion:

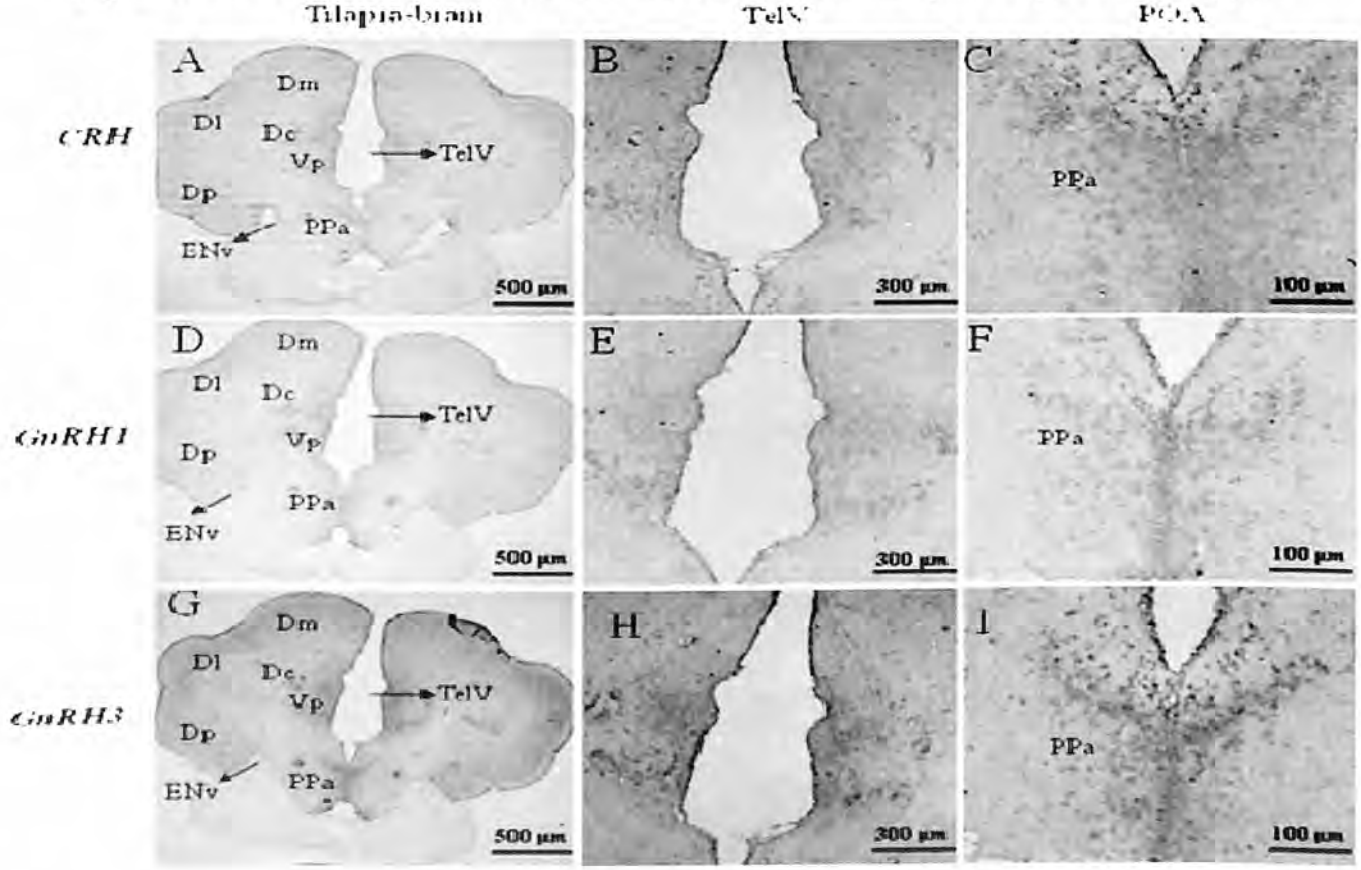
These results suggested that the activation of stress axis (hypothalamus- pituitary- interrenal axis, HPI) suppressed the hypothalamus- pituitary- gonadal (HPG) axis to affect reproduction in tilapia during salinity stress. The elevated transcripts of CRH and CRH-R in both brain and gill suggest that it may be essential for tilapia SW acclimation. Further, the expression of β-NKA transcripts and α-NKA proteins were increased in the gill of SW fish than the FW fish, which implied that the gill play an essential key role during salinity stress response. The *in situ* hybridization study demonstrated that the transcripts of CRH and GnRH3 were more abundant in the POA of brain than the GnRH1.

References:

[1]ARUNA, A., NAGARAJAN, G., CHANG, C.F. Long-term salinity effects on stress genes in the brain-pituirary-gill of tilapia, *Oreochromis mossambicus*. Gen. Comp. Endocrinol. (Submitted)

[2]TOMY, S., CHANG, Y.M., CHEN, Y.H., CAO, J.C., WANG, T.P., CHANG, C.F. 2009. Salinity effects on the expression of osmoregulatory genes in the euryhaline black porgy *Acanthopagrus schlegeli*. Gen. Comp. Endocrinol., 161: 123-132.

Fig. 2. Localization of CRH, GnRH1 and GnRH3 mRNA in the tilapia brain by *in situ* hybridization analysis.



CLONING AND CHARACTERIZATION OF VASOTOCIN IN THE CATFISH *HETEROPNEUSTES FOSSILIS*: SEASONAL AND TISSUE EXPRESSION STUDIES

Banerjee P°, Chaube R.* and Joy K.P.°

°Department of Zoology, Banaras Hindu University, Varanasi, U.P. - 221005, India
*Zoology Department, MMV, Banaras Hindu University, Varanasi, U.P. - 221005, India
chaubersadhak@rediffmail.com, kpjoy@bhu.ac.in

Introduction:

Vasotocin (VT) is the basic neurohypophysial nonapeptide in teleosts as in other nonmammalian tetrapods. It is classically known to be synthesized by the nucleus preopticus (NPO) of the hypothalamus, and then transported via axons to neurohypophysis where it is stored until release [1]. Various studies have shown that VT is secreted/distributed in brain and other peripheral organs such as ovary, uterus, testis, adrenal, thymus and sympathetic nervous system of higher vertebrates [3]. In teleost fish, VT has been known to mediate diverse actions such as osmoregulation, cardiovascular function, reproduction, stress response, social behavior and circadian and seasonal biology [2]. Recently, Singh and Joy (2008) demonstrated distribution of VT in the catfish brain and ovary by immunolocalisation, HPLC and EIA through different phases of the reproductive cycle of the catfish. Seasonal dynamics of vasotocin in relation to the reproductive cycle in *Heteropneustes fossilis*, have shown that there is significant seasonal and sexual difference. VT immunoreactivity has also been demonstrated in ovary, which varied with the growth of the follicles implicating a direct role of the peptide in reproduction. These initial reports need to be further supported by molecular studies to get an overall status of vasotocin gene expression - its transcription, translation, posttranslational processing, storage and finally release and how each of these steps get affected with respect to the changing reproductive seasons. In the present study, molecular cloning and characterization of vasotocin gene was done to obtain partial cDNA sequence, followed by its tissue distribution and seasonal expression profile by semi-quantitative RT-PCR.

Methods:

Adult female catfish were obtained from a local fish market and acclimatized to laboratory conditions in ambient photoperiod and temperature. The acclimatized fish were sacrificed by decapitation; various tissues were dissected out *viz*. brain, gill, liver, muscle, kidney, ovary, and snap frozen in liquid nitrogen. For cloning of vasotocin gene, total RNA was extracted from adult brain using the Qiagen RNAesy lipid tissue mini kit, following manufacturer's protocol. 5µg RNA was reverse transcribed using random hexamer primers and Revert Aid M-MuLV reverse transcriptase (first strand cDNA synthesis kit Fermantas). From 1µl of the resulting cDNA, partial vasotocin cDNA was amplified using the degenerate primer set- FP- tccgcttgttaatccaraaytgycc, RP- acatcccagtccctctccrcarcdat. The amplified product was purified using Hipura PCR product purification kit and cloned using Promega pGEM-T Easy kit. Recombinant colonies were selected by blue-white screening and plasmid was extracted from recombinant colonies, by alkaline lysis method. Sequencing of recombinant plasmid was got done at Xceleris laboratory, Ahmedabad to get the partial cDNA sequence of VT. Specific primers were designed from the partial sequence (FP- gttacatccagaactgccccaga RP- acatcccagtccctctccgcagca) and used to study tissue expression and seasonal expression of vasotocin by semi-quantitative RT-PCR.

Results and Discussion:

The degenerate primers amplified a 145 bp fragment of VT cDNA, that codes for the 9 aa hormonal moiety and the N-terminal part of neurophysin. The sequence analysis of this cDNA clone reveals a significant homology with most of the nucleotide sequences of teleost vasotocin precursor available on the NCBI database, with a maximum identity of 81% with *Epinephlus adscensionis* VT preprohormone (Acc. No. HQ 141397) in the blast search. The brain was the main site of vasotocin expression, followed by ovary which was shown to be a major ectopic site for transcription of the hormonal gene, known conventionally as a neurohypophysial peptide. This result supports the earlier findings of our lab, demonstrating VT immunoreactivity in ovarian follicle [4] . However, there was no expression in gill, liver, kidney and muscle tissues. Brain VT mRNA transcript showed no significant seasonal variation, though its transcript level was higher in comparison to ovarian VT mRNA transcript. Ovarian VT mRNA transcript showed significant seasonal variation; resting phase had low transcript level whereas preparatory, prespawning and spawning season had significantly higher level, though insignificant among the respective seasons. Further studies are needed to get a thorough picture of the dynamics of expression of VT precursor over the different phases of the reproductive cycle. In accordance, quantifying of absolute transcript level by real time

qPCR will be planned.

Conclusion:

In conclusion, VT mRNA is expressed both in brain and ovary. Brain showed higher level than ovary. Thus, the study signifies differential role of VT gene in regulating sexual behavior, spawning and reproduction at molecular level.

References:

[1]ACHER R. 1985. Biosynthesis, processing and evolution of neurohypophysial hormone precursors.Jap. Sci. Soc. Press, 11-25

[2]BALMENT R. J., LU E. W., WEYBOURNE W., WARNE J. M. 2006. Arginine vasotocin: a key hormone in fish physiology and behaviour: A review with insights from mammalian models. Gen. Comp. Endocrinol., 147: 9-16

[3]BARTH S.W., SAITO N., GROSSMANN R. 1997. Gene expression of the neurohypophysial hormone arg-vasotocin in the reproductive system of the hen. Eur. J. Physiol., 1: 434 -454

[4]SINGH V., JOY K.P. 2008. Immunocytochemical localization, HPLC characterization and seasonal dynamics of vasotocin in the brain, blood plasma and gonads of the catfish *Heteropneustes fossilis*. Gen. Comp. Endocrinol.,159: 214- 225

THE NEUROENDOCRINE CONTROL OF PUBERTAL DEVELOPMENT IN ATLANTIC BLUEFIN TUNA (*THUNNUS THYNNUS*)

Berkovich, N.[1,2], Corriero, A.[3], Santamaria, N.[3], Mylonas, C.C.[4], Bridges C.R.[5], Vassallo-Aguis, R.[6], De La Gándara, F.[7], Belmonte, A.[8], Mislov, K.[9], Katavic, I.[10], Elizur, A.[11] Meiri-Ashkenazi, I.[1], Gordin, H.[1], and Rosenfeld, H.[1]

[1]Israel Oceanographic and Limnological Research, National Center for Mariculture, P.O. Box 1212, Eilat 8112, Israel. Fax +972-8-6375761; e-mail: hannarosenfeld@gmail.com
[2]Department of Life Science, Ben-Gurion University of the Negev, P.O. Box 653, Beer-Sheva 84105
[3]Department of Animal Health and Welfare, University of Bari, Italy
[4]Institute of Aquaculture, Hellenic Center for Marine Research, Crete, Greece
[5]Heinrich-Heine Universität, Institut für Zoophysiologie, Düsseldorf, Germany
[6]Malta Center for Fisheries Sciences, Marsaxlokk, Malta
[7]Instituto Español de Oceanografía, Centro Oceanográfico de Murcia, Puerto de Mazarrón, Spain
[8]Tuna Graso S.A., Carretera de la Palma Km 7, Paraje la Estrella, Cartagena (Murcia), Spain
[9]Kali tuna D.O.O. Put Vele Luke bb, 23272 Kali, Croatia
[10]Laboratory for Aquaculture, Institute of Oceanography and Fisheries, Split, Croatia
[11]Faculty of Science, Health and Education, University of the Sunshine Coast, Qld., Australia

Introduction:

The bluefin tuna (BFT, *Thunnus thynnus*), a large pelagic fish with remarkably high commercial value, has become an endangered species due to overfishing [1, 2]. Recently, significant progress on spawning induction in captive BFT has been achieved providing the basis for the species' domestication [3]. To further promote the development of a self- sustained BFT aquaculture, we investigated first sexual maturity in BFT reared from an immature stage in captivity. Accordingly, our major objectives were to evaluate: (i) maturational status of the brain-pituitary-gonadal (BPG) axis, and (ii) responsiveness of the BPG to exogenous hormones. Special emphasis was given to characterize the KiSS/GPR54 system that constitutes the trigger of puberty onset, and the gonadotropins follicle stimulating hormone (FSH) and luteinizing hormone (LH) that act as central regulators of gonadal development and gamete maturation.

Methods:

In vivo studies - In a first trial (July, 2009), sexually immature BFT juveniles (n=20) and sexually mature individuals (n=19), were sampled during BFT natural spawning season within the Mediterranean Sea. In a second trial (June-July, 2010), sexually immature BFT juveniles were treated with poly [ethylene-vinyl acetate] (EVAc) implants containing KiSS-peptides (n=6). The remaining fish (n=14) were used as untreated controls. Three weeks post implantation all fish were sampled. Morphometric parameters were recorded and the relative gonadosomatic index values were calculated. The first spiniform ray of the dorsal fin was removed to determine the age of the fish, while brain, pituitary and gonad tissues were removed and stored frozen until use for gene expression and hormonal measurements. Additionally, gonad slices were fixed in Bouin's fixative for histological analysis.

In vitro studies - Uniformly sized gonad fragments derived from treated- or untreated BFT juveniles (trial 2) were challenged (16 h) with graded doses of recombinant BFT LH or FSH (rLH and rFSH, respectively) previously produced at IOLR-NCM using the methylotrophic yeast *Pichia pastoris* expression system. At the end of the experiment, media and tissues were collected for further analyses. Gonadal sections were subjected to immunohistochemical staining using anti-proliferating cell nuclear antigen (PCNA). Quantification of germ cell proliferation was performed by measuring the surface of anti-PCNA positive germ cells using the QWIN image analysis software.

Hormonal and gene expression analyses - Sex steroid were extracted from gonadal tissue with diethyl ether. The dry extract was assayed for 11-ketotestosterone or estradiol levels using the specific ELISAs. The LH levels were measured in pituitary extract using an ELISA modified for tuna species [4]. The pituitary FSH levels were measured using our newly calibrated Immuno-Dot Blot assay. Expression levels of target genes were measured in relevant tissues employing the quantitative real time PCR technique.

Results and Discussion:

The growth parameters recorded for the captive BFT juveniles are consistent with the length-weight relationship established for wild Mediterranean BFT stocks. The histological analyses of the gonads indicate advanced sexual maturation in BFT males compared to females, yet it is not yet clear whether this phenomenon

typifies wild stocks or is induced due to the culture conditions. The hormone measurements show expression and accumulation of both gonadotropins in the pituitaries of immature and mature BFT. The pituitary LH content increased concomitantly with the age of the fish, exhibiting sex dimorphic patterns (i.e. 3-fold higher levels in females) in adult but not in juvenile BFT. The pituitary FSH levels, however, were elevated in 2Y immature males and in fully mature adults. Comparable to mammals [5], the intra-pituitary FSH/LH ratio was found to be higher (>1) in sexually immature than in maturing or pubertal BFT. Nevertheless, in the 3Y BFT females, which were all immature, the onset of puberty appears to require some other prerequisites, such as a rise in the LH storage above a minimal threshold. Our *in vitro* trials further demonstrated the capacity of rFSH and to a lesser extent that of rLH to stimulate cell proliferation in the immature ovarian and testicular fragments. Both rFSH and rLH have failed to stimulate steroidogenesis, yet pre-treatment with KiSS containing EVAc implants (trial 2) appeared to potentiate FSH-stimulated steroidogenesis in the immature testes.

Conclusions:

Our results highlight the importance of the intra-pituitary FSH/LH ratio during the period of peripubertal/pubertal transition. Similarly to what was established for mammals, the fact that such ratio in sexually immature BFT is higher than in pubertal stages indicates that it may be used as an important endocrine clinical marker in fish as well. Our *in vivo* and *in vitro* studies further attest the growing notion that KiSS and FSH are critical regulators of the initiation of pubertal development in general and of testis maturation in particular.

Future studies testing the effects of captivity and hormone-based treatments on precocious maturity at relatively small body size are expected to facilitate the handling in confined environments, and to greatly improve the cost-efficiency of BFT farming.

References:

[1]FROMENTIN, J.M., POWERS, J.E. 2005. Atlantic bluefin tuna: population dynamics, ecology, fisheries and management. Fish Fisher., 6: 281-306.

[2]OTTOLENGHI, F. 2008. Capture-based aquaculture of bluefin tuna. In: Capture-Based Aquaculture, 508 (Eds. Lovatelli, A., Holthus, P.F.), pp. 169-182, Food and Agriculture Organization of the United Nations, Rome.

[3]MYLONAS, C.C., DE LA GANDARA, F., CORRIERO, A., RIOS, A.B. 2010. Atlantic Bluefin Tuna (*Thunnus thynnus*) Farming and Fattening in the Mediterranean Sea. Rev. Fish. Sci., 18: 266-280.

[4]ROSENFELD, H., KLENKE, U., GORDIN, H., ZOHAR, Y. 2003. Preliminary study of the key hormones regulating reproduction in the bluefin tuna (BFT): the brain gonadotropins-releasing hormones (GnRHs) and the pituitary gonadotropins, FSH and LH. Cah. Options Mediterr., 60:177-181.

[5]WAŃKOWSKA, M., POLKOWSKA, J. 2010. The pituitary endocrine mechanisms involved in mammalian maturation: maternal and photoperiodic influences. Reprod. Biol., 10: 3-18.

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

CHARACTERIZATION OF THE KISSPEPTIN SYSTEMS IN THE BRAIN OF THE EUROPEAN SEA BASS (*Dicentrarchus labrax*): RELATIONSHIPS WITH OESTROGEN RECEPTORS

Escobar S.[1], Servili A.[2], Felip A.[1], S Zanuy S.[1], Carrillo M.[1] and Kah O[2].

[1]Department of Fish Physiology and Biotechnology, Institute of Aquaculture of Torre de la Sal (IATS), Spanish National Research Council (CSIC), Ribera de Cabanes s/n 12595, Castellón, Spain. E-mail: carrillo@iats.csic.es
[2]Neurogenesis and Œstrogens, UMR CNRS 6026, IFR 140, University of Rennes 1, Campus de Beaulieu, 35042 Rennes cedex, France.

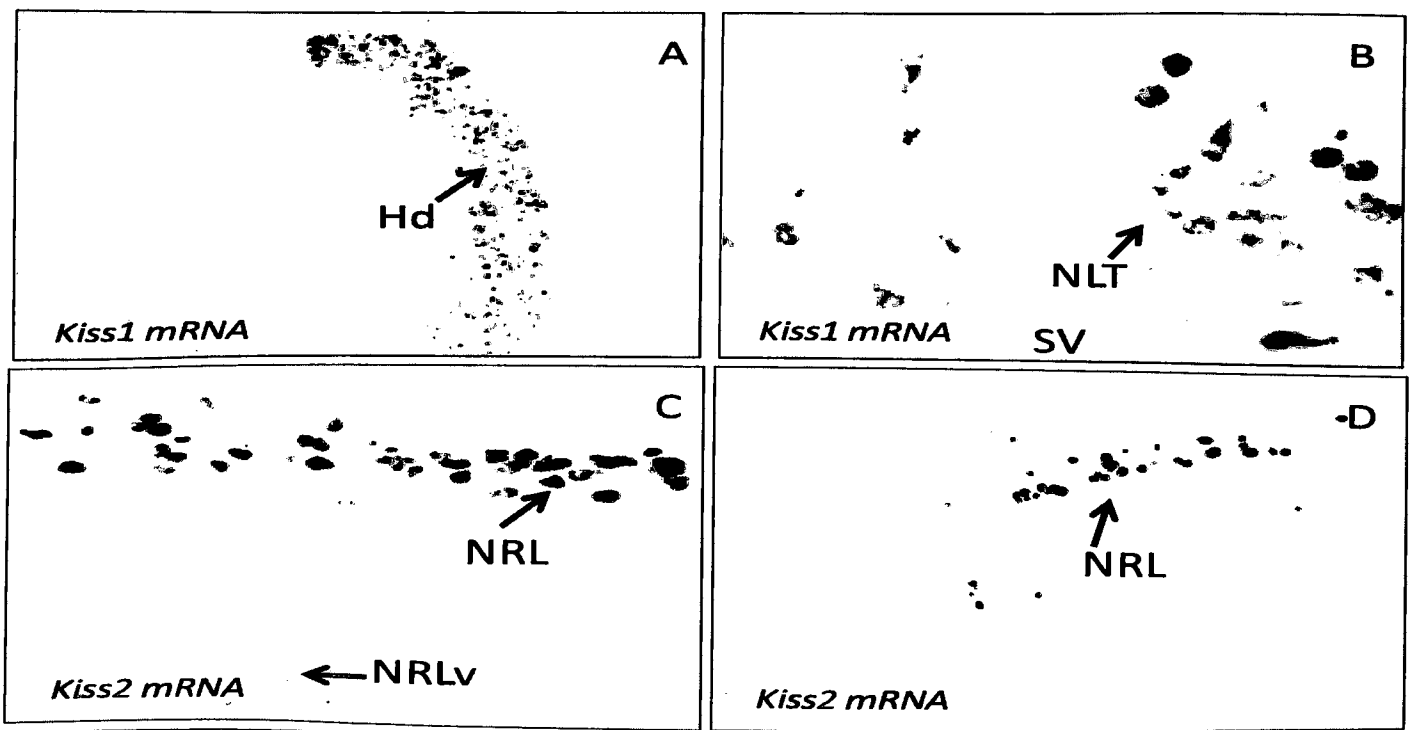
Introduction:

Due to the increasing importance of sea bass (*Dicentrarchus labrax*) in the European aquaculture, puberty control is the focus of intense research. While the GnRH systems have been well described in this species, little information regarding the kisspeptin systems exists. Kisspeptins belong to a family of peptides first identified for their capacity to inhibit tumor metastasis through the receptor GPR54 in mammals. It has been largely demonstrated that the kisspeptin system plays an essential role in the neuroendocrine control of puberty and reproduction by stimulating the GnRH neurons and subsequently releasing gonadotropin hormones. Therefore, the kisspeptin system could be the gatekeeper in the onset of reproduction on fish. In sea bass two ligands and two receptors coded by different genes have been identified and molecularly characterized [1]. Additionally, several sex steroids including œstradiol-17β have been well studied in sea bass and clear evidence exists regarding these hormones play during the period of reproduction. In sea bass, three oestrogen receptors (ERs) have been characterized, named as esr1 (ERα), esr2a (ERβ2) and esr2b (ERβ1) [2, 3]. Here, we report the neuroanatomical distribution of the cells expressing *kiss1* and *kiss2* and their cognate receptors in the brain of adult sea bass and their relationship with the three oestrogens receptors by *in situ* hybridization.

Methods:

Four brains of adult sea bass maintained under a natural of photoperiod were perfused, embedded in paraffin and transversally cut. Specific probes were synthesized using Dig-RNA labeling Mix according to [4]. The relationships between kisspeptin receptors and

Fig 1. Photomicrograph of *kiss1* and *kiss2* expressing cells in the brain of sea bass by *in situ* hybridization. (A) High number of *kiss1* mRNA expressing cells in the habenular nucleus (Hd). (B) Cells located at the caudal lateral tuberal nucleus (NLT) above the saccus vasculosus (VS). In (C-D) a high number of *kiss2* mRNA expressing cells located above the nucleus of the lateral recess (NRL) and a lower expression in cells of the ventral part of nucleus of the lateral recess (NRLv) in the hypothalamus.



the GnRH systems were studied using specific antibodies raised against the GAP portion of the preproGnRH1 [5].

Results and Discussion:

Our findings indicate that *kiss1*-expressing cells are located at the level of the habenular nucleus (Hd) (Fig. 1A) and the mediobasal hypothalamus where a strong expression of *kiss1* messengers is observed just above the pituitary stalk (Fig.1B), which is in line with the *kiss1* messenger distribution in the medaka fish [6]. Additionally, *kiss1* messengers were also detected in the proximal pars distalis of the pituitary gland. In contrast, *kiss2* expressing cells were observed around the nucleus of the lateral recess (NRL) (Fig.1 C-D) and in the anterior preoptic area. Interestingly, similar to what shown in zebrafish [7] the *kiss1r* was also expressed in the habenular nucleus and in the anterior ventral preoptic region. On the other hand, the *kiss2r*-mRNA had a much wider expression in the ventral, lateral and dorsal parts of the telencephalon, the entopeduncular nucleus, the preoptic region and the hypothalamus. Furthermore, a double staining showed that GnRH1 neurons express the *kiss2r*- mRNA in the anterior ventral preoptic area.

Conclusion:

In summary, this work represents the first neuroanatomical analysis of the kisspeptin systems and their relationships with the three oestrogens receptors in the sea bass brain and suggests a critical role of this system in the control of reproduction.

Supported by the EU Project LIFECYCLE (FP7-222719-1) to O. Kah and S. Zanuy. S. Escobar and A. Servili were sponsored by JAE-Predoc CSIC (Spain) and the LIFECYCLE project, respectively.

References:

[1]FELIP, A., ZANUY, S., PINEDA, E., PINILLA, L., CARRILLO, M., TENA-SAMPERE, M., GÓMEZ, A. 2009. Evidence for two distinct KiSS genes in non-placental vertebrates that encode kisspeptins with different gonadotropin-releasing activities in fish and mammals. Mol. Cell. Endocrinol., 312:61-71

[2]MURIACH, B., CARRILLO, M., ZANUY, S., CERDÁ-REVERTER, J.M. 2008. Distribution of estrogen receptor 2 mRNAs (Esr2a and Esr2b) in the brain and pituitary of the sea bass (*Dicentrarchus labrax*). Brain Res., 1210:126-141.

[3]MURIACH, B., CERDÁ-REVERTER, J.M., GÓMEZ, A., ZANUY, S., CARRILLO, M. 2008. Molecular characterization and central distribution of the estradiol receptor alpha (ERalpha) in the sea bass (*Dicentrarchus labrax*). J. Comp. Neurol., 35(1):33-48.

[4]ANGLADE, I., MAZURAIS, D., DOUARD, V., LE JOSSIC-CORCOS, C., MAÑANOS, E.L., MICHEL, D., KAH O. 1999. Distribution of glutamic acid decarboxylase mRNA in the forebrain of the rainbow trout as studied by in situ hybridization. J. Comp. Neurol., 410: 277-89.

[5]GONZÁLEZ-MARTÍNEZ, D., ZMORA, N., MAÑANOS, E., SALIGAUT, C., ZANUY, S., ZOHAR, Y., ELIZUR, A., KAH, O., MUÑOZ-CUETO, J.A. 2002. Immunohistochemical localization of three different prepro-GnRHs in the brain and pituitary of the European sea bass (*Dicentrarchus labrax*) using antibodies to the corresponding GnRH-associated peptides. J. Comp. Neurol., 446(2):95-113.

[6]KANDA, S., AKAZOME, Y., MATSUNAGA, T., YAMAMOTO, N., YAMADA, S., TSUKAMURA, H., MAEDA, K., OKA, Y. 2008. Identification of KiSS-1 Product Kisspeptin and Steroid-Sensitive Sexually Dimorphic Kisspeptin Neurons in Medaka (*Oryzias latipes*). Endocrinology, 149(5):2467-2476.

[7]SERVILI, A., LE PAGE, Y., LEPRINCE, J., CARATY, A., ESCOBAR, S., PARHAR, I.S., YONG SEONG, J., VAUDRY, H, KAH, O. 2011. Organization of two independent kisspeptin systems derived from evolutionary-ancient kiss genes in the brain of zebrafish. Endocrinology, 152(4):1527-1540

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

EFFECTS OF ETIOCHOLANOLONE GLUCURONIDE, A PUTATIVE PHEROMONE ON BRAIN AND PLASMA VASOTOCIN LEVELS IN THE CATFISH *HETEROPNEUSTES FOSSILIS*

Chaube R.*, Singh R.K. and Joy K.P.°

*Zoology Section, MMV, Banaras Hindu University, Varanasi, U.P. - 221005, India
°Department of Zoology, Banaras Hindu University, Varanasi, U.P. - 221005, India
chauberadha@rediffmail.com, kpjoy@bhu.ac.in

Introduction:

Teleost gonads have been demonstrated to be an active source of sex (reproductive) pheromones having releaser or primer functions. Steroid hormones, prostaglandins and their metabolites have received considerable attention in this regard. Etiocholanolone glucuronide (EG) is a natural metabolite of etiocholanolone generated in the liver by UDP gluconyl transferase. Etiocholanolone (or 5-isoandrosterone) is a metabolite of testosterone, classified as a ketosteroid; it causes fever, immunostimulation and leukocytosis. Glucuronidation is a means of excretion of toxic substances, drugs or other substances that cannot be used as an energy source. In teleosts, EG is secreted by males (testis and seminal vesicle) and exerts pheromonal roles via olfactory stimulation of females [1]. This steroid has got pheromonal properties and is responsible for regulation of spawning behavior and reproduction. Vasotocin (VT) is the basic neurohypophysial nonapeptide in nonmammalian vertebrates and is involved in diverse functions like osmoregulation, reproduction and behavior [2]. The administration of VT has been shown to influence or modify reproductive/seasonal behavioral activities (spawning, courtship, egg laying, clasping and song production) in a variety of vertebrates like fish, amphibians, reptiles and birds [3, 4, 5]. The reproductive role of VT was investigated in the catfish and ovarian steroids were found to modulate VT secretion in vitro [6]. We, therefore, hypothesize that pheromone exposure may induce brain VT release and influence catfish reproductive behavior. To test this hypothesis we perfused female catfish with different concentrations of EG and measured brain and plasma vasotocin levels.

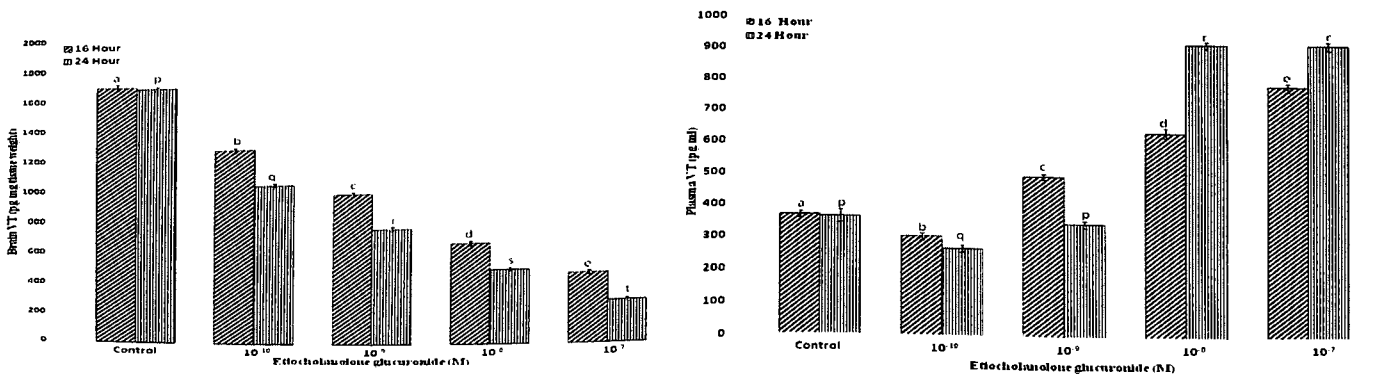
Methods:

Female catfish *Heteropneustes fossilis* were collected from local fish markets during spawning phase (June - July) and acclimatized in laboratory conditions for 48hr. After acclimatization, intact or olfactory organ-ablated fish were perfused with different concentrations of EG (10^{-7}, 10^{-8}, 10^{-9} and 10^{-10} M) for 16 or 24 hr in a static system at a flow rate of 4ml/hr, individually (n = 5 fish). EG was dissolved in 100µl of methanol and then final concentrations were made in fish saline. After blood plasma collection, the fish were sacrificed by decapitation and brains along with pituitary were removed and dropped into cold anhydrous acetone and stored at -70°C. Brains and plasma were processed for VT extraction and quantification by EIA kit (Bachem Peninsula Laboratories, California, USA; Catalogue No. S - 1239, EIAH - 8121). Data were presented as mean ± SEM and analyzed by one way ANOVA, followed by Tukeys' test.

Results and Discussion:

The perfusion of the olfactory organ-intact fish with different concentrations of EG produced an overall significant effect on brain and plasma VT levels (Fig. 1). EG decreased brain VT levels significantly in a concentration-dependent manner in comparison to the

Fig. 1. Effects of perfusion of different concentrations of etiocholanolone glucuronide on brain (A) and plasma (B) VT levels during spawning phase



control group but increased plasma VT levels at both 16 and 24 hr. In the olfactory organ-ablated fish, there was no significant effect compared to the control group. The results suggest that EG perfusion increases the release of VT from the pituitary to the circulation causing a decline in concentration in the brain and an increase in the plasma. Thus, pheromonal stimulation can regulate VT secretion and influence reproductive behavior in the catfish. The pheromonal chemosensory system can transmit social stimuli between conspecific individuals that can induce reproductive behavior in the recipients, in this case females. VT has been shown to stimulate the synthesis of the MIS and induce ovulation in the catfish at ovarian level. It has been reported that sensory organs are the filters through which organisms determine the context of their environment and respond accordingly. Pheromones have diverse roles in modulating vertebrate behavior [7]. Social stimuli often evoke stereotypical behavioural responses in conspecifics, potentially by activating endogenous neuropeptide systems, notably those related to vasotocin (VT) and its mammalian derivative, vasopressin (VP). These peptides influence a variety of species-specific social behaviors across vertebrates [8] and social stimuli can activate VT/VP cells and drive release of the peptides in the brains of at least some species.

Conclusion:

In conclusion, the present study demonstrates that EG, as a putative pheromone can regulate VT function, thereby, influencing reproductive behavior and spawning activity in the catfish. This is the first report correlating functional interplay between pheromonal and VT systems.

References:

[1]RESINK, J.W., VOORTHUIS, P.K., VAN DEN HURK, R., PETERS, R.C., VAN OORDT, P.G.W.J. 1989. Steroid glucuronides of the seminal vesicle as olfactory stimuli in African catfish, *Clarius gariepinus*. Aquaculture, 83, 153-166.

[2]BALMENT, R.J., LU, W., WEYBOURNE, E., WARNE, J.M. 2006. Arginine vasotocin a key hormone in fish physiology and behaviour: a review with insights from mammalian models. Gen. Comp. Endocrinol. 147, 9-16.

[3]PICKFORD, G.E., STRECKER, E.L. 1977. The spawning reflex response of the killifish, *Fundulus heteroclitus*: isotocin is relatively inactive in comparison with arginine vasotocin. Gen. Comp. Endocrinol. 32, 132-137.

[4]GUILLETTE, L.J.J., JONES, R.E. 1982. Further observation of arginine vasotocin-induced oviposition and parturition in lizards. J. Herp. 16, 140-144.

[5]MOORE, F.L., WOOD, R., BOYD, S.K. 1992. Sex steroids and vasotocin interact in a female amphibian (*Taricha granulosa*) to elicit female-like egg-lying behavior or male-like courtship. Horm. Behav. 26, 156-166.

[6]SINGH, V., JOY, K.P. 2009. Effects of hCG and ovarian steroid hormones on vasotocin levels in the female catfish *Heteropneustes fossilis*. Gen. Comp. Endocrinol. 162, 172-178.

[7]BRENNAN, P.A., ZUFALL, F. 2006. Pheromonal communication in vertebrates. Nature, 444, 308-315.

[8]GOODSON, J.L., BASS, A.H. 2001. Social behavior functions and related anatomical characteristics of vasotocin/vasopressin systems in vertebrates. Brain. Res. Rev. 35, 246-265.

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

ONTOGENIC EXPRESSION PROFILES OF GONADOTROPINS (*fshb*, *lhb*) AND GROWTH HORMONE (*gh*) DURING SEXUAL DIFFERENTIATION AND PUBERTY ONSET IN FEMALE ZEBRAFISH

Chen W. T. and Ge W.

School of Life Sciences, The Chinese University of Hong Kong, Shatin, HKSAR, China
Fax: (852) 2603-5646 email: weige@cuhk.edu.hk

Introduction:

The reproductive process in teleosts is mainly regulated by the hypothalamus-pituitary-gonad (HPG) axis. As a primary factor in the axis, gonadotropin-releasing hormone (GnRH) from the hypothalamus acts at the pituitary to control the expression and release of gonadotropins (GTHs), follicle-stimulating hormone (FSH) and luteinizing hormone (LH), which in turn regulate gonadal activities [1]. Growth hormone (GH), another pituitary hormone, has also been demonstrated to play an important role in reproduction, so has been termed co-gonadotropin [2-4]. The timing of the appearance of FSH and LH-expressing cells during reproductive development appeared to be species-specific. In most species studied such as the salmonids [5, 6], gilthead seabream [7] and cichlids [8], the FSH cells appeared earlier than LH cells. In the gilthead seabream, both FSH and LH cells appeared prior to sexual differentiation whereas in the cichlids, the LH cells were detected at the onset of sex differentiation [7]. Both FSH and LH cells also appeared before sex differentiation in the pejerry, while LH cells were detected earlier than FSH cells [9]. Puberty is the period in vertebrate life cycle that marks the transition from sexual immaturity to maturity to enable the animal to acquire adult reproductive function. As an important point in vertebrate reproductive life, puberty has been under extensive study in the past decades. Although it is well accepted that the puberty onset requires an increased pulsatile release of GnRH which in turn activates GTHs, the two types of gonadotropins seem to have different roles in controlling puberty in different species. In mammals, the onset of puberty is associated with an increase in the amplitude and frequency of LH [10]. However, evidence in teleosts points to the importance of FSH signaling in puberty onset.

Methods:

The zebrafish were collected at different time points of early development including 4, 6, 8, 10, 13, 16, 19, 22, 25, 28, 38, 48 and 53 days post fertilization (dpf). The head of each fish including the brain and pituitary was fixed in 4% paraformaldehyde at 4°C overnight for the Fluorescent In Situ Hybridization (FISH), whereas the bodies were fixed in Bouin's solution at room temperature overnight for histological examination of gonadal developmental stage.

Results and discussion:

Using double-colored in situ hybridization, we demonstrated that the expression of *fshb* was earlier than *lhb* with its mRNA signal detectable (~2-3 cells/pituitary) shortly after hatching (4 dpf). *Lhb* expression became detectable, albeit at very low level, at the time of sex differentiation (~25 dpf). In female zebrafish, the first morphological sign for puberty is the first wave of follicle transition from the primary growth (PG) to previtellogenic stage (PV), which is marked by the appearance of cortical alveoli in the oocytes. Our data showed that the expression of *lhb* was very low (~5-6 cells/pituitary) before this transition but increased dramatically during and after the transition. In contrast, the expression of *fshb* was abundant before puberty with only a slight increase during puberty onset. Interestingly, the location of *fshb*-expressing cells changed significantly during puberty from predominantly peripheral to central location. As a control, the expression of *gh* was abundant throughout prepubertal and pubertal periods. Our result strongly suggests an important role for LH at the puberty onset of female zebrafish, similar to the situation in mammals, and its expression could be a sign for puberty at the pituitary level. However, the significance of location change of FSH cells during this period will be interesting to investigate. The increased expression of *fshb* and *lhb* at puberty was also supported by real-time qPCR analysis.

References:

[1]Swanson P., Dickey J.T., Campbell B. 2003. Biochemistry and physiology of fish gonadotropins. Fish Physiol. Biochem., 28:53-59

[2]Hull K.L., Harvey S. 2001. Growth hormone: roles in female reproduction. J Endocrinol 168:1-23

[3]Hull K.L., Harvey S. 2002. GH as a co-gonadotropin: the relevance of correlative changes in GH secretion and reproductive state. J. Endocrinol., 172:1-19

[4]Childs G.V. 2000. Growth hormone cells as co-gonadotropes: partners in the regulation of the reproductive system. Trends Endocrinol. Metab. 11:168-175

[5]Nozaki M., Naito N., Swanson P., Dickhoff W.W., Nakai Y., Suzuki K., Kawauchi H 1990. Salmonid pituitary gonadotrophs. II. Ontogeny of GTH I and GTH II cells in the rainbow trout (*Salmo gairdneri irideus*). Gen. Comp. Endocrinol. 77:358-367

[6]Saga T., Oota Y., Nozaki M., Swanson P. 1993. Salmonid pituitary gonadotrophs. III. Chronological appearance of GTH I and other adenohypophysial hormones in the pituitary of the developing rainbow trout (*Oncorhynchus mykiss irideus*). Gen. Comp. Endocrinol., 92:233-241

[7]Garcia Ayala A., Villaplana M., Garcia Hernandez M.P., Chaves Pozo E., Agulleiro B. 2003. FSH-, LH-, and TSH-expressing cells during development of *Sparus aurata L.* (Teleostei). An immunocytochemical study. Gen. Comp. Endocrinol., 134:72-79

[8] Pandolfi M., Lo Nostro F.L., Shimizu A., Pozzi A.G., Meijide F.J., Vazquez G.R., Maggese M.C. 2006. Identification of immunoreactive FSH and LH cells in the cichlid fish *Cichlasoma dimerus* during the ontogeny and sexual differentiation. Anat. Embryol. (Berl), 211:355-365

[9]Miranda L.A., Strussmann C.A., Somoza G.M. 2001. Immunocytochemical identification of GtH1 and GtH2 cells during the temperature-sensitive period for sex determination in pejerrey, *Odontesthes bonariensis*. Gen. Comp. Endocrinol., 124:45-52

[10]Watanabe G., Terasawa E. 1989. In vivo release of luteinizing hormone releasing hormone increases with puberty in the female rhesus monkey. Endocrinology, 125:92-99

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

SEX-DEPENDENT EXPRESSION OF ESTROGEN AND ANDROGEN RECEPTOR GENES IN THE MEDAKA BRAIN

Hiraki T., Okubo K.

Department of Aquatic Bioscience, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo, Tokyo 113-8657, Japan
Fax: +81-3-5841-5289, e-mail: hiraki@marine.fs.a.u-tokyo.ac.jp.

Introduction:

In vertebrates, estrogen and androgen play central roles in sexual differentiation of the brain [1, 2, 3, 7]. These steroid hormones control a variety of physiological and behavioral traits, including neuroendocrine hormone secretion, reproductive behavior, and aggression, in a sex-dependent manner. Most of these effects depend on activation of nuclear estrogen and androgen receptors (ER and AR) in the brain. Since there are sex differences in the effects of estrogen and androgen on some of these traits, ER and AR are supposedly expressed in a sex-dependent manner in the brain. In fact, sex differences in their expression have been identified in several areas of mammalian and avian brains that could underlie the differences in traits [4, 5, 6]. In contrast, virtually nothing has been known about sex differences in the expression of ER and AR in the brain of teleost fishes. In the present study, therefore, we investigated whether the expression of ER and AR is sexually differentiated in the brain of medaka *Oryzias latipes*.

Methods:

Sex differences in the expression of three ER and two AR genes in the medaka brain was examined in relation to several factors, including genetic sex, phenotypic sex, and steroid hormone stimulation. We also identified the brain nuclei that were the sources of sex differences in the ER and AR expression.

Results:

Each of all five receptor genes in medaka was differentially expressed between the sexes in specific brain nuclei of the telencephalon, preoptic area, and hypothalamus. The most conspicuous sex differences could be seen in several nuclei of the ventral telencephalon and dorsal preoptic area, where ER and AR expression was prominent in females but almost completely absent in males. It is thus highly likely that these nuclei represent female-specific estrogen and androgen target sites in the medaka brain. Subsequent analysis revealed that the sex-dependent expression of the medaka ER and AR was not under the direct control of sex chromosomal genes. Instead, their expression depended mostly on the stimulation by their ligands, estrogen and androgen.

Conclusion:

Here we report sexually dimorphic expression patterns of ER and AR in the medaka brain. Sex differences in the expression of these genes are supposedly responsible for some sex differences in physiological and behavioral traits.

References:

[1]ARNOLD A.P. 2009. The organizational-activational hypothesis as the foundation for a unified theory of sexual differentiation of all mammalian tissues. Horm. Behav., 55: 570-578.

[2]KUDWA A.E., MICHOPOULOS V., GATEWOOD J.D., RISSMAN E.F. 2006. Roles of estrogen receptors alpha and beta in differentiation of mouse sexual behavior. Neuroscience, 138: 921-928.

[3]MCCARTHY M.M. 2010. How it's made: organisational effects of hormones on the developing brain. J. Neuroendocrinol., 22: 736-742.

[4]SCOTT C.J., TILBROOK A.J., SIMMONS D.M., RAWSON J.A., CHU S., FULLER P.J., ING N.H., CLARKE IJ. 2000. The distribution of cells containing estrogen receptor-alpha (ERalpha) and ERbeta messenger ribonucleic acid in the preoptic area and hypothalamus of the sheep: comparison of males and females. Endocrinology, 141(8): 2951-62.

[5]SHAH N.M., PISAPIA D.J., MANIATIS S., MENDELSOHN M.M., NEMES A., AXEL R. 2004. Visualizing sexual dimorphism in the brain. Neuron, 43: 313-319.

[6]VOIGT C., BALL G.F., BALTHAZART J. 2009. Sex differences in the expression of sex steroid receptor mRNA in the quail brain. J. Neuroendocrinol., 21: 1045-1062.

[7]ZULOAGA D.G., PUTS D.A., JORDAN C.L., BREEDLOVE S.M. 2008. The role of androgen receptors in the masculinization of brain and behavior: what we've learned from the testicular feminization mutation. Horm. Behav., 53: 613-626.

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

THE BRAIN-PITUITARY AXIS STRUCTURE IN CAPTIVITY REARED FEMALES OF *STEINDACHNERIDION PARAHYBAE* (SILURIFORMES)

Honji R. M.*, Caneppele D.†, Pandolfi M.‡, Lo Nostro, F.‡ and Moreira R. G.*

* Institute of Biosciences, University of São Paulo (IB-USP). Rua do Matão, trav. 14, 321, São Paulo, 05508-090, Brazil. Fax: +55-11-30918095, email: honjijp@usp.br

† Energetic Company of São Paulo State (CESP), Paraibuna, SP, Brazil

‡ University of Buenos Aires (UBA), Buenos Aires, Argentina

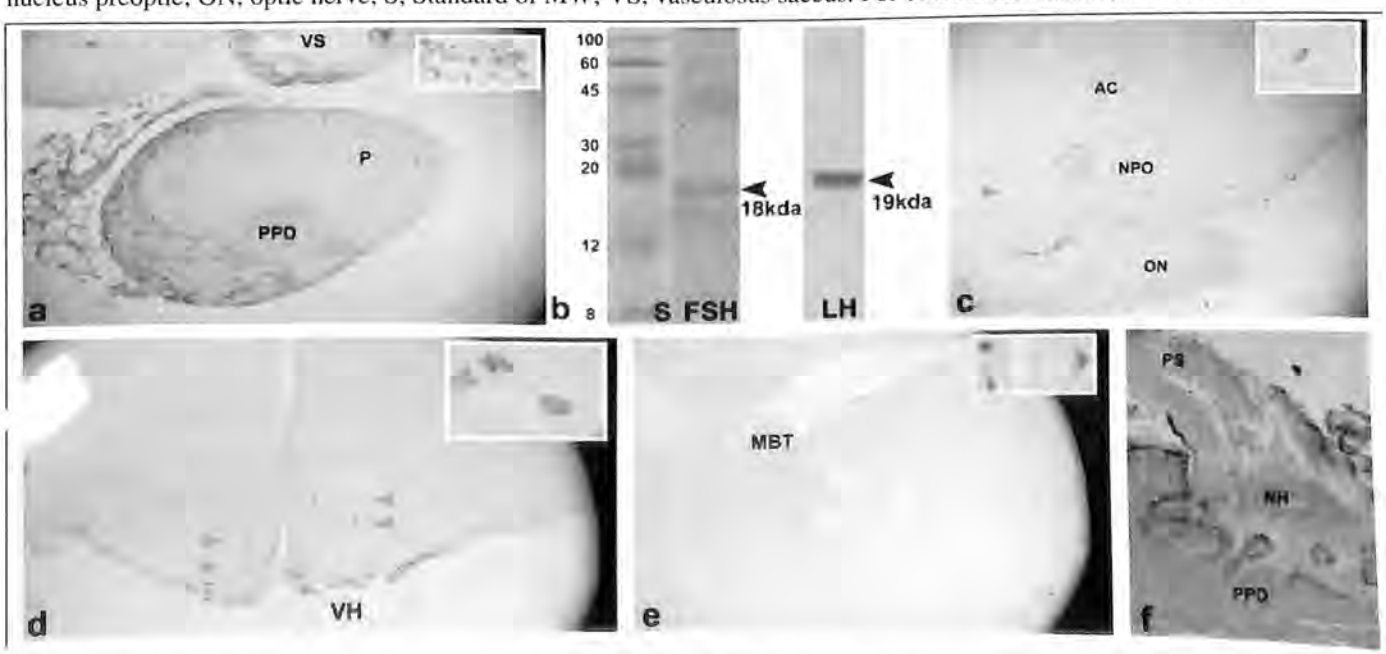
Introduction:

Steindachneridion parahybae is a freshwater catfish, endemic to the Paraíba do Sul River Basin, Brazil, which has been seriously threatened by environmental disturbances in the last decades [1]. This species is in the conservation program undertaken by the Energetic Company of São Paulo State (CESP) that depends on the collection of wild broodstock and their transference to captivity for artificial reproduction. However, the domestication of this broodstock is not completely successful, because they exhibit reproductive impairments in captivity [2]. The endangered status and endocrine dysfunction showed by this species in captivity deserves special attention and urgent action addressing the knowledge of its reproductive physiology, which is the basic premise for fish restocking programs. Captivity negatively impacts on the brain-pituitary axis (BP), mainly on the gonadotropin-releasing hormone (GnRH) production, which is the main regulator of gonadotropins (GtHs): follicle-stimulating hormone (FSH) and luteinizinghormone (LH) release from pituitary. Although fish pituitary has been studied for many years [3], there is no information about the anatomy and pituitary cell types in Neotropical catfishes yet, mainly in species, which show reproductive dysfunctions in captivity. Thus, the BP axis structure and cytoarchitecture is the key to understand the physiological causes of reproductive dysfunctions; therefore, our studies focused on the GnRH neurons, FSH and LH cells in *S. parahybae* sexually mature females in captivity.

Methods:

S. parahybae adult females were sampled during the annual reproductive cycle at CESP fish farm: brain and pituitary were dissected, fixed in Bouin solution (immunohistochemistry (IH) or histology), or frozen (Western blott). Serial sections (12µm) were obtained and stained with hematoxylin acid, Mallory's trichrome and periodic acid-Schiff. For IH, sections were immunostained using several primary antisera: catfish

Figure: 1. a) LH-ir cells in PPD, and detail (insert); b) MW for FSH (18kda) and LH (19kda); c) cfGAP-ir in the VT (arrowhead), and neurons details (insert); d) cfGAP-ir in the VH (arrowhead), and neurons details (insert); e) cIIGAP-ir in the MBT (arrowhead), and neurons details (insert); f) cfGAP-ir in the PS, mainly in PPD. AC, anterior commissure; NPO, nucleus preoptic; ON, optic nerve; S, Standard of MW; VS, vasculosus saccus. For other abbreviations, see the text.



GnRH and GAP (cfGnRH, cfGAP), chicken-II GnRH and GAP (cIIGnRH, cIIGAP), and GtHs antisera: mummichog FSH and LH (fhFSH, fhLH), and visualized with DAB (diaminobenzidine). Frozen samples were used for molecular weight (MW) data and to confirm the specificity of the immunostaining.

Results and Discussion:

The morphology of the adenohypophysis (AH) of *S. parahybae* does not differ from the basic scheme found in other teleosts [3]. Briefly, the AH is divided in three anterior-posterior areas: rostral pars distalis (RPD), proximal pars distalis (PPD) and pars intermedia (PI). The neurohypophysis (NH) interdigitates into all regions of the AH. Immunostaining method allowed to observe only LH immunoreactive (-IR) cells (Fig. 1a). This lack of immunoreaction (ir) for FSH could be attributed to the protein conformation of catfish FSH, which does not permit that this fhFSH antisera recognized the active site. This result can be linked to the fact that the molecular structure is more conserved, both in structure and primary sequence, in fish LH than in FSH [3]. The estimated MW was 18kda (FSH) and 19kda (LH) (Fig. 1b), results that are similar to the catfish *Ictalurus punctatus* (20kda, FSH and 21kda, LH) [3]. Regarding the GnRH, due to the colocalization of cfGnRH-cfGAP and cIIGnRH-cIIGAP we used GAP antisera instead of GnRH antisera. Neurons immunostained with cfGAP were found in the forebrain, extending to the antero-posterior direction, from ventral telencephalon (VT) to the caudal, mainly in ventral hypothalamus (VH). cfGAP was strongly immunoreactive in VH fibers, pituitary stalk (PS) and pituitary gland (Pt), mainly in PPD (Figs. 1c, d, e, f). cIIGAP-immunoreactive cell bodies appeared restricted to the anterior dorsal midbrain tegument area (MBT), close to the ventricular surface. Small cfGAP neurons were identified in the anterior region (*i.e.*, VT) compared with cfGAP neurons identified in the posterior region (*i.e.*, VH). This topography of GnRH neurons is similar to the catfish *Clarias gariepinus*, that showed two cfGnRH populations in ventral region (VT and VH), and cIIGnRH population in the MBT [4], and *S. parahybae* seems to have the same distribution pattern.

Conclusion:

This data provide an important comparative information for further investigations on the reproduction in this Neotropical catfish. Furthermore, these results can be useful tools to fulfill the proposal scheme to understand the physiological basis of reproductive dysfunction exhibited by many migratory fish, when in captivity. Our data, together with the findings previously described by Caneppele *et al.* [1], who artificially induced *S. parahybae* to spawning, contribute to the recovery of threatened species, using biological information to predict and solve future problems identified by conservationists.

References:

[1]CANEPPELE, D., HONJI, R.M., HILSDORF, A.W.S., MOREIRA, R.G. 2009. Induced spawning of the endangered Neotropical species *Steindachneridion parahybae* (Siluriformes: Pimelodidae). Neotrop. Ichthyol., 7: 759-762.

[2]ZOHAR, Y., MUÑOZ-CUETO, J.A., ELIZUR, A., KAH, O. 2010. Neuroendocrinology of reproduction in teleost fish. Gen. Comp. Endocrinol., 165: 438-455.

[3]LEVAVI-SIVAN, B., BOGERD, J., MAÑANÓS, E.L., GÓMEZ, A., LAREYRE, J.J. 2010. Perspectives on fish gonadotropins and their receptors. Gen. Comp. Endocrinol., 165: 412-437.

[4]DUBOIS, E.A., ZANDBERGEN, M.A., PEUTE, J., BOGERD, J., GOOS, H.J.T. 2001. Development of three distinct GnRH neuron populations expressing two different GnRH forms in the brain of the African catfish (*Clarias gariepinus*). J. Comp. Neurol. 437: 308-320.

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

EXPRESSION OF DEEP-BRAIN PHOTORECEPTOR, VERTEBRATE ANCIENT LONG OPSIN, IN THE JAPANESE MEDAKA

Kitahashi T. and **Parhar I.S.**

Brain Research Institute, Jeffrey Cheah School of Medicine, Monash University Sunway campus, PJ 46150, Malaysia

Fax: +60-3-5638-7830 E-mail: takashi.kitahashi@monash.edu

Introduction:

Light plays an important role in the regulation of animal reproduction. Recent advances in genomic information have shown the existence of several non-image-forming photoreceptors in the genome of non-mammalian species [1]. Although it has been known that fish brain itself is a photoreceptive organ, detailed mechanism remains unclear. We hypothesize that medaka brain expresses non-visual photoreceptors to regulate reproduction by light.

Methods:

Primers for RT-PCR were designed based on predicted coding sequence of vertebrate ancient opsin (VALop) in the genomic database. The full-length cDNA of medaka VALop was further obtainedby 5'- and 3'-RACE using the cloned partial cDNA sequence. *In situ* hybridization for VALop mRNA in the brain was performed using digoxigenin-labeled RNA probes. The sections were then counterstained with cresyl violet to confirm the position of labeled cells.

Results and Discussion:

Cloned medaka cDNA was about 2.8 kbp in length and contains an open reading frame of 1164 bp. High homology with VALop cDNAs in other animals confirms that the cloned cDNA encodes medaka VALop. The deduced amino acid (aa) sequence contains 387 aa with putative 7 transmembrane domains, which are common to all G protein-coupled receptors including all types of photoreceptors. *In situ* hybridization showed the expression of VALop mRNA in a limited number of neurons that are bilaterally scattered from the area near to the ventral habenula and extended caudally and ventrally.

Conclusion:

In the current study, it is shown that the medaka brain directly receives light information via VALop.

References:

[1]PEIRSON S.N., HALFORD S., FOSTER R.G. 2009. The evolution of irradiance detection: melanopsin and the non-visual opsins. Philos. Trans. R. Soc. Lond. B Biol. Sci., 364:2849-2865

injection (gdx), while the second group received 17β-estradiol (gdx+E; 2 mg kg^{-1}) and a third received 11-ketoandrostenedione (gdx+K; 2 mg kg^{-1}) via microspheric delivery systems. During mid-vitellogenesis, one group was given a vehicle injection (gdx), while the second group received testosterone (gdx+T; 4 mg kg^{-1}) via microspheric delivery systems. Fish were sacrificed on day 10 post-surgery. The transcript levels of *kiss1*, *kiss2*, *kiss2r* and *gnrh1* were measured in the brain using real-time fluorescence-based quantitative RT-PCR. To determine differences between experimental groups, the data were subjected to Analysis of Variance (ANOVA) followed by a post hoc test (Scheffe's). Statistical significance was set at $p < 0.05$.

Results:

In female striped bass, steroid levels gradually rise throughout the reproductive cycle. During early recrudescence, when primary oocytes are entering the growth phase, steroid levels are very low compared to the mid-vitellogenic stage, when steroid hormone production increases to stimulate vitellogenin production [4]. To investigate the responsiveness of the kisspeptin/GnRH1 neuronal network to steroid feedback regulation at these stages, we tested the effects of gonadectomy and estrogen/androgen replacement on expression levels of *kiss1*, *kiss2*, *kiss2r* and *gnrh1* expression in the brain. In the control group, we observed significantly higher transcription levels of all four genes when comparing the recrudescent stage with samples from mid-vitellogenic females (Fig. 1). During recrudescence, expression of the investigated genes was not significantly different from controls in any of the treatment groups (Fig. 2A), indicating no gonadal feedback to the hypothalamus at this stage. However, at mid-vitellogenesis gonadectomy significantly reduced expression of *kiss1* by ~30% and *gnrh1* by ~35%, while the reduction in *kiss2* and *kiss2r* (both ~25%) mRNA levels did not reach significance (Fig. 2B). T-replacement further reduced transcript levels of *kiss1* (~40%), *kiss2* (~35%), and *kiss2r* (~30%). These data indicate that during mid-vitellogenesis scgonadectomy removed a positive gonadal feedback from the kisspeptin/GnRH1 system. However, testosterone treatment further reduced gene expression, suggesting a negative steroidal feedback effect on the kisspeptin system.

Conclusion:

Our data supports the conclusion that the hypothalamic kisspeptin/GnRH1 system in female striped bass is not under gonadal control during recrudescence, while later in the reproductive cycle, the kisspeptin/GnRH1 system is regulated by the gonadal factors. There appear to be two simultaneous regulatory mechanisms controlling the kisspeptin/GnRH system: a positive feedback and a negative feedback, the later in the form of T/E_2. The positive effect may be exerted by another gonadal factor such as activin. The close correlation between the *gnrh1, kiss1/2,* and *kiss2r* response in this study may suggest that the kisspeptin system plays a mediatory role in gonadal feedback regulation of the HPG axis in female striped bass.

References:

[1]HERBISON, A.E. 2006. Physiology of the gonadotropin-releasing hormone neuronal network. In: Physiology of Reproduction 3rd Edition (ed. Neill, J.D.), pp 1415-1482, Elsevier, New York.

[2]OAKLEY, A.E., CLIFTON, D.K., STEINER, R.A. 2009. Kisspeptin signaling in the brain. Endocrine Rev., 30: 713-743.

[3]AKAZOME, Y., KANDA, S., OKUBO, K., OKA, Y. 2010. Functional and evolutionary insights into vertebrate kisspeptin systems from studies in fish brain. J. Fish Biol., 76: 161-182.

[4]SULLIVAN, G.V., BERLINSKY, D.L., HODSON, R.G. 1997. Reproduction. In: Striped bass and other *Morone* culture (ed. Harrell R.M.). Developments in Aquaculture and Fisheries Science, Vol. 30, pp. 11-73, Elsevier, Amsterdam.

RELEASING-HORMONE AND MELATONIN RECEPTORS IN THE BRAIN OF A VENEZUELAN CATFISH, "SIERRA NEGRA" (*Oxydoras sifontesi*)

Marcano D.[1], Espinoza A.[1], Cardillo E.[1], Poleo G.[2] and Guerrero H.Y[1].

[1]Laboratorio de Neuroendocrinología Comparada, Facultad de Medicina, Universidad Central de Venezuela. Caracas 1041-A. Venezuela. Fax: +58-212-6053668, e-mail: dtmarcano@cantv.net.
[2]Estación de Piscicultura, Decanato de Agronomía, Universidad Centro Occidental Lisandro Alvarado. Barquisimeto. Venezuela.

Introduction:

Oxydoras sifontesi has an annual gonadal cycle closely related to changes in the environmental conditions of Venezuelan floodplain. In fish, melatonin receptors have been detected in all of the different brain areas. Physiological data have suggested an involvement of melatonin in the synchronization of rhythmic processes, including the annual cycle of reproduction and behaviour. *O. sifontesi* presents an annual variation in plasma melatonin concentration inversely related to the annual gonadal maturation cycle [1], which suggest a putative role for melatonin as the endocrine messenger of daily and seasonal photic information to the neuroendocrine system. On the other hand, it is well known that gonadotropin releasing hormone (GnRH) is involved in the hypothalamic control of reproduction in fishes. However, there is currently little evidence supporting a major role for melatonin in the control of reproduction in teleost. In this work we aimed to localize melatonin receptors and gonadotropin releasing hormone (GnRH) producing neurons in the telencephalon and diencephalon of *O. sifontesi*.

Methods:

Adults male and female *O. sifontesi* were sampled from the Portuguesa River (Portuguesa State, Venezuela). The brains were rapidly dissected after decapitation of anaesthetized fish. Tissue was freeze in dry ice and processed by cryostat sectioning for autoradiography or previously fixed by intracardiac perfusion for immunohistochemical techniques. Detection of melatonin receptors were realized by in vitro autoradiography, using 2-[$^{(125)}$I] iodomelatonin (I-mel) as a radioligand. The analysis of immunoreactive neurons to GnRH (GnRHir) was performed using anti-LHRH antibody (Sigma).

Results and Discussion:

Telencephalon: Melatonin receptors and GnRHir were detected mainly in the dorsal and ventral telencephalon of *O. sifontesi*. The existence of both melatonin receptors and GnRH producing hormones in the same telencephalic areas, suggests that in *O. sifontesi* both systems are associate, and that melatonin acts like an inhibitor and/or stimulator GnRH production.

Diencephalon: GnRHir was located in all nuclei of the preoptic area, however, melatonin receptors were only detected in the *nucleus parvocellularis anterioris* and *preopticus magnocellularis* of *O. sifontesi*. The GnRHir present in the preoptic area of *O. sifontesi*, as in other vertebrates, may act like a hipophysiotropic factor [2]. Therefore, Falcon et al [3] have suggested that preoptic area of fish may be a key point for the photoneuroendocrine regulation. In this sense, melatonin, in *O. sifontesi*, may have a role in the integration of the photoperiod information.

Hypothalamus: High density of GnRHir somas was found in the following hypothalamic nuclei: *anterioris hipotalamis* (NAH), *difussus lobi inferioris* (NDLI), *lateralis tuberis* (NLT), *anterioris tuberis* (NAT), *diffusus torus lateralis* (NDLT), *posterioris tuberis* (NPT) and *recessus posterioris* (NRP). In teleosts, the NLT has been described as the main area that communicates directly with the pituitary gland [4]. Melatonin receptors were located in NAT, NDLT, NRL and NLT. The presence of immunoreactive somas and fibres and melatonin receptors in common areas of the hypothalamus, suggests that in *O. sifontesi* both systems may be associated and to act of direct form in the modulation of the pituitary secretions.

Conclusion:

These findings agree with the concept that melatonin has a role in the modulation of the production of GnRH in this species like has been reported in various species of teleosts.

This work was supported by grants from FONACIT-MCTII (N° 2002000292) and CDCH-UCV (N° 09-00-6975).

References:

[1]GUERRERO H.Y., CARDILLO E., POLEO G., MARCANO D. (2008). Daily and annual variations of plasma melatonin in the tropical catfish "Sierra Negra" (*Oxydoras sifontesi*), related to the annual gonadal maturation. Cybium, (suppl) 32: 22-24.

[2]ZOHAR Y, MUÑOZ-CUETO JA, ELIZUR A, KAH O. (2010). Neuroendocrinology of reproduction in teleost fish. Gen. Comp. Endocrinol., 165(3): 438-55.

[3]FALCÓN J., BESSEAU L., SAUZET S., BOEUF G. (2007). Melatonin effects on hypothalamo-pituitary axis in fish. Trends Endocrinol. Metab., 18(2): 81-88.

[4]STRIEDTER G.F. (1990). The diencephalon of the channel catfish, *Ictalurus punctatus*. I. Nuclear organization. Brain Behav. Evol., 36: 329-354.

EFFECT OF THERMAL REGIME ON THE EXPRESSION OF SEX-RELATED GENES IN FEMALE EUROPEAN EEL

Mazzeo I[1]., Peñaranda D.S[1]., Gallego V[1]., Nourizadeh-Lillabadi R[2]., Tveiten H[3]., Weltzien F.A[2]., Asturiano J.F[1]., Pérez L[1].

[1]Grupo de Acuicultura y Biodiversidad. Instituto de Ciencia y Tecnología Animal. Universidad Politécnica de Valencia. Camino de Vera s/n. 46022, Valencia, Spain. mlpereig@dca.upv.es
[2]Norwegian School of Veterinary Science, Oslo, Norway.
[3]Norwegian Institute of Fisheries and Aquaculture, Tromsø, Norway.

Introduction:

Due to decrease in natural population, European eel (*Anguilla anguilla*) reproduction is becoming more and more urgent. Detailed information about natural reproduction is unknown, but first evidences indicate depth-temperature changes during eel migration [1]. Previously, we have observed that a variable thermal regime caused higher gonadotropin (FSHβ, LHβ) expression, and higher E2 plasma levels compared to a constant high temperature regime [2]. The present study was aimed to test the effect of thermal regime on the expression of genes related with reproduction.

Methods:

Female eels were hormonally induced to maturation by CPE injection during 12 weeks. Three thermal groups were assessed: T10, T15 (starting at 10 and 15 °C and increasing along the hormonal treatment to 15 or 18 °C, respectively) and T18 (constant 18 °C). Eight females were sacrificed before the transfer to seawater (control group); then, eels were acclimatized to seawater (1 week) and maintained one week more at 10, 15 or 18 °C (T10, T15, T18 groups) before sacrifice 6 eels/group (week 0). Also, 6 females were sacrificed after 4, 8 and 12 CPE injections. Brain expression of GnRH Receptor 1 (GnRH-R1), and Androgen Receptors (ARα, ARβ) were studied by qPCR, as well as pituitary GnRH-R1, ARα, ARβ, FSHβ, and LHβ expression. Ovarian expression of ARα, ARβ, and Zona Radiata proteins (ZR2 and ZR3) were also studied. ZR expression was also studied in liver. Brain expression was studied in olfactory bulbs (O.B.), telencephalon (Tel), and mesencephalon-diencephalon (M-D). Plasma E2 levels were measured by RIA. Developmental stages were established by gonad histology.

Results and Discussion:

Two way ANOVA (thermal treatment and time) showed that thermal regime affected significantly brain ARβ expression, pituitary ARβ, FSHβ, and LHβ expression, E2 plasma levels, GSI (Fig. 1A) and sex development velocity. Sex development under T15 conditions was similar or even faster than that observed at 18 °C (T18) until 8th week of hormonal treatment (60% of T15 females were in mid-vitellogenesis *vs* 20% in T18). Later on, T18 females developed faster, reaching 83% late vitellogenic stage after 12 CPE injections. That means that a 15 °C temperature does not inhibit the sexual development, at least until mid-vitellogenic stage.

After 4 injections, some mRNA (M-D ARα and ARβ, pituitary ARβ) expression increased with the temperature, while others (pituitary and Tel GnRH-R1, ovary aromatase) decreased with the temperature. After 8 injections, mid-temperatures (T15) originated higher pituitary FSHβ, LHβ, aromatase and ARβ expression (Fig. 1B), as well as higher M-D ARα and ARβ expression. After 12 injections, T18 originated higher pituitary FSHβ, LHβ, ARβ and aromatase expression, and lower E2 plasma levels. These higher expressions in T15 at week 8 and in T18 at week 12 could be related to the maturation stage, more advanced in T15 at week 8 (60% females in mid-vitellogenesis) and in T18 at week 12 (83% in late vitellogenesis). ZR2 liver expression showed a different profile, being the highest in T10 at weeks 8 and 12, coinciding with the temperature increase in the T10 group from 10 to 15°C (Fig. 1C).

On other hand, ovarian ARα and β expression increased with development in all the groups (data not shown), supporting the crucial role of androgens on the whole vitellogenesis in eels [3].

Conclusion:

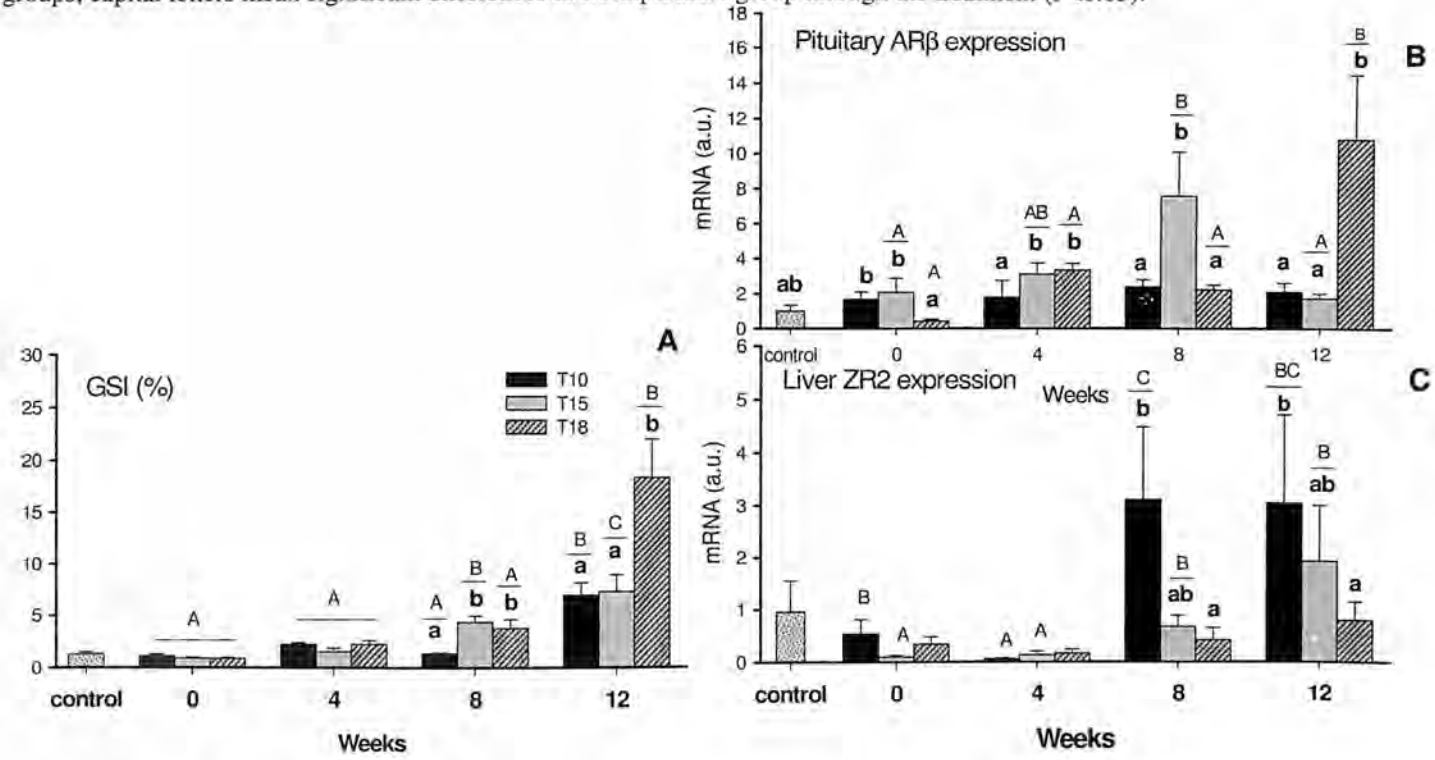
Eels matured at constant 18 °C developed faster after 12 weeks of CPE treatment, but the higher expression of some genes (GnRH-R1, FSHβ, LHβ, liver ZR2) observed in groups under lower and variable thermal regimes (T10, T15) could indicate that a slower egg development was more appropriate for successful maturation. In the case of liver ZR2 the lower expression at high temperatures could be related with the fragility of some batches of eggs obtained under captivity.

Acknowledgements

Funded from the European Community's 7th Framework Programme under the Theme 2 "Food, Agriculture and Fisheries, and Biotechnology", grant agreement n°245257 (Pro-Eel). D.S.P. have a UPV postdoc grant. I.M. and V.G. have predoctoral grants

from Generalitat Valenciana and Spanish MICINN, respectively.

Fig. 1. GSI, Pituitary ARβ expression, liver ZR2 expression. Small letters show significant differences between temperature groups; capital letters mean significant differences in a temperature group through the treatment (P<0.05).



References:

[1]AARESTRUP, K., ØKLAND, F., HANSEN, M.M., RIGHTON, D., GARGAN, P., CASTONGUAY, M., BERNATCHEZ, L., HOWEY, P., SPARHOLT, H., PEDERSEN, M.I., MCKINLEY, R.S. 2009. Oceanic spawning migration of the European Eel (*Anguilla anguilla*). Science, 325 (5948): 1660

[2]PEREZ, L., PEÑARANDA, D.S., DUFOUR, S., BALOCHE, S., PALSTRA, A.P., VAN DEN THILLART, G., ASTURIANO, J.F. Influence of temperature regime on endocrine parameters and vitellogenesis during experimental maturation of European eel (*Anguilla anguilla*) females. Gen. Comp. Endocrinol., In revision.

[3]TOSAKA, R., TODO, T., KAZETO, Y., LOKMAN, P.M., IJIRI, S., ADACHI, S., YAMAUCHI, K. 2010. Expression of androgen receptor mRNA in the ovary of Japanese eel, *Anguilla japonica*, during artificially induced ovarian development. Gen. Comp. Endocrinol., 168: 424–430.

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

EFFECTS OF DAILY HIGH WATER TEMPERATURE FLUCTUATIONS ON BRAIN-PITUITARY-GONAD AXIS OF PEJERREY (*Odontesthes bonariensis*) DURING ITS REPRODUCTIVE SEASON

Miranda, L.A.[*], **Chalde T.**[o] **and Elisio, M.**[o]

Laboratorio de Ictiofisiología y Acuicultura, IIB-INTECH (CONICET-UNSAM).Camino de Circunvalación Laguna Km 6, (B7130IWA) Chascomús, provincia de Buenos Aires, Argentina, Fax: +54-2241-430323 email: lmiranda@intech.gov.ar

Introduction:

The increases noted in the temperature of many water bodies which are considered to be consequences of global warming have already started to affect fish reproduction [1]. Pejerrey is a multiple spawner and its reproductive activity begins when water temperatures reach around 13 to15° C and peaks at temperatures between 18 to 20° C with a major spawning period during spring [2]. Recently, it has been demonstrated that constant temperature regimes higher than 23° C impair pejerrey reproduction [3]. In the last years, in shallow lakes from pampasic region of Argentina (the natural habitat of *O. bonariensis*), were reordered daily temperature fluctuations from 19° C to 27° C during some days in spring. The aim of this study was to examine the effects of short-term pulses of warm water on brain-pituitary-gonad axis of pejerrey during spawning season.

Methods:

Three groups of 10 fish of both sexes with external signs of active reproductive status were kept in tanks and subjected to constant 19° C (reproductive control), daily temperature fluctuation from 19 to 25° C and from 19 to 27° C. After 8 days, all fish were sacrificed and gene expression of brain GnRH variants, pituitary GtH subunits (FSH-β, LH-β, GPH-α) and gonad GtH receptors (FSH-R and LH-R) were analyzed by real-time quantitative PCR. Also, a portion of the gonad of each fish was processed by routine histological methods.

Results:

Fish exposed to pulses of warm water stop to spawn and the histological analysis of their testis and ovaries showed clear signs of regression. In general, in those experimental fish it was observed that GnRHs (Fig. 1), GtH subunits (Fig. 2), and GtH-Rs (Fig. 3) mRNA levels decreased when compared with fish from control group. However, only pjGnRH (GnRH-I) and FSH-β in both sexes, LH-β and GPH-α in males and FSH-R in females showed statistically significant differences.

Fig.1. GnRH-I, GnRH-II, and GnRH-III gene expression in the brain of pejerrey males and females kept at 19°C (control), 19-25, and 19-27°C for 8 days. Values are mean ± SE. Different letters represent significant differences between temperatures ($p<0.05$).

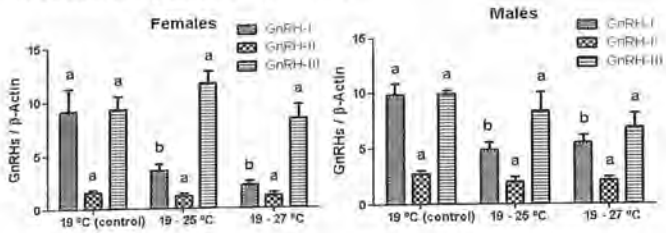
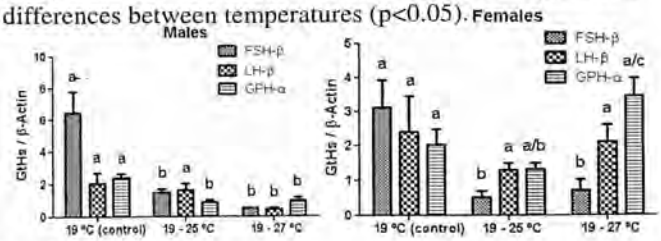
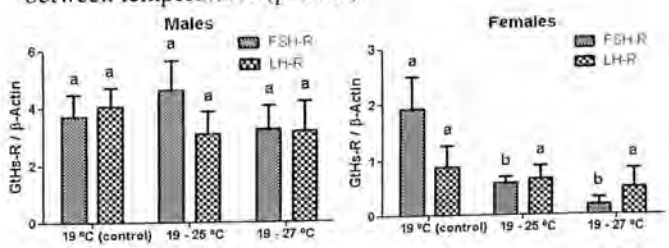


Fig. 2. FSH-β, LH-β, and GPH-α gene expression in the pituitary of pejerrey males and females kept at 19° C (control), 19-25° C, and 19-27° C for 8 days. Values are mean ± SE. Different letters represent significant differences between temperatures ($p<0.05$).



Conclusion:

Fig. 3. FSH-R, and LH-R gene expression in the brain of pejerrey males and females kept at 19° C (control), 19-25° and, 19-27° C for 8 days. Values are mean ± SE. Different letters represent significant differences between temperatures ($p<0.05$).



The results obtained demonstrated that daily high water temperature fluctuations during spawning season suppressed the gene expression of different genes in the brain-pituitary-gonad axis blocking pejerrey reproduction. Global warming may increase the frequency of exposure to harmful temperatures causing shortening or overall disruption of spawning activity affecting pejerrey wild populations.

References:

[1]PANKHURST, N.W., KING, H.R. 2010. Temperature and salmonid reproduction: implications for aquaculture. J .Fish Biol., 76: 69–85.

[2]STRUSSMANN, C.A., CONOVER, D.O., SOMOZA, G.M., MIRANDA, L.A. 2010. Implications of climate change for the reproductive capacity and survival of atherinopsid fish species. J. Fish Biol., 77: 1818-1834.

[3]SORIA, F.N., STRÜSMANN, C.A., MIRANDA, L.A. 2008. High water temperatures impair the reproductive ability of the pejerrey fish *Odontesthes bonariensis*: effects on the hypophyseal-gonadal axis. Physiol. Biochem. Zool., 81: 898-905.

COMPARATIVE ANALYSIS OF YELLOWTAIL KINGFISH AND ZEBRAFISH *KISS* AND *KISS RECEPTOR* GENE PROMOTERS

Nocillado, J.N., Mechaly, A.S. and Elizur, A.

Faculty of Science, Health and Education, University of the Sunshine Coast
Sippy Downs, Queensland, Australia, 4556
Fax: +61-7-54302889 email: JNocilla@usc.edu.au

Introduction:

The kisspeptin system is now accepted as an essential component of the reproductive system in most vertebrates [1]. Recently, additional physiological functions have been attributed to the system including metabolism, cardiovascular function, circadian rhythm and behaviour [2, 3, 4, 5]. It is therefore intriguing to investigate the molecular basis regulating these diverse functions. This paper will describe the molecular architecture of the promoters of the genes comprising the kisspeptin system in a perciform, *Seriola lalandi* (yellowtail kingfish, YTK) and a cyprinid, *Danio rerio* (zebrafish, ZF).

Methods:

Promoter sequences of ytk*Kiss2* and ytk*Kiss1R* were isolated from genomic DNA by genome walking PCR. The ZF *Kiss1* and *Kiss2* gene promoter sequences were identified *in-silico*. Sequences were analysed for conserved regulatory elements using bioinformatic tools (TFSearch, Signal Scan).

Results and Discussion:

We have obtained and analysed 380 bp of ytk*Kiss2* and 420 bp of ytk*Kiss1R* putative promoter sequences. We have analyzed 1.5 kb of the zf*Kiss1* and zf*Kiss2* sequences upstream of the putative transcriptional initiation site. Bioinformatic analysis of the putative promoter sequences revealed the most highly represented and significant regulatory elements (Table 1).

All promoter sequences investigated showed multiple AP-1 motifs, which has been shown to be activated by oestrogen receptors [6], suggesting the sensitivity of the kisspeptin system to oestrogen and to oestrogen-like compounds. Interestingly, C/EBP, which corresponds to the light-responsive D-box regulatory motif [7], is also present in all promoter sequences, pointing to responsiveness of the kisspeptin system to photoperiod. The GR elements in zf*Kiss1* and zf*Kiss2* gene promoters imply stress pathway regulation. The promoter sequences of the ZF *Kiss* receptors will be investigated for further comparative studies.

Acknowledgments:

This work was supported by the Australian Seafood CRC and the University of the Sunshine Coast.

References:

[1]AKAZOME Y, KANDA S, OKUBO K, OKA Y. 2010. Functional and evolutionary insights into vertebrate kisspeptin systems from studies of fish brain. J. Fish Biol., 76: 161–182

[2]MILLAR RP, ROSEWEIR AK, TELLO JA, ANDERSON RA, GEORGE JT, MORGAN K, PAWSON AJ. 2010. Kisspeptin antagonists: unravelling the role of kisspeptin in reproductive physiology. Brain Res., 1364: 81-89.

[3]KIM J. SEMAAN SJ, CLIFTON DK, STEINER RA, DHAMIJA S, KAUFFMAN AS. 2011. Regulation of Kiss1 expression by sex steroids in the amygdala of the rat and mouse. Endocrinology, Doi: 10.1210/en.2010-1498.

[4]CHAN YM, BUTLER JP, PINNEL NE, PRALONG FP, CROWLEY WFJR, REN C, CHAN KK, SEMINARA SB. 2011. Kisspeptin resets the hypothalamic GnRH clock in men. J. Clin. Endocrin. Metab., Doi: 10.1210/jc.2010-3046.

[5]SAWYER I, SMILLIE SJ, BODKIN JV, FERNANDES E, O'BYRNE KT, BRAIN SD. 2011. The vasoactive potential of kisspeptin-10 in the peripheral vasculature. PLoS one, 6:e1 4671.

[6]KUSHNER PJ, AGARD DA, GREENE GL, SCANLAN TS, SHIAU AK, UHT RM, WEBB P. 2000. Estrogen receptor pathways to AP-1. J. Steroid Biochem. Mol. Biol., 74: 311– 317.

[7]WEGER BD, SAHINBAS M, OTTO GW, MRACEK P, ARMANT O, DOLLE D, LAHIRI K, VALLONE D, ETTWILLER L, GEISLER R, FOULKES NS, DICKMEIS T. 2011. The light responsive transcriptome of the zebrafish: function and regulation. PloS one, 6: e17080.

Table 1. *Conserved promoter regulatory elements of ytkKiss1R, ytkKiss2, zfKiss1 and zfKiss2 genes*

Gene promoter	Regulatory elements
ytk*Kiss1R*	Sp1, AP-1, C/EBP, CdxA, GATA-1, GATA-2
ytk*Kiss2*	AP-1, AP-4, ER, C/EBP, CdxA, GATA-1, GATA-2
zf*Kiss1*	AP-1, AP-2, Sp1, Cre-BP, C/EBP, SRY, CdxA, GATA-1, GATA-2, n-MYC, GR
zf*Kiss2*	AP-1, AP-4, C/EBP, GATA-1, PR, GR, Cre-BP/CREB, SRY, CdxA, SRE-BP

KISSPEPTIN SIGNALING AND REPRODUCTION IN FLATFISH

Mechaly, A.S.*, Viñas, J. , Piferrer, F.***

* Institut de Ciències del Mar, Consejo Superior de Investigaciones Científicas (CSIC), Passeig Marítim, 37-49, 08003 Barcelona, Spain. piferrer@icm.csic.es

** Laboratori d'Ictiologia Genètica, Departament of Biology, University of Girona, Spain.

Introduction:

The recently discovered decapeptide kisspeptin and its G-protein coupled receptor form a signaling system expressed ubiquitously and are implicated in a variety of still poorly characterized functions [1]. In the brain, kisspeptin is produced by specific neurons and its receptor is localized in GnRH neurons [2]. Kisspeptin signaling has been fully established in the control of the onset of puberty in vertebrates, from fish to mammals [3-6]. To obtain a better understanding of the mechanisms associated with kisspeptin signaling within the reproductive axis we used Senegalese sole (*Solea senegalensis*), an important valuable flatfish species for aquaculture, as a model. We aimed at the study of the expression of kisspeptin and its receptor in the different components of the reproductive axis in relation to other important genes, according to sex and during a full reproductive cycle.

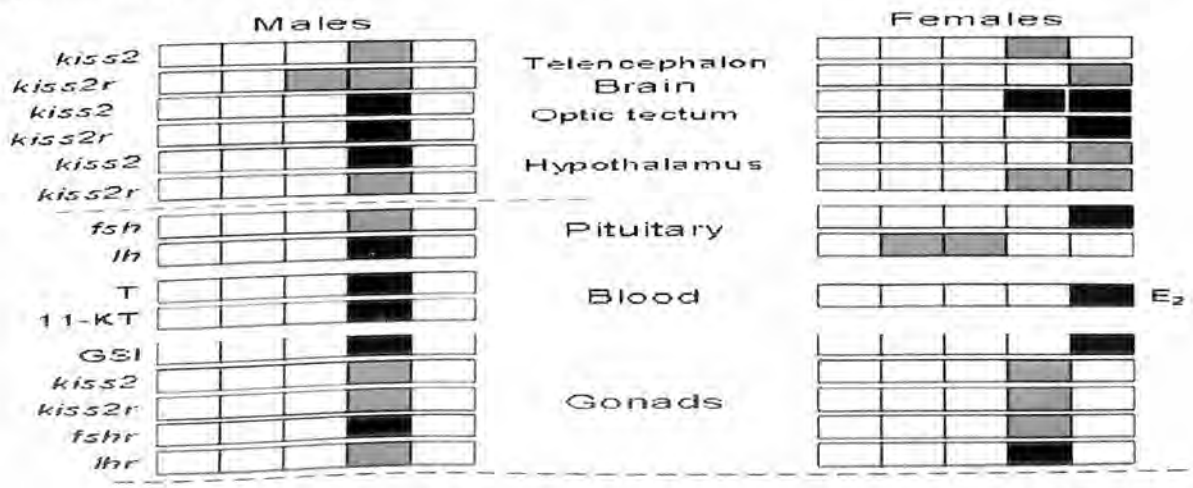
Methods:

Senegalese sole (F1 generation) were reared from eggs spawned by different stocks of wild fish (F0) and acclimated to captivity. Seasonal changes in plasma levels of testosterone (T) and 11-ketotestosterone (11-KT) were determined in males, whereas estradiol-17ß (E2) plasma levels were determined in females, using commercially available enzyme immunoassay (EIA) kits. The expression patterns of *kiss2* and *kiss2r* in different brain areas (including hypothalamus, telencephalon and optic tectum) and gonads, mRNA levels of *lh* and *fsh* in pituitary, and *lhr* and *fshr* in gonads were analyzed by quantitative real-time PCR (qRT-PCR) in males and females at the five different samplings periods (spring, summer, fall, winter and a second spring) comprising a full reproductive cycle. To calculate relative changes in gene expression, we analyzed the data using the comparative Ct method (also known as the DDCt method). Fold change (the relative quantification, RQ) was calculated from the DDCt and normalized by the endogenous reference gene *ßactin*.

Results:

In males, plasma levels of T and 11-KT followed the same pattern as the GSI, with a clear and significant peak in winter (Fig. 1). In females, E2 plasma levels remained low until the fall and then increased through winter and the following spring. Regarding the gene expression analysis, in the telencephalon, *kiss2* increased after summer and peaked in winter in both males and females. In contrast, *kiss2r* mRNA levels were highest during the fall and winter in males but not until the following spring in females. In the optic tectum of males, significant changes in both *kiss2* and *kiss2r* mRNA levels were observed, with a clear peak of expression in winter. In females, in contrast, *kiss2* mRNA levels started to increase in the fall and reached

Fig. 1. Summary of the changes observed in the variables measured in this study. The five boxes correspond, from left to right, to spring, summer, fall, winter and the following spring. The level of expression is related to the intensity of shadowing in each box: white, low expression, grey, high expression (but not significantly different), black, highest expression with significant differences.



maximum levels in winter, and then started to slightly decrease, whereas *kiss2r* mRNA levels kept increasing until they reached maximum values in the following spring. In the hypothalamus, a similar pattern was observed, i.e., low levels of both *kiss2* and *kiss2r* in males throughout except in winter, when a clear peak was observed in males but only a progressive increase in females. In the gonads, both *kiss2* and *kiss2r* shared a similar expression pattern, with maximum mRNA levels in the winter, although no significant differences were observed. Regarding the expression levels of gonadotropin genes in the pituitary, *lh* in males peaked in winter and *fsh* of females in the second spring. Non-statistically significant changes were observed in *fsh* mRNA levels in males and *lh* mRNA levels in females. In the gonads, mRNA levels of *fshr* and *lhr* remained low during most part of the study, but were consistently higher in winter. However, the inverse situation was found with respect to the levels of mRNA for *fsh* and *lh* observed in the pituitary, i.e., significant differences were observed for *fshr* in the testis and *lhr* in the ovaries (Fig. 1).

Conclusion:

Analysis of the temporal and spatial changes in expression of kisspeptin, gonadotropins and their respective receptors in the Senegalese sole during a full reproductive cycle shows that, in males, *kiss2* agrees with what one would expect according to its proposed role as a major regulator of the onset of reproduction. However, in females the situation is not so clear, since *kiss2* and *kiss2r* expression was highest either before or during the reproductive season. The origin and physiological significance of these differences, which could also apply to other fish, deserve further investigation.

References:

[1]TENA-SEMPERE, M. 2006. GPR54 and kisspeptin in reproduction. Hum. Reprod. Update, 12: 631–639.

[2]OAKLEY, A.E., CLIFTON, D.K., STEINER, R.A. 2009. Kisspeptin signaling in the brain. Endocr. Rev., 30: 713–743.

[3]TARANGER, G.L., CARRILLO, M., SCHULZ, R.W., FONTAINE, P., ZANUY, S., FELIP, A., WELTZIEN, F.A., DUFOUR, S., KARLSEN, O., NORBERG, B., ANDERSSON, E., HANSEN, T. 2009. Control of puberty in farmed fish. Gen. Comp. Endocrinol., 165: 483–515.

[4]MECHALY, A.S., VIÑAS, J., PIFERRER, F. 2009. Identification of two isoforms of the kisspeptin-1 receptor (kiss1r) generated by alternative splicing in a modern teleost, the Senegalese Sole (*Solea senegalensis*). Biol. Reprod., 80: 60–69.

[5]MECHALY, A.S., VIÑAS, J., MURPHY, C., REITH, M., PIFERRER, F. 2010. Gene structure and regulation of the Kiss-1 receptor-2 (Kiss1r-2) in the Atlantic halibut: Insights into the evolution and regulation of Kiss1r genes. Mol. Cell. Endocrinol., 317: 78–89.

[6]MECHALY,A.S.,VIÑAS,J.,PIFERRER,F. 2011. Gene structure analysis of kisspeptin-2 (Kiss2) in the Senegalese sole (*Solea senegalensis*): Characterization of two splice variants of Kiss2, and novel evidence for metabolic regulation of kisspeptin signaling in non-mammalian species. Mol. Cell. Endocrinol., 339: 14-24.

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

PARTIAL CLONING AND CHARACTERIZATION OF VASOTOCIN1A RECEPTOR GENE IN THE CATFISH *HETEROPNEUSTES FOSSILIS*: SEASONAL AND TISSUE EXPRESSION STUDY

Rawat A. Chaube R.*, Joy K. P.

Department of Zoology, Banaras Hindu University, Varanasi, U.P. - 221005, India
*Zoology Section, MMV, Banaras Hindu University, Varanasi, U.P. - 221005, India
email: chauberadha@rediffmail.com, kpjoy@bhu.ac.in

Introduction:

Vasotocin (VT), a neurohypophysial hormone of non-mammalian vertebrates is the ortholog of vasopressin (VP) in the mammals. VT is released from the neurohypophysis of teleost fish involved in the regulation of various physiological processes such as reproduction, behavior, osmoregulation, blood pressure, cardiovascular regulation etc [9]. In mammalian species, at least three subtypes of receptor for VP (V1a, V2, V3/V1b) have been reported and characterized [8]. The VP V1a receptor is mainly expressed in vascular smooth muscle cells and hepatocytes [5], the V2 receptor is expressed in the kidney, where it regulates water reabsorption [4], and the V3/ V1b receptor is exclusively located in the pituitary gland [2]. In birds and firebelly newt (*Cynops pyrrhogaster*) three distinct VT receptors have been identified [3]. VT receptors have been isolated and described in several teleost fishes ranging from flatfishes to trout [1]. There is still lack of information related to VT receptor types, its presence, amino acid structure identities and intracellular signal transduction mechanisms in teleosts. In the present study, we cloned and characterized VT1a receptor gene in the catfish *Heteropneustes fossilis* and seasonal and tissue specific expression were studied.

Methods:

Adult female *H. fossilis* (40–50 g) were collected and maintained in the laboratory for 48 h under natural photoperiod and temperature to overcome stress due to transportation and fed daily with goat liver *ad libitum*. The fish were weighed and sacrificed by decapitation. Brain, ovary, kidney, gill, liver and muscle were dissected out from mature catfish and were stored in RNA later solution (Ambion, Austin, Tx) at -80^{0}C until used. Total RNA was extracted using RNeasy for lipid tissues (QIAGEN) mini kit and cDNA was synthesized from 5µg total RNA with oligo (dT) primers using Revert Aid H-minus M-MuLV reverse transcriptase (Fermentas, Life Sciences, USA) according to the manufacturer's instructions. Degenerate primers were designed to match highly conserved regions of full-length cDNA sequences available from NCBI databases for VT1a receptor (amphibian, fish, and mammal sequences).The forward and reverse degenerate VT1a receptor primers were: 50-GACCGATACATCGCTATCTGYCAYCCNYT-30 and 50-AAGGCTGGATGAAGTGASCCCARCARTC-3 (Amplicon size-192bp). The PCR product was further purified using Hipura PCR product purification kit and sequenced. Purified PCR products were cloned into the pGEM-T Easy Vector (Promega) and sequencing was done at Xcelris Labs Ltd. Bodakdev, Ahmedabad-380054, India. Seasonal changes in VT1a expression in brain and ovary were studied during different reproductive phases: resting (January), preparatory (April), pre-spawning (June), and spawning (July). Total RNA was extracted and first strand cDNA synthesized as described above. Semi-quantative RT-PCR was performed using gene specific primers designed from the partial sequence. Following are the forward and reverse primer sequences 5'-GACCGATACATCGCTATCTGTCACCCGCT-3' and 5'-AGGTTGGATGAAGTGAGCCCAGCAGTC-3'. Primers for catfish b-actin gene, 5'-TGGCCGTGACCTGACTGAC-3' (forward) and 5'-CCTGCTCAAAGTCAAGAGCGAC-3' (reverse), were used as an internal control. The gel images were analyzed by Alpha Ease FC software (Alpha Innotech Corporation).

Results and Discussion:

The partial VT1a receptor gene sequence shown the highest identity (82%) with cDNA sequence encoding the *Takifugu rubripes* VT receptor (V1-β) gene complete cds (accession no. AY027887.1).. Our result shows that VT1a transcript was express in all tissues like brain, liver, gill, kidney, ovary and muscle but significant changes were shown only in brain and ovary across the reproductive phase. Densitometric analysis showed that VT1a expression was high in the brain than ovary. Brain and ovarian VT1a mRNA showed significant seasonal variations. In the brain, the transcript levels were relatively higher in the resting and preparatory (previtellogenic stage) phases, but decreased conspicuously in the pre-spawning phase (early post-vitellogenic stage) and spawning phase (late post-vitellogenic stage). On the other hand, VT1a expression in the ovary was relatively low in both resting and preparatory phases, but increased in the pre-spawning and spawning phases giving the highest transcript levels. All identified teleost VT receptors constitute a single VT

receptor type that groups within the V1a type class of mammalian VP receptor. VT receptor mRNA is present in the brain, where VT has been reported to be involved in behavior and regulation of pituitary hormone release [6]. In the catfish *H. fossilis* VT1a receptor seems to be mainly involved in final oocyte maturation and ovulation by stimulating the secretion of the maturation-inducing steroid (MIS), [7].

Conclusion:

In conclusion, VT1a receptor gene was characterized in the brain and ovary of catfish. It showed extensive tissue expression & seasonal activity. The distribution and seasonal activity suggest that VT is involved in a variety of functions in catfish.

References:

[1]AN, K.W., SHIN, H.S., AN, M.I., JO, P.G., CHOI, Y.K., CHOI, C.Y., 2008B. Physiological response and expression of arginine vasotocin receptor, prolactin and prolactin receptomRNA in olive flounder Paralichthys olivaceus during osmotic stress. Mar. Fresh. Behav. Physiol., 41: 191–203.

[2 DE KEYZER, Y., LENNE, F., AUZAN, C., JEGOU, S., RENE, P., VAUDRY, H., KHUN, J-M., LUTON, J-P., CLAUSER, E., BERTAGNA, X., 1996. The pituitary V3 vasopressin receptor and the corticotroph phenotype in ectopic ACTH syndrome. J. Clin. Invest., 97: 1311–1318.

[3]HASUNUMA, I., SAKAI, T., NAKADA, T., TOYODA, F., NAMIKI, H., KIKUYAMA, S., 2007. Molecular cloning of three types of arginine vasotocin receptor in the newt, Cynops pyrrhogaster. Gen. Comp. Endocrinol., 151: 252-258.

[4]LOLAIT, S.J., O'CARROL, A-M., MCBRIDE, O.W., KONIG, M., MOREL, A., BROWNSTEIN,M.J., 1992. Cloning and characterization of a vasopressin V2 receptor and possible link to nephrogenic diabetes insipidus. Nature, 357: 336–339.

[5] MOREL, A., O'CARROLL, A-M., BROWNSTEIN, M.J., LOLAIT, S.J., 1992. Molecular cloning and expression of a rat V1a arginine vasopressin receptor. Nature, 356: 523–526.

[6]PIERSON, P.M., GUIBBOLINI, M.E., LAHLOU, B., 1996. A V1-type receptor for mediating the neurohypophyseal hormone-induced ACTH release in trout pituitary. J. Endocrinol., 149: 109–115.

[7]SINGH, V., JOY, K.P., 2010. An involvement of vasotocin in oocyte hydration in the catfish Heteropneustes fossilis: a comparison with effects of isotocin and hCG. Gen. Comp. Endocrinol., 166:504–512.

[8]SUGIMOTO, T., SAITO, M., MOCHIZUKI, S., WATANABE, Y., HASHIMOTO, S., KAWASHIMA, H., 1994. Molecular cloning and functional expression of a cDNA encoding the human V1b receptor vasopressin receptor. J. Biol. Chem., 269: 27088–27092.

[9]WARNE JM, BALMENT RJ (1997) Changes in plasma arginine vasotocin (AVT) concentration and dorsal aortic blood pressure following AVT injection in the teleost, Platichthys flesus. Gen. Comp. Endocrinol., 105:358–364.

THE CELL CONTEXT INFLUENCES RAINBOW TROUT GONADOTROPIN RECEPTORS' SELECTIVITY

Saha A.*, Sambroni E.[1], Bogerd J.[2], Schulz R.W.[2], Le Gac F.[1], Lareyre J.J.[1]

*Central Institute of Freshwater Aquaculture, Kausalyaganga, Bhubaneswar, 751002, India
Fax: +91-674-2465407 email:ashis73in@yahoo.co.in
[1] INRA SCRIBE, UPR 1037, 35042 Campus de Beaulieu, Rennes, France
[2] Utrecht University, Utrecht, The Netherlands

Introduction:

Vertebrate reproduction is tightly regulated by glycoprotein hormones produced by the pituitary gland. Two gonadotropins, FSH and LH are present in tetrapod vertebrates and the duality of gonadotropins has become an accepted principle also for fishes, the largest group of vertebrates. The presence of two distinct gonadotropin receptors (GtHRs) in a single fish species was confirmed by the molecular cloning of two different cDNAs in several fish species: salmon, catfish, zebrafish, sea bass, eel and trout. The ligand selectivity of mammalian GtHRs is well defined: FSHR and LHR bind their respective ligands specifically and show little cross-activation (0.01–0.1%). In contrast to the situation in mammalian species, the bioactivity of fish gonadotropins seems to be less well separated as a result of promiscuous hormone–receptor interactions. Depending on the species, hormones, and hormone concentrations used, a promiscuous activation of one or the other fish GtHRs was reported in functional studies using mammalian cell lines expressing fish receptors. In African catfish, recombinant cfFSH and cfLH activated FSHR with a similar biopotency [1] whereas in amago salmon, only FSH was able to activate FSHR. In zebrafish, recombinant zfFSH stimulated only FSHR, whereas recombinant zfLH stimulated both FSHR and LHR [2]. In trout, gonadotropins purified from trout or salmon pituitaries specifically activate their cognate receptor [3]. In summary, data from studies using different bioassays do not allow drawing general conclusions on the responsiveness of the piscine receptors to GtHs. In the present study, we report that the apparent discrepancy in fish gonadotropin receptors cross-selectivity originates mainly in the choice of the cell line used for receptor expression, and also from the heterologous or homologous origin of the hormones tested. The COS-7 cell line led to a highly selective responsiveness of the GtHRs whereas HEK cells show strong cross-reactivity.

Methods:

Rainbow trout FSH and LH receptor expression vectors were constructed using pcDNA3.1/V5-His-TOPO expression vector (Invitrogen) and the GtHR cDNA placed upstream from the polyadenylation site of the bovine growth hormone gene and downstream the cytomegalovirus (CMV) promoter. The HEK293/CREB-Luc cells (Panomics), and COS -7 cells were used for transient transfection assays. HEK293 cells were cultured in presence of 0.2 % hygromycin B (50mg/ml) in the DMEM culture medium in 24 well culture plate at a density of 20000 cell/well for 96 hours. COS-7 cells were cultured in a 24 well culture plate at a density of 70,000 cells/well for 24 hours. After that cells were transfected with either pcDNA3.1/V5-His-FSHR or pcDNA3.1/V5-His-LHR (10ng/well). Cells were stimulated with purified chinook FSH (cFSH), chinook LH(cLH), recombinant zebrafish FSH (zfFSH), recombinant zebrafish LH (zfLH), human FSH (hFSH), human LH (hLH) and human chorionic gonadotropin (hCG) for six hours. Luciferase activity was measured from 40µl lysates using the luciferase assay kit (Promega). Cultures of testicular tissue explants were carried out to analyse the effect of different salmonid and non-salmonid hormones on 11-ketotestosterone (11-KT) production.

Results and discussion:

Functional characteristics of trout GtHRs were analysed in two different cell contexts: COS-7 and HEK-293 cell lines. FSH receptor was efficiently activated by both cFSH and cLH in the HEK293 cell line and similar inductions of FSHR were obtained at 800 ng/ml (maximal fold induction of the luciferase reporter gene was 6.4 with cFSH vs. 6.6 with cLH). The cLH potency appeared to be lower, with an effective half-maximum concentration (EC_{50}) equal to 349 ng/ml versus 111 ng/ml for cFSH. In contrast, in the COS-7 cell line, FSH receptor was only activated by cFSH, with an effect similar to that observed in HEK cells (5.9 fold induction at 800 ng/ml and EC_{50} =133 ng/ml). These results reveal that FSHR response to LH is strongly dependent on the cell context.

LH receptor was mainly stimulated by cLH in both HEK293 and COS-7 cell lines. The maximal fold induction was significantly higher in HEK293 cell compared to COS-7 cell (x79 vs. x14). In addition, the potency of cLH appeared almost twice higher in HEK293 cells than in COS-7 cells, with an EC_{50} value of 5 ng/ml versus 9.8 ng/ml, respectively. This indicates

that HEK cells were more favorable to LH receptor response, as compared to COS cells. Although trout LHR was mainly stimulated by cLH, high doses of cFSH (1600 ng/ml) efficiently activated LHR in the HEK293 cell line but not in the COS cell line. So, our results indicate that the cellular context modulates the selectivity and the amplitude of the LH receptor response.

To address further whether the cell context could modulate ligand-receptor selectivity, we tested the trout GtHRs responsiveness to mammalian and zebrafish gonadotropins. In COS-7 cells, FSH receptor was activated by salmonid FSH but not by the zebrafish or human gonadotropins tested. In HEK cells, FSH receptor was activated not only by salmonid FSH and LH, but also by all heterologous LH and hCG. In COS cells, LH receptor was efficiently stimulated by salmonid LH and moderately by non-salmonid LH but never by any FSH or hCG. On the contrary, in HEK cells, LH receptor was activated by all types of LH and hCG. Interestingly, it was also induced by salmonid and non-salmonid FSH, although to a lower magnitude.

Finally, additional studies revealed that mammalian hormones including hCG, up to 1600 ng/ml, did not induce 11-KT production from rainbow trout testicular explants cultured *ex vivo*.

Conclusions:

We demonstrate that cross-selectivity of the trout GtHRs' responsiveness depends on the cellular context. Trout GtHRs show high ligand selectivity when expressed in COS-7 cells, but not when expressed in HEK cells. Trout receptors selectivity in COS-7 cells seems to reflect better the *ex vivo* conditions. Altogether, we propose that trout GtHRs are highly selective and that the mammalian cell lines used reflect only partially this high selectivity.

References:

[1]BOGERD, J., BLOMENROHR, M., ANDERSSON, E., VAN DER PUTTEN, H.H., TENSEN, C.P., VISCHER, H.F., GRANNEMAN, J.C., JANSSEN-DOMMERHOLT, C., GOOS, H.J., SCHULZ, R.W. 2001. Discrepancy between molecular structure and ligand selectivity of a testicular follicle-stimulating hormone receptor of the African catfish (*Clarias gariepinus*). Biol. Reprod., 64: 1633–1643

[2]KWOK, H.-F., SO, W.-K., WANG, Y., GE, W. 2005. Zebrafish gonadotropins and their receptors: I. Cloning and characterization of zebrafish follicle-stimulating hormone and luteinizing hormone receptors – evidence for their distinct functions in follicle development. Biol. Reprod., 72: 1370–1381.

[3]SAMBRONI, E., LE GAC, F., BRETON, B., LAREYRE, J.J. 2007. Functional specificity of the rainbow trout (*Oncorhynchus mykiss*) gonadotropin receptors as assayed in a mammalian cell line. J. Endocrinol., 195: 213–228.

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

STEROIDOGENIC GENE EXPRESSION IN THE BRAIN OF AN INDIAN MAJOR CARP, *LABEO ROHITA* (HAM.)

Saravanan, N., Uma, T., Pratheeba F.J., Sarumathi, P., Sundari, G., Inbaraj, R.M.

N. Saravanan, Endocrinology Unit, Department of Zoology, Madras Christian College, Tambaram, Chennai-600059.
Email: saran_mscmphil@yahoo.co.in

Introduction:

Brain is a steroidogenic organ that produces steroidogenic enzymes [1]. These enzymes play an important role in the pathway of steroidogenesis. The internalization of cholesterol by the mitochondria is the rate-limiting step for the general steroidogenic pathway, and is mediated by Steroidogenic acute regulatory protein (StAR). Once inside the mitochondria cholesterol is converted to pregnenolone by the enzyme P450 side chain cleavage (P450scc). Pregnenolone is converted to progesterone by 3beta-hydroxysteroid dehydorgenase (3β-HSD). The conversion of pregnenolone and progesterone to their 17α-hydroxylated products and then to either DHEA or androstenedione, respectively is mediated by a single enzyme P450c17α-hydroxylase (P450c17). Pregnenolone is the first steroid in the pathway and is the common precursor for all species and all tissues, from this point the converted cholesterol is committed to becoming a steroid. Such steroids synthesized de novo in the brain, as well as other areas of the nervous system, are called neurosteroids. The enzyme 3alpha-hydroxy steroid dehydrogenase (3α-HSD) catalyzes the conversion of 5α-dihydroxytestosterone (5α-DHT) and 5α-dihydroxyprogesterone (5α-DHP) into 3α-androstanediol and 3α,5α-tetrahydroxyprogesterone (3α,5α-THPROG), respectively. The pathways of steroidogenesis differ from organism to organism. Steroids play an important role in the onset of sexual characters and development of gametes [2]. To understand the enzyme pathway and neurosteroid actions in the brain, we need data on the specific gene expression of steroidogenic enzymes in the brain of an Indian major carp, *Labeo rohita*. We have identified the gene expression of StAR protein and enzyme P450scc, P450c17, 3beta-HSD and 3alpha-HSD in the brain of *L. rohita*. This is the first report of gene expression study in this fish brain.

Methods:

Labeo rohita was collected from Sathanoor reservoir. The total RNA extraction was done in brain tissue by guanidine thiocyanate method. The RNA was reverse transcribed into cDNA using M-MuLV, RT-PCR kit (Medox). The gene specific primers were constructed and obtained from sigma (USA). RT-PCR product with gene specific primer of StAR forward 'GGTACAGTGAAACTGCGAATGG', reverse 'TGGTGCCCTTCCGTCAATTCC', P450scc forward 'GAGGAGGGTAGGAGCCA', reverse 'CCTTGTGGGACTCTGGT', P450c17 forward 'CCAGAGAGGTTCTCCTGCTG', reverse 'TGGACAACAGCTCCTCACAG', 3α-HSD forward 'CTGTGCCTGAGAAGGTTGCT' reverse CATGTGTCACAGATATCCAC, 3β-HSD forward 'CTCTGCAGGAACATCCCAAT', reverse 'TGATCCACAGCATCCACACT' was subjected to PCR by Taq polymerase kit (Genei, Bangalore). The PCR products were visualized by gel electrophoresis using ethidium bromide. The DNA amplified through PCR was quantified before sending for sequencing. The PCR amplified samples were sent for sequencing to Bangalore Genei, India.

Results and Discussion:

Total RNA was isolated and reverse transcribed to synthesized first strand cDNA. Using the RT-PCR samples the ordered primers was used to identify and amplify StAR protein, enzymes P450scc, P450c17, 3α-HSD and 3β-HSD responsible gene. After the PCR they were loaded into the agarose gel. The result of PCR products along with 100bp marker DNA is shown in Fig. 1a-e. The results confirm the presence and expression of


Fig. 1a


Fig. 1b

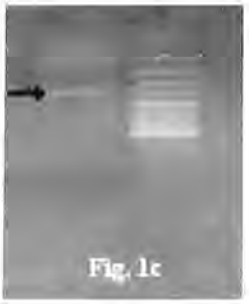


Fig. 1c


Fig. 1d


Fig. 1e

steroidogenic enzymes in the brain of *Labeo rohita*. The formation of neurosteroids in the brain was originally demonstrated in mammals and subsequently in other vertebrates, such as birds, amphibians and fish. Thus *de novo* neurosteroidogenesis in the brain from cholesterol is a conserved property of vertebrates [3]. This is the first demonstration of steroidogenic enzyme gene expression in the brain of *L. rohita*. Our studies on steroidogenic protein and enzyme gene expression have provided the opportunity to understand biosynthesis of steroidogenic protein and enzymes in the brain of *L. rohita*.

Acknowledgement:

The authors thank FIST Lab, Endocrinology Unit, Department of Zoology, Madras Christian College, Tambaram, Chennai-600059. India.

References:

[1]Costa, E., Paul, S. (eds). 1991. Neurosteroids and brain function. Fidia Research Foundation symposium series. Thieme New York, Vol 8.

[2]Ebner, M.J., Corol, D.I., Havlikova, H., Honour, J.W., Fry, J.P. 2000. Identification of Neuroactive steroids and Their precursors and metabolites in adult male rat brain. Endocrinology, 147: 179-190.

[3]Kazuyoshi Tsutsui, 2006. Biosynthesis, mode of action and functional significance of neurosteroids in the developing Purkinje cell. J. Steroid Biochem. Mol. Biol. 102: 187–194.

REGULATION OF ONSET OF SEXUAL MATURATION BY MELANOCORTIN RECEPTOR 4 POLYMORPHISMS IN *XIPHOPHORUS*

Schartl M.[1], Schories S.[1], Schmidt C.[1], Fischer P.[1], Lampert K.[2], Volff J.-N.[3], C. Hoffmann C.[4] and Lohse M.[4]

[1]Physiological Chemistry I, Biocenter, University of Wuerzburg, Germany, Fax: +49-931-3184150 email: phch1@biozentrum.uni-wuerzburg.de

[2]Evolutionary Ecology and Biodiversity of Animals, University of Bochum, Germany, Fax: +49-234-32-14114 email: kathrin.lampert@ruhr-uni-bochum.de

[3]Institut de Génomique Fonctionnelle de Lyon, Ecole Normale Supérieure de Lyon, France, Fax: +33-472-728992 email: Jean-Nicolas.Volff@ens-lyon.fr

[4]Pharmacology and Toxicology, University of Wuerzburg, Germany, Fax: +49-931-201-48539 email C.Hoffmann@toxi.uni-wuerzburg.de, and Fax: +49-931-201-48411 email: lohse@toxi.uni-wuerzburg.de

Introduction:

The onset of sexual maturation marks an important point in the reproductive life of a fish. Moreover, precocious or delayed puberty can have profound negative effects in fish farming and broodstock management. Our knowledge about the molecular processes involved in regulation of puberty in fish are still fragmentary. To identify still unknown components of puberty regulation, experimental model systems that exhibit genetic defects or genetic polymorphisms affecting the onset of sexual maturity provide useful tools for further analyses. A prominent example is the polymorphism in adult male body size in some species of platyfish and swordtails of the genus *Xiphophorus*. A single Mendelian locus (*P*) was shown to be responsible for the observed polymorphism. *P* determines the onset of sexual maturity of males and results, due to the fact that males stop to grow after reaching puberty, in a marked size polymorphism. Strikingly the different male size classes show pronounced differences in behavior and females prefer large over small males.

Results:

We show that a polymorphism of the melanocortin receptor 4 (mc4r) comprising functional and non-signal transducing versions and the copy number variation of *mc4r* genes on the Y-chromosome underlie the polymorphism of the *P*-locus. Non-functional copies of *mc4r* that are only present in some males act as dominant negative mutations and delay the onset of puberty leading to the large phenotype. The co-expression of dominant –negative receptor versions with wildtype mc4r leads to a reduction of cAMP production and decreased expression of mc4r effector genes. Copy number variation, as a regulating mechanism, makes the system extremely flexible and is responsible for the large variety of phenotypes.

Conclusion:

Our analysis shows that a gene polymorphism of mc4r is linked to size differences of adult males and explains the molecular mechanisms maintaining phenotypic variation. As mc4r is a critical component of the hypothalamus-pituitary axis involved in regulation of body weight and appetite, a novel link between the physiological system controlling energy balance and the regulation of reproduction becomes apparent.

KISSPEPTIN AND GnRH EXPRESSION PROFILES IN THE BRAIN AND PITUITARY OF FEMALE CHUB MACKEREL (*SCOMBER JAPONICUS*) DURING THE SPAWNING CYCLE

Selvaraj S.[1], Kitano H.[1], Amano M.[2], Nyuji M.[1], Ohga H.[1], Yamaguchi A.[1], Yoneda M.[3], Shimizu A.[3] and Matsuyama M.[1]

[1]Faculty of Agriculture, Kyushu University, Fukuoka 812-8581, Japan; [2]Kitasato University, Ofunato, Iwate 022-0101, Japan ; [3]Fisheries Research Agency, Kanazawa, Yokohama 236-8648, Japan
Tel/Fax. +81 92 642 2888; E-mail. sraj146@gmail.com

Background:

Kisspeptins have emerged as key modulator of reproduction in mammals, through its regulation of gonadotropin-releasing hormone (GnRH) neurons. Unlike placental mammals, few teleosts express *kiss1* and *kiss2* [1]. Moreover, teleosts brain expresses multiple GnRH forms. The chub mackerel show asynchronous type of ovarian development, where a small percentage of first clutch, late vitellogenic oocytes undergo final oocyte maturation (FOM) and spawning with successive progression of mid- and early vitellogenic oocytes. Our previous study found that the chub mackerel brain expresses *kiss1* and *kiss2*. Further, this species expresses three GnRH forms (GnRH1 or seabream GnRH; GnRH2 or chicken GnRH-II; GnRH3 or salmon GnRH) and their neuroanatomical distribution revealed that GnRH1-immunoreactive axonal fibers project mainly to the anterior pituitary, where gonadotropic cells are localized [3]. In the present study, to clarify whether multiple *kiss* and GnRH1 forms are involved in the regulation of female spawning cycle, their mRNA expression profiles in the brain as well as corresponding GnRH peptides in the pituitary were analyzed.

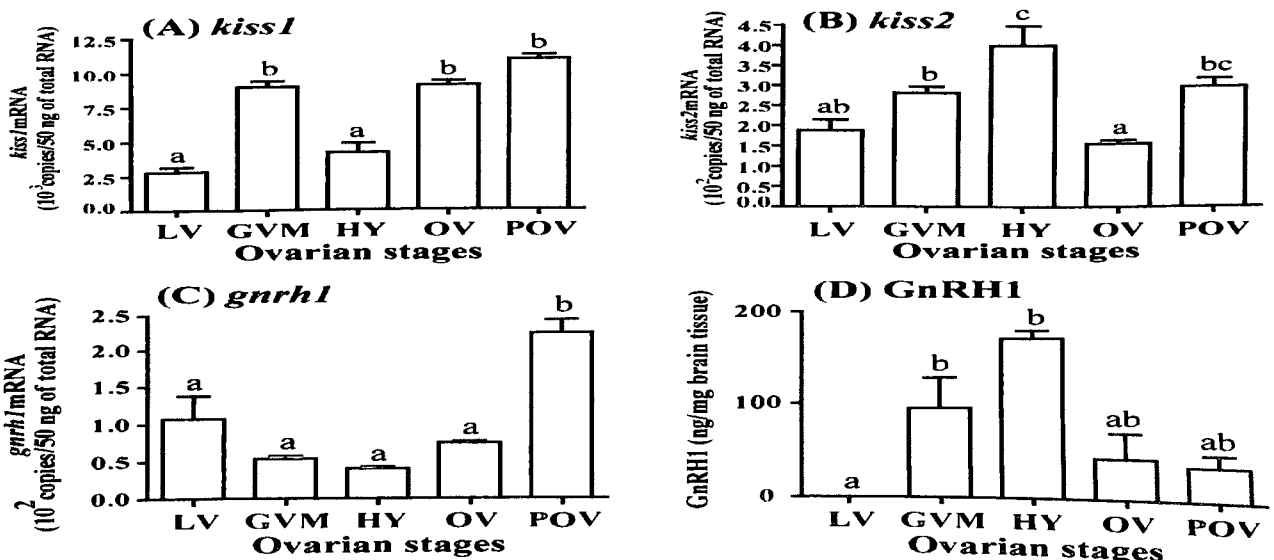
Methods:

Chub mackerel females fail to undergo FOM and ovulation spontaneously in the captivity. During the natural spawning season (April-June), females (2-year-old) with late vitellogenic oocytes (600-650 µm in diameter) were selected by ovarian biopsy and induced to undergo FOM, ovulation, and spawning in 3-ton outdoor concrete tanks. Induction protocol was based on sustained release formulation of GnRH agonist (Des-Gly10, [D-Ala 6] LH-RH Ethylamide). The first spawning was observed 34-36 h post-injection, and subsequent daily spawning occurred between 22.00 and 24.00 h. After one week, fish sampling was performed at 13.00, 16.00, 20.00, and 6.00 h of the day, corresponding to different stages (n=4-6 for each stage) of spawning cycle. SYBR Green quantitative real-time PCR (qRT-PCR) and time-resolved fluoroimmunoassay (TR-FIA) was used to analyze the changes in *kiss* and *gnrh* mRNA levels in the whole brain and GnRH peptides in the whole brain and pituitary, respectively.

Results and Discussion:

Daily spawning activity in chub mackerel females after one week of GnRH agonist administration suggests

Fig. 1. Expression profiles of *kiss1* (A), *kiss2* (B), and *gnrh1* (C) mRNAs in the brain and GnRH1 peptides in the pituitary (D) at different ovarian stages during the spawning cycle (LV, late vitellogenesis; GVM, germinal vesicle migration; HY, hydration; OV, ovulation; POV, post-ovulation). Different letters above the bars represent significant differences between stages.



that the endogenous hormonal pathway of Brain-Pituitary-Gonad (BPG) axis is involved in the regulation of multiple spawning, without the influence of externally administered GnRH agonist. Results show that brain *kiss1* and *kiss2* mRNA levels fluctuates significantly ($p<0.05$) during GVM and HY stages, respectively (Figs. 1A and B). Similarly, brain GnRH1 peptide levels were highly elevated during GVM and HY stages (Fig. 1D). In contrast, pituitary GnRH1 peptide levels were elevated throughout the spawning cycle (data not shown). These results suggest that increased synthesis of brain GnRH1 peptides is responsible for sustained release of the peptides in the pituitary and regulates pituitary GtH synthesis and secretion during the spawning cycle.

Interestingly, an increase in *kiss1*, *kiss2*, and *gnrh1* levels was evident during the post-ovulation (POV) stage. Histologically, females sampled at POV period (06.00 h), showed the presence of 6- to 8-h old post-ovulatory follicles in their ovarian tissue, which indicated that these fish had undergone spawning on the previous night. Moreover, during the POV period, it is most likely that the standing stock of late vitellogenic oocytes in the ovary is ready to undergo germinal-vesicle migration (GVM) for the next spawning. So, we propose that an increased level of *kiss1*, *kiss2*, and GnRH1 in the brain regulates spawning cycle in female chub mackerel.

Conclusion:

The results of expression profiles suggest that *kiss1*, *kiss2*, and GnRH1 forms are involved in the regulation of spawning cycle in female chub mackerel. Future studies on circulating GtH levels will help to clarify the significance of sustained GnRH release into the pituitary.

References:

[1]AKAZOME, Y., KANDA, S., OKUBO, K., OKA, Y. 2010. Functional and evolutionary insights into vertebrate kisspeptin systems from studies of fish brain. J. Fish Biol., 76: 161–182.

[2]SELVARAJ, S., KITANO, H., FUJINAGA, Y., AMANO, M., TAKAHASHI, A., SHIMIZU, A., YONEDA, M., YAMAGUCHI, A., MATSUYAMA, M. 2009. Immunological characterization and distribution of three GnRH forms in the brain and pituitary gland of chub mackerel (*Scomber japonicus*). Zool. Sci., 26: 828–839.

[3]SELVARAJ, S., KITANO, H., FUJINAGA, Y., OHGA, H., YONEDA, M., YAMAGUCHI, A., SHIMIZU, A., MATSUYAMA, M. 2010. Molecular characterization, tissue distribution, and mRNA expression profiles of two *Kiss* genes in the adult male and female chub mackerel (*Scomber japonicus*) during different gonadal stages. Gen. Comp. Endocrinol., 169: 28–38.

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

NEUROANATOMICAL CHARACTERIZATION OF TWO INDEPENDENT KISSPEPTIN SYSTEMS DERIVED FROM EVOLUTIONARY-ANCIENT KISS GENES IN THE BRAIN OF ZEBRAFISH

Servili A[1], Page YL[1], Leprince J[2], Caraty A[3], Escobar S[4], Parhar IS[5], Seong YJ[6], Vaudry H[2], Kah O[1]

[1]Neurogenesis and OEstrogens, Unité Mixte de Recherche Centre National de la Recherche Scientifique 6026, Institut Fédératif de Recherche 140, Université de Rennes 1, 35042 Rennes, France
E-mail: arianna.servili@univ-rennes1.fr;
[2]Différenciation et Communication Neuronale et Neuroendocrine, Institut National de la Santé et de la Recherche Médicale U982, PRIMACEN, IFR Multidisciplinaires sur les Peptides 23, Université de Rouen, France;
[3]Physiologie de la Reproduction et des Comportements, INRA, Nouzilly, France;
[4]Instituto de Acuicultura de Torre de la Sal, CSIC, Castellón, Spain;
[5]School of Medicine and Health Sciences, Monash University, Malaysia and
[6]Graduate School of Medicine, Korea University, Seoul, Republic of Korea.

Introduction:

Before kisspeptins became new players in the field of reproductive biology, GnRH was acknowledged in all vertebrates as the major initiator of the hormonal cascade modulating the reproductive axis. Originally identified as a metastasis suppressor in mammals [1], the KISS1 gene produces several peptides named kisspeptins (kisspeptin -54, 14, 13, 10), which activate the KISS1 receptor (GPR54 or KISSR) previously known as an orphan receptor [2]. Recent phylogenetical analyses provided evidence that the number of kiss genes and kiss receptors varies from one class of vertebrate to the other. According to these studies [3] modern mammals have only one KISS gene, monotrems have two, birds would have none, reptiles have one, amphibians have three and fishes have two KISS genes. Similarly, the number of genes encoding GPR54 receptors (or Kissr) also varies from one class to the other. With the aim to enlarge our knowledge on organization and potential functions of Kiss systems in relation to GPR54 receptors in non-mammalian species, so far very poorly investigated, we have focused our interest on the elucidation of these systems in zebrafish, taken as model of study. Zebrafish have two kiss genes, kiss1 and kiss2 and two kiss receptors (GPR54), kiss1r and kiss2r.

Methods:

To be able to provide detailed information regarding the organization of the Kiss1 and Kiss2 systems in zebrafish brain we produced specific antibodies (raised against the C-terminus of zebrafish preproKiss1 and preproKiss2) that unambiguously distinguish zebrafish preproKiss1 from preproKiss2. Immunohistochemical analysis was fully confirmed by *in situ* hybridization and transgenic approaches.

Results and Discussion:

Our findings show that kiss1-expressing neurons are only located in the habenular nucleus, while kiss2-expressing neurons are found in the dorsal and ventral hypothalamus. Kiss1-expressing cells project only to the interpeduncular and raphe nuclei, and strongly expressed the kiss1r receptor. In contrast, kiss2-expressing cells are mostly present in the dorsal and ventral hypothalamus and project widely into the subpallium, the preoptic area, the thalamus, the ventral and caudal hypothalamus and the mesencephalon. All these regions strongly expressed the kiss2r messengers. In the pituitary gland, no proKiss2- positive fibers were detected, while positive cells were observed in the pars intermedia.

Furthermore, the potential relationships between Kiss neurons and GnRH neurons have been investigated. Briefly, Kiss2 fibers profusely innervate the ventral forebrain and notably made close apposition with GnRH3 neurons. Moreover, estrogen treatment of juvenile fish with estradiol causes increase in kiss2 and kiss2r expression.

Conclusion:

In addition of providing a successful strategy to develop antibodies to kisspeptins, this study provides for the first time detailed information on the organization of two separate kisspeptin systems derived from evolutionary-ancient kiss genes in the brain of a vertebrate (see Figure 1 for a schematic representation). These data will be discussed in relation with steroids, melatonin and leptin signaling.

Supported by the EU Project LIFECYCLE (FP7-222719-1) to O. Kah and the NEMO project to O. Kah. A. Servili and S. Escobar were sponsored by LIFECYCLE project and the JAE-Predoc CSIC (Spain), respectively.

References:

[1]LEE JH, MIELE ME, HICKS DJ, PHILLIPS KK, TRENT JM, WEISSMAN BE, WELCH DR. 1996. KiSS-1, a novel human malignant melanoma metastasis-suppressor gene. J. Natl. Cancer Inst 88:1731–1737.

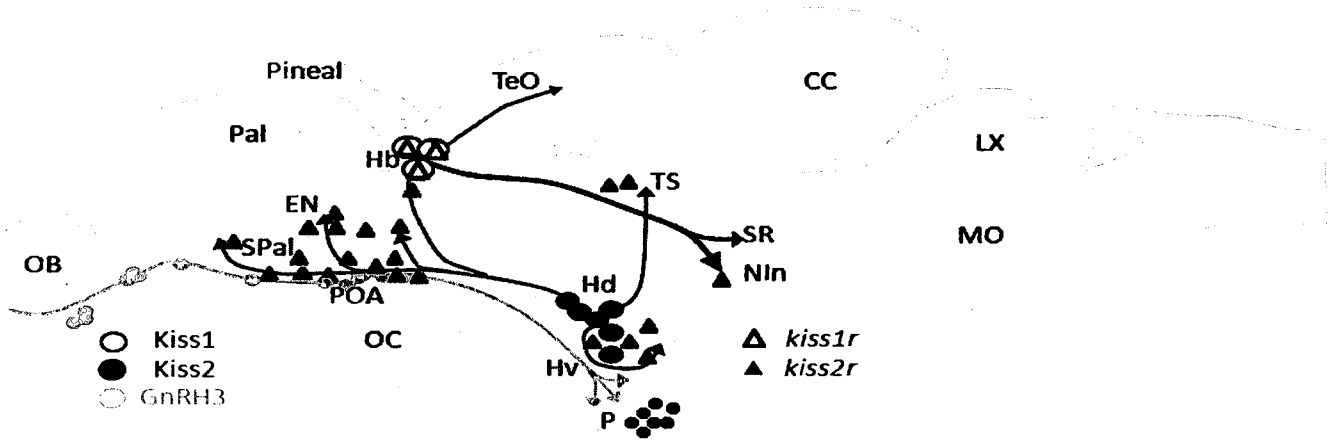
[2]KOTANI M, DETHEUX M, VANDENBOGAERDE A, COMMUNI D, VANDERWINDEN JM, LE

POUL E, BRE´ZILLON S, TYLDESLEY R, SUAREZ-HUERTA N, VANDEPUT F, BLANPAIN C, SCHIFFMANN SN, VASSART G, PARMENTIERM. 2001. The metastasis suppressor gene KiSS-1 encodes kisspeptins, the natural ligands of the orphan G protein-coupled receptor GPR54. J. Biol. Chem., 276:34631–34636.

[3]LEE YR, TSUNEKAWA K, MOON MJ, UM HN, HWANG JI, OSUGI T, OTAKI N, SUNAKAWA Y, KIM K, VAUDRY H, KWON HB, SEONG JY, TSUTSUI K. 2009. Molecular evolution of multiple forms of kisspeptins and GPR54 receptors in vertebrates. Endocrinology, 150(6): 2837-2846.

[4]SERVILI A, LE PAGE Y, LEPRINCE J, CARATY A, ESCOBAR S, PARHAR IS, SEONG JY, VAUDRY H, KAH O. 2011. Organization of two independent kisspeptin systems derived from evolutionary-ancient kiss genes in the brain of zebrafish. Endocrinology, 152(4): 1527-1540.

Fig 1. Schematic representation of the organization of the Kiss1 and Kiss 2 systems in the brain of zebrafish. (Adapted from [4]). Kiss 1 neurons are restricted to the habenula (Hb). They express also kiss1r and project only into the interpeduncular nucleus (IPN) and the raphe (SR). Kiss 2 neurons are located in the dorsal (Hd), lateral and ventral hypothalamus (Hv). These neurons send extensive projections towards the subpallium, the entopeduncular nucleus (EN) the preoptic region (POA), the thalamus, the ventral and caudal hypothalamus and the torus semicircularis (TS). In all these regions, kiss2r are widely expressed. Kiss2 fibers make direct contacts with GnRH3 neurons that project to the pituitary. In contrast, no Kiss fibers were seen in the pituitary (P), whereas Kiss2 positive cells are present in the pars intermedia. CC: Crista cerebellui; LX: ··agal lobe; MO; medulla oblongata; OB: Olfactory bulb; OC: Optic chiasma; Pal: Palium; TeO: optic tectum



Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

EFFECTS OF CATECHOLAMINES ON BRAIN AND PLASMA VASOTOCIN LEVELS IN THE CATFISH *HETEROPNEUSTES FOSSILIS*

Singh R.K.*,° Chaube R.* and Joy K.P.°

*Zoology Department, MMV, Banaras Hindu University, Varanasi, U.P. – 221 005, India
°Department of Zoology, Banaras Hindu University, Varanasi, U.P. – 221 005, India
email: chauberadha@rediffmail.com, kpjoy@bhu.ac.in

Introduction:

Vasotocin(VT) is the basic neurohypophysial nonapeptide hormone in non-mammalian vertebrates having the ring structure of oxytocin and side chain part of Arg (8) – vasopressin and hence showing biological activity of both vasopressin and oxytocin. Vasotocin is involved in various functions like osmoregulation, metabolism, reproduction and behavior [2]. The peptide was characterized in catfish brain and ovary and showed seasonal activity [6]. In the ovary VT is involved in stimulating steroidogenesis, follicular maturation and ovulation [7]. The role of catecholamines in the release of vasopressin has been demonstrated in the past [3, 4]. McNeill *et al.* [5] have described the interaction between the monoamine – and neurophysin – containing pathways. Studies in rat of the vasopressin response to dopamine have yielded varied results (increase, decrease or no effect). However, studies on the catecholaminergic regulation of VT in lower vertebrates (teleosts) are meagre. In teleosts, central catecholamines are involved in the regulation of gonadotropin secretion. The objective of the present study was to investigate the role of catecholamines in VT secretion in the catfish *Heteropneustes fossilis*, since VT has a gonadotropin-like role.

Methods:

Adult female catfish *Heteropneustes fossilis* were collected from local fish markets during preparatory (previtellogenic) phase and acclimatized in laboratory conditions for 48hr. After acclimatization, the fish were injected intraperitoneally with different concentrations of α -methylparatyrosine (α-MPT; 25, 50, 100 and 250µg/g BW), catecholamines – dopamine (DA), norepinephrine (NE), epinephrine (E) (0.5, 1.0, 10 and 100ng/g BW) and L-DOPA (0.5, 1.0, 10 and 100ng/g BW) alone or in combination. After 24hr of the injection, blood samples were taken by caudal puncture and plasma was separated. The fish were sacrificed by decapitation and brains along with pituitary were removed and dropped into cold anhydrous acetone and stored at -70°C. Tissues were processed for VT extraction and quantification by an EIA kit (Bachem Peninsula Laboratories, California, USA; Catalogue No. S- 1239, EIAH - 8121). Data were presented as mean ± SEM and analyzed by one way ANOVA, followed by Tukeys' test.

Results and Discussion:

In the present study the administration of L-DOPA, DA, NE, E and β -MPT (an irreversible inhibitor of CA, rate –limiting enzyme) produced overall significant effects on brain and plasma VT levels ($P \leq 0.05$). In the brain and plasma L-DOPA and DA inhibited VT levels in a concentration – dependent manner. Norepinephrine exhibited biphasic effects, low dose (1ng/g BW) stimulated and higher doses (10 and 100ng/g BW) inhibited VT levels in a concentration – dependent manner. Epinephrine stimulated VT levels in a concentration – dependent manner in both brain and plasma. α-MPT inhibited VT levels in a concentration – dependent manner in both brain and plasma. The administration of α-MPT (250µg/g BW), L-DOPA and DA (10ng/g BW) significantly decreased the VT levels in comparison to the control groups. The percentage decrease was 78.43, 27.78, 22.27 in the brain and 89.05, 32.69 and 29.47 in the plasma, respectively. In the α -MPT + L-DOPA and α-MPT + DA groups, the brain VT level was significantly decreased compared to the α-MPT, L-DOPA or DA groups, although the percentage decrease was not significant in plasma. In the α-MPT + L-DOPA + DA group, the VT level was decreased further significantly. On the other hand, the administration of NE and E (5ng/g BW) significantly increased VT levels in comparison to the control groups. The percentage increase was 31 and 46 in the brain and 16 and 32 in the plasma, respectively. In the α-MPT + NE and α-MPT + E groups, the VT level was significantly decreased compared to the NE or E alone groups, but the level was significantly higher than α-MPT alone group. In the α-MPT + NE + E group, the VT level was significantly increased in comparison to the control group, amine alone group or combination group. Thus, the present study suggests that brain and plasma VT levels (synthesis and release) are differentially modulated by catecholamines, NE stimulating and DA inhibiting. The physiological significance of the inhibitory effect of DA has been reported by Baggio and Ferrari [1] who demonstrated that DA could influence water handling by the kidney and decrease AVP concentration in animals undergoing maximal diuresis. The functional significance of the catecholaminergic regulation of VT, however, needs further study.

Conclusion:

In conclusion, the present data suggests that catecholamines act differentially to modulate vasotocin secretion in the catfish, as in mammals

References:

[1]BAGGIO, C., FERRARI, F. 1981. DPI, a supposed selective agonist of dopamine receptors, strongly increases rat diuresis through α-adrenergic receptor activation. Life Sci., Oxford., 28: 1449-1456.

[2]BALMENT, R. J., LU, E. W., WEYBOURNE, W., WARNE, J. M. 2006. Arginine vasotocin, a key hormone in fish physiology and behavior: A review with insight from mammalian models. Gen. Comp. Endocrinol. 147: 9-16.

[3]FORSLING, M. L. 1976. Pathways and transmitters in vasopressin release. In: Antidiuretic Hormone, pp. 33–39. Churchill Livingstone, Edinburgh.

[4]FORSLING, M. L., WILLIAMS, H. 1983. The effect of intracerebroventricular injections of dopamine on vasopressin release in the normally hydrated and water loaded rat. J. Physiol., 339: 33P.

[5]McNEILL, O. H., HOFFMAN, D. L., KOZLOWSKI, G. P. 1977. Correlative fluorescence-immunocytochemical technique for the localization of monoamines and neurophysin. Am. J. Anat., 149: 613-618.

[6]SINGH, V., JOY, K. P. 2008. Immunocytochemical localization, HPLC characterization, and seasonal dynamics of vasotocin in the brain, blood plasma and gonads of the catfish *Heteropneustes fossilis*. Gen. Comp. Endocrinol., 159: 214-225.

[7]SINGH, V., JOY, K.P. 2009. Effects of hCG and ovarian steroid hormones on vasotocin levels in the female catfish *Heteropneustes fossilis*. Gen. Comp. Endocrinol., 162: 172-178.

GnRH ELEVATES THE CYTOSOLIC CA^{2+} CONCENTRATION IN MEDAKA LH-PRODUCING GONADOTROPE CELLS

Strandabø R.A.U. [1*], **Weltzien F-A.** [1,2] **and Haug T.M.** [1]

[1] Department of Molecular Biosciences, University of Oslo, Oslo, Norway.
* e-mail: ronnaug.strandabo@imbv.uio.no
[2] Department of Basic Sciences and Aquatic Medicine, Norwegian School of Veterinary Science, Oslo, Norway.

Introduction:

Puberty and sexual maturation in vertebrates are regulated through increased activity in the brain-pituitary-gonad axis (BPG). Gonadotropin releasing hormone (GnRH) is the major hypothalamic factor stimulating synthesis and secretion of FSH and LH. In teleosts, FSH and LH are expressed in separate gonadotropes, in contrast to mammals, where a single gonadotrope cell type produces both hormones. The existence of two gonadotrope cell types in teleosts facilitates investigation of how GnRH separately controls secretion of FSH and LH. We have developed a transgenic line of medaka (*Oryzias latipes*) that has GFP coupled to the promoter of LH beta subunit (LHß), thus allowing easy identification of LH-producing cells (see Hildahl *et al.* this meeting). Our overall aim is to unravel the signaling pathways involved in the response to GnRH, eventually comparing FSH and LH expressing cells. Exocytosis of peptide hormones is triggered by elevation of the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$), which is thus an interesting parameter to track during exposures to GnRH. The present study focuses on the immediate increase in $[Ca^{2+}]_i$ observed after application of GnRH2 (cGnRH-II).

Methods:

Pituitaries were dissected out and enzymatically dissociated to establish primary cell cultures, in which the LH-producing gonadotrope cells were easily identified by their expression of GFP. $[Ca^{2+}]_i$ was monitored during exposure to GnRH using an imaging system based on the fluorophore fura-2. The sources of $[Ca^{2+}]_i$ elevation during the GnRH response were investigated by applying either Ca^{2+}-free extracellular solution, the SERCA-pump blocker thapsigargin (Tg), the L-type Ca^{2+} channel blocker nifedipine, or a combination of these options.

Results:

Exposure to GnRH2 (10^{-7} M) elicited a pronounced elevation of $[Ca^{2+}]_i$ in the GFP-positive LH-expressing cells. The time course of the response to GnRH2 was often, but not always, biphasic, with an instant peak followed by a somewhat delayed second peak in $[Ca^{2+}]_i$. In mammalian gonadotropes, a similar pattern of fluctuations in $[Ca^{2+}]_i$ is well known, and the biphasic response is caused by different sources of Ca^{2+}; The initial increase in $[Ca^{2+}]_i$ is due to release from intracellular stores, whereas the second increase comes from influx of external Ca^{2+}. In Ca^{2+}-free extracellular solution, only one phase was observed in the medaka LH-expressing cells following GnRH2 exposure. After emptying the intracellular Ca^{2+} stores by incubation with thapsigargin, we still observed an elevation of $[Ca^{2+}]_i$. This elevation was persistent to adding the L-type Ca^{2+} channel blocker nifedipine to the extracellular solution. No response was detected in thapsigargin-treated cells in Ca^{2+}-free solution.

Conclusion:

GnRH2 induces an increase in $[Ca^{2+}]_i$ in medaka LH-expressing gonadotropes. This increase in $[Ca^{2+}]_i$ probably induces secretion of LH by exocytosis. The elevated $[Ca^{2+}]_i$ depends on both intracellular and extracellular sources. L-type Ca^{2+} channels seem not to have a major role in the increase of $[Ca^{2+}]_i$, because blocking of this channel with nifedipine in thapsigargin-treated cells does not abolish the response to GnRH2. The identity of the Ca^{2+} influx channels involved in the response to GnRH remains to be elucidated.

ANALYSIS OF GENES REGULATED BY HIGH TEMPERATURE IN MEDAKA.

Uchimura T., Hayashi Y., Tashiro S., Shiraishi E., Kitano T.

Department of Biological Sciences, Graduate School of Science and Technology, Kumamoto University, 2-39-1 Kurokami, Kumamoto 860-8555, Japan. e-mail: 107d8079@st.kumamoto-u.ac.jp

Introduction:

Medaka (*Oryzias latipes*) is a teleost fish that has an XX/XY sex determination system. The sex determining gene DMY, which is located on the Y chromosome, has been identified in medaka [1]. Recently, we showed that the XX medaka masculinized by high temperature or cortisol treatment, and high temperature treatment elevated whole-body levels of cortisol. These results suggest that high temperature induces masculinization of XX medaka through elevation of cortisol levels [2]. Cortisol is one of glucocorticoids produced by the interrenal cells in fishes. Cortisol secretion is induced through elevation of adrenocorticotropic hormone (ACTH) levels by corticotrophin-releasing hormone (CRH), which is secreted from hypothalamus [3]. However, the molecular mechanism which regulates CRH secretion in hypothalamus remains unclear. In this study, to elucidate the regulational mechanism of CRH expression in medaka, we analyzed the expression profiling of genes regulated by high temperature in the hypothalamus using DNA microarray analysis.

Methods:

We used *neurogenin3-GFP* transgenic medaka line, which expresses specifically GFP in the hypothalamus. Medaka embryos were reared in ERM (17 mM NaCl, 0.4 mM KCl, 0.27 mM $CaCl_2 2H_2O$, 0.66 mM $MgSO_4$, pH 7) at 26°C or 33°C from 0 days post-fertilization (dpf) to 4 dpf. To investigate the expression profiling of genes regulated by high temperature (33°C) in the hypothalamus, we performed DNA microarray analysis using mRNAs extracted from hypothalamic regions in 4-dpf embryos. After that, we performed quantitative real-time PCR analysis using SYBR Green I Master (Roche) on a LightCycler 480 (Roche) to confirm the expression patterns of genes isolated by DNA microarray.

Results:

As results, the expression of 2,512 genes was increased more than twice by high temperature treatment, and 65 genes, containing *heat shock protein 70-kDa* and *heat shock protein 30-kDa*, had a more than ten-fold increase in their expressions. On the other hand, the expression of 1,981 genes was decreased more than twice by the treatment, and 58 genes, containing *keratin* and *choriolysin H*, had a more than ten-fold decrease in their expressions. The expression patterns of these genes were similar to results by quantitative real-time PCR analysis.

Conclusion:

In this study, we isolated many genes regulated by high temperature in the hypothalamic regions of medaka embryos. These results suggest that their genes isolated here might regulate CRH expression in hypothalamus under the high temperature treatment. In the future, we will analyze the functions of these genes on the sexual differentiation in medaka.

References:

[1]MATSUDA M., NAGAHAMA Y., SHINOMIYA A., SATO T., MATSUDA C., KOBAYASHI T., MORREY C. E., SHIBATA N., ASAKAWA S., SHIMIZU N., HORI H., HAMAGUCHI S., SAKAIZUMI M. (2002) DMY is a Y-specific DM-domain gene required for male development in the medaka fish. Nature, 417, 559-563.

[2]HAYASHI Y., KOBIRA H., YAMAGUCHI T., SHIRAISHI E., YAZAWA T., HIRAI T., KAMEI Y., KITANO T. 2010. High temperature causes masculinization of genetically female medaka by elevation of cortisol. Mol. Reprod. Dev., 77:679–686.

[3]PAPADIMITRIOU A., PRIFTIS K.N. (2009) Regulation of the hypothalamic-pituitary-adrenal axis. Neuroimmunomodulation, 16, 265-271.

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

FSH PLASMA LEVELS DURING TESTICULAR RECRUDESCENCE OF PRECOCIOUS AND NON PRECOCIOUS MALE EUROPEAN SEA BASS USING A NEWLY DEVELOPED SPECIES SPECIFIC ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

Molés G. Rocha A., Espigares F., Gómez A., Carrillo M. and **Zanuy S.**

Department of Fish Physiology and Biotechnology, Institute of Aquaculture of Torre de la Sal (IATS), Spanish National Research Council (CSIC), Ribera de Cabanes s/n 12595, Castellón, Spain. zanuy@iats.csic.es

Introduction:

Sea bass (*Dicentrarchus labrax*) is an important cultured marine fish in Europe in which puberty is intensively studied. In this species large males attain puberty earlier than small ones [1]. Therefore the high rate of growth achieved under intensive culture, results in a high proportion of precocious males before attaining marketable size. At puberty the individual becomes able to reproduce for the first time implying the functional competence of the brain–pituitary–gonad (BPG) axis [2]. Gonadotropins, the follicle-stimulating hormone (FSH) and luteinizing hormone (LH), are components of this axis and are known to be fundamental in the regulation of steroideogenesis and gametogenesis. Although the duality of gonadotropins in fish is well established, the distinct role of piscine FSH and LH is still not clear for most species studied so far mainly due to the scarcity of assays to measure FSH in plasma. By comparing FSH and LH plasma levels in male sea bass that decide or not to mature, we aimed to gain knowledge on the functional discrimination of sea bass gonadotropins in the regulation of the onset of puberty. These data was generated using a newly developed species specific ELISA for FSH.

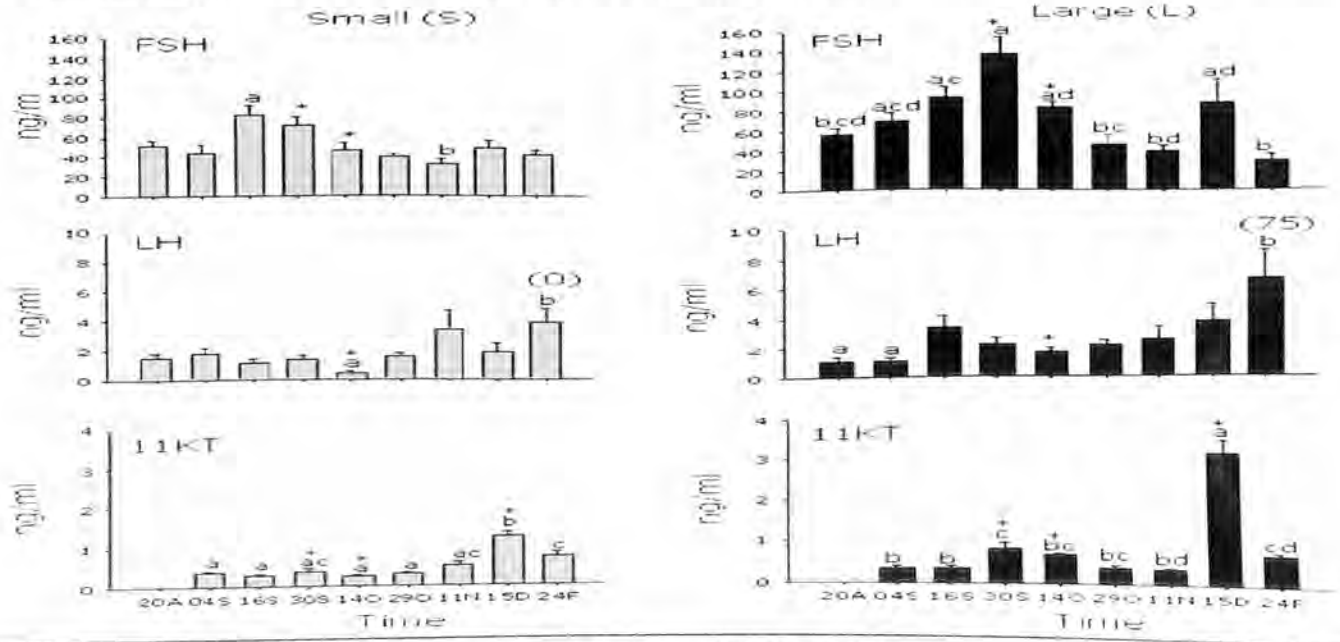
Methods:

Animals and experimental conditions: In early March six month old juvenile male sea bass were placed under simulated natural photoperiod (NP, 40° NL). At sampling fish were size sorted into two groups, one consisting of small (S) and the other of large fish (L). Fish were sampled from August to February, blood collected and plasma used for FSH, LH [3] and 11ketotestosterone (11KT) [4] analysis. Gonads were dissected, processed and testicular growth stages classified according to [1]. *FSH Assay:* The ELISA developed for sea bass FSH used polyclonal antibodies raised against recombinant FSHβ produced in yeast (*Pichia pastoris)* and recombinant FSH dimer produced in the baculovirus system as standard curve and coating.

Results and Discussion:

Small fish displayed a remarkable delay in the progression of testicular development that prevented them from achieving spermiation in February. Large fish entered in spermatogonial mitosis towards meiosis in

Fig.1. Plasma profiles of FSH, LH and 11KT of S and L yearling male sea bass during the first reproductive cycle. Different letters indicate differences among sampling points. Asterisks denote differences between groups at the same sampling time. In brackets percentage of spermiating males in February.



September, almost 1.5 month earlier than S group attaining full spermiogenesis in February. The highest FSH plasma levels were observed in L fish which almost doubled the FSH values of S fish at this time ($p<0,05$). These high levels of FSH were linked to spermatogonial proliferation and associated with the onset of gametogénesis, as in other teleosts, [5]. A transient FSH peak ($p<0.05$) was observed at mid December in L fish coincident with increasing levels of LH suggesting a possible collaborative role of both gonadotropins in eliciting the 11KT surge observed at this time that in turn would drive active spermatogenesis forward and meiosis of germinal cells [2, 4,]. Finally, in L fish LH increased from mid October onwards peaking in February coinciding with full spermiation, revealing its prominent role at late gametogenesis. In S fish levels of LH were lower, started to increase one month later and peaked in February but with about half of the value shown by L fish at this date. In both groups 11-KT exhibited the highest levels by middle of December although values of L doubled those of S ($p<0.05$).

In L fish a transient but significant elevation of 11KT was observed by the end of September coincident with the highest levels of FSH observed at this date, confirming the steroidogenic role of FSH in sea bass [6] and its mediation in stimulating early stages of spermatogenesis as previously suggested in other fish [5]. Though small fish had similar endocrine profiles as L fish, their amplitude were much lower being most likely the reason why functional competence of the BPG axis in former fish did not occur. Apparently fish size is a permissive condition to guaranty full effectiveness of the hormonal actions. The ELISA for sea bass FSH showed a high degree of parallelism between the standard curve and serially diluted plasma and pituitary samples of this species. The assay has a high sensitivity and reliability.

Supported by the EU (LIFECYCLE; FP7-222719-1), the GV (ACOMP/2010/086 and PROMETEO/2010/003) and MICINN (AQUAGENOMICS; CSD2007-00002) Projects to S.Z. F. Espigares was sponsored by a JAE-Predoc CSI

References:

[1]BEGTASHI, I., RODRÍGUEZ, L., MOLES, G., ZANUY S. AND CARRILLO, M. 2004. Long-term exposure to continuous light inhibits precocity in juvenile male European sea bass (*Dicentrarchus labrax* L.). I. Morphological aspects. Aquaculture, 241:539-559

[2]TARANGER, G.L., CARRILLO, M., SCHULZ, R.W., FONTAINE, P., ZANUY, S., FELIP, A., WELTZIEN, F-A., DUFOUR, S., KARLSEN, Ø., NORBERG, B., ANDERSSON, E. AND HANSEN T. 2010. Control of puberty in farmed fish. Gen. Comp. Endocrinol., 165: 483–515.

[3]MATEOS, J. MAÑANOS, E., SWANSON, P., CARRILLO, M. AND ZANUY S. 2006. Purification of luteinizing hormone (LH) in the sea bass (*Dicentrarchus labrax*) and development of *a specific immunoassa*y. Cien. Mar., 32: 271-283.

[4]RODRÍGUEZ, L., BEGTASHI, I., ZANUY, S. AND CARRILLO, M. 2005. Long-term exposure to continuous light inhibits precocity in European male sea bass (*Dicentrarchuslabrax* L.): hormonal aspects. Gen. Comp. Endocrinol., 140: 116–125.

[5]SCHULZ, R.W., DE FRANÇA, L.R., LAREYRE , J.J., LEGAC, F., CHIARINI-GARCIA, H., NOBREGA, R. H., AND MIURA, T. 2010. Spermatogenesis in fish. Gen. Comp. Endocrinol., 165: 390-411

[6]MOLÉS, G., GÓMEZ, A., ROCHA, A., CARRILLO, M. AND ZANUY, S. 2008 Purification and characterization of follicle-stimulating hormone from pituitary glands of sea bass (*Dicentrarchus labrax*). Gen. Comp. Endocrinol., 158: 68-76.

Sex Determination & Gonad Differentiation

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

SEX IN SALMONIDS: FROM GONADAL DIFFERENTIATION TO GENETIC SEX DETERMINATION

Yano A.[1], Nicol B.[1], Valdivia K.[1], Juanchich A.[1], Desvignes, T.[1], Caulier, M.[1], Vazir Zadeh A.[1], Guerin A.[1], Jouanno E.[1], Nguyen T.[1], Mourot B.[1], Rime, H.[1], Bodinnier P.[1], Cauty C.[1], Quillet E.[2], Guyomard R.[2], Bobe J.[1], Fostier A[1], Guiguen Y. [1].

[1] Institut National de la Recherche Agronomique, UR 1037, INRA-SCRIBE, IFR 140, Campus de Beaulieu, Rennes, France. Fax: +332223465820. Yann.Guiguen@rennes.inra.fr

[2] Institut National de la Recherche Agronomique, UMR1313, GABI, Domaine de Vilvert, 78352 Jouy en Josas Cedex, France.

Introduction:

Members of the salmonid family are present worldwide and many of them are species of major importance for aquaculture, wild stock fisheries or recreational sport fisheries. Sex differentiation and sex determination in salmonid species have been studied since the early twenties [1, 2] and, from that moment on, a lot of data has been gathered on this topic both because of its importance for fisheries and aquaculture and because salmonids can be excellent research models [3]. This communication will review some of the available published knowledge on sex differentiation and sex determination in salmonid species along with some results obtained from genomic, genetic and expression screens studies that have been carried out recently in our laboratory.

All salmonid species in which sex determination has been studied in details thus far belong to the Salmoninae subfamily and they possess a male heterogametic sex determination system (XY/XX) (reviewed in [4]). This strict genetic system has been used to produce genetic all-male and all-female populations (all XX or all XY individuals) that are now one major experimental feature allowing to work on male and female sex differentiation at very early developmental stages when the gonad is not yet histologically differentiated. We extensively used these monosex populations to compare the early steps of rainbow trout (*Oncorhynchus mykiss*) male and female gonadal differentiation using microarrays, *in situ* hybridization screens and next generation sequencing (NGS).

Results and Discussion:

Our recent results first demonstrated that gonadal differentiation starts prior to any histological differentiation and much earlier than initially thought. The first sexually dimorphic gene expression patterns were detected around hatching (30 days post fertilization = 30 dpf). Among these early actors, the estrogen synthesis potentiality of the differentiating ovary was confirmed to be crucial [5], with an early and female-specific expression of ovarian aromatase (*cyp19a1a*). We also found that follistatin (*fst*) is the earliest gene co-expressed with *cyp19a1a* in the differentiating ovary suggesting that *fst* is also a key player in ovarian differentiation and a potential regulator of *cyp19a1a* expression (Nicol et al., this meeting). *In situ* hybridization screens revealed an unexpected complexity of the early differentiating gonads but also that these gonads are already well structured. For instance, we identified that transforming growth factor beta 2 (*tgfb2*) expression pattern clearly delimits the ventral epithelium of the differentiating gonads. A lot of these new actors of the gonadal sex differentiation cascade were identified from microarray screens, but we recently switched to NGS to identify novel genes that would be specifically expressed in embryonic male or female gonads. Using Roche's 454 Titanium sequencing technology we compared cDNA libraries made from male and female embryonic gonads sampled a few days after hatching. We were thus able to characterize a novel gene that could be a prime candidate as a rainbow trout master sex determining gene. This gene is expressed only during testicular differentiation and tightly linked to the Y chromosome (Yano et al., this meeting). Furthermore, even though rainbow trout sex determination is mainly genetic, some recent studies also report that temperature can influence gonadal sex differentiation leading to skewed sex ratio [6, 7]. Using a genetic all-female rainbow trout population carrying a masculinizing mutation [8] we demonstrated that rearing larvae at high temperature can increase the masculinization rate of this population (Valdivia et al., this meeting) and that *cyp19a1a* was down-regulated during gonadal differentiation in these mutated populations.

Conclusion:

By combining various genomics approaches we now have a better understanding of the early steps of gonadal sex differentiation in rainbow trout including the first sex determination switch acting at the top of the gene cascade.

References:

[1]MRSIC W. 1923. Die spatbefruchtung und deren Einfluss auf Entwicklung und Geschlechtsbildung. Arch. Micr. Anat., 98: 129-209.

[2]MRSIC W. 1930. Uber die Eireifung bei der Forelle und deren Bedeutung fur die ubliche Methode der

kunstlichen Laichgewinnung. Arch. Hidrobiol., 21: 649-678.

[3]THORGAARD G.H., BAILEY G.S., WILLIAMS D., BUHLER D.R., KAATTARI S.L., RISTOW S.S., HANSEN J.D., WINTON J.R., BARTHOLOMEW J.L., NAGLER J.J., et al. 2002. Status and opportunities for genomics research with rainbow trout. Comp. Biochem. Physiol. B Biochem. Mol. Biol., 133: 609-646.

[4]DAVIDSON W.S., HUANG T.K., FUJIKI K., VON SCHALBURG K.R., KOOP B.F. 2009. The sex determining loci and sex chromosomes in the family salmonidae. Sex. Dev., 3: 78-87.

[5]GUIGUEN Y., FOSTIER A., PIFERRER F., CHANG C.F. 2010. Ovarian aromatase and estrogens: a pivotal role for gonadal sex differentiation and sex change in fish. Gen. Comp. Endocrinol., 165: 352-366.

[6]MAGERHANS A., HORSTGEN-SCHWARK G. 2010. Selection experiments to alter the sex ratio in rainbow trout (*Oncorhynchus mykiss*) by means of temperature treatment. Aquaculture, 306: 63-67.

[7]MAGERHANS A., MULLER-BELECKE A., HORSTGEN-SCHWARK G. 2009. Effect of rearing temperatures post hatching on sex ratios of rainbow trout (*Oncorhynchus mykiss*) populations. Aquaculture, 294: 25-29.

[8]QUILLET E., AUBARD G. , QUEAU I. 2002. Mutation in a sex-determining gene in rainbow trout: Detection and genetic analysis. J. Heredity, 93: 91-99.

PROTANDROUS BLACK PORGY AS A MODEL ANIMAL TO INVESTIGATE GONADAL SEX DIFFERENTIATION AND SEX CHANGE

Wu G.C., Chang C.F.

Department of Aquaculture, National Taiwan Ocean University, Keelung 20224, Taiwan
Fax: +886-2-2462-1579 e-mail: B0044@mail.ntou.edu.tw

Introduction:

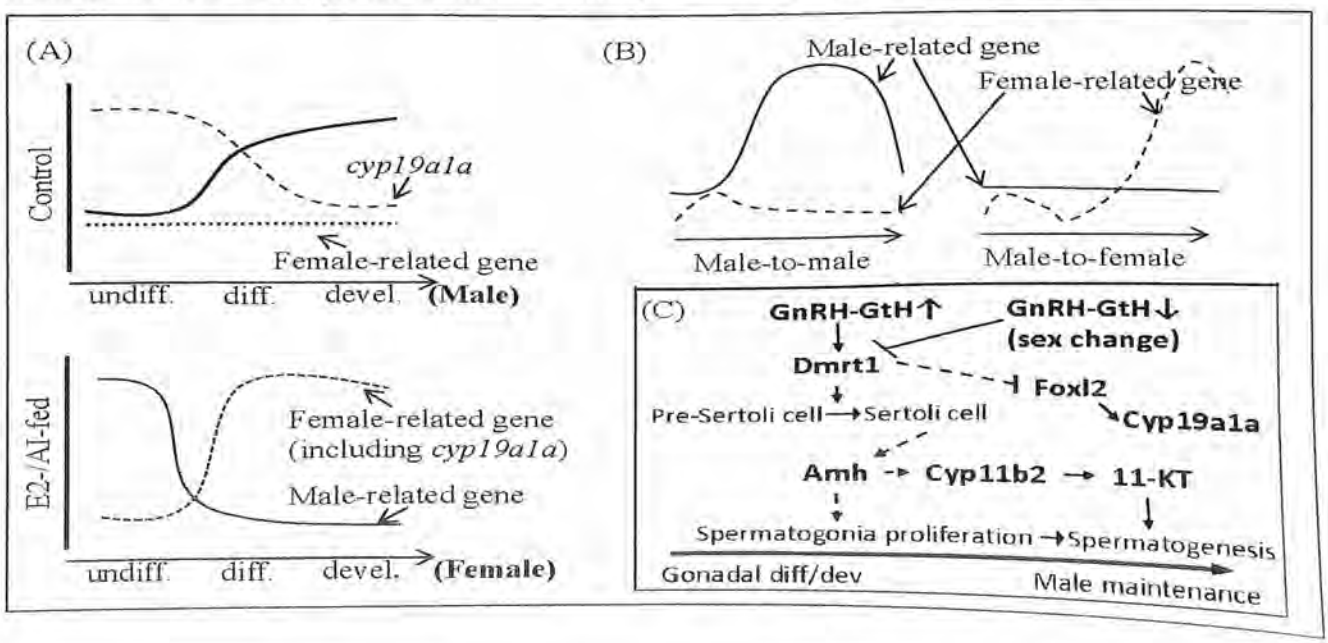
Animals have two distinct systems that define sexual fate: gonochores (which have fixed sexes) and hermaphrodites (which can have both sexes during their life). This sex change occurs in animals, but sex change is lost from amphibians to mammals in vertebrates. Thus, if we wish to clarify the etiology of diverged sex determination strategies, an alternative approach is required. The teleosts, which include gonochores and hermaphrodites, represent an ideal case for this type of study, as the sex determination pathway (male: *dmrt1*, *amh*, *amhr2*, *sf1*, and *P45011β*; female: *wnt4*, *foxl2*, and *P450arom*) has been well studied in gonochoristic fishes, and a great deal of information is available on hermaphrodite fish (black porgy, *Acanthopagrus schlegeli*). Black porgies are functional males for the first two years of life, but about 50% of them transform into females during the third year. There is no clear difference in body length/size or outer characteristics between a male and female in the 3-yr-old black porgy. Therefore, black porgies are a unique model to study the sex differentiation of the gonad and development of the bisexual gonad, especially due to the close interaction of the testicular and ovarian tissues in the gonad. Thus, we chose the protandrous black porgy to investigate the mechanisms of sex development at the molecular, cellular and organismic levels.

Methods:

In this report, we examined the consequences of sex determination pathway genes (male: *dmrt1*, *amh*, *amhr2*,and *sf1*; female: *wnt4*, *foxl2*, and *cyp19a1a*) in relation to 1) gonadal sex differentiation, i.e., from undifferentiated to differentiated juveniles (0^+-yr-old fish), given the functional mono-male sex pattern observed in this species during the first and second reproductive seasons; 2) the effects estradiol (E2) on sex differentiation and gonad development using exogenous E2- and aromatase inhibitor (AI)-treatment in 0^+-yr-old fish; 3) the annual profile of gene expression in the bisexual gonad of 1^+- to 3-yr-old fish, especially related to the natural sex change; 4) the testicular effects on the development of ovarian tissue in the bisexual gonad and the expression of related genes by *in vivo* surgical manipulation of testicular tissue and ovarian tissue; and 5) gonadotropin hormone effects during different gonadal stages. Gene expression was quantified by real-time PCR and RT-PCR. Localization and expression of gene/protein was performed by *in situ* hybridization and immunohistochemical staining.

Results and Discussion:

Male-related genes (*dmrt1*, *amh*, *amhr2*, and *sf1*) have long been considered important for male sexual



dmrt1 ↔ Fol 2

differentiation in vertebrates. Based on quantitative real-time PCR data, transcription levels of male-related genes were significantly increased after testicular differentiation (Fig. A)[1-4]. Transcription levels of female-related genes (*wnt4*, *foxl2*, and *cyp19a1a*) had no changes during testicular differentiation, but expressions of female-related genes were significantly increased at E2- and AI-stimulated ovarian differentiation (Fig. A)[1-3]. These data showed that male/female-related genes did correlate with testicular/ovarian differentiation and development in black porgy. After gonadal differentiation, male-related genes were continued to highly express in the testicular tissue in 0^+-, 1^+- and 2^+-yr-old fish, and the expression was significantly decreased in testicular tissue at female phase (Fig. B)[1,4]. By contrast, female-related genes were weakly expressed in ovarian tissue at male phase (0^+-, 1^+- and 2^+-yr-old fish), and expression was significantly increased in ovarian tissue at female phase (testis-removal sex change in 1^+-yr-old fish and natural sex change in 2^+-yr-old fish) (Fig. B)[1-3], respectively. hCG could regulate *amh* and *amhr2* expression in testicular tissue but not in ovarian tissue [4]. After hCG injection, the responses of many genes were differential between active and inactive gonadal tissues [4]. The data suggest that the testicular/ovarian tissues in a single bisexual gonad had differential responses to the endogenous stimulation.

Conclusion:

Protandrous black porgy with predictably and dramatically anatomical changes in the reproductive system, thus making it is a unique model fish to study sex differentiation and development. This study described the sequential expressions in certain male and female related genes. According to the data from sex change in the testis-removal fish, we suggest that maintaining the male phase will inhibit sex change in the bisexual gonad (Fig. B). This male phase maintenance may be regulated by GnRH-GtH-gonadal dmrt1 axis as summarized in the Fig. C. Further, a transition in functional sex from a male to female requires one or more changes in the sex determination pathway to occur.

References:

[1]WU, G.C., TOMY, S., CHANG, C.F. 2008. The expression of *nrOb1* and *nr5a4* during gonad development and sex change in protandrous black porgy fish, *Acanthopagrus schlegeli*. Biol. Reprod., 78: 200-210.

[2]WU, G.C., TOMY, S., NAKAMURA, M., CHANG, C.F. 2008. Dual roles of cyp19a1a on gonadal diffrentiation and development in the protandrous black porgy, *Acanthopagrus schlegeli*. Biol. Reprod., 79: 1111-1120.

[3]WU, G.C., CHANG, C.F. 2009. wnt4 is associated with the development of ovarian tissue in the protandrous black porgy, *Acanthopagrus schlegeli*. Biol. Reprod., 81: 1073-1082.

[4]WU, G.C., CHIU, P.C., LYU, Y.S., CHANG, C.F. 2010. The Expression of *amh* and *amhrII* is associated with the development of gonadal tissue and sex change in the protandrous black porgy, *Acanthopagrus schlegeli*. Biol. Reprod., 83: 443-453.

TRANSCRIPTOME ANALYSIS OF THE MOLECULAR MECHANISMS FOR TILAPIA SEX DETERMINATION, DIFFERENTIATION AND SEX REVERSAL

Wang D.S.

Key Laboratory of Freshwater Fish Reproduction and Development (Ministry of Education), Key Laboratory of Aquatic Science of Chongqing, School of Life Science, Southwest University, Chongqing, 400715, China.
Fax: +86-2368253005, Email : wdeshou@swu.edu.cn

Introduction:

The Nile tilapia, a worldwide cultured fish, is a good model for the study of sex determination and differentiation, due to the availability of monosex offsprings and short (14days) spawning cycle. In order to elucidate the molecular mechanism for tilapia sex determination, differentiation and sex reversal, analysis of EST sequences from juvenile and adult gonads, and transcriptome analysis of gene expression profile in gonads during the induced sex reversal were performed.

Methods:

Animal: All genetic female (XX) and male (XY) tilapia (*Oreochromis niloticus*) were obtained by artificial fertilization of eggs from normal females (XX) with sperm from either sex-reversed males (XX) or super males (YY), respectively. Tilapias were reared in 0.5-ton tanks with re-circulating aerated fresh water at 26°C under natural photoperiod.

Drug treatment

The aromatase inhibitor Letrozole was dissolved in 95% ethanol, and then added to the feed for long-term treatment of all 3-month-old XX female tilapia until sex reversal.

Transriptome, ESTs and genome sequences

Gonads from Letrozole treated group and control group were sampled at 0, 10, 20, 45, 70, 90 dat (days after treatment). Histological examination was performed to confirm the sex reversal. Total RNA was extracted from all the samples using TRIzol reagent (TaKaRa, Japan) and cDNA was prepared for the real-time PCR. Total RNA extracted from gonad of 0, 45, 90 dat fish and control fish were also used for transcriptome analysis by RNA sequencing. The Illumina RNA-seq was performed by BGI (Beijing Genomics Institute). EST sequences of 5-35 dah (days after hatching) tilapia gonads were from Prof. Nagahama (NIBB, Japan). EST sequences of adult tilapia gonads were downloaded from NCBI EST database. Tilapia genome sequences were downloaded from Broad Institute http://www.broadinstitute.org/ftp/pub/assemblies/fish/tilapia/Oreni11/. Based on these sequences, three database, i.e. Gonad-EST, Transcriptomes, Genome, were established for local blast analysis using the Local Blast software downloaded from NCBI.

Results and Discussion:

Totally 22385 ESTs from 5-35dah male and female gonad and 16059 ESTs from adult ovary and testis were used for the construction of the database. RNA sequencing data of the gonad of 0, 45, 90 dat fish and control fish were used for transcriptome database. Tilapia genome sequences (5899 scaffolds) were used for genome database. The three databases were correlated with each other for gene expression pattern in gonad. Some important gene families, such as FOX (forkhead), SOX, WNT, FGF, GATA genes, DM domain genes, NR (nuclear receptor), and genes involved in steroidogenesis, meiosis, protein synthesis were analyzed in detail. Our data indicate that these gene families are important for tilapia sex determination, differentiation and sex reversal. Many members from these gene families were found to be sexual dimorphically expressed in tilapia gonad, and their expression pattern reversed during the induced sex reversal. For example, 29 FOX genes were found to be expressed in the 3-month-old tilapia gonad, with 12 and 17 genes being expressed much higher in the ovary and testis, respectively. Some of them even showed sexual dimorphic expressions in gonad from as early as 5dah, indicating that like Foxl2, these FOX genes might also play important roles in fish gonad differentiation and development. Most interestingly, some translation elongation factors and ribosome genes showed significant sexual dimorphic expression profiles in gonad during normal development and induced sex reversal, which suggests for the first time that sex specific protein synthesis in male and female gonad might be undertaken by different translation elongation factors and ribosome proteins. Many new genes or duplicated genes, which were never reported in teleosts or even other vertebrates, were discovered. The combination of transcriptome data with the genome sequences also enabled us to discover novel alternatively spliced forms which have never been reported before. Histological examination of the gonad samples fixed at different time during Letrozole treatment revealed that sex reversal was a gradual process, started from ventral to dorsal and from posterior to anterior of the gonad. Transcriptome analysis showed that *Sf-1* and *Cyp19a1a* were significantly up-regulated during the early period of treatment (0-45 dat), which

was in consistence with the aromotase protein level, as revealed by immunohisto-staining with tilapia aromatase anti-sera. Foxl2 was found to be down regulated, while Dmrt1 was up-regulated gradually during the treatment period. It is worth noting that the up-regulation of *Cyp11b1*, the key enzyme responsible for 11KT production, was up-regulated only at the late treatment period.

Conclusion:

Our data indicate that estrogen is not only critical for the ovarian differentiation, but also for ovarian maintenance, while androgen might be the consequence, instead of being the reason for the Letrozole induced sex reversal. Besides Sf-1, Foxl2, Dmrt1, numerous factors were found to be critical for the sex determination and sex reversal. The establishment of tilapia local blast databases also enabled us to investigate the expression profile of numerous genes in gonadal development. The completion of the tilapia genome sequencing and gonad transcriptome sequencing will surely promote the elucidation of the molecular mechanism for tilapia sex determination and differentiation.

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

A DUPLICATED, Y-LINKED COPY OF THE ANTI-MULLERIAN GENE DETERMINES TESTICULAR FORMATION IN PATOGONIAN PEJERREY *ODONTESTHES HATCHERI*

Hattori R.S.[(1)], **Murai Y.**[(2)], **Oura M.**[(2)], **Masuda S.**[(2)], **Majhi S.K.**[(2)], **Sakamoto T.**[(2)], **Fernandino J.I.**[(3)], **Somoza G.M.**[(3)], **Yokota M.**[(2)] and **Strüssmann C.A.**[(2)]

(1) Departamento de Genética e Biologia Evolutiva, Laboratório de Ictiogenética, University of Sao Paulo, Matao Street 227, Cidade Universitária 05508-090, São Paulo, Brazil, Fax +55-11-3091-7553 email:shuheibio@yahoo.co.jp
(2) Graduate School of Marine Science and Technology TUMSAT, Tokyo, Japan.
(3) Instituto de Investigaciones Biotecnologicas INTECH, Chascomús, Argentina.

Introduction:

In many vertebrates, gonadal sex determination follows a sequence of genetically programmed events. However, only a handful of genes have been described as the ultimate triggers of differentiation and the mechanism of genotypic sex determination still remains elusive for most vertebrates. In order to clarify the process of genotypic sex determination in the Atherinopsid fish Patagonian pejerrey (*Odontesthes hatcheri*), a species with the XX-XY sex determining system, we have conducted transcriptomic analyses of several sex-related genes during early sex differentiation. Those studies lead to the identification of a duplicated copy of the anti-Müllerian hormone (*amh*) gene which plays a critical role in sex determination of this species.

Methods:

The complete cDNA sequences of the two forms of *amh* were obtained by degenerated RT-PCR and RACE PCR using larvae and adult samples. The non-transcribed sequence of the male-specific *locus* was obtained by PCR of genomic DNA from an XY individual. Fluorescence *in situ* hybridization (FISH) was performed for chromosome mapping. mRNA expression was analyzed by RT-PCR and *in situ* hybridization during gonadal sex differentiation. Functional analysis was conducted by microinjection of antisense morpholinos.

Results and Discussion:

The male-specific (*amhy*) (termed *amhy*) gene showed 92.2% of amino acid identity in relation to the autosomal one (termed *amha*). Physical mapping of *amhy* allowed the identification of the Y chromosome in this species and its transcripts were detected from 6 days after fertilization in presumptive Sertoli cells. *Amhy* knockdown in XY embryos resulted in the development of ovaries.

Conclusion:

These results establish *amhy* as a candidate for the master sex-determining gene in *O. hatcheri* and for the first time associate a hormone-related gene, a member of the TGF-β superfamily, with such a role.

* knock down
* [illegible]

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

THE FATE OF OVARIAN SOMATIC CELLS DURING SEX CHANGE IN THE PROTOGYNOUS WRASSE, *HALICHOERES TRIMACULATUS*

Nozu R.*, Horiguchi R.°, Murata R.°, Kobayashi Y.° and **Nakamura M.°**

*Graduate School of Engineering and Science, University of the Ryukyus, Motobu, Okinawa 905-0227, Japan
°Tropical Biosphere Research Center, University of the Ryukyus, Motobu, Okinawa 905-0227, Japan Fax: +81-980-47-6072 email: masaru@lab.u-ryukyu.ac.jp

Introduction:

The three-spot wrasse (*Halichoeres trimaculatus*) changes sex from female to male. Oocytes disappear completely, and male germ cells and somatic cells comprising testicular tissue arise anew during the process of sex change [1]. However, little is known about the fate of germ and somatic cells during sex change. In our previous study, we analyzed cellular behavior (apoptosis and cell proliferation) in the sex changing gonads [2]. Unexpectedly, few apoptotic somatic cells were found to be present during sex change. Interestingly, in gonads at an early stage of sex change, cell proliferation was detected in many granulosa cells surrounding the degenerating oocytes, a few epithelial cells covering ovigerous lamella and somatic cells associated with gonial germ cells. These results suggest that ovarian somatic cells survive during the regression of ovarian tissue. In the present study, we investigated the fate of ovarian somatic cells during sex change by tracking proliferating somatic cells.

Methods:

To track proliferating cells, Bromodeoxyuridine (BrdU) pulse-chase analysis was performed (Fig. 1). BrdU was injected into the abdominal cavity of an early stage of sex change fish. After 2 h injection, we carried out gonadal biopsy to check localizations of BrdU-incorporated cells. Two weeks later, the identical gonads were examined for BrdU by immunodetection.

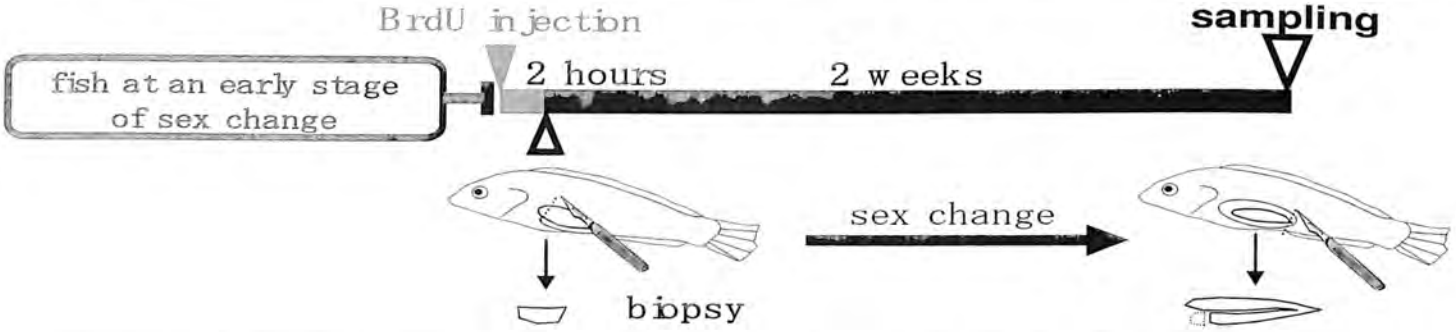


Fig. 1. Strategy of BrdU pulse chase analysis. To confirm the localizations of BrdU-incorporated cells, a piece of gonads were sampled by biopsy at 2 hours after BrdU injection. The identical gonads were examined for BrdU by immunodetection after 2 weeks.

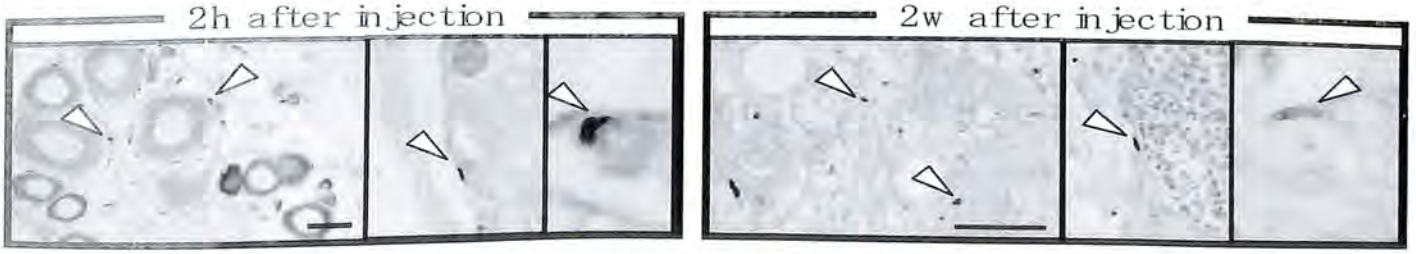


Fig. 2. BrdU pulse chase analysis. BrdU-incorporated cells at an early stage of sex change remain in the gonads at 2 weeks after BrdU injection. Arrowheads indicate BrdU positive cell.

Additionally, to identify the kinds of BrdU-incorporated cells in the gonads, we immunohistochemically determined the locations of Foxl2 (marker for granulosa cells), DMRT1 (marker for Sertoli cells) and P450scc (marker for steroid producing cells).

Results and Discussion:

At the early stage of sex change, BrdU-immunoreactive somatic cells were of three types, granulosa cells, epithelial cells and somatic cells associated with gonial germ cells (Fig. 2). Two weeks after BrdU injection, fish had gonads containing almost testicular tissue. In the spermatogenic gonads (2 weeks after injection), BrdU signal was detected in somatic cells in the central region of the lamella, Sertoli cells surrounding the cyst of spermatogenic germ cells, and somatic cells associated with gonial germ cells (Fig. 2). Foxl2 immunoreactivity was detected in somatic cells in the central region of the lamella, and some of the Foxl2-positive cells were also BrdU-positive. These results imply that proliferating granulosa cells at the early stage of sex change remain in the gonads during sex change. DMRT1 immunoreactivity was detected in Sertoli cells, some of which were BrdU-positive. P450scc immunoreactivity was detected in interstitial cells, and some of the somatic cells in the central region of the lamella were BrdU-positive. These results indicate that cells proliferating during the early stage of sex change might be source of testicular somatic cells in the three-spot wrasse.

Conclusion:

The present study examines the fate of ovarian somatic cells during sex change in the three-spot wrasse. From the present results, we conclude that some functional somatic cells of the ovary are reused as testicular somatic cells (i.e., supporting cells and steroid producing cells) in the restructuring that forms the testes.

References:

[1]NOZU, R., KOJIMA, Y., NAKAMURA, M. 2009. Short term treatment with aromatase inhibitor induces sex change in the protogynous wrasse, *Halichoeres trimaculatus*. Gen. Comp. Endocrinol., 161:360-364.

[2]NOZU, R., KOJIMA, Y., NAKAMURA, M. 2009. Cellular behavior in the gonad during sex change in the protogynous wrasse. 16th International Congress of Comparative Endocrinology Abstract Book, pp. 112.

GROWTH AND SEX DIFFERENTIATION RELATIONSHIP IN THE EUROPEAN SEA BASS (*DICENTRARCHUS LABRAX*)

Díaz, N., Ribas, L., Piferrer, F.

Institut de Ciències del Mar, Consejo Superior de Investigaciones Científicas (CSIC) Passeig Marítim, 37-49, 08003 Barcelona, Spain. Email: piferrer@icm.csic.es

Introduction:

Phenotypic sex is the result of the processes of sex determination (genetic or environmental) [1] and sex differentiation [2], and is highly labile in fish, since in several species it is affected by environmental factors such as temperature, pH or density. The European sea bass (*Dicentrarchus labrax*) is a gonochoristic species where its polygenic sex determination mechanism [3] is influenced by temperature [4]. Growth dimorphism is present in teleost fish, but while in some species males are the largest fish [5] in others, such as the European sea bass, females are larger than males [3, 6, 7, 8]. A correlation between growth and final sex has been found in studies where size-grading of sea bass populations applied at the early stages of development generated two well differentiated populations: one female-dominant population (the largest fish) and one male-dominant (the smallest fish) [9]. It has been suggested that gonadal differentiation is dependent on whether a certain size is reached by a certain developmental time [10, 11], suggesting that early growth is the factor controlling sex. However, in fish it has been also observed that growth can depend on phenotypic rather than genotypic sex [8, 12], which would suggest that the course of sex differentiation determines early growth. The objective of this study was to investigate these two alternative possibilities in the European sea bass.

Methods:

European sea bass larvae were reared under standard conditions. Fish were size-graded at ~4 cm into three experimental groups based on growth (fast, F; medium, M and slow, S). When fish were ~6.5 cm the M-group was further subdivided in three groups: control, Estradiol (E2)- and Fadrozole (Fz)-treated groups, whereas the F and S groups were in turn subdivided into fast-fast (FF), fast-slow (FS), slow-fast (SF) and slow-slow (SS) groups based on subsequent forced growth rates achieved by either *ad libitum* feeding or food restriction until 232 dph (Fig. 1). Fish were periodically sampled and weight and length determined. At 337 dph the sex ratio was determined visually and histologically.

Results:

Significant differences ($P<0.05$) between groups in length and weight at 232 dph were observed according to the regime experienced, i.e., FF >SF>FS>SS (Table 1). Males predominated in the Ctrl group as is typical in cultured sea bass. However, groups initially exposed to fast growth (FF and FS) had the highest percentage of females and a sex ratio significantly different ($P<0.05$) from that of the Ctrl group. Groups initially exposed to slow growth (SS and SF) had a sex ratio in between that of the Ctrl group ($P>0.05$) and the FF and FS groups ($P<0.05$). In addition, there was a relationship between growth and sex ratios in the treated groups (Growth and percent males: Ctrl = Fz > E2 (Table 1).

Conclusions:

Together, the results of this study suggest that sex ratios not only are established well before the first signs of sex differentiation but, importantly, that they are essentially not affected by growth rates during the period of gonadal differentiation. Further, due to the low number of females in the Ctrl group we could not compare the growth of Ctrl females with E2-treated females. Nevertheless, the fact that right after the end of the treatment E2-treated fish (~94% females) were smaller than Ctrl fish (~2% females) indicates that sex can also influence growth, although a deleterious effect

Figure 1. Growth and sex differentiation experimental design (F, Fast; M, Medium; S, Slow growth).

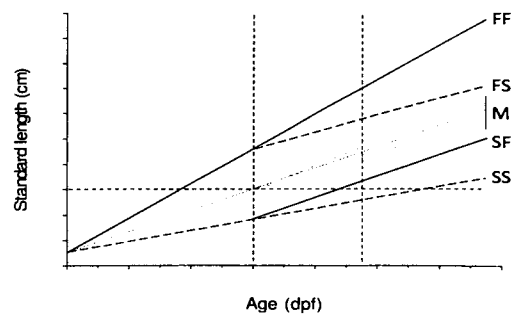


Table 1. Weight and length of European sea bass at 232 dph and sex ratios at 337 dph. Groups with different letters are significantly different ($P<0.05$)

	Length ± SEM	Weight ± SEM	Male percent
FF	$12,93^{a} \pm 0,14$	$35,51^{a} \pm 0,99$	$66,15^{b}$
FS	$10,89^{b} \pm 0,08$	$18,36^{b} \pm 0,34$	$61,19^{b}$
Ctrl	$12,00^{c} \pm 0,08$	$27,30^{c} \pm 0,53$	$97,83^{a}$
Fz	$11,93^{c} \pm 0,09$	$27,06^{c} \pm 0,64$	100^{a}
E2	$11,53^{d} \pm 0,41$	$23,11^{d} \pm 0,07$	$5,97^{c}$
SF	$11,18^{b} \pm 0,15$	$25,00^{c} \pm 0,63$	$90,57^{a}$
SS	$9,39^{e} \pm 0,09$	$12,93^{e} \pm 0,23$	$92,19^{a}$

of E2 treatment cannot be excluded.

References:

[1]PENMAN, D.J., PIFERRER, F. 2008. Fish gonadogenesis. Part 1. Genetic and environmental mechanisms of sex determination. Rev. Fish Sci., 16: 14-32

[2]PIFERRER, F., GUIGUEN, G. 2008. Fish Gonadogenesis. Part II: Molecular Biology and Genomics of Sex Differentiation. Rev. Fish Sci., 16: 35-55.

[3]VANDEPUTTE, M., DUPONT-NIVET, M., CHAVANNE, H., CHATAIN, B. 2007. A polygenic hypothesis for sex determination in the European sea bass (*Dicentrarchus labrax*). Genet., 176:1049-1057.

[4]PIFERRER, F., BLÁZQUEZ, M., NAVARRO-MARTÍN, L., GONZÁLEZ, A. 2005. Genetic, endocrine, and environmental components of sex determination and differentiation in the European sea bass (*Dicentrarchus labrax* L.). Gen. Comp. Endocrinol., 142:102-110.

[5]TOGUYENI, A., FAUCONNEAU, B., BOUJARD, T., FOSTIER, A., KUHN, E.R., MOL, K.A., BAROILLER, J.F. 1997. Feeding behaviour and food utilisation in tilapia, *Oreochromis niloticus*: effect of sex ratio and relationship with the endocrine status. Physiol. Behav., 622:273-279.

[6]IMSLAND, A.K., FOLKVORD, A., GRUNG, G.L., STEFANSSON, S.O., TARANGER, G.L. 1997. Sexual dimorphism in growth and maturation of turbot, *Scophthalmus maximus* (Rafinesque, 1810). Aquacult. Res., 28:101-114.

[7]BLÁZQUEZ, M., CARRILLO, M., ZANUY, S., PIFERRER, F. 1999. Sex ratios in offspring of sex-reversed sea bass and the relationship between growth and phenotypic sex differentiation. J. Fish Biol., 55:916-930.

[8]SAILLANT, E., FOSTIER, A., MENUA, B., HAFFRAYD, P., CHATAIN, B. 2001. Sexual growth dimorphism in sea bass *Dicentrarchus labrax*. Aquacult., 202:371-387.

[9]PAPADAKI, M., PIFERRER, F., ZANUY, S., MAINGOT, E., DIVANACH, P., MYLONAS, C.C. 2005. Growth, sex differentiation and gonad and plasma levels of sex steroids in male- and female-dominant populations of *Dicentrarchus labrax* obtained through repeated size grading. J Fish Biol., 66:938-956.

[10]MITTWOCH, U. 1989. Sex differentiation in mammals and tempo of growth. Probabilities vs switches. J. Theor. Biol., 137:445-455.

[11]KRAAK, S.B.M., DELOOZE, E.M.A. 1993. A new hypothesis on the evolution of sex determination in vertebrates - Big females ZW, Big males XY. Neth. J. Zool., 43:260-273.

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

MOLECULAR CHARACTERIZATION OF TESTIS DIFFERENTIATION IN THE SIBERIAN STURGEON, *ACIPENSER BAERII*

Berbejillo J.[1], Martinez-Bengochea A.[1], Bedó G.[2] a, Vizziano-Cantonnet D.[1]

[1]Laboratorio de Fisiología de la Reproducción y Ecología de Peces, [2]Sección Genética Evolutiva, Instituto de Biología, Facultad de Ciencias, Uruguay.
Laboratorio de Fisiología de la Reproducción y Ecología de Peces, Facultad de Ciencias, Universidad de la República, Iguá 4225, Montevideo, 11400, Uruguay. vizziano@gmail.com

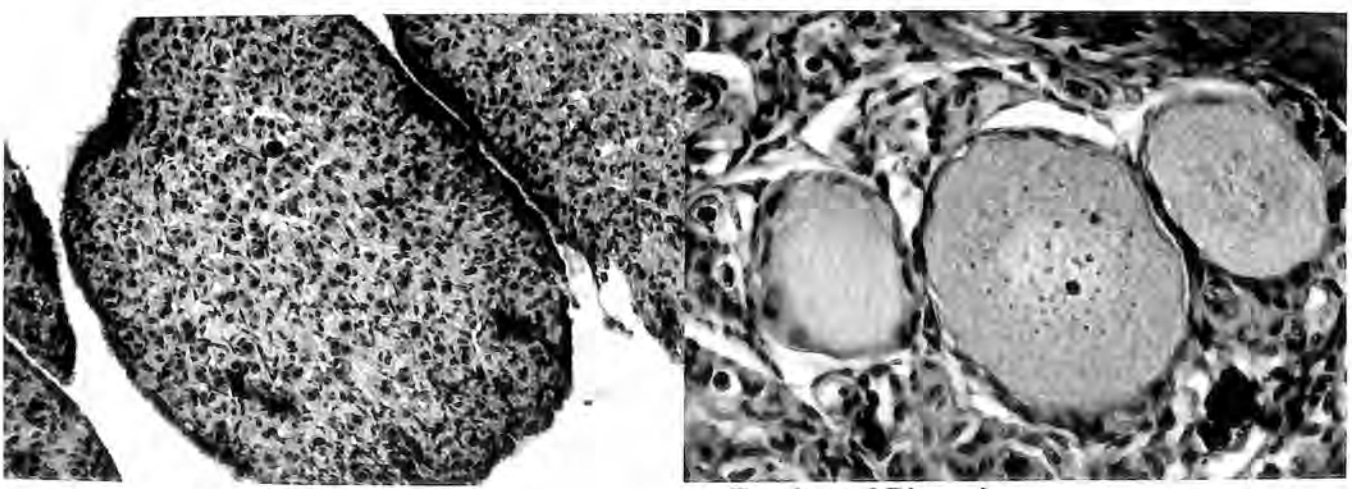
Introduction:

The morphological differentiation of vertebrate gonads is preceded by molecular changes that are not completely understood in the different vertebrate taxa. The most studied genes involved in the masculine pathway were Sox9, Dmrt1, Amh, Nr5a1, NrOb1 and Wt1 [1]. Besides transcription factors and other proteins, the endogenous steroids have been accepted as key players for gonad sex differentiation in fishes. In fact, *cyp19a1* (gonadal aromatase) is a key gene for ovarian differentiation [2], and androgens seem to have a key role during testis determination-differentiation in temperature-sensitive species [3]. However, the molecular mechanisms underlying gonad differentiation in phylogenetically ancient fishes as sturgeons remain poorly understood. As the time of sex differentiation was not reported for the species in rearing conditions in Uruguay, we started by an identification of this period by gonad morphology. For the molecular characterization of testis differentiation seven genes were studied, including testis differentiation markers as *dmrt1, ar, sox9, igf1* [1][4], and some markers of steroid synthesis (i.e. *cyp17, star*) and *lh*. A complete study on sex differentiation was difficult due to the scarce information on genes markers of gonad development in sturgeons, specially the ovarian markers. In this work *dmrt1*, *cyp17* and *star* sequences were characterized for *A. baerii*. The expression of genes was studied around the sex differentiation period as well as in already differentiated fish.

Methods:

Siberian sturgeons were obtained from producers of Uruguay. One gonad was fixed for histology, and the contralateral gonad was frozen in liquid nitrogen and transferred at -80°C until RNA extraction. For gene characterization, primers were designed from fish data bases at conserved sequence regions and used to amplify cDNA of post-differentiated testis. The fragments amplified were sequenced, and blasted in order to confirm their identity. The partial cDNAs sequences encoding for *dmrt1* (HQ110106), *ar* (HQ110107), *cyp17* (HQ026486) and *star* were characterized. Primers for qPCR were designed based on the gene sequences characterized here (*dmrt1*, *cyp17*) and on sequences previously submitted to GenBank for *ar* (DQ388357.1), *sox9* (EU241882.1), *igf1* (FJ428828.1), *lh* (AJ251656.1). For qPCR we studied 12 undifferentiated gonads, 10 just differentiated ovaries and 4 just differentiated testis (16 months old), and 4 ovaries at stage III (oocyte diameter of 2 mm) using 18S (AY904445.1) or ß-actin (FJ205611.1) for standardization. The validity of qPCR was confirmed by analysis of melting curves and checking amplified fragments in agarose gel.

Fig.1. Histological sections of gonads of undifferentiated fish (left) and just differentiated females (right).



Results and Discussion:

The histological analysis of gonads collected from 22 animals at age 16 months showed that 55% sampled fishes had undifferentiated gonads (Fig. 1 A) and 45% had differentiated ovaries with early pre-vitellogenic oocytes (Fig. 1 B). Four fish samples at the age 16 months had differentiated testes. This differs from data reported for other sturgeon species in which the differentiation occurs at 9 months [5].

Comparative expression in ovaries and testis of 16 months old showed a male up-regulation of 67 fold for *igf1* ($p<0.0001$) and of more than 200 fold for *dmrt1* ($p<0.001$), *ar* ($p<0.001$), *lh* ($p<0.001$), *cyp17* ($p<0.001$). *star* was not dimorphic at this stage. This trend was also observed when just differentiated testis was compared to ovaries at stage III, except for *sox9* and *star* that were slightly over-expressed in females.

The undifferentiated gonads showed low level of expression in all genes analyzed. For undifferentiated gonads the trends of *dmrt1*, *ar*, *lh* and *cyp17* were similar, with some fish showing high levels of expression and the others very low expression level, suggesting over expression in those probably undergoing male differentiation. Among the genes selected the transcription factor *dmrt1* seems to play a central and conserved role in fish testis differentiation [6]. The higher expression of this gene in Siberian sturgeon testis suggests that the function of *dmrt1* appeared already in this ancient fish and was conserved in teleost. Androgens play essential roles in sex differentiation and sexual maturation in vertebrates and their actions are mediated through androgen receptors that seem important during *A. baerii* testis differentiation. The *sox9*, a direct downstream target of SRY in mammals [7], seems not to play and essential role in testis development of chondrostean fish. The steroid synthesis capacity seems to be higher in just differentiated testis when compared to ovaries, but more efforts must be made to conclude if undifferentiated gonads are able to produce steroids.

Conclusion:

Four genes (*dmrt1*, *ar*, *lh*, *cyp17)* can be considered as potential markers of testis differentiation before we can identify sex of Siberian sturgeon juveniles by the testis morphology.

References:

[1]BARON, D., HOULGATTE, R., FOSTIER, A., GUIGUEN, A. 2005. Large scale temporal gene expression profiling during gonadal differentiation and early gametogenesis in rainbow trout. Biol. Reprod. 73: 959—966.

[2]GUIGUEN, Y., FOSTIER, A., PIFERRER, F., CHANG, C.F. 2010. Ovarian aromatase and estrogens: A pivotal role for gonadal sex differentiation and sex change in fish. Gen. Comp. Endocrinol.,165: 352-366.

[3]BLASCO, M., SOMOZA, G.M. AND VIZZIANO, D. 2010. Rol de los andrógenos como mediadores del desarrollo testicular en el pejerrey *Odontestes bonariensis.* Res, I Congreso Uruguayo de Zoología, 5-10 December. Montevideo, Uruguay.

[4]VIZZIANO, D., RANDUINEAU, G., BARON, D., CAUTY, CH., GUIGUEN, Y. 2007. Characterization of early molecular sex differentiation in rainbow trout, *Oncorhynchus mykiss.* Dev. Dyn., 236: 2198-2206.

[5]GRANDI, G., CHICCA, N. 2008. Histological and Ultrastructural Investigation of early gonad development and sex differentiation in Adriatic Sturgeon (*Acipenser naccarii*, Acipenseriformes, Chondrostei). J. Morphol., 269: 1238–1262.

[6]PIFERRER, F., GUIGUEN, Y. 2010. Fish Gonadogenesis. Part II: Molecular Biology and Genomics of Sex Differentiation. *Rev Fish Science*, 16(S1): 35–55, 2008.

[7]BRENNAN, J., CAPEL, B. 2004. One tissue, two fates: Molecular genetic events that underlie testis versus ovary development. Nat. Rev. Genet., 5: 509-521.

GENE EXPRESSION PROFILING DURING GONADAL DIFFERENTIATION IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) USING A NEXT GENERATION SEQUENCING (NGS) APPROACH

Ayaka Yano (1), Elodie Jouanno (1), Christophe Klopp (2), Yann Guiguen (1)

(1) INRA-SCRIBE, 37000 Rennes, France
(2) INRA-SIGENAE, 31326 Castanet-Tolosan, France
Fax : +33-2-2348- 5020 email:ayaka.yano@rennes.inra.fr

Introduction:

Knowledge on the molecular mechanisms of sex determination and sex differentiation in fish is still limited. Rainbow trout (*Oncorhynchus mykiss*) is an economically important fish species and also one of the most studied fish species with many experimental advantage including the availability of all-male (XY) and all-female (XX) genetic populations, a relatively large body size and a slow development that makes possible to sample embryonic gonads at the very beginning of the sex differentiation process (around hatching i.e., 30 days post fertilization (dpf)). In order to better understand the molecular mechanisms of sex determination and sex differentiation in fish, we used a NGS approach to characterize gene expression profiles during early gonadal sex differentiation in rainbow trout.

Methods:

Doubled-strand cDNA libraries were constructed from pooled testis and from pooled ovaries sampled in 35 dpf rainbow trout embryos. These libraries were sequenced using the Roche 454 Titanium technology and the resulting sequences have been assembled and mapped onto the existing rainbow trout transcriptome from SIGENAE. Some of these transcripts were further characterized during early gonadal development (from 28-70 dpf) using qPCR and whole mount *in situ* hybridization (WISH)

Results and Discussion:

The sequencing yielded 350,638 reads from the ovary library and 349,465 reads from the testis library. Mapping of these sequences results in the identification of 42,044 "transcripts" (bioinformatics contigs) expressed in differentiating gonads. To better characterize sex dimorphic genes, we selected transcripts (74 for ovary and 48 for testis) containing more than 30 total reads with at least a 2 fold change between sexes. Among these selected transcripts, *cyp19a1a*, a well known key gene for ovarian differentiation [1], was predominantly expressed in the ovary library (84 reads and 5 reads in the testis library), demonstrating the efficiency of the NGS approach. Among these transcripts, we identified one previously uncharacterized gene with a very restricted expression pattern in some epithelial and somatic peri-germinal cells of the differentiating testis. This gene was specifically expressed in the testis at very early stages of gonadal development, with no expression detected in the female differentiating ovaries. These expression patterns suggest that this novel gene plays important role in testis development.

Conclusion:

The identification of novel testis specific genes in rainbow trout demonstrates the interest of characterizing gene expression profile in early gonadal development using a NGS approach. Therefore, analyzing more of this subset of sequence information will lead to a better knowledge of the molecular mechanisms of sex determination and sex differentiation in fish.

References:

[1]Vizziano, D., Randuineau, G., Baron, D., Cauty, C., Guiguen, Y. 2007. Characterization of early molecular sex differentiation in rainbow trout, Oncorhynchus mykiss. Dev. Dyn., 236: 2198-2206.

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

FOLLISTATIN IS EXPRESSED ALONG WITH AROMATASE IN FEMALE GONADS DURING SEX DIFFERENTIATION IN THE RAINBOW TROUT

Barbara Nicol, Ayaka Yano, Elodie Jouanno, Adèle Branthonne, Alexis Fostier, Yann Guiguen

Institut National de la Recherche Agronomique (INRA), UR1037 SCRIBE, campus de Beaulieu, 35000 Rennes, France

email: barbara.nicol@rennes.inra.fr; fax: +33 2 23 48 50 20

Introduction:

Follistatin (Fst) is an inhibitor of some members of the TGFβ family that was first identified in mammalian ovary. This gene has been shown to be expressed in granulosa cells and oocyte of mammalian and fish species, and is involved in folliculogenesis and oocyte development. In addition to these functions in ovarian development, *fst* is also expressed in the mouse embryonic ovary, and its inactivation induces partial female-to-male sex reversal, suggesting that *fst* plays a significant role in early ovarian differentiation in mammals. However, only little is known about its potential role in early gonadal differentiation in teleost fish.

Methods:

Rainbow trout (*Oncorhynchus mykiss*) is a teleost fish with a strict male heterogametic sex determination system (XX/XY). Genetic all-male (XY) and all-female (XX) experimental populations can be produced in that species, allowing the sex-specific characterization of the early molecular events that occur before histological differentiation of the gonads. We then used the rainbow trout model to investigate the expression pattern of *fst* in embryonic gonads by In Situ Hybridization and qPCR, and to determine its regulation during sex differentiation in fish.

Results and discussion:

Shortly after hatching that occurs around 30 days post-fertilization (30 dpf), *fst* is already expressed in female gonads in some clusters of somatic cells, whereas no expression is detected in male gonads. Later, its expression becomes restricted to some somatic cells lining the future ovarian lamellae. This expression pattern is very similar to what is observed for the *aromatase* gene (*cyp19a1a*) coding for a key steroidogenic enzyme needed for ovarian differentiation in fish. Given that *cyp19a1a* is the earliest known gene to be expressed in a sexually dimorphic fashion in female gonads of rainbow trout, we compared *fst* and *cyp19a1a* expressions several days before hatching. Both *fst* and *cyp19a1a* expression begins around 26 dpf in somatic cells, in a punctiform pattern throughout the female gonads. Then, around hatching, this expression becomes continuous throughout the gonads. These results suggest that *fst* is implicated along with *cyp19a1a* in the early steps of ovarian differentiation in teleost fish. As *fst* expression is known to be induced by the Wnt/β-Catenin pathway in mammals, we treated embryos *in vivo* with IWR-1, an inhibitor of the Wnt pathway, and we analysed by qPCR the effects of this treatment on *fst* expression. We found that the IWR-1 treatment decreases *fst* expression in female gonads, suggesting a role of the Wnt/β-Catenin pathway in regulating expression of *fst* in rainbow trout.

Conclusion:

These results demonstrate that *follistatin* is one of the earliest markers of ovarian differentiation in rainbow trout and its co-expression with *cyp19a1a* suggests that *fst* could be implicated along with *cyp19a1a* in the ovarian differentiation process. Furthermore, expression of *fst* is downregulated by an inhibitor of the Wnt/β-Catenin pathway, suggesting a wide conservation of *fst* regulation across vertebrates.

GONADAL EXPRESSION PATTERNS OF STEROIDOGENESIS-RELATED GENES DURING SEX DIFFERENTIATION IN ZEBRAFISH

Caulier M. (1) (2), Guérin A. (2), Cauty C. (2), Hinfray N. (1), Brion F. (1) Guiguen Y. (2)

(1) INERIS, Unité d'écotoxicologie in vitro et in vivo, Verneuil-en-Halatte, France, e-mail : morgane.caulier@rennes.inra.fr

(2) Institut National de la Recherche Agronomique, UR 1037, INRA-SCRIBE, IFR 140, Campus de Beaulieu, Rennes, France

Introduction:

Zebrafish is an important animal model recommended by the Organisation for Economic Co-operation and Development (OECD) for detecting and assessing the effects of pollutants. In fish, sex differentiation is often used to evaluate the effect of endocrine disrupting chemicals (EDCs) as many of them are known to alter gonadal differentiation. Steroidogenesis has been shown to be highly sensitive to hormonal perturbations and is also of importance for natural fish gonadal differentiation. In zebrafish, there is still however a lack of knowledge regarding the steroidogenic gene expression during gonadal differentiation leaving unexplored the precise mechanism of action of EDCs on this physiological process. To fulfill these gaps, we then investigated the normal expression profiles of some selected genes involved in steroidogenesis during early gonadal development in zebrafish. This information will serve as a basis to investigate the molecular mechanism of EDCs on steroidogenic gene expression during early gonadal development in zebrafish.

Methods:

Genes were selected for their well known implication in steroidogenesis including steroid enzymes *(cyp11b2* and *cyp17a1)*, and a cholesterol mitochondrial transfer protein (*star*). Expression patterns were characterized using whole mount *in situ* hybridization in males and females zebrafish sampled at different stages of development (20, 30, 40 and 60 dpf or days post-fertilization). Fish were fixed overnight in 4% paraformaldehyde in PBS and hybridizations were performed with DIG-labeled probes on 10 different fishes for each gene and developmental stage investigated.

Results and discussion:

At the later stages of development (40 and 60 dpf), *star* and *cyp11b2* were more expressed in male gonads whereas *cyp17a1* was more strongly expressed in female gonads. At earlier stages (20 and 30 dpf) the precise identification of the sex phenotype of the gonads was not possible but similar differences were suspected as labeling was only found in some gonads. Interestingly, *star* and *cyp11b2* displayed a rather similar expression pattern characterized by small internal clusters of positive cells in male gonads and scattered positive cells mostly located at the periphery of the female gonads. For *cyp17a1* a similar male expression pattern was recorded but in female gonads expression was localized in the cytoplasm of previtellogenetic oocytes.

Conclusion:

Our study confirm previous results showing that some genes involved in steroidogenesis are differentially expressed during early development between male and female gonads. We will now extend this analysis to other genes involved in steroidogenesis including for instance *amh*, *cyp19a1a*, *cyp11a1*, *hsd3b1*, *foxl2a*, *nr0b1*, and *nr5a1b*. These precise gene expression patterns will set grounds for future studies with an ecotoxicological prospective.

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

MASCULINIZATION IN RAINBOW TROUT CARRYING THE *MAL* MUTATION IS TEMPERATURE SENSITIVE

Valdivia K.[1], Jouanno E.[1], Mourot B.[1], Quillet E.[2], Guyomard R.[2], Volff J-N.[3], Galiana-Arnoux D.[3], Cauty C.[1], Fostier A.[1] and Guiguen Y.[1]

[1] INRA, UR 1037-SCRIBE, IFR 140, Ouest Génopole, 35000 Rennes, France
Fax: 33 (0) 2 23 48 50 20 , email: Yann.Guiguen@rennes.inra.fr
[2] GABIE, INRA, Jouy-en-Josas France ; [3] IGFL / ENS, Lyon, France.

Introduction:

Sex determination in rainbow trout is mainly genetic with a XY male heterogametic system. However, a maleness mutation (*mal*) was discovered in an all female (XX) rainbow trout population obtained by endomitotic gynogenesis [1]. Some of these XX fish can display functional masculinization features of their gonads with various phenotypes such as: a full masculinization of both gonads, a left-right asymmetry of masculinization or intersexual gonads [1,2]. Interestingly, preliminary experiments on these *mal*-carrying populations suggested that masculinization could be sensitive to water temperature. Therefore, we investigated under controlled conditions whether low or high temperature applied after hatching during the sex differentiation period could affect the masculinization of different *mal*-carrying progenies.

Methods:

Oocytes from four different *mal*-carrying females were fertilized with sperm from one XX *mal*-carrying male (sex inverted by the *mal* mutation) and these eggs were kept separately at 10°C until hatching when these were mixed (100 individuals from each progeny) before the application of the different temperature treatments. These temperature treatments started from hatching (30 days post-fertilization) for different periods in order to compensate the temperature effects on development: 6 months at 8°C, 3 months at 12°C, and 2 months at 18°C. For each temperature condition both left and right gonads were sampled between 9 to 12 months post-fertilization for histological processing in order to analyze the sex ratio. Furthermore, in order to assign each fish to its family, a fin clip was sampled and stored in 90% ethanol before microsatellite genotyping.

Results and Discussion:

Overall, our results demonstrate that the highest temperature treatment (18°C) produced a significant and higher proportion of masculinized animals (28.9% at 18°C, 15.2% at 12°C and 16.2% at 8°C). Interestingly the masculinization effect was even more pronounced in one of the four families studied in which the 18°C treatment induced a two fold increase of masculinization (72%) compared with the 8°C (33%) and the 12°C (37%). These family differences could be explained by a "genetic parental effect" as previously described in Atlantic silverside [3], Nile tilapia [4] and rainbow trout [5]. In agreement with previous observations in intersexual *mal*-carrying animals [2], the right gonad was shown to be more frequently masculinized in animals at 8°C and 12°C. However, at the 18°C this left-right asymmetry of masculinization was no more detectable.

Conclusion:

These results demonstrate that masculinization in *mal*-carrying rainbow trout is dependant on female genotype and is responsive to high temperature.

References:

[1]QUILLET , E., AUBARD, G. , QUEAU, I. 2002. Mutation in a sex-determining gene in rainbow trout: detection and genetic analysis, J. Hered., 93: 91-99.

[2]QUILLET, E., LABBE, L., QUEAU,I. 2004. Asymmetry in sexual development of gonads in intersex rainbow trout, J. Fish Biol., 64: 1147-1151.

[3]LAGOMARSINO, I. V., CONOVER, D. O. 1993. Variation in environmental and genotypic sex-determining mechanisms across a latitudinal gradient in the fish, *Menidia menidia*. Evolution, 47: 487-494.

[4]BAROILLER, J. F., D'COTTA, H. , BEZAULT, E., WESSELS, S., HOERSTGEN-SCHWARK, G. 2009. Tilapia sex determination: Where temperature and genetics meet. Comp. Biochem. Physiol. Part A: Mol. Integr. Physiol., 153: 30-38.

[5]MAGERHANS, A., HORSTGEN-SCHWARK, G. 2010. Selection experiments to alter the sex ratio in rainbow trout (*Oncorhynchus mykiss*) by means of temperature treatment, Aquaculture, 306: 63-67.

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

MOLECULAR CLONING OF COMMON CARP GONADAL SOMA DERIVED FACTOR (GSDF)

Fujimoto T.*, Ohmae T.*, Sato S.°, Horiguchi R.+, Nagahama Y.+ and Hirai T.*

*Department of Biosciences, Teikyo University of Science and Technology, Yamanashi 409-0193, Japan. Fax +81-554-634450 e-mail: t-hirai@ntu.ac.jp

°Niigata Prefectural Inland Water Fisheries Experiment Station, Niigata 940-1137, Japan. +Laboratory of Reproductive Biology, National Institute for Basic Biology, Okazaki 444-8585 and Institution for Collaborative Relations, Ehime University, Matsuyama 790-8577, Japan.

Introduction:

Gonadal soma derived factor (GSDF) is a teleost-specific member of TGF-beta superfamily originally isolated from gonads of rainbow trout (*Oncorhynchus mykiss*), and known to promote proliferation of primordial germ cells (PGCs) and spermatogonia[1]. Recently, it has been demonstrated that this molecule plays a crucial role in early testicular differentiation of medaka (*Oryzias latipes*)[2]. It has been suggested that somatic cells play a leading role in gonadal sex differentiation. However, there are no useful molecular markers available to date for sex differentiation of somatic cells in common carp *Cyprinus carpio* . In this study, we cloned common carp GSDF cDNAs and examined their expression profiles during gonadogenesis.

Methods:

cDNA fragments were obtained from maturing testis and ovary by RT-PCR using degenerate primers designed for consensus sequences of known GSDFs. The entire structure of GSDF cDNAs were determined by 5'- and 3'-RACE. Their gene expressions were investigated by semi-quantitative RT-PCR and in situ hybridization (ISH) with colorimetric or fluorescent imaging.

Results and Discussion:

Two types of full-length GSDF cDNAs encoding 198 and 201 amino acids have been identified (designated as KoiGSDF1 and 2). They were expressed dominantly in the gonads and with the overall superiority of KoiGSDF1. Localizations of their messages were basically identical. In 4-month-old fish just after the gonadal sex differentiation, GSDF mRNA intensively expressed in the testicular somatic cells while somewhat lower levels were also observed in the ovarian somatic cells surrounding germ cells. In 16-month-old fish, GSDF expression came to be restricted to the testicular somatic cell surrounding spermatogonia. Meanwhile in the ovary (at the early stage of oogenesis), a weak signal was detected in the follicle layer of early-vitellogenic oocytes while a significant level of expression was observed in somatic cells at dorsal and ventral margins in some case.

Conclusion:

These results suggest that GSDF supports not only early step of spermatogenesis but also sex differentiation and/or maintenance of sexual plasticity.

References:

[1]SAWATARI, E., SHIKINA, S., TAKEUCHI, T., YOSHIZAKI, G. 2007. A novel transforming growth factor-beta superfamily member expressed in gonadal somatic cells enhances primordial germ cell and spermatogonial proliferation in rainbow trout (*Oncorhynchus mykiss*). Dev. Biol., 301: 266-275.

[2] Shibata, Y., Paul-Prasanth, B., Suzuki, A., Usami, T., Nakamoto, M., Matsuda, M., Nagahama, Y. 2010. Expression of gonadal soma derived factor (GSDF) is spatially and temporally correlated with early testicular differentiation in medaka. Gene Expr. Patterns, 10: 283-289.

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

TRANSCRIPTOME ANALYSIS OF ARTIFICIALLY INDUCED SEX REVERSAL IN THE NILE TILAPIA

Huang B.F., Sun Y.L., Wang D.S.

Key Laboratory of Freshwater Fish Reproduction and Development (Ministry of Education), Key Laboratory of Aquatic Science of Chongqing, School of Life Science, Southwest University, Chongqing, 400715, China. Fax: +86-2368253005, E-mail : wdeshou@swu.edu.cn

Introduction:

The Nile tilapia, a worldwide cultured fish, is a good model for the study of sex determination and differentiation, due to the availability of monosex offsprings and short (14days) spawning cycle. In order to elucidate the molecular mechanism for induced sex reversal, transcriptome analysis of gonads (testis and ovary) from 3-month-old tilapia was carried out by RNA sequencing.

Methods:

Animal- All genetic female (XX) and male (XY) tilapia (*Oreochromis niloticus*) were obtained by artificial fertilization of eggs from normal females (XX) with sperm from either sex-reversed males (XX) or super males (YY), respectively. Tilapias were reared in 0.5-ton tanks with re-circulating aerated fresh water at 26°C under natural photoperiod until 3 months old. Total RNA was extracted from female and male gonad, respectively, using TRIzol reagent (TaKaRa, Japan). The Illumina RNA-seq was performed by BGI (Beijing Genomics Institute). *Drug treatment*- The aromatase inhibitor Letrozole was dissolved in 95% ethanol, and then added to the feed for long-term treatment of all 3-month-old genetic female tilapia until sex reversal. *Histological studies*- After 0, 10, 20, 45, 70, 90 days of Letrozole treatment, the gonads were dissected out and fixed in Bouin's solution for 24 h at room temperature, and subsequently dehydrated, embedded in paraffin, and then serially sectioned at 6μm thickness. The sections were stained with hematoxylin and eosin. *Real time PCR*- Gonads from Letrozole treated group and control group were sampled at 0, 10, 20, 45, 70, 90 days after treatment Total RNA was extracted from all the samples and cDNA was prepared for the real-time PCR.

Results and Discussion:

Totally about 70000 unigenes were found to be expressed in 3 month old gonads. Out of 11765 annotated unigenes, about 10935 were expressed in both gonads, while 193 and 593 were found to be expressed specifically in the ovary and testis, respectively. Comparative study of the transriptome data from the testis and ovary revealed 14066 differentially expressed genes, in the 3-month-old tilapia gonads, with 10111 expressed higher in testis, while 3955 were expressed higher in ovary. Therefore, more genes are expressed in much higher levels in the testis compared with the ovary. Functional classification of unigenes was done by COG and KEGG. Predominant classifications like cell cycle control, posttranslational modification, ribosomal structure, energy production and conversion, suggest that the fundamental metabolism and development were the main processes in the 3-month-old tilapia gonads. The predominant classes of genes expressed in the ovary were fatty acid-binding protein, Cyclin, Actin and Zona pellucida sperm-binding protein, indicating it as the nutritive and energy accumulation period for oocyte growth. While, in the testis, the predominant classes were NADH-ubiquinone oxidoreductase, ATP-dependent DNA helicase and Cell differentiation factor, indicating an active cell division phase for spermatogenesis in the 3-month-old gonad. Analysis of the expression profiles of the steroidogenic enzyme genes in the transcriptome revealed that only the estrogen synthesis related genes, such as *Cyp19a1a*, *Cyp19a1b*, *3beta-HSD-II* and *17 beta -HSD-I* were expressed higher in the ovary than in the testis, while the majority of steroidogenic enzymes, such as *Cyp11b1*, *P450scc*, *Star1*, *Star2*, *3beta-HSD-I*, *11 beta -HSD-II* and *17beta-HSD12-II*, were expressed higher in the testis. This indicates the differential synthesis of steroid hormone between ovary and testis, with much active steroidogenesis in the testis. Some important gene families were analyzed thoroughly with the transcriptome data. For example, 29 Fox genes were found to be expressed in the 3-month-old tilapia gonad, with 12 and 17 genes being expressed much higher in the ovary and testis, respectively, indicating that like Foxl2, these Fox genes might also play important roles in fish gonad differentiation and development.

In addition, sex reversal from ovary to functional testis was successfully induced by long term treatment of 3-month-old female tilapia with aromatase inhibitor, Letrozole. Histological examination of the gonad samples fixed at different time during treatment revealed that sex reversal was a gradual process, which started from ventral to dorsal and from posterior to anterior of the gonad. Real time PCR was employed to check the profiles of genes which were found in the transcriptome and were known to play important roles in fish sex determination and differentiation. The results showed that *Sf-1* and *Cyp19a1a* were significantly up-regulated during the early period of treatment (0-45 dat, days after

treatment), followed by a moderate expression levels in the remaining period, which was in consistence with the aromotase protein level, as revealed by immunohisto-staining with tilapia aromatase anti-sera. Foxl2 was found to be down regulated, while Dmrt1 was up-regulated gradually during the treatment period. It is worth noting that the un-regulation of *Cyp11b1*, the key enzyme responsible for 11KT production, was up-regulated only at the late treatment period. The transcriptome analysis of the sex reversal process is still in progress.

Conclusion:

Our data indicate that estrogen is not only critical for the ovarian differentiation, but also for the maintenance of the ovary, while androgen might be the consequence, instead being the reason of the Letrozole induced sex reversal.

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

MICRORNAs EXPRESSION DURING FISH OOGENESIS AND SEX DIFFERENTIATION

Juanchich A. (1), Klopp C. (2), Guiguen Y. (1), Bobe J. (1)

(1) INRA, UR1037 SCRIBE, Campus de Beaulieu, F-35042 Rennes, FRANCE.
Fax +33-223485020 e-mail : amelie.juanchich@rennes.inra.fr
(2) INRA SIGENAE, UR875 Biometrie et Intelligence Artificielle, F-31326 Castanet-Tolosan, FRANCE

Introduction:

The molecular biology dogma that proteins are the only effectors of gene function has evolved since the discovery of genes that do not encode proteins. Non-coding RNAs (ncRNAs) such as microRNAs (miRNAs) or short interfering RNAs (siRNAs) recently emerged as key regulators of physiological functions. MicroRNAs are small endogenous ncRNAs that are important in the post-transcriptional regulation of gene expression. Previous studies have shown the implication of microRNAs in the regulation of development and oogenesis in mammals but data in fish are very scarce. The main goal of this study was to characterize the microRNA repertoire in fish using the rainbow trout, *Oncorhynchus mykiss*, as a model species in order to identify microRNAs of importance for sex differentiation and final oocyte maturation.

Methods:

To characterize the microRNAs repertoire, a Next Generation Sequencing (NGS) analysis was performed. Small size RNA samples isolated from rainbow trout undergoing differentiation gonads and ovaries at different stages of final oocyte maturation were subjected to deep sequencing using Illumina technology. The aim was to compare these data to a wider sample group of tissues in order to identify gonad-specific or gonad-predominant miRNAs. In addition, as microRNAs are highly conserved among species, a candidate miRNA approach was initiated using miRNA data available in mammals and other vertebrates. Quantitative real-time PCR was used to quantify miRNAs in different tissue samples and ovary samples at different stages of final oocyte maturation.

Results:

These two complementary techniques highlight the presence of miRNAs in the trout ovary. Firstly, the ongoing sequencing analysis widens and specifies the current rainbow trout repertoire. Then, fourteen microRNAs were screened using the candidate approach and some of them show a stage-dependent expression profile during follicular differentiation during rainbow trout oogenesis.

Conclusion:

This work will provide a first comprehensive overview of the miRNA repertoire in rainbow trout and will set grounds of future studies aiming at understanding miRNA-regulated gene expression during sex differentiation and oogenesis in fish.

INVOLVEMENT OF OLFACTION IN SEXUAL BEHAVIOR IN GOLDFISH

Kawaguchi, Y., Mitsuhashi, T., Kitami, A., Nagaoka, A., Hayakawa, Y., and Kobayashi, M.

Department of Life Science, International Christian University, Osawa, Mitaka, Tokyo, 181-8585, Japan
FAX: +81-422-33-1449, email: g136031a@yamata.icu.ac.jp

Introduction:

Physiological and environmental regulation of sexual behavior (spawning behavior) has been intensively studied in goldfish, *Carassius auratus* [1, 2]. Prior to spawning of goldfish, ovulated females produce prostaglandin $F_2\alpha$ (PG) in the ovary. This PG triggers female spawning behavior (oviposition act) by acting on the brain of the female. Meanwhile, a portion of the PG produced by the ovulated females is released into the water as a pheromone, and stimulates the olfactory systems of males, eliciting male spawning behavior (chasing and ejaculation act) in sexually mature males. Thus, olfaction is essential for the occurrence of sexual behavior in male goldfish. However, the involvement of olfaction in female sex behavior has not been examined in goldfish. In the present study, we have examined whether olfaction is involved in sexual behavior of female goldfish by blocking female olfactory pathways.

Methods:

Spawning behavior of male and female goldfish was induced by injecting PG in non-ovulated female goldfish [1]. The PG-injected females performed female spawning behavior. The injected PG was released into the water and induced male spawning behavior. Obstruction of olfactory function was conducted in two separate methods: nasal occlusion with glue which blocked the reception of olfactants in the olfactory epithelium, and olfactory tract section (OTX) which blocked the transmission of olfactory information from the olfactory bulbs to the telencephalon.

Results and Discussion:

Male spawning act was significantly reduced by nasal occlusion (control; 60.0±7.9, mean±SE, 60 min test, N=12: nasal occlusion; 0.6±0.4, N=14, $p<0.05$) and OTX (Sham; 80.9±18.6, mean±SE, 90 min test, N=15: OTX;16.3±8.3, N=18, $p<0.05$) in males. These results confirm the importance of the pheromone in male sex behavior in goldfish. In the PG-injected females, nasal occlusion (control; 60.8±9.3, mean±SE, 60 min test, N=16: nasal occlusion; 0±0, N=9, $p<0.05$) significantly reduced female spawning act. This result indicates that olfaction is also essential in females for the occurrence of sex behavior although it is not known whether there is a specific olfactant or a pheromone from males that stimulates the females. Interestingly, OTX females preformed spawning act after PG injection although these fish could receive no olfactory information. (sham; 137.7±11.5, mean±SE, 90 min test, N=17: OTX; 118.8±16.1, N=19) The results of these experiments suggest the existence of the following interaction between olfaction and sexual behavior in females. The olfactory input is essential for female sex behavior, and when this input is interrupted, sex behavior is inhibited by the neurons in the olfactory bulbs which project fibers (mitral cells) to the telencephalon, a control center of sexual behavior [3].Therefore, even though information of the olfactory input is blocked by OTX, females can perform sexual behavior by the stimulation of PG because the inhibitory system of mitral cells is removed.

Conclusion:

The results of present study showed the involvement of olfaction in sexual behavior both in male and female goldfish. Male goldfish sense pheromones from ovulated females for the promotion of sexual behavior, but in contrast, it is not known whether female goldfish sense specific olfactants for the initiation of behavior. The results suggest that there is an inhibitory system of sexual behavior in the olfactory bulbs of female goldfish. This inhibitory system continues to function when the olfaction is blocked by nasal occlusion, but inhibition is ceased by an olfactory tract section.

References

[1]KOBAYASHI, M. SORENSEN, P. W., AND STACEY, N. E. 2002. Hormonal and pheromonal control of spawning behavior in the goldfish. Fish Physiol. Biochem., 26: 71-84.

[2]MUNAKATA, A. AND KOBAYASHI, M. 2009. Endocrine control of sexual behavior in teleost fish. Gen. Comp. Endocrinol., 165: 456-468.

[3]KOYAMA, Y., SATOU, M., AND UEDA, K. 1985. Sexual behavior elicited by electrical stimulation of the telencephalic and preoptic areas in the goldfish, *Carassius auratus*. Zool. Sci., 2: 565-570.

HIGH TEMPERATURE CAUSES MASCULINIZATION OF GENETICALLY FEMALE MEDAKA BY ELEVATION OF CORTISOL LEVEL

Kitano T.*, Hayashi Y.*, Yamaguchi T.*, Shiraishi E.*

*Department of Biological Sciences, Graduate School of Science and Technology, Kumamoto University, Kumamoto 860-8555, Japan. E-mail: tkitano@kumamoto-u.ac.jp

*National Research Institute of Aquaculture, Fisheries Research Agency, Mie 516-0193, Japan.

Introduction:

In poikilothermic vertebrates, sex determination is sometimes influenced by temperature. Many studies of teleost fish show that environmental factors such as pH, density, and social factors also can change the sex ratio. However, little is known about the molecular mechanisms underlying environmental sex determination in these species. More recently, in the Japanese flounder (*Paralichthys olivaceus*), which exhibits temperature-dependent sex determination (TSD), cortisol caused female-to-male sex reversal [1]. These observations suggest that cortisol may be involved in TSD of various species.

Medaka (*Oryzias latipes*) is a small laboratory fish with an XX/XY sex determination system. Recently, it was reported that XX medaka can be sex-reversed into phenotypic males by high water temperature (HT) treatment during the sex differentiation period [2]. To elucidate molecular mechanisms underlying TSD in medaka, we investigated the effects of cortisol, HT and metyrapone (2-methyl-1,2-di-3-pyridyl-1-propanone), an inhibitor for 11β-hydroxylase which is involved in cortisol synthesis, on sex differentiation in medaka.

Methods:

The FLFII medaka stock was used [3]. This stock allows identification of genotypic sex by the appearance of leucophores before the onset of sex differentiation. Medaka embryos and larvae were reared in ERM (17 mM NaCl, 0.4 mM KCl, 0.27 mM $CaCl_2 2H_2O$, 0.66 mM $MgSO_4$, pH 7) mixed with or without cortisol (Sigma-Aldrich, Gillingham, UK) at 26°C from 0 days post-fertilization (dpf) to 5 days post-hatching (dph). On the other hand, HT experiment was carried out by rearing them in ERM mixed with or without metyrapone (Sigma-Aldrich) at 33°C from 0 dpf to 5 dph. After treatments, these fishes were maintained up to adults at 26°C under a 14 h light and 10 h dark cycle. Steroid hormones in five pooled fishes were extracted in diethyl ether as described previously [4]. Cortisol levels were measured using a cortisol EIA kit (Cayman Chemical, Ann Arbor, MI) according to the manufacturer's instructions. The phenotypic sex of each adult fish was determined by histological observation of gonads [5].

Results and Discussion:

HT causes elevation of whole-body levels of cortisol, while metyrapone inhibits the elevation by HT treatment during sexual differentiation of medaka

Whole-body levels of cortisol were measured by enzyme-immunoassay at 0 dph in the FLFII medaka larvae reared at 26°C and 33°C during gonadal sex differentiation. At 0 dph, cortisol levels in the cortisol-treated larvae reared at 26°C or untreated fishes reared at 33°C were significantly higher than those in the untreated larvae reared at 26°C or metyrapone-treated fishes reared at 33°C. This indicates that HT causes elevation of cortisol level, whereas metyrapone inhibits the elevation by HT treatment in the medaka larvae during sex differentiation.

Cortisol causes female-to-male sex reversal and metyrapone inhibits HT-induced masculinization of XX medaka

To investigate whether cortisol induces masculinization of XX medaka in a similar way to HT treatment, we treated the FLFII medaka with or without cortisol at 26°C, or with or without metyrapone at 33°C from 0 dpf to 5 dph. Histological observations at the adult stage showed that both cortisol and 33°C treatments caused female-to-male sex reversal and that the sex-reversed XX fishes had typical testes. Moreover, masculinization by 33°C treatment was completely counteracted by administration of metyrapone. Therefore, we suggest that masculinization of XX medaka by HT treatment may be attributable to an elevation of cortisol level during gonadal sex differentiation.

Conclusion:

This study has presented the first evidence of the involvement of cortisol in TSD of the medaka. We have demonstrated that cortisol causes female-to-male sex reversal and that metyrapone inhibits HT-induced masculinization of XX medaka. HT treatment caused elevation of whole-body levels of cortisol, while metyrapone suppressed the elevation by HT treatment during sexual differentiation. Therefore, these findings strongly suggest that the masculinization of XX medaka induced by HT is mediated by the elevation of cortisol level.

References:

[1]YAMAGUCHI T. YOSHINAGA N., YAZAWA T., GEN K., KITANO T. 2010. Cortisol is involved in temperature-dependent sex determination in the Japanese flounder. Endocrinology, 151: 3900-3908.

[2]SATO T., ENDO T., YAMAHIRA K., HAMAGUCHI S., SAKAIZUMI M. 2005. Induction of female-to-male sex reversal by high temperature treatment in medaka, *Oryzias latipes*. Zool. Sci., 22: 985-988.

[3]WAKAMATSU Y., INOUE C., HAYASHI H., MISHIMA N., SAKAIZUMI M., OZATO K. 2005. Establishment of new medaka (*Oryzias latipes*) stocks carrying genotypic sex markers. Environ. Sci., 10: 291-302.

[4]KITANO T., TAKAMUNE K., KOBAYASHI T., NAGAHAMA Y., ABE S. 1999. Suppression of P450 aromatase gene expression in sex-reversed males produced by rearing genetically female larvae at a high water temperautre during a period of sex differentiation in the Japanese flounder (*Paralichthys olivaceus*). J. Mol. Endocrinol., 23: 167-176.

[5]HAYASHI Y., KOBIRA H., YAMAGUCHI T., SHIRAISHI E., YAZAWA T., HIRAI T., KAMEI Y., KITANO T. 2010. High temperature causes masculinization of genetically female medaka by elevation of cortisol level. Mol. Reprod. Dev., 77: 679-686.

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

ZEBRAFISH SEX IS GENETICALLY DETERMINED WITHOUT DIFFERENTIATED SEX CHROMOSOMES

Liew W.C.[1,2], Bartfai R.[1,3], Lim Z.[1,3], Sreenivasan R.[1], Orban L.[1,3]

[1]Reproductive Genomics Group, Temasek Life Sciences Laboratory, 1 Research Link, National University of Singapore, Singapore. Fax:+65-68727517. Email: wcliew@tll.org.sg
[2]School of Biological Sciences, Nanyang Technological University, Singapore
[3]Department of Biological Sciences, National University of Singapore, Singapore

Introduction:

Zebrafish is a popular research organism however its sex determination mechanism is still unknown. Recent publications suggest that environmental factors have minimal impact on zebrafish sex ratio.

Results and Discussion:

Using classical breeding experiment we found that repeated single pair crossing produced broods of similar sex ratio. On the other hand, sex ratio across families were wide ranging (4.8% to 97.3% male). Further examination by array comparative genomic hybridization using a custom-designed high density oligo array in 4 families failed to find universal sex-linked differences between the male and female genomes.

Conclusion:

Taken together, our study suggests that zebrafish sex is genetically determined without differentiated sex chromosomes. Therefore we hypothesize that several autosomal factors might be involved in the sex determination of zebrafish.

MOLECULAR CLONING OF WT1a AND WT1b AND THEIR POSSIBLE INVOLVEMENT IN FISH SEX DETERMINATION AND DIFFERENTIATION

Mohapatra S., Liu Z.H., Zhou L.Y., Zhang Y.G., Wang D.S.

Key Laboratory of Freshwater Fish Reproduction and Development (Ministry of Education), Key Laboratory of Aquatic Science of Chongqing, School of Life Science, Southwest University, Chongqing, 400715, China
Fax: +86-2368253005, E-mail : wdeshou@swu.edu.cn

Introduction:

The Nile tilapia is considered as a good experimental animal for the sex determination and differentiation, owing to its monosex offsprings availability and a short (14 days) spawning period. Feral Southern catfish, an endemic fish to China, has a sex ratio 1:1, while the fry obtained by artificial propagation were all female, thus making it a good experimental fish to be studied. It is well demonstrated that estrogens play decisive role in the ovarian differentiation of the teleosts. It has been reported earlier that Wt1 is involved in the mammalian sex determination and differentiation *via* regulation of *Sry*, *Sox9*, *Amh* and *Cyp19a1*. Mice carrying homozygous knockout mutations of Wt1 lack kidneys, gonads and adrenal glands, and have defectively formed heart and spleen. Mutation in Wt1 changes the +KTS/-KTS ratio and causes male to female sex reversal (Frasier syndrome) in mice. However, +KTS and -KTS ablated mice show clearly different phenotypes, indicating distinct functions of the two splicing variants, in particular, in the sex determination pathway. Although *Wt1* duplication [designated as *Wt1a* and *Wt1b*] were found in different teleosts, but their role in early gonadal development is poorly understood. To understand the role of Wt1 in fish sex determination/differentiation, the two Wt1 genes were cloned, both in tilapia and Southern catfish, and the expression pattern in the developing gonad and kidney was studied. The ability of Wt1 in transcriptional regulation of *Cyp19a1a* and *Dmrt1* gene expression was also investigated.

Methods:

All genetic female (XX) and male (XY) tilapia (*Oreochromis niloticus*) were obtained by artificial fertilization of eggs from normal females (XX) with sperm from either sex-reversed males (XX) or super males (YY), respectively. Southern catfish (*Silurus meridionalis*), collected from Hechuang to Beibei section of the Jialing River, was used for the experiment. Gonads of adult tilapia and Southern catfish were dissected and total RNA was extracted. Different alternatively spliced isoforms (cDNAs) of Wt1a and Wt1b were cloned using RT-PCR and RACE. The body (after the removal of the yolk sac, intestine, head and tail) of the 3, 5, 20, 40 dah (days after hatching) fish and the gonads of the 8 month old fish were fixed and embedded in paraffin. Digoxigenin- labeled sense and antisense probes of Wt1a and Wt1b were used for *in situ* hybridization which was performed as previously described.

Approximately, 2kb promoter region of *Cyp19a1a* and *Dmrt1* were amplified from the tilapia genome and cloned into pGL3-basic vector. Different isoforms of Wt1 a/b were cloned into pcDNA3.1 plasmid. pRL-TK was used as an internal control plasmid. HEK293/TM4 cells were used for transcription analysis. Luciferase assay was performed using Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions.

Results and Discussion:

Both the *Wt1a* and *Wt1b* cDNAs were cloned from tilapia and Southern catfish. 5 variants of *Wt1a* and 3 variants of *Wt1b* were isolated from tilapia, which were derived from alternative splicing within the ORFs, including either a +KTS or –KTS isoforms. *Wt1a* of tilapia and Southern catfish comprises an ORF of 1245 and 1257bp, encoding 415 and 419aa, respectively. Tilapia Wt1b has a 1272bp ORF, encoding 424aa while the Southern catfish Wt1b has a 1209bp ORF, encoding 402aa. Multiple alignment analysis revealed that Wt1a was more conserved than Wt1b, as it shared a higher similarity to other teleosts (85-90%) as compared to mammals (70%). On the contrary, Wt1b shared a homology of 66% and 61% to other fishes and mammals, respectively. However, Wt1a and Wt1b of both the species showed less homology (<70%) among themselves. Phylogenetic analysis also revealed that fish Wt1a and Wt1b clustered into two different clads, due to the fish specific genome duplication.

In situ hybridization results showed that until 40dah, Wt1 homologues were found to express in the gonadal somatic cells and pronephrons of the kidney. Wt1a showed higher expression in the XY gonad, while Wt1b expression was found to be highest in both the XX and XY gonad. Wt1a and Wt1b expression was found to be down regulated with the development from 3dah to 40dah. In adulthood, (8 months after hatching) Wt1a/b expression was concentrated in the theca and interstitial cells of the ovary and Sertoli cells of the testis. Interestingly, Wt1b expression was seen to be higher in the testicular Sertoli cells that colocalizes with *Dmrt1*, a

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

gene critical for male sex determination, in testis. Promoter analysis showed that in various transcription factor tested, only the -KTS form of Wt1b activated *Dmrt1* in a dose dependent manner. Addition of Foxl2 and Sf1 could not change the Wt1b (-KTS) dependent *Dmrt1* activation. This shows the potential role of Wt1b in testis development via *Dmrt1* regulation. On the other hand, we failed to correlate *cyp19a1a* activation with either Wt1a or Wt1b homologues, which is different from the situation found in mammals.

Conclusion:

In the present study, transcription factor Wt1a and Wt1b were cloned in the Southern catfish and tilapia. Both genes were found to be expressed higher in the XY gonad compared with the XX gonad in the critical period for tilapia sex determination and differentiation. Furthermore, –KTS isoform of Wt1b can activate the *Dmrt1* transcription. Taken together, our data highlight the role of Wt1 in testicular differentiation. Even though several alternatively spliced forms of Wt1a and Wt1b were isolated, future detail analysis is necessary to understand the roles of individual homologue of Wt1 in the gonad developmental process.

SOME LIKE IT HOT: TEMPERATURE SELECTION DURING THE CRITICAL PERIOD OF THERMOLABILE SEX DETERMINATION IN PEJERREY (*ODONTESTHES BONARIENSIS*)

Kaiga, J.*, Strüssmann, C.A.*, Hattori, R.S.*,°, Oura, M.*, Yokota, M.*

*Graduate School of Marine Science and Technology, Tokyo University of Marine Science and Technology, 4-5-7 Konan, Minato, Tokyo, 108-8477, Japan. Tel/Fax +81-3-5463-0541 e-mail carlos@kaiyodai.ac.jp.
°Institute of Biosciences, University of Sao Paulo, Brazil

Introduction:

Many fish species have thermolabile sex determination (TSD) and the sex ratios of wild populations may be influenced by the thermal conditions during the reproductive season. The pejerrey (*Odontesthes bonariensis*) is an Atherinopsid fish that shows a remarkable level of TSD at environmentally relevant temperatures [1]. Thus, gonadal sex is determined between 1 and 5 weeks after hatching and the frequency of males varies from 0% at temperatures below 19°C to 100% at 29°C [2]. As a first step to model the effects of temperature on the sex ratios of wild populations and to clarify the adaptive significance of TSD, we examined the thermal preference of pejerrey larvae during the time of gonadal sex determination and how sex ratios vary under a situation where larvae can freely select the temperature.

Methods:

Newly hatched pejerrey larvae were stocked in a horizontal thermal gradient consisting of five interconnected aquaria kept at constant temperatures of 17, 20, 23, 26, and 29°C. Larvae were able to move freely through the entire gradient. Similar amounts of live food (*Artemia* nauplii) were delivered to each aquarium three times a day regardless of the presence of larvae. Larval behavior was monitored 24-hour/day for 5 weeks using overhead cameras and the number of larvae and time spent at each temperature was calculated. Sex ratios were analyzed at the end of the experiments by light histology.

Results and Conclusion:

The behavioral analysis revealed a preference for high, masculinizing temperatures (26-29°C) and the resulting sex ratios were highly male-biased (80-100%). We also observed a diurnal rhythm of temperature selection, with fish preferring higher temperatures at night compared to daytime. These results provide important clues to understand the ecology of TSD for this species and predict the sex ratios of wild populations as a function of temperature. They could be also useful for modeling the effects of global warming on pejerrey resources.

References:

[1]STRÜSSMANN, C.A., CONOVER, D.O., SOMOZA, G.M., MIRANDA, L.A. 2010. Implications of climate change for the reproductive capacity and survival of Atherinopsid fish species. J. Fish Biol., 77: 1818-1834.

[2]STRÜSSMANN, C.A., SAITO,T., USUI, M., YAMADA, H., TAKASHIMA, F. 1997. Thermal thresholds and critical period of thermolabile sex determination in two atherinid fishes, *Odontesthes bonariensis* and *Patagonina hatcheri*. J. Exp. Zool., A 278: 167-177.

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

ISOLATION OF SEX-SPECIFIC MARKERS IN THE NILE TILAPIA, *OREOCHROMIS NILOTICUS*, BY AFLP

Sun Y.L., Zeng S., Ye K., Yang C., Wang D.S.

Key Laboratory of Freshwater Fish Reproduction and Development (Ministry of Education), Key Laboratory of Aquatic Science of Chongqing, School of Life Science, Southwest University, Chongqing, 400715, China
Fax: +86-2368253005, E-mail: wdeshou@swu.edu.cn

Introduction:

Fish and other aquatic products have been widely recognized as a safe source of protein for the world's rapidly growing population, and aquaculture has become one of fastest growing industries in China. The Nile tilapia (*Oreochromis niloticus*) is one of the most important species among the farmed fish (FAO 2007). The growth rate of male individuals is significantly faster than that of females after gonadal differentiation, and the average weight of the 1-year-old male tilapia is about 30% higher than that of the same-age female. Therefore, breeding and culturing all-male population of the Nile tilapia is a good way to rapidly increase yields. A major challenge in commercial production of tilapia is to control reproduction, as the fish often begins to breed before reaching the marketable size. The traditional method to control reproduction was to culture only phenotypic males produced by hormonal sex reversal, artificial selection, interspecific cross etc. However, all these manipulations had some drawbacks. An effective method for producing all genetic male tilapia (GMT) on a large scale is to cross normal females (XX) with supermales (YY) broodstock. In general, for GMT production, identification of the sex specific molecular marker is the key point. To date, no sex-specific markers have been found in tilapia, and therefore, identification of the genotypic sex in the Nile tilapia requires extensive progeny testing. The goal of this study was to identify gender-related DNA markers in the Nile tilapia by AFLP fingerprinting. Subsequently, these markers were used to establish a molecular method to distinguish gender in this species.

Methods:

The constructive strain of the Nile tilapia, *Oreochromis niloticus*, was obtained from Prof. Nagahama (Laboratory of Reproductive Biology, National Institute for Basic Biology, Japan). Genomic DNA from no less than 5 individuals of XX, XY and YY tilapia were taken to construct genomic DNA pools. The AFLP procedure used for isolation of sex-specific markers was as described by Vos et al. (1995) with minor modifications, 256 different primer combinations in total were analyzed, with samples electrophoresed on an BIO-RAD Sequi-Gen GT DNA sequencer and gel analysis carried out using GeneScan.

Results and Discussion:

In our study, using bulked segregant analysis we identified one male specific AFLP marker, named as NtY1 that showed a sex-specific pattern of segregation. After sequence analysis, a different male specific SCAR marker NtY2 and a female specific SCAR marker NtX1 were designed. PCR combined with H.E. stained gonad section examination were performed to further confirm the accuracy using different SCAR markers for amplification of the expected fragments from 131 individual (78 males、53 females and 5 YY supermale genomic DNA). The NtY1 and NtY2 male specific fragment were amplified in all 78 XY males and 5 YY supermale but only in 8 of the 58 XX females (94.1% overall efficiency). The NtX1 female specific fragment was amplified in all XY males and XX females but not in 5 YY males (100% accuracy). This finding has important implications for commercial aquaculture, not only in shortening the breeding period and improving the economic benefits, but is also conducive in excluding the sex reversal in fish, induced by environmental effects or autosomal factors. Access to sex-specific molecular markers allowed a rapid detection (selection) of genetic XX, XY and YY tilapia for the establishment of mono-sex tilapia (XX, XY and YY) production systems.

Conclusion:

Unisex cultivation is an important trend in the modern fish breeding industry. In this study, we obtained two sex specific markers, one is X specific and the other is Y specific. Combined use of these two markers allowed us to distinguish genetic XX, XY and YY tilapia, no matter it is normal or sex reversed in phenotype. This is a critical step for successful development of all male and supermale tilapia system. Sex specific markers could also help us to identify the sex chromosome and clone the sex determine gene of the Nile tilapia, and deepen our understanding of the molecular mechanism for fish sex determination and differentiation.

Acknowledgments:

This work was supported by grants from the National High Technology Research and Development Program (863 program) of China (No. 2007AA10Z165), the National Basic Research Program of China (2009CB941200 and 2010CB134405), the Program for Changjiang Scholars and Innovative Research Team in University (IRT0859) and the Specialized Research Fund for the Doctoral Program of Higher Education of China (No. 20090182110008), the Natural Science Foundation Project of Chongqing, Chongqing Science and Technology Commission (Nos. CQ CSTC 2008BB1006 and 2008AC1016), and the Science and Technology Innovation Fund for the Graduates of Southwest University (Nos. kb2009009 and kb2010009).

SEXUALLY DIMORPHIC EXPRESSION OF AROMATASE IN THE MEDAKA BRAIN

Takeuchi A, Okubo K.

Department of Aquatic Bioscience, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo, Tokyo 113-8657, Japan
Fax: +81-3-5841-5289, e-mail: takeuchi@marine.fs.a.u-tokyo.ac.jp.

Introduction:

Male and female vertebrates, including teleost fishes, exhibit differences in a variety of behavioral and physiological traits, including reproductive behavior, aggression, and endocrine secretion patterns. Several types of neuroanatomical and neurochemical sex differences have been identified in mammals and birds that could underlie these functional differences [3, 5]. In contrast, very little is known about sex differences in the brain of teleosts. Teleosts should, however, provide unique models for the study of sex differences and sexual differentiation of the brain in that their brains exhibit a considerable degree of sexual plasticity throughout their lifetime [7]. To uncover the molecular basis of sex differences in the teleost brain, we examined sex differences in the expression of the brain-predominant form of aromatase, the rate-limiting enzyme in the conversion of androgen to estrogen [2, 4, 6, 8], in the brain of medaka *Oryzias latipes*.

Methods:

We searched for genes exhibiting sexually dimorphic patterns of expression in the medaka brain, and identified the aromatase gene as being predominantly expressed in females. Sex differences in its expression were examined in relation to several factors, including reproductive stages, genetic sex, phenotypic sex, and gonadal steroids. We also determined which brain area and cell type were the source of the sex difference in aromatase expression.

Results:

A series of analyses revealed that aromatase expression in the medaka brain was not under the direct control of sex chromosome genes, but increased secretion of estrogen from the ovary, but not from the testis, after the onset of puberty led to higher expression of aromatase in the female than male brain in a reversible and transient manner. The medaka aromatase was expressed throughout the ventricular zones in the brain, where, in most regions, females have a greater degree of expression compared to males. The most prominent sex difference was observed in the optic tectum, where expression was almost specific to females only. We also found that contrary to what is known in other teleost species, radial glial cells was not the source of aromatase expression in the medaka brain.

Conclusion:

Since it is widely accepted, although not proven in teleosts, that aromatase in the brain plays an important role in sex differences and sexual differentiation of the vertebrate brain [1, 6, 9], the reversible and transient regulatory system for aromatase expression in medaka may be able to account for the conspicuous sexual plasticity of the teleost brain. Further studies are needed to understand what determines the unique cell specificity of aromatase expression in the medaka brain and what physiological significance this will lead to.

References:

[1]BALTHAZART J., CORNIL C.A., CHARLIER T.D., TAZIAUX M., BALL G.F. 2009. Estradiol, a key endocrine signal in the sexual differentiation and activation of reproductive behavior in quail. J. Exp. Zool. A Ecol. Genet. Physiol., 311: 323-345.

[2]CALLARD G.V., TCHOUDAKOVA A.V., KISHIDA M., WOOD E. 2001. Differential tissue distribution, developmental programming, estrogen regulation and promoter characteristics of cyp19 genes in teleost fish. J. Steroid Biochem. Mol. Biol., 79: 305-314.

[3]DE VRIES G.J., SÖDERSTEN P. 2009. Sex differences in the brain: the relation between structure and function. Horm. Behav., 55: 589-596.

[4]DIOTEL N., LE PAGE Y., MOURIEC K., TONG S.K., PELLEGRINI E., VAILLANT C., ANGLADE I, BRION F., PAKDEL F., CHUNG B.C., KAH O. 2010. Aromatase in the brain of teleost fish: expression, regulation and putative functions. Front. Neuroendocrinol., 31: 172-192.

[5]FORGER N.G. 2009. Control of cell number in the sexually dimorphic brain and spinal cord. J. Neuroendocrinol., 21: 393-399.

[6]FORLANO P.M., SCHLINGER B.A., BASS A.H. 2006. Brain aromatase: new lessons from non-mammalian model systems. Front. Neuroendocrinol., 27: 247-274.

[7]GODWIN J. 2010. Neuroendocrinology of sexual plasticity in teleost fishes. Front. Neuroendocrinol., 31: 203-216.

[8]LE PAGE Y., DIOTEL N., VAILLANT C., PELLEGRINI E., ANGLADE I., MÉROT Y., KAH O. 2010. Aromatase, brain sexualization and plasticity: the fish paradigm. Eur. J. Neurosci., 32: 2105-2115.

[9]ROSELLI C.F. 2007. Brain aromatase: roles in reproduction and neuroprotection. J. Steroid Biochem. Mol. Biol., 106: 143-150.

ANALYSIS OF REGULATIONAL MECHANISM OF FOLLICLE-STIMULATING HORMONE RECEPTOR (FSHR) EXPRESSION USING FSHR-GFP TRANSGENIC MEDAKA.

Uchikawa T.*, Kobira H. *, Hirai T.°, Kitano T. *

*Department of Biological Sciences, Graduate School of Science and Technology, Kumamoto University, 2-39-1 Kurokami, Kumamoto 860-8555, Japan.e-mail: kumautuchi@yahoo.co.jp

°Department of Bioengineering, Teikyo University of Science and Technology, Yamanashi 409-0193, Japan.

Introduction:

Medaka (*Oryzias latipes*) is a teleost fish that has a XX/XY sex determination system. The fish has several desirable features, including a short generation time, small genome size, and the availability of several useful strains [1], suggesting that the medaka is an excellent fish model for the molecular genetic analysis of various biological phenomena, including embryonic development and sex differentiation. We previously showed that the expression level of follicle-stimulating hormone receptor (*fshr*) mRNA in XX medaka was higher than that in XY fishes at the hatching stage (the sexual differentiation stage). Moreover, cortisol, a glucocorticoid which induces masculinization of XX medaka [2], suppressed the expression of *fshr* mRNA in XX fishes, suggesting that FSHR may be involved in the ovarian differentiation in medaka. However, the molecular mechanism of transcriptional regulation of *fshr* gene in medaka remains unclear.

Methods:

The FLFII medaka stock was used [3]. This stock allows identification of genotypic sex by the appearance of leucophores before the onset of sex differentiation. *Fshr-GFP* transgenic (Tg) medaka line was generated by injecting the EGFP vector fused to the regulatory region of the medaka *fshr* gene (DDBJ accession no. AB526251) into fertilized eggs of the FLFII medaka. All injected embryos were bred to adults, and only F1 embryos possessing GFP fluorescence were selected and used to produce succeeding generations. We then mated the *fshr-GFP* Tg XX medaka with *olvas-DsRed* Tg XY fishes [2], where the germ cells could be visualized by DsRed fluorescence. Medaka embryos and larvae were reared in ERM (17 mM NaCl, 0.4 mM KCl, 0.27 mM $CaCl_2 2H_2O$, 0.66 mM $MgSO_4$, pH 7) mixed with or without cortisol (Sigma-Aldrich) at 26°C from 0 days post-fertilization (dpf) to 9 dpf (0 days post-hatching (dph)). Immunohistochemistry was performed with mouse monoclonal anti-GFP antibody (Roche) or rabbit polyclonal anti-FSHR antibody using the larvae at 0 dph as described previously [4]. To quantify expression levels of *GFP* and *fshr* mRNAs in the larvae at 0 dph, quantitative real-time PCR analysis was performed using SYBR Green I Master (Roche) on a LightCycler 480 (Roche).

Results and Discussion:

GFP signals were observed in the gonadal somatic cells surrounding the DsRed-positive germ cells in the larvae at 0 dph under a laser scanning microscope (FV10i, Olympus). Additionally, immunohistological observations of gonads in the adult medaka showed that both FSHR and GFP signals were co-localized in the gonadal somatic cells. These results suggest that GFP expression in the *fshr-GFP* Tg medaka line reflects FSHR expression in gonads. On the other hand, quantitative real-time PCR analysis showed that both *GFP* and *fshr* mRNAs in cortisol-treated XX larvae were lower than those in normal XX larvae at 0 dph. Moreover, we observed GFP expression petterns in the cortisol-treated medaka at 0 dph under a fluorescent microscope (MZFLIII, Leica). GFP signals in the gonadal somatic cells of cortisol-treated XX larvae were weaker than those of normal XX larvae. These results suggest that cortisol inhibits *fshr* mRNA expression via the promoter *in vivo*.

Conclusion:

In the present study, we established *fshr-GFP* Tg medaka line that expresses GFP specifically in the gonadal somatic cells. Additionally, cortisol inhibited the expression of GFP and *fshr* mRNAs in the Tg XX larvae. These results show that this Tg line provides a useful model for analyzing gonadal development and sex differentiation.

References:

[1]ISHIKAWA Y. 2000. Medakafish as a model system for vertebrate developmental genetics. BioEssays, 22:487–495.

[2]HAYASHI Y., KOBIRA H., YAMAGUCHI T., SHIRAISHI E., YAZAWA T., HIRAI T., KAMEI Y., KITANO T. 2010. High temperature causes masculinization of genetically female medaka by elevation of cortisol. Mol. Reprod. Dev. 77:679–686.

[3]WAKAMATSU Y., INOUE C., HAYASHI H., MISHIMA N., SAKAIZUMI M., OZATO K. 2005. Establishment of new medaka (*Oryzias latipes*) stocks carrying genotypic sex markers. Environ. Sci. 10: 291-302.

[4]YAMAGUCHI T. YOSHINAGA N., YAZAWA T., GEN K., KITANO T. 2010. Cortisol is involved in temperature-dependent sex determination in the Japanese flounder. Endocrinology, 151: 3900-390.

UNDIFFERENTIATED GONADS OF PEJERREY (*ODONTESTHES BONARIENSIS*) LARVAE EXPOSED TO MASCULINIZING TEMPERATURES PRODUCE 11-KETOTESTOSTERONE WHEN INCUBATED WITH A TRITIATED PRECURSOR

Blasco, M.[1], Somoza, G.M.[1] and Vizziano-Cantonnet, D.[2]

[1]IIB-INTECH (CONICET-UNSAM), Chascomús, Argentina
[2]Laboratorio de Fisiología de la Reproducción y Ecología de Peces, Facultad de Ciencias. Iguá 4225, Montevideo (11400), Uruguay. FAX +598-2 5258617
e-mail: vizziano@gmail.com

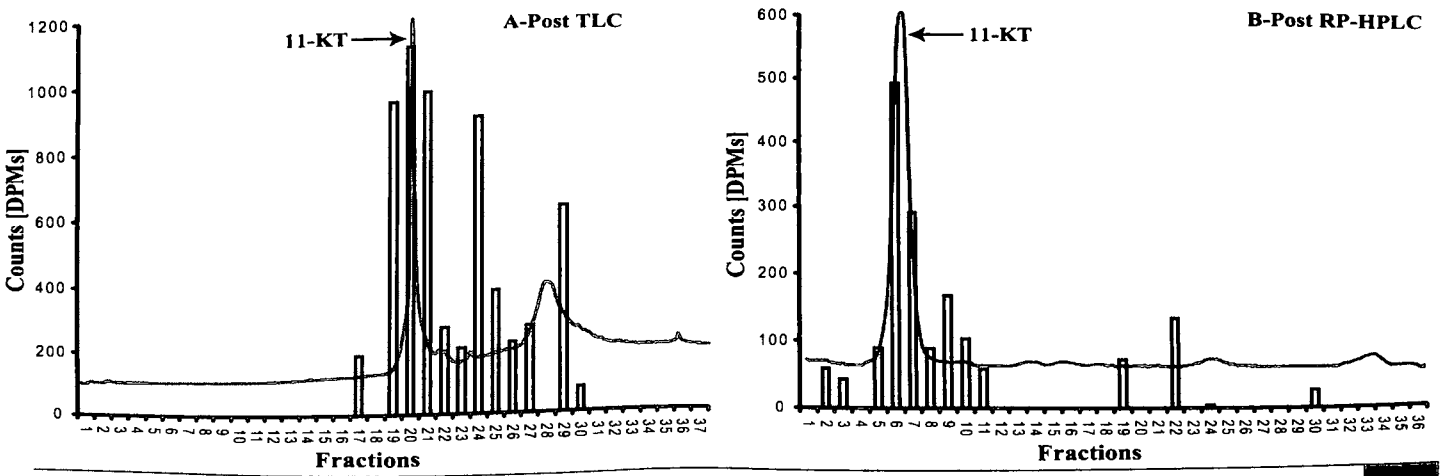
Introduction:

The involvement of androgens as early mediators of testis differentiation in fish is still a matter of debate [1]. Larval gonadal size limits the possibility to establish whether an early production of 11-oxigenated androgens precedes testicular differentiation. In a previous study we reported the expression of genes involved in androgen synthesis (i.e. *cyp11a* y la *cyp11b)* in larval trunks [2] and the production of 11-ketotestosterone was reported in larval trunks by EIA [3]. In this context the aim of the present work was to study the androgen synthesis capacity of gonads and peritoneum of *Odontesthes bonariensis* in larvae exposed to masculinizing temperatures (29°C) before morphological differentiation of the testis. First we validate the nature of androgens produced by adult testis, liver and spleen and then we studied the androgen production in larval gonads at 29°C.

Methods:

Pejerrey larvae were raise for 5 weeks at 29°C in order to obtain a whole male population. At 5 that moment, 100 fish were sampled and individually immerse in ice-cold water until movement ceased. Due to technological constrains in taking then apart, the gonads and peritoneum were dissected together under binocular microscope and pooled in ice cold L-15 medium. Adult testis at the onset of spermatogenesis, spleen and liver were also obtained and placed on L-15 medium. Tissue samples were incubated for 18 hs in 500 µL of L-15 medium in the presence of tritium labeled precursors. 17P was used for gonad and peritoneum, and A4 for spleen and liver. The reaction was arrested by ethanol addition to 80% followed by tissue disaggregation using a glass-glass grinder. The supernatant was evaporated under nitrogen until 20% its original volume qws reached and then extracted three times with dichloromethane (DM): methanol (M) (9DM:1M). This fraction was re-suspended and applied to a silica TLC plate, cleaned by a non-polar mobile phase run and then resolved by using benzene acetone (2:1). The resolved radioactive steroids were revealed by autoradiography, the characteristic Rf computed and radioactive zones isolated by scratching the silica followed by extraction using three rounds of 9DM: 1M. The bands extracts corresponding to 11-ketotestosterone (11-KT) and 11β-hydroxyandrostenedione (11β-OHA4) were separately analyzed by HPLC using a RP-C18 column and 0.5 mL fractions were obtained and their radioactivity content determined by scintillation counting. The 11β-OHA4 obtained from kidney extracts was analyzed by co-crystallization to constant specific activity.

Figure 1: Chromatogram is shown as a continuous line and the fraction radioactivity in bars. A: 11-KT co-eluting fraction from the TLC resolved by RP-HPLC. B: For fractions 19-21 from A resolved by RP-HPLC.



Results:

The results of the incubation of the 5 larval gonads and peritoneum from male promoting temperature (29°C) showed that these tissues were able to metabolize 17P to 11-KT (Figure 1). Furthermore, the analysis of the zone corresponding to 11β-OHA4 (fractions 19-21) resulted in no radioactivity after resolution by RP-HPLC. In the adult testis the major androgen observed in the presence of 17P was 11β-OHA4. On the other hand the incubation of spleen with A4 resulted in 11β-OHA4 whereas liver did not show production of neither 11-KT nor 11β-OHA4 (data not shown).

Discussion and Conclusion:

In fish, 11-oxygenated androgens are considered the biologically active androgens, but their roles in early gonadal development are not fully understood. Here it was observed that undifferentiated gonads (and/or nearby tissues) of larvae exposed to masculinizing temperatures were able to produce 11-KT from 17P. This data, together with previous information, suggests that 11KT can act as early mediator of testis differentiation. Furthermore, it was observed a clear difference in the 11-oxygenated androgen profile produced by mature male fish gonads, were 11β-OHA4 dominates, indicating that steroidogenic pathways are different in non-differentiated compared to adult fish. Furthermore, as adult spleen is able to produce 11β-OHA4 by using A4, other tissues would be cooperating in the production of 11-oxygenated androgens in fish during early sex differentiation. 11-KT would be involved in the gonadal differentiation process driven by temperature in pejerrey.

References:

[1]VIZZIANO, D., RANDUINEAU, G., BARON, D., CAUTY, C., GUIGUEN, Y. 2007. Characterization of early molecular sex differentiation in rainbow trout, *Oncorhynchus mykiss*. Dev. Dyn., 236: 2198–2206.

[2]BLASCO, M., FERNANDINO, J.I., GUILGUR, L.G., STRÜSSMANN, C.A., SOMOZA, G.M., VIZZIANO–CANTONNET, D. 2010. Molecular characterization of *cyp11a1* and *cyp11b1* and their gene expression profile in pejerrey (*Odontesthes bonariensis*) during early gonadal development. Comp. Biochem. Physiol., *A* 156: 110-118.

[3]HATTORI, R.S., FERNANDINO, J.I., KISHII, A., KIMURA, H., KINNO, T., OURA, M., SOMOZA, G.M., YOKOTA, M., STRÜSSMANN, C.A., WATANABE, S. 2009. Cortisol-Induced Masculinization: Does thermal stress affect gonadal fate in pejerrey, a teleost fish with temperature-dependent sex determination? PLOS ONE, 4(8): e6548.

Reproductive Cycles, Behaviour and Migration

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

OVARIAN STRUCTURES THAT SUPPORT REPRODUCTIVE CYCLES- GERMLINE STEM CELLS AND THEIR NICHE STRUCTURE IN OVARY

Tanaka, M., Nakamura, S., Saito, D., Kobayashi, K. and Nishimura, T.

Lab. of Molecular Genetics for Reproduction, National Institute for Basic Biology
Higashiyama, Myodaiji, Okazaki 444-8787, Japan
Fax: +81-564-59-5851, email: mtanaka@nibb.ac.jp

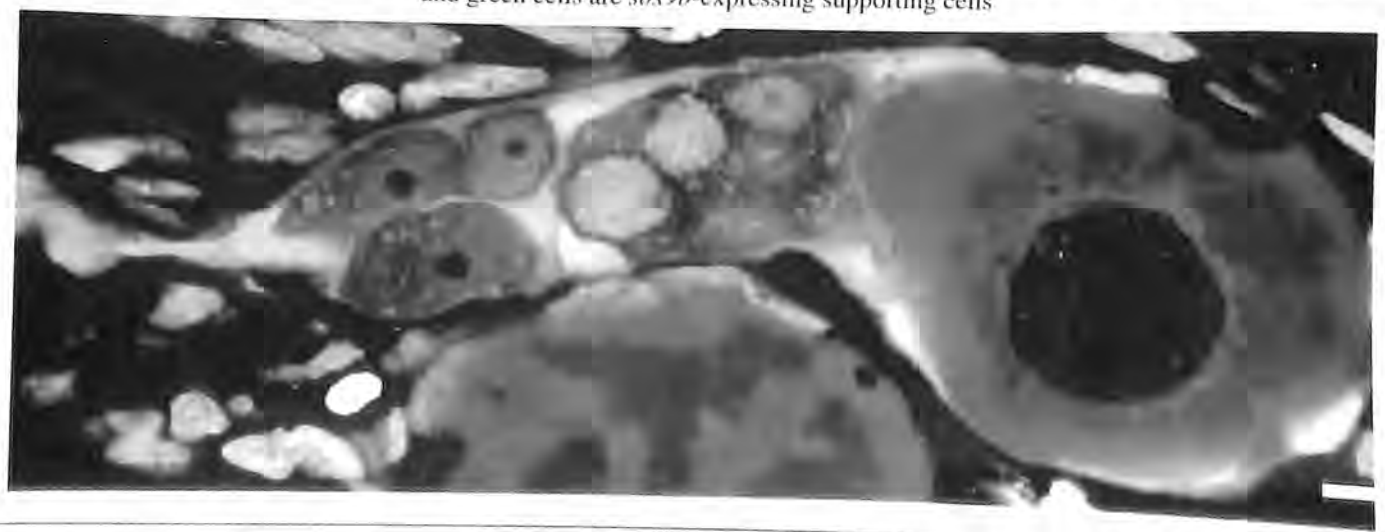
Fish species form a largest group in vertebrates and different species show a variety of reproductive cycles, dependent on their reproductive strategies. Some species spawn eggs everyday but another species exhibit periodical spawning over years. From the intensive analyses of histology, it has been thought that the regulation at the level of oogonia and spermatogonia is a key to proper reproductive cycles. However, little is known about the characters of these gonial cells in the process of production of eggs and sperms.

Stem cells are the conceptual cells that have the capability both to keep producing differentiated cells and to retain self-renewal. In the reproductive organs, there should be many types of stem cells that could explain not only the continuous production of eggs and sperms (germline stem cells) but also the maintenance and growth of its structure (somatic stem cells). Especially in mammals, germline stem cells have been investigated intensively. Recent research has proved the presence of germline stem cells in testis and begins to reveal their characters and regulations while it is well accepted that there are no germline stem cells in ovary because all primordial germ cells in the developing ovary differentiate into oocytes and make a pool of oocytes during fetal stages for adult reproductive term. Therefore the presence of germline stem cells that accounts for many aspects of reproduction in ovary has not been identified in vertebrates.

Medaka (*Oryzias latipes*) spawns a dozen of eggs everyday at the onset of light during a couple of months of reproductive term. Our previous analysis indicated that *nos2* gene is expressed in both oogonial and spermatogonial cells and that, unlike mammalian gonads, medaka *sox9* orhtologue, *sox9b*, is expressed in both ovary and testis. With combination of heat-inducble cre recombinase and *nos2*-promoter driven fluorescent protein (GFP and RsRed), we have developed transgenic medaka that allow clonal analysis of germ cells. This analysis clearly indicated that *nos2*-expressing oogonia have the ability to produce eggs continuously for a reproductive term and these eggs are found fertile to develop into embyos. Therefore the *nos2*-expressing oogonia completely fulfill the criteria for germline stem cells.

The germline stem cells reside in a special structure, called germinal cradle, composed of *sox9b*-expressing cells (Figure 1). The germinal cradles are interconnected to each other with a thin cellular process of *sox9b*-expressing cells, thus forming networks, called ovarian cords. This networks spread within germinal epithelium on the dorsal surface of ovary. The germinal epithelium is the epithelial tissues where many researchers more than 50 years ago postulated the presence of oogonia in adult mammalian ovary and one of the important tissues

Figure 1. An ovarian niche for germline stem cells (germinal cradle): Red cells are germ cells at the early stages of oogenesis and green cells are *sox9b*-expressing supporting cells



Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

for the origin of ovarian cancer. Oogenesis proceeds from germline stem cells to very early stages of oocytes in the germinal cradles. Cystic division of amplifying oogonia occurs in the cradles, which is evidenced by imaging technique. The imaging also reveals that some populations of oogonia are eliminated during the cystic division. All these observations indicated that a germinal cradle is a very important place to ensure and regulate reproduction according to both internal and external environment. The sex of medaka is determined genetically. However, some treatment, for example causing the change of hormonal environment, exerts sex reversal. In addition, it is indicated by other group that germ cells are sexually reversible or bipotent. These evidences suggest that the germinal cradles are also important for regulation and maintenance of sex.

References:

[1]NAKAMURA, S., KOBAYASHI, K., NISHIMURA, T., HIGASHIJIMA, S., TANAKA, M. 2010. Identification of germline stem cells in the ovary of teleost medaka. Science, 328: 1561- 1563.

[2]AOKI, Y., NAKAMAURA, S., ISHIKAWA, Y., TANAKA, M. 2009. Expresssion and systemic analysis of four *nanos* genes in medaka. Zool. Sci., 26: 112-118.

[3]NAKAMURA, S., AOKI, Y., SAITO, D., KUROKI, Y., FUJIYAMA, A., NARUSE, K., TANAKA, M. 2008. *Sox9b/sox9a2*-EGFP transgenic medaka reveals the morphological reorganization of the gonads and a common precursor of both the female and male supporting cells. Mol. Reprod. Dev., 75: 472-476.

[4]SAITO, D., MORINAGA, C., AOKI, Y., NAKAMURA, S., MITANI, H., FURUTANI-SEIKI, M., KONDOH, H., TANAKA, M. 2007. Proliferation of germ cells during gonadal sex differentiation in medaka: insights from germ cell depleted mutant *zenzai*. Dev. Biol., 310: 280-290.

[5]KUROKAWA, H., SAITO, D., NAKAMURA, S., KATOH-FUKUI, Y., OHTA, K., AOKI, Y., BABA, T., MOROHASHI, K., TANAKA, M. 2007. Germ cells are essential for sexual dimorphism in the medaka gonad. Proc. Acad. Natl. Sci. USA, 104: 16958-16963.

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

NEUROPHYSIOLOGICAL MECHANISMS OF IMPRINTING AND HOMING MIGRATION IN SALMON

Hiroshi UEDA

Field Science Center for Northern Biosphere, Hokkaido University, North 9 West 9, Kita-ku, Sapporo, Hokkaido 060-0809, Japan.

Introduction:

Salmon have an amazing ability to migrate thousands of kilometers from the open ocean to their natal stream for reproduction after several years of oceanic feeding migration. It is now widely accepted that specific factors of the natal stream are imprinted to particular nervous systems of juvenile salmon during downstream migration and that adult salmon evoke these factors to recognize the natal stream during upstream homing migration.

Methods:

Three different research approaches from behavioral to molecular biological studies have been applied to clarify neurophysiological mechanisms of mysterious salmon imprinting and homing migration using four anadromous Pacific salmon (*Oncorhynchus* species; pink, chum, sockeye, and masu salmon) migrating from the North Pacific Ocean to Hokkaido, as well as lacustrine sockeye and masu salmon in Lake Toya, Hokkaido, where the lake serves as the model oceanic system.

Results:

The first approach was behavioral research by means of biotelemetry techniques. We tracked homing migration of sensory function-disturbed lacustrine sockeye and masu salmon in Lake Toya, and found that both visual and olfactory cues were important for these lacustrine salmon. The second approach was endocrinological research on hormone profiles in the brain-pituitary-gonadal axis of chum salmon migrating from the Bering Sea to the spawning ground in Hokkaido. Levels of two types of gonadotropin-releasing hormone (sGnRH and cIIGnRH), two types of gonadotropin (LH and FSH) in the pituitary, and gonadal steroid hormones were measured by specific time-resolved fluoroimmunoassay. We also carried out *in vitro* brain slice experiment to clarify possible roles of N-Methyl-D-Aspartate (NMDA) receptor on GnRH functions. The third approach was molecular and physiological research on olfactory functions that play a crucial role in imprint and discrimination of the natal stream odorant during downstream and upstream migration, respectively. We cloned and characterized a salmon olfactory system imprinting-related gene and salmon odorant receptor genes. We also compared whether dissolved free amino acid (DFAA) mixture of their natal stream have attractive effects on upstream selective movement among four Pacific salmon.

Conclusion:

These results demonstrate the navigation ability in open water, the hormone profiles in the brain-pituitary-gonadal axis, and the olfactory functions related with imprinting and discriminating ability of natal stream odorants in salmon. These recent findings are discussed in relation to neurophysiological mechanisms of the amazing imprinting and homing migration in anadromous and lacustrine salmon.

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

REPRODUCTIVE BEHAVIOUR, THE ABSENCE OF REPRODUCTIVE BEHAVIOUR IN CULTURED (G1 GENERATION) AND CHEMICAL COMMUNICATION IN THE SENEGALESE SOLE (SOLEA SENEGALENSIS)

Ignacio Carazo[1], Ignacio Martin[2], Peter Hubbard[3], Olvido Chereguini[2], Evaristo Mañanós[4], Adelino Canário[3], and Neil Duncan[1]*

[1]IRTA-Sant Carles de la Rápita, Tarragona, Spain
[2]Instituto Español de Oceanografía (IEO), Barrio de Corbanera, Santander, Spain.
[3]CCMAR, Universidade do Algarve, Campus de Gambelas, 8005-139, Faro, Portugal
[4]CSIC-IATS, Torre la Sal s/n, 12595-Cabanes, Castellón, Spain.
*Corresponding author: IRTA-Sant Carles de la Rápita, Ctra. Poble Nou Km 6, 43540-Sant Carles de la Rápita, Tarragona, Spain Fax +34-977744138 email: neil.duncan@irta.cat

Introduction:

Wild caught sole held in captivity spawn sufficient quality and quantity for commercial culture whereas cultured sole (G1 generation) held in the same conditions spawn infrequently producing eggs of poor quality. The correct sequence of reproductive behaviour or courtship is a requirement in many species of fish for the successful completion of spawning. The present study examined the reproductive behaviour of wild and cultured Senegalese sole and the potential for chemical communication.

Methods:

In the present studies the reproductive behaviour of groups of wild and G1 Senegalese sole was observed and recorded with cameras and low intensity red light night illumination. In the IEO Santander, two different tanks (14,000 L with 29 fish: 14 females and 15 males) of wild Senegalese sole that spawned frequently large volumes (>100 ml floating eggs with >75% fertilisation) of fertilised eggs were each observed for a period of eight days. A series of different experiments with groups of cultured G1 sole were observed: Exp. Nat: In el IEO Santander a tank of G1 broodstock that spawned infrequently but spontaneously were observed over a period of two months. Exp. GnRH: Two tanks of ten sole (five females and five males) were treated with a single injection of 10 $\mu g\ kg^{-1}$ GnRHa and observed during the following week. Exp. hCG: Six tanks each with two females and three males were set up. Females were treated with 50 μg implant of GnRHa $fish^{-1}$ and males were given three different treatments in duplicate, a) 50 μg implant of GnRHa $fish^{-1}$ (two tanks) b) three injections of 1000 IU hCG (two tanks) and controls with no treatment (two tanks). Exp. PG: Six tanks each with two females and three males were set up. In experimental groups (four tanks), females and males were treated with repeated injections of 5 $\mu g\ kg^{-1}$ of GnRHa and after spawning started with 100 $\mu g\ kg^{-1}$ $PGF_{2\alpha}$. Controls were not treated (two tanks). Finally in the IEO Santander, two tanks of mixed stocks were monitored and observed when spawning was obtained, one tank contained 6 wild caught males and 10 G1 and G2 females and a second tank contained 6 G1 males and 10 wild females.

The olfactory potency of conspecific skin mucus, urine and intestinal fluids was measured by the electro-olfactogram. Skin mucus, urine and intestinal fluids were collected from immature and mature G1 Senegalese sole. Urine and intestinal fluids were filtered, fractionated (C-18 extraction cartridge) and diluted [1]. The potency of diluted filtrate and eluate was assessed by the electro-olfactogram as has been described [2] and using juvenile Senegalese sole.

Results:

The wild Senegalese sole were observed to spawn eight times and on each occasion over 300 mL of fertilised eggs were collected in the morning. The associated reproductive behaviour was observed and described and was divided into 3 periods that can be briefly described as: 1) A period of intense activity between a potential female spawner, males and amongst males. 2) The female swims from the bottom of the tank accompanied by a male. 3) The female and male swim in synchrony at the water's surface with the genital ducts held closely together and release and fertilise the gametes. In all the experiments with only cultured G1 sole broodstock the eggs that were collected did not develop and appeared to not have been fertilised. No G1 females were observed to swim to the surface in the company of a male. In Exp. Nat six spawns were collected, in Exp. GnRH a total of five spawns of more than 20 mL of eggs were collected, in Exp. hCG a total of 31 spawns of more than 20 mL of eggs were collected with a range of four to seven spawns per group and in Exp. PG a total of 9 spawns of more than 20 mL of eggs were collected. These observations indicated that G1 Senegalese sole have a reproductive behavioural dysfunction that resulted in the infrequent liberation of ova that were not fertilised by the mature males and, therefore, the eggs did not develop. The group of mixed

stocks that contained wild males and G1 and G2 females produced fertilised spawns and during a period of observation of one month five spawns were collected of which two contained fertilised eggs. Observations confirmed that the on each night before a fertilised spawn was collected a wild male and a captive breed female successfully completed the reproductive behaviour and spawned. No spawns were obtained from G1 males and wild females. These observations suggest that the behavioural problem associated with captivity breed broodstock maybe in particular a problem with G1 males.Electro-olfactogram responses were significantly higher to the eluate fraction from urine compared to other fractions and all fractions from mucus and intestinal fluids. The sensitivity to the eluate fraction from urine could be ranked highest sensitivity to lowest: mature female > mature male > immature female > immature male. The eluate fraction contains non-polar lipids which include the sterol lipids such as estrogens, androgens (testosterone and androsterone), the progestogens and prostaglandins.

Conclusion:

Wild Senegalese sole have a complex reproductive behaviour where pairs spawn together in synchrony and which is similar to the spawning behaviour of other flat fish. The cultured G1 Senegalese sole do not complete the reproductive behaviour observed in wild broodstock and eggs spawned were not fertilised. This behavioural dysfunction may be in particular a G1 male problem as captivity breed females spawned with wild males, but no G1 male spawned with a wild female. The G1 broodstock urine contained non-polar chemicals that act as potent odorants, suggesting that chemical communication may be important during spawning of the sole

References:

[1]HUERTAS *ET AL.*, (2007) . J. Fish Biol.,70: 1907-1920

[2]VELEZ *ET AL.*, (2005). Physiol.Biochem Zool., 78: 756-765

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

MALE TILAPIA URINARY PHEROMONE INDUCES ENDOCRINE SEX RESPONSES IN CONSPECIFIC FEMALES

Hubbard P.C., Huertas M., Almeida, O.G. and Canário, A.V.M.

Centro de Ciências do Mar, Universidade do Algarve, Campus de Gambelas, 8005-139 Faro, Portugal
Fax: +351- 289 800 051; e-mail: phubbard@ualg.pt

Introduction:

The study of control of reproduction by sex pheromones in fish is mostly restricted to behavioural responses to sex cues. Our aim was to test whether male Mozambique tilapia (*Oreochromis mossambicus*) urinary pheromone modulates the steroidogenic - physiological - control of female reproduction. This hypothesis was addressed because: male tilapia can discern between olfactory stimuli of pre-ovulated and post-ovulated females [1]; male tilapia increase their urination rate in the presence of ovulated females [2]; female tilapia release eggs when exposed to male urine (unpublished). To test this hypothesis we used a non-invasive method, by measuring release of cortisol, testosterone, estradiol and 17,20βP by pre-ovulated and post- ovulated tilapia female into the water after exposure to pheromonal stimulus (male urine). Moreover, blood and urine samples were taken from the females at the end of the experiment for measurement of steroid concentration and plasma steroid binding properties to understand the dynamics of steroid transport and release in this species.

Methods:

A pre- or post-ovulatory female was placed in a tank with clean water (10 g fish.l^{-1}). One litre of water was taken at 0h, 1h, 2h and 6h after male urine exposure (final dilution 1:10 000) or distilled water (control) and replaced with 1l clean water. At the end of the experiment (6h) urine and blood samples were taken. Steroid extraction from water and biological fluids and steroid quantification by radio-immuno assay (RIA) were carried out according Huertas *et al.* [3]. All steroid measurements were validated for recovery, specificity and identity following the guidelines of Scott *et al.* [4]. To characterize steroid binding affinity, capacity and specificity in female plasma, we followed the method of Scott *et al.* [5].

Results and Discussion:

The exposure to male urine dramatically affected the release of 17,20βP to the water by female tilapia with a rapid response within 1h of treatment. The release rate of free 17,20βP rose to 113±24 pg.$h^{-1}kg^{-1}$ fish after 1h. The release rate remained significantly higher than controls for both pre- and post-ovulatory females over 6h, and higher in post-ovulatory than pre-ovulatory females. The peaks of sulphated and glucoronidated 17,20βP were 61±9 and 171±45 pg.$h^{-1}.kg^{-1}$ fish, respectively. The release of both conjugated forms returned to control values after 2h in all experimental groups. The release rate of free cortisol and estradiol (E_2) ranged from 5 to 98 pg.$h^{-1}kg^{-1}$ fish and 10 to 200 pg.$h^{-1}kg^{-1}$ fish respectively over 6h period and was unaffected by pheromone treatment or maturation state. Only E_2 showed significantly higher release of conjugated *vs* free. Free testosterone (T) release rate showed significant differences between pre-ovulatory (361±80 ng.$h^{-1}kg^{-1}$ fish) and post-ovulatory females (99±23 ng.$h^{-1}kg^{-1}$ fish). The increase of maturing inducing steroid 17,20βP after pheromone treatment strongly suggests a synchronization of spawning by inducing final maturation of the eggs [6]. As the number of immature follicles in post-ovulatory females is higher, this is consistent with the higher release, and presumably production, of 17,20βP in this group.

The steroid concentrations in plasma or urine after 6h was unaffected by the treatment and only T was significantly different between pre- and post-ovulatory females. In plasma and urine, concentrations of 17,20βP (free and conjugated) were below 0.8 ng.ml^{-1}. Plasma had a specific binding protein for E_2 with high affinity (14nM) and moderate capacity (54nM). The relative binding affinity was E2 (100%) > T (35%) > 17,20β P (3.3%) > F and conjugated forms (<1%).

Plasma sex steroid concentration is used as an indicator of reproductive status in fish. However, their concentrations in plasma are determined by the capacity and specificity of transport by steroid binding proteins (SBP). Thus, 17,20βP can be produced in significant amounts in the gonads but, due to its low affinity for SBP, it is rapidly metabolized and excreted (probably by the gills [6]). This leads to low concentrations in body-fluids but release rates as high as that of estradiol and suggests point measurement in the water as the most appropriate assay to evaluate changes.

Conclusion:

Urine from male tilapia contains a pheromone that dramatically affects 17,20βP metabolism in conspecific females, less than 1h after exposure. This pheromonal effect was only visible in non-invasive measurement of steroids in the water and can only be understood after evaluation of steroid binding proteins.

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

References:

[1]MIRANDA, A., ALMEIDA, O.G., HUBBARD, P.C., BARATA, E.N., CANÁRIO, A.V.M. 2005. Olfactory discrimination of female reproductive status by male tilapia (*Oreochromis mossambicus*). J. Exp. Biol., 208: 2037-2043.

[2]ALMEIDA, O.G., MIRANDA, A., FRADE, P., HUBBARD, P.C., BARATA, E.N., CANARIO, A.V.M. 2005. Urine as a social signal in the Mozambique tilapia (*Oreochromis mossambicus*). Chem. Senses, 30: i309-i310.

[3]HUERTAS, M., SCOTT, A.P., HUBBARD, P.C., CANARIO, A.V.M., CERDA, J. 2006. Sexually mature European eels (*Anguilla anguilla* L.) stimulate gonadal development of neighbouring males: Possible involvement of chemical communication. Gen. Comp. Endocrinol., 147: 304-313.

[4]SCOTT, A.P., HIRSCHENHAUSER, K., BENDER, N., OLIVEIRA, R., EARLEY, R.L., SEBIRE, M., ELLIS, T., PAVLIDIS, M., HUBBARD, P.C., HUERTAS, M., CANARIO, A. 2008. Non-invasive measurement of steroids in fish-holding water: important considerations when applying the procedure to behaviour studies. Behaviour, 145: 1307-1328.

[5]SCOTT, A.P., PINILLOS, M.L., HUERTAS, M. 2005. The rate of uptake of sex steroids from water by *Tinca tinca* is influenced by their affinity for sex steroid binding protein in plasma. J. Fish Biol., 67: 182-200.

[6]SCOTT, A.P., SUMPTER, J.P., STACEY, N. 2010. The role of the maturation-inducing steroid, 17,20 beta-dihydroxypregn-4-en-3-one, in male fishes: a review. J. Fish Biol., 76: 183-224.

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

INTER-POPULATIONAL VARIATION IN THE REPRODUCTIVE BEHAVIOUR OF THE PEACOCK BLENNY

Saraiva, J.L.*+, Gonçalves, D.+ & Oliveira, R.F.+

*Centro de Ciências do Mar, Universidade do Algarve, Campus de Gambelas, 8005-139 Faro, Portugal
Tel & Fax: +351289800051
+Instituto Superior de Psicologia Aplicada, Lisboa, Portugal

Introduction:

The inter-populational variation in the reproductive behaviour of the peacock blenny *Salaria pavo*, particularly the influence of the ecologic environment, was investigated in the present work. Two populations of this species inhabiting contrasting environments were studied: the Ria Formosa population, a coastal lagoon with sandy/muddy substrate located in the south of Portugal, and the Gulf of Trieste, an area presenting rocky substrate located in the northern Adriatic sea.

This species presents high sexual dimorphism: males are larger than females and exhibit conspicuous secondary sex characters, like a head crest and anal gland, that consists of modified rays of the anal fin. These characters develop mainly in the breeding season. The mating system is promiscuous, with exclusive male parental care. In rocky shore populations, males nest in crevices or holes in the rock and defend a courting territory around the entrance of the nest where they perform elaborate courtship displays, assuming a bourgeois reproductive tactic. Females usually have a passive role in courtship, responding with changes in colouration and a few displays before they enter the nest to spawn.

The Ria Formosa population, however, presents severe changes to this pattern. In this coastal lagoon, nesting substrates are scarce and the only adequate sites to establish nests are bricks located in artificial reefs, used by clam culturists to delimit their fields. During the breeding season, nests are highly aggregated, rendering the maintenance of the typical bourgeois territory impossible. Sex-role reversal has been described for this population, with females displaying intense and elaborate courtship and males assuming an almost passive role in courtship. In the Ria Formosa there is also a high proportion of small parasitic males that mimic female morphology and courtship, and try to achieve parasitic fertilizations during sapwning episodes. These alternative reproductive tactics are sequential and males that assumed a parasitic tactic in one breeding season usually acquire a nest in the next season.

In the present study, some questions regarding the emergence of these behavioural differences between populations were addressed. The main hypothesis was that the variations in the mating system should be due to high behavioural plasticity under the influence of the ecological environment, namely the abundance and dispersion of nesting sites. More precisely, the following questions were studied:

1. Are the sex-roles dynamic in this species and can they be modulated through experimental manipulation of nest-site aggregation?
2. How does the mating system work in populations inhabiting sites with different nest availability and dispersion? Are there alternative reproductive tactics in populations where nest-sites are abundant?
3. Are there morphological changes in females and different male morphs between populations? What are the endocrine correlates of such differences?
4. What are the differences in the neuroendocrine regulation of reproductive behaviour between the two populations?

Methods:

An integrative approach was used to address these questions: transects (to quantify abundance and dispersion of nest sites), behavioural observations in the field and in the lab (to assess behavioural differences between populations and the ecological factors responsible for them), morphometry (to identify correlates of sexual preference and condition-dependent traits), radio-immuno assays and quantitative RT-PCRs (to identify the underlying physiological factors in control of reproductive behaviour).

Results and Discussion:

The results point to a high behavioural plasticity in this species, with a strong influence of nest-site abundance and dispersal in the modulation of the mating system and reproductive behaviour. In fact, the aggregation and scarcity of nest sites in Ria Formosa apparently promote a strong competition for access to nests sites, favouring larger males and probably promoting the development of more pronounced secondary sex characters as intra and intersexual signalling. As only the largest males acquire nests, the operational sex-ratio (number of mature females/ number of males qualified to mate) will be biased towards females, limiting their reproductive potential and causing sex-role reversal. On the other hand, a large proportion of sexually mature males cannot breed and the smallest adopt alternative reproductive tactics.

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

Although bourgeois males in the Gulf of Trieste are smaller and have less developed secondary sex characters than in Ria Formosa, they present relatively larger gonads. It is probable that sperm competition is higher in the Gulf of Trieste. This can be explained by the longer periods outside of the nest that bourgeois males from this population spend, increasing the chance for nest-takeovers or stealing fertilizations by rivals.

The analysis of circulating levels of androgens revealed higher concentrations of 11-keto-testosterone in bourgeois males from Ria Formosa, suggesting on one hand a correspondence between this hormone and the development of secondary sex characters, and on the other that androgens are highly sensitive to the social environment. The study on the aromatase enzyme, that converts testosterone into estradiol and is involved in the regulation of courtship and aggressive behaviours, reveals a higher expression of this enzyme in the brains of bourgeois males from Ria Formosa. A higher local testosterone-estradiol conversion rate may be down-regulating the aggressiveness and courtship in these males, allowing them to cohabitate with neighbours.

Conclusion:

The data present in this work allow an integrated understanding of some of the mechanisms that regulate behavioural plasticity in *S. pavo*, whose expression of reproductive behaviours seem to be closely related to ecological factors.

REPRODUCTIVE BIOLOGY OF GIANT KOKOPU, *GALAXIAS ARGENTEUS.*

Wylie M. J., Closs G. P., Lokman P. M.

Department of Zoology, University of Otago, 340 Great King Street, Dunedin 9016, New Zealand. Fax +64-34797584, e-mail: wylma731@student.otago.ac.nz

Introduction:

In New Zealand, the whitebait fishery is comprised of five amphidromous species of crystalline juveniles that belong to the genus *Galaxias*, a group of Southern hemisphere Salmoniformes that have a marine larval feeding phase. Whitebait is a seasonal and valuable delicacy [6,7]. Progress towards the commercial production of whitebait using *G. maculatus*, the most abundant galaxiid species [7,9], has been slow due to technical constraints (e.g. low fecundity and poor larval survival). The giant kokopu *(G. argenteus)*, is a promising alternative, although it is considered threatened [8]. Specimens over 40cm in length and 1 kg in weight have been recorded [2], with a far greater fecundity and egg diameter compared to *G. maculatus* [8,11]. However, no observations of spawning or egg deposition have been documented [5] and essentially nothing is known about the reproductive physiology of this fish. To fill these voids, oocyte diameters and plasma levels of estradiol-17β (E_2) were determined from wild fish at monthly intervals throughout the reproductive cycle until ovulation. Ovarian mRNA levels of cathepsin D, a protein associated with yolk processing in developing oocytes [3,4], were also measured. In order to assess the potential of this fish for cultivation, the same data were obtained from fish held in captivity via repeated biopsies. The outcomes were compared, and where possible, gametes collected from wild and captive fish to obtain data on fertilization and hatching rates following incubation of eggs at a range of different salinities.

Methods:

Wild fish - Ovarian tissue was biopsied and blood samples were collected from four to six wild fish (~300 g) at monthly intervals until ovulation. *Captive fish* - The first group of wild-sampled fish were transferred to a holding facility and maintained under simulated natural photoperiod and water temperatures. Ovarian tissue was repeatedly biopsied and blood samples were collected at near-monthly intervals until ovulation. Subsequent to sample collection, oocyte diameters were measured using light microscopy. Methacrylate resin sections were stained with haematoxylin and eosin to determine the developmental stage of the oocytes. Cathepsin D mRNA levels were quantified using real-time PCR and plasma E_2 levels were measured using radioimmunoassay. *Artificial propagation* - Mature fish were checked for ovulation daily. Stripped eggs were dry-fertilised with milt from wild fish prior to activation of gametes in water of different salinities (0 ppt, 9 ppt, 15 ppt and 30 ppt). Water was subsequently drained from treatments and eggs were incubated semi-dry at 10 ± 1°C under 100% humidity. In an additional treatment, eggs were fertilised and incubated fully submerged in local dechlorinated tap water. Dead eggs were removed daily. Hatch rates were calculated for each treatment.

Results and Discussion:

Oocyte growth started in December and continued in wild fish until late June, when ovulated eggs could be obtained. Levels of E_2 increased as vitellogenesis progressed, followed by a dramatic reduction to undetectable levels on completion of oocyte growth, a pattern that is reminiscent of that in salmonids. Messenger RNA levels for cathepsin D increased as vitellogenesis progressed in giant kokopu. In contrast, mRNA levels were highest at the onset of vitellogenesis in gilthead sea bream *(Sparus aurata)* and rainbow trout *(Oncorhynchus mykiss)* [3,4]. In captivity, fish also reached the ovulatory stage, but it lagged wild fish by more than six weeks, presumably reflecting the stresses of repeated biopsy and a protozoan infection in March; indeed, stress through the action of corticosteroids can reduce immunocompetence and alter levels of hormones [1]. High levels of cathepsin D seen during mid vitellogenesis may similarly reflect fish illness, as over-activity has been associated with poor-quality eggs [12]. Viable eggs were collected from both groups and indications of compromised egg quality in captive fish were further reinforced by low hatch rates (26%), especially when compared to hatch rates of submerged eggs from wild fish (71%). Regardless of salinity, incubation of eggs under semi-dry conditions was unsuccessful due to an unidentified disease.

Conclusion:

Oocyte growth and estradiol-17β plasma levels were documented throughout the reproductive cycle of giant kokopu until spontaneous ovulation in both wild and captive fish. We conclude that captive fish ovulated and produced viable eggs, but later in the season than fish from the wild. Larvae only hatched from eggs fertilised and fully submerged in fresh water until hatching, and hatch rate was higher in eggs from wild fish than from captive fish. In addition to the greater fecundity and larger egg size of *G. argenteus* compared to *G.*

maculatus these novel results provide a foundation for which the controlled propagation of the species can be optimised for both conservation and aquaculture purposes.

References:

[1]BARTON, B. A., IWAMA, G. K. 1991. Physiological changes in fish from stress in aquaculture with emphasis on the response and effects of corticosteroids. Annu. Rev. Fish Dis., 1: 3-26.

[2]BONNETT, M. L., MC DOWALL, R. M., SYKES, J. R. E. 2002. Critical habitats for the conservation of giant kokopu, *Galaxias argenteus* (Gmelin, 1789). Science for Conservation 206. 50p.

[3]BROOKS, S., TYLER, C. R., CARNEVALI, O., COWARD, K., SUMPTER, J. P. 1997. Molecular characterisation of ovarian cathepsin D in the rainbow trout, *Oncorhynchus mykiss*. Gene, 201: 45-54.

[4]CARNEVALI, O., CIONNA, C., TOSTI, L., CERDA, J., GIOACCHINI, G. 2008. Changes in cathepsin gene expression and relative enzymatic activity during gilthead sea bream oogenesis. Molecular Reprod. Develop., 75: 97-104.

[5]CHARTERIS, S. C., ALLIBONE, R. M., DEATH, R. G. 2003. Spawning site selection, egg development, and larval drift of *Galaxias postvectis* and *Galaxias fasciatus* in a New Zealand stream. NZ J . Mar. Freshw. Res., 37: 493-505.

[6]HAGGERTY, J. H. 2007. "I'm not a greenie but": Environmentality, eco-populism and governance in New Zealand experiences from the Southland whitebait fishery. J. Rural Stud., 23: 222-237.

[7]JEFFS, A. 2003. Assessment of the potential for aquaculture development in Northland. National Institute of Water and Atmospheric Research Ltd. http://www.enterprisenorthland.co.nz

[8]JELLYMAN, D. J. (1979). Observations on the biology of the giant kokopu *Galaxias argenteus* (Gmelin, 1789). Mauri. Ora., 7: 53-61.

[9]MARDONES, A., VEGA, R., ENCINA, F. 2008. Cultivation of whitebait in *(Galaxias maculatus)* in Chile. Aquacult. Res., 39: 731-737.

[10]MC DOWALL, R. M. 2010. New Zealand freshwater fishes. Fish Fisheries Serie, 32: 1-33.

[11]MITCHELL, C. P. (1989). Laboratory of *Galaxias maculatus* and potential applications. NZ J. Mar. Freshw. Res., 23: 325-336.

[12]RIME, H., GUITTON, N., PINEAU, C., BONNET, E., BOBE, J., JALABERT, B. 2004. Post-ovulatory aging and egg quality: A proteomic analysis of rainbow trout coelomic fluid. Reprod. Biol. Endocrin., 2: 26.

MOLECULAR CHARACTERIZATION AND EXPRESSION PATTERNS OF ATLANTIC BLUEFIN TUNA (*TUNNUS THYNNUS*) LEPTIN DURING THE REPRODUCTIVE SEASON

Yanowski E.[1,2], Mylonas, C.C.[3], Corriero, A.[4], Bridges C.R.[5], Vassallo-Aguis, R.[6], De La Gándara, F.[7], Belmonte, A.[8], Meiri-Ashkenazi, I.[1], Gordin, H.[1], and Rosenfeld, H.[1]

[1]Israel Oceanographic and Limnological Research, National Center for Mariculture, P.O. Box 1212, Eilat 8112, Israel. Fax +972-8-6375761; e-mail: hannarosenfeld@gmail.com
[2]Department of Life Science, Ben-Gurion University of the Negev, P.O. Box 653, Beer-Sheva 84105
[3]Institute of Aquaculture, Hellenic Center for Marine Research, Crete, Greece
[4]Department of Animal Health and Welfare, University of Bari, Italy
[5]Heinrich-Heine Universität, InstitutfürZoophysiologie, Düsseldorf, Germany
[6]Malta Center for Fisheries Sciences, Marsaxlokk, Malta
[7]Instituto Español de Oceanografía, Centro Oceanográfico de Murcia, Puerto de Mazarrón, Spain
[8]Tuna Graso S.A., Carretera de la Palma Km 7, Paraje la Estrella, Cartagena (Murcia), Spain

Introduction:

The Atlantic bluefin tuna (BFT, *Thunnus thynnus*) is a highly prized fish comprising one of the world's most valuable commercial fisheries. Recently, with the intention of reducing fishing pressure on wild stocks, successful BFT breeding protocols were established, paving the way towards a self-sustained industry [1]. To further understand the endocrine mechanisms regulating reproduction in BFT, which is notable among fishes for its pseudo- endothermic metabolism and for its capacity for rapid lipid mobilization, the current study was focused on the molecular characterization of leptin. In mammals the latter hormone appears to transmit signals of the energy stores to the central nervous and endocrine systems allowing the onset of energy demanding situations such as puberty and reproduction [3, 4]. In order to look for parallels to these roles of leptin in BFT, our specific goals were to clone the cDNA encoding for BFT leptin (*bft*Lep), and follow the temporal expression profiles of the related gene during BFT natural spawning season within the Mediterranean Sea.

Methods:

Experimental fish and sampling procedures - Mature BFT captured at the Balearic Islands and held in floating cages (25-m diameter, 20-m deep) by the coast of Murcia, Spain (Tuna Graso S.A.), were sampled at three characteristic stages (April, n=15; May, n=12; July, n=12; 2008) during the natural spawning season within the Mediterranean Sea. Morphometric parameters were recorded and organ (i.e. gonad, liver, fat and viscera) indices (GSI, HIS, FSI and VSI, respectively) were calculated for individual fish. In addition, pituitary, brain, gonad and liver tissues were collected for gene expression analyses.

Cloning and gene expression analyses - The cDNA encoding for the BFT leptin (*bft*Lep) was cloned using the 5`-and 3`-RACE technique and degenerate primers that were designed according to the most conserved regions of leptin sequences available at the NCBI database. The identity of the isolated amplicon was confirmed as BFT leptin by BLAST (www.ncbi.nlm.nih.gov) and the tertiary structure of the hormone was validated using the I-TASSER server for protein 3D structure prediction (http://zhanglab.ccmb.med.umich.edu/I-TASSER/). The expression levels of *bft*Lep were profiled using real time-PCR quantification and were normalized to 18S as a housekeeping gene.

Results and Discussion:

The full length *bft*LepcDNA was 1126 bp long, encoding a precursor peptide of 160 amino acids including 19 residues of signal peptide. At the amino acid level, the *bft*Lep shows low homology with cognate tetrapod (~16%) and teleost (~26%) proteins, whereas its predicted tertiary structure reveals the four-alpha-helix bundle, characteristic of the class-I helical cytokine family.

The *bft*Lep mRNA was detectable in the liver and to a lesser extent in the hypothalamic, pituitary, fat and spleen tissues. Interestingly, similar temporal patterns were observed in both liver and hypothalamic tissues, with relatively low*bft*Lep transcript levels in fully mature fish that were sampled in May (GSI=1.85 ± 0.2 %), and elevated levels in fish sampled in July with regressed gonads (GSI= 0.62 ± 0.1 %). No sexual dimorphic patterns were observed. The organ indices HIS and FSI, declined concomitant with the progression of the reproductive season reaching minimal values during July, the end of the spawning season. Further regression analysis revealed a significant negative correlation between the HIS values and the hypothalamic*bft*Lep mRNA levels.

Conclusions:

The wide spectrum of tissues expressing the *bft*Lep gene may suggest the hormone's multiple physiological functions in BFT. The mechanisms by which leptin regulates reproductive functions are not yet fully

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

understood. However, the fact that leptin is being produced in the central nervous system (hypothalamus and pituitary) may suggest autocrine and/or paracrine regulation of the hypothalamic-pituitary-gonadal axis. Our striking findings that reveals a clear association between regressed gonads, depleted energy resources (as indicated by the low HIS and FSI values) and increased transcription of the *bft*Lep gene in the hypothalamic tissue, further attest to this notion. Nevertheless, since expression levels do not necessarily mirror the circulating hormone levels, further studies employing homologous immunoassays, are required to clarify the specific physiological roles of leptin in the control of gonadal function in BFT.

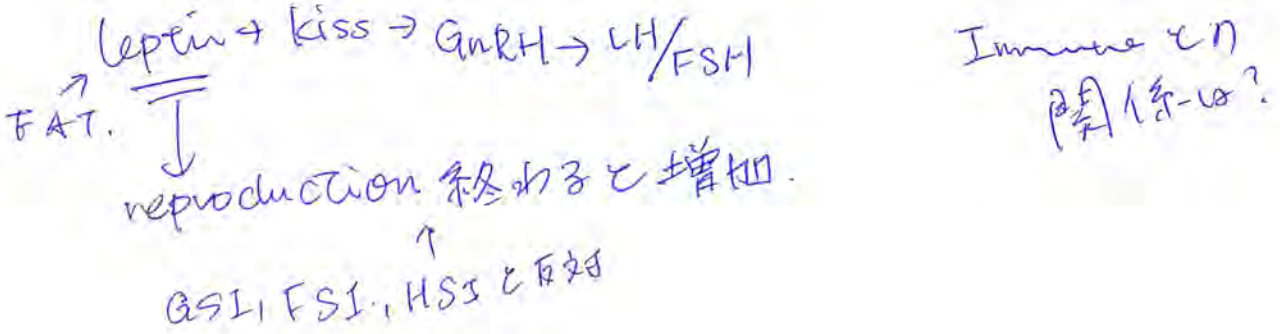
References:

[1]MYLONAS, C.C., DE LA GANDARA, F., CORRIERO, A., RIOS, A.B. 2010. Atlantic Bluefin Tuna (*Thunnusthynnus*) Farming and Fattening in the Mediterranean Sea. Rev. Fish. Sci., 18: 266-280.

[2]BLANK, J.M., FARWELL, C.J., MORRISSETTE, J.M., SCHALLERT, R.J. & BLOCK, B.A. 2007. Influence of swimming speed on metabolic rates of juvenile pacific bluefin tuna and yellowfin tuna. Physiol. Biochem. Zool., 80: 167-177

[3]ROSENBAUM, M., LEIBEL, R.L. 1998. Leptin: A molecule integrating somatic energy stores, energy expenditure and fertility. Trends Endocrinol. Metab., 9:117–124.

[4]FERNANDEZ-FERNANDEZ, R., MARTINI, A.C., NAVARRO, V.M., CASTELLANO, J.M., DIEGUEZ, C., AGUILAR, E., ET AL. 2006. Novel signals for the integration of energy balance and reproduction. Mol. Cell. Endocrinol., 254– 255:127–132.



Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

AROMATASE ACTIVITY OF BRAIN AND OVARY IN RELATION TO ANNUAL REPRODUCTIVE CYCLE OF THE INDIAN CATFISH, *HETEROPNEUSTES FOSSILIS*

Aggarwal, N., Goswami, S.V., Sehgal, N.

Department of Zoology, University of Delhi, Delhi 110 007, India
E-mail address : neerja.agg@gmail.com

Introduction:

Aromatization of androgens by cytochrome P450 aromatase is the key step for the synthesis of estrogens. Although cytochrome P450 aromatase is predominantly expressed in brain and ovary, some other tissues such as liver, kidney, adipose tissue and skin of fish also show its presence. It occurs mainly in two isoforms, ovarian and brain types, encoded by *cyp*19a and *cyp*19b, respectively. Expression and activity of aromatase *cyp*19a in the ovary regulates ovarian production of E_2 during reproductive cycle of fish. In addition, developmental or seasonal fluctuations in brain aromatase activity are also observed in several teleosts, suggesting its involvement in the control of reproductive cycle. Moreover, the aromatase activity is maximum in the brain areas associated with reproductive functions. Estrogen synthesized from circulating substrate in these areas binds to nuclear receptors and is therefore physiologically important. In the present study an attempt has been made to investigate changes in aromatase activity in ovary and brain and their relationship with circulating levels of E_2 and T at different stages of reproductive cycle in the annually reproducing Indian catfish, *Heteropneustes fossilis*.

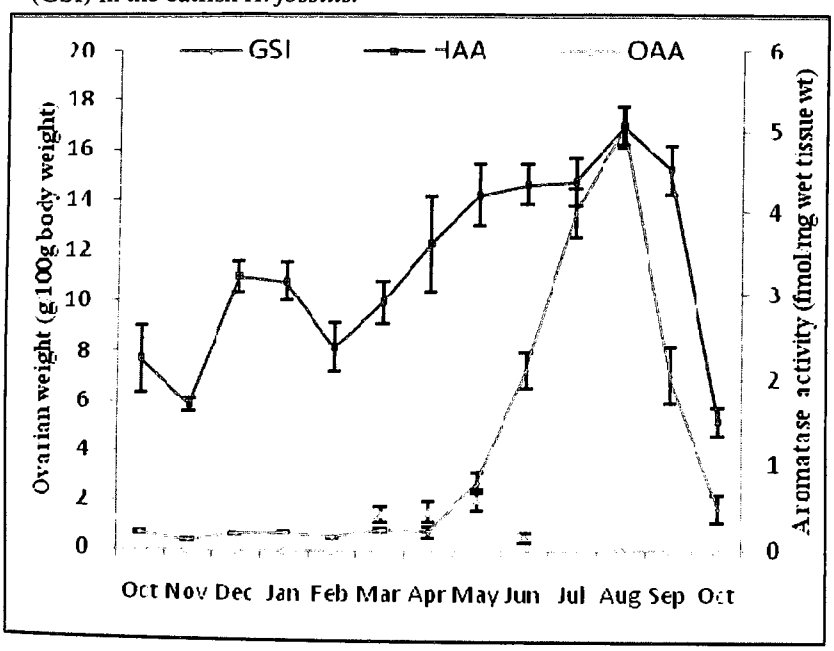
Methods:

The catfish, *Heteropneustes fossilis* were collected every month from backwaters of river Yamuna and acclimated to laboratory conditions (25°C; L:D::12:12) for at least 7 days. At the time of sampling, female fishes were anaesthetized and weighed to the nearest 0.5 gm. Blood samples were collected from caudal artery and processed for estimation of vitellogenin by alkali-labile phosphorus method and steroid hormones (E_2 and T) by radioimmunoassay [2]. The fishes were decapitated, ovaries were removed and weighed for calculating GSI. The brain was exposed and the hypothalamic region was excised. A piece of ovarian tissue and brain (hypothalamic region) were weighed to the nearest 0.1 mg and processed for estimation of aromatase activity [1].

Results and Discussion:

Aromatase activity in hypothalamus of the female catfish was detectable throughout the year and registered two peaks during the annual ovarian cycle. The hypothalamic aromatase activity starts increasing during the preparatory period, along with increase in ovarian weights. The increase in enzyme activity continues till the spawning period; the activity declines immediately after spawning in September and October. The second peak of smaller magnitude was recorded in late post spawning period (December-January) when the photoperiod and temperature are low and the fish is sexually quiescent. Aromatase activity in the ovary was detectable only during the preparatory and prespawning periods, the time of the year when the production of ovarian estrogen is needed in order to promote hepatic synthesis of vitellogenin and its incorporation in the growing oocytes. At the completion of vitellogenesis, ovarian aromatase activity declined sharply resulting in elevation of plasma testosterone levels, which in turn could be utilized as substrate by the hypothalamic aromatase whose activity was highest in the postvitellogenic catfish. The ovaries at this stage are maintained in a viable state by a tonic release of gonadotropins. Also at this stage of reproductive cycle, the pituitary stores

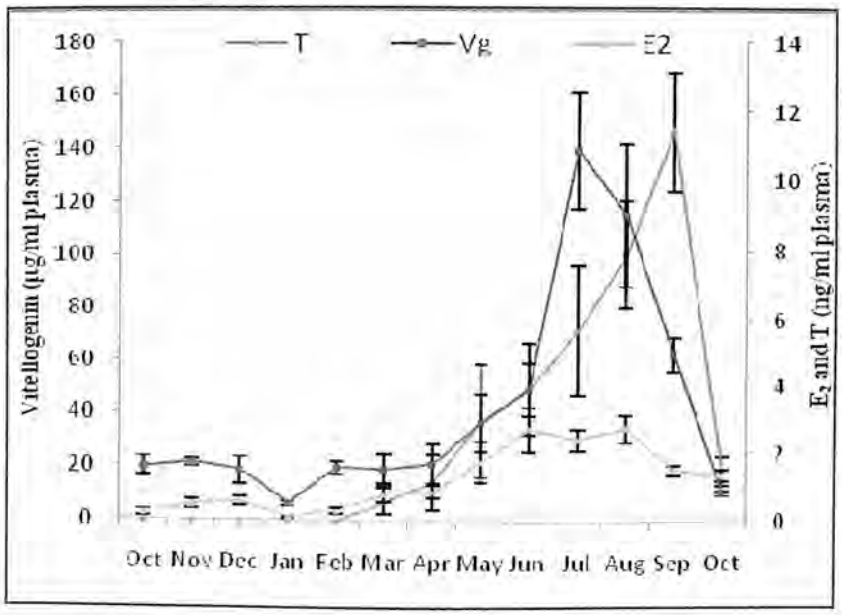
Fig.1. Seasonal variations in ovarian aromatase activity (OAA), hypothalamic aromatase activity (HAA) and gonadosomatic index (GSI) in the catfish *H. fossilis*.



Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

gonadotropin for subsequent release as a surge at the onset of the maturational phase. It is reasonable to assume that the extremely high levels of circulating testosterone observed in the female catfish in the present study, coupled with elevated aromatase activity in the hypothalamus are responsible for promoting storage of gonadotropins in the pituitary.

Fig.2. Seasonal variation in plasma vitellogenin (Vg), estradiol-17β (E_2) and testosterone (T) in the catfish *H.fossilis*



Conclusion:

The present study establishes the existence of seasonally related variations in plasma sex steroids and aromatase activity in the brain and the ovaries of the catfish, *H. fossilis*. Furthermore, changes in circulating steroids are responsible for the changes in both brain and ovarian aromatase activity. Marked seasonal variations in hypothalamic aromatase activity suggest that estrogen synthesis in central nervous system plays a pivotal role in reproduction.

References:

[1]CALLARD, G.V., PETRO, Z. AND RYAN, K.J. 1981. Estrogen synthesis *in vitro* and *in vivo* in the brain of a marine teleost (Myoxocephalus). Gen. Comp. Endocrinol., 43: 243-255.

[2]LAMBA, V.J., GOSWAMI, S.V. AND SUNDARARAJ, B.I. 1982. Radioimmunoassay for plasma cortisol, testosterone, estradiol-17β and estrone in the catfish, *Heteropneustes fossilis* (Bloch): Development and validation. Gen. Comp. Endocrinol., 47: 170-181.

PLASMA SEX STEROID PROFILES IN MEAGRE (*ARGYROSOMUS REGIUS*)

Vallés, R.[1], Bayarri, M.J.[2], Mañanós, E.[2] and Duncan, N.[1]

[1] Institut de Recerca i Tecnologia Agroalimentàries (IRTA), Sant Carles de la Ràpita, Tarragona, Spain. Fax: +34-977744138. e-mail: neil.duncan@irta.cat

[2] Instituto de Acuicultura de Torre la Sal (CSIC), Torre la Sal, Castellón, Spain

Introduction:

Meagre is a new species for Mediterranean aquaculture and the reproduction is still relatively unknown. The aim of this work is to obtain the profile of sex steroids levels during one year and 8 months to gain knowledge on the meagre reproductive system.

Methods:

A meagre broodstock (first generation, G1, in captivity) of mean weight 6,79±0,19 kg was held in IRTA, Sant Carles de la Ràpita, Spain, under natural photoperiod and temperature (min 12.8°C, max 23.7°C). Every month, from February 2009 to November 2010, fish were anesthetized with MS-222 (70 mg L^{-1}) and a blood sample of 1.5 mL collected. Plasma was obtained by centrifugation (3000g, 15min, 4°C) and stored at -80°C for steroid analysis. Levels of estradiol (E_2), testosterone (T) and 11-ketotestosterone (11-KT) were quantified by ELISA. Oocyte samples were obtained by canulation biopsy and sperm samples by abdominal pressure.

Results:

During the first year, female plasma levels of E_2 were elevated during February and increased reaching the maximum in April (0.54±0.09 ng ml^{-1}), which coincided with the presence of vitellogenic oocytes in the ovary. The E_2 plasma levels then returned to basal levels in June. In the second year, the E_2 started to increase in December and the highest levels were observed in March (1.15±0.52 ng ml^{-1}). A similar pattern was observed in plasma levels of 11-KT in males, during the first year levels were significantly higher in February (0.64±0.07 ng ml^{-1}) and decreased to a low in June. The highest percentage of spermiating males was encountered in April. In the second year, 11-KT began to increase in November with the maximum in March (0.27±0.11 ng ml^{-1}) and decreased in May when 100% of males had flowing sperm. Plasma levels of T in males did not show significant differences over the sampling period.

Conclusion:

In conclusion, this study provides the profiles of three sex steroids that clearly identify the period of gametogenesis (December – March) before the spawning period (April-May).

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

THE EFFECT OF TEMPERATURE ON OOGENESIS AND BRAIN GENE EXPRESSION OF HORMONES INVOLVED IN REPRODUCTION AND GROWTH IN THE FEMALE BLUE GOURAMI (*TRICHOGASTER TRICHOPTERUS*)

Gad Degani[1, 2], Dalia David[1, 2] and Gal Levy[1, 2, 3]

[1]Faculty of Science and Technology, Tel-Hai Academic College, Upper Galilee 12210, Israel
[2]MIGAL- Galilee Technology Center, Kiryat Shmona 11016, Israel, Fax: +972-4-6944980, E. mail: gad@migal.org.il;
[3]Department of Neurobiology, George S. Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv, Israel

Introduction:

The blue gourami (*Trichogaster trichopterus*) female has an asynchronic ovary development and the FOM will occur only in the presence of the male. Thus, each stage of its gonadal development can be controlled and examined separately in the laboratory [4]. Previous studies in our laboratory have shown variations in hypothalamic and pituitary hormonal gene expression during reproduction stages. Gonadotropin releasing hormone 3 (GnRH3), and pituitary adenylate cyclase activating polypeptide (PACAP - long and short form [PRP]) gene expression increased in reproductively active females [5,6], and beta-follicle stimulating hormone (beta FSH) and beta-luteinizing hormone (beta LH) gene expression were increased in females in the vitellogenic stage [4]. Accumulated data indicate an involvement of growth factors in the regulation of reproduction, e.g., growth hormone (GH) and prolactin (PRL) [2, 3]. The present study examined the effect of temperature on reproduction and growth-related factors in blue gourami (*Trichogaster trichopterus*) females under non-reproductive (NRC) and reproductive conditions.

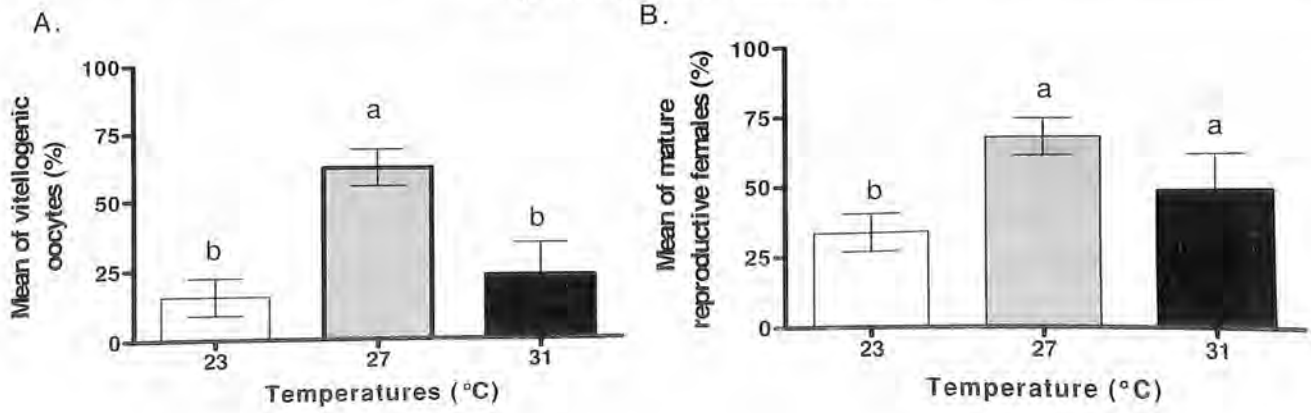
Methods:

The females, maintained in three different containers at the temperatures as described in prevue study [1], gonads were sampled and mRNA levels of GnRH3, PACAP, PRP, IGF1, GH, beta-LH, beta-FSH and PRL were mustered[1].

Results:

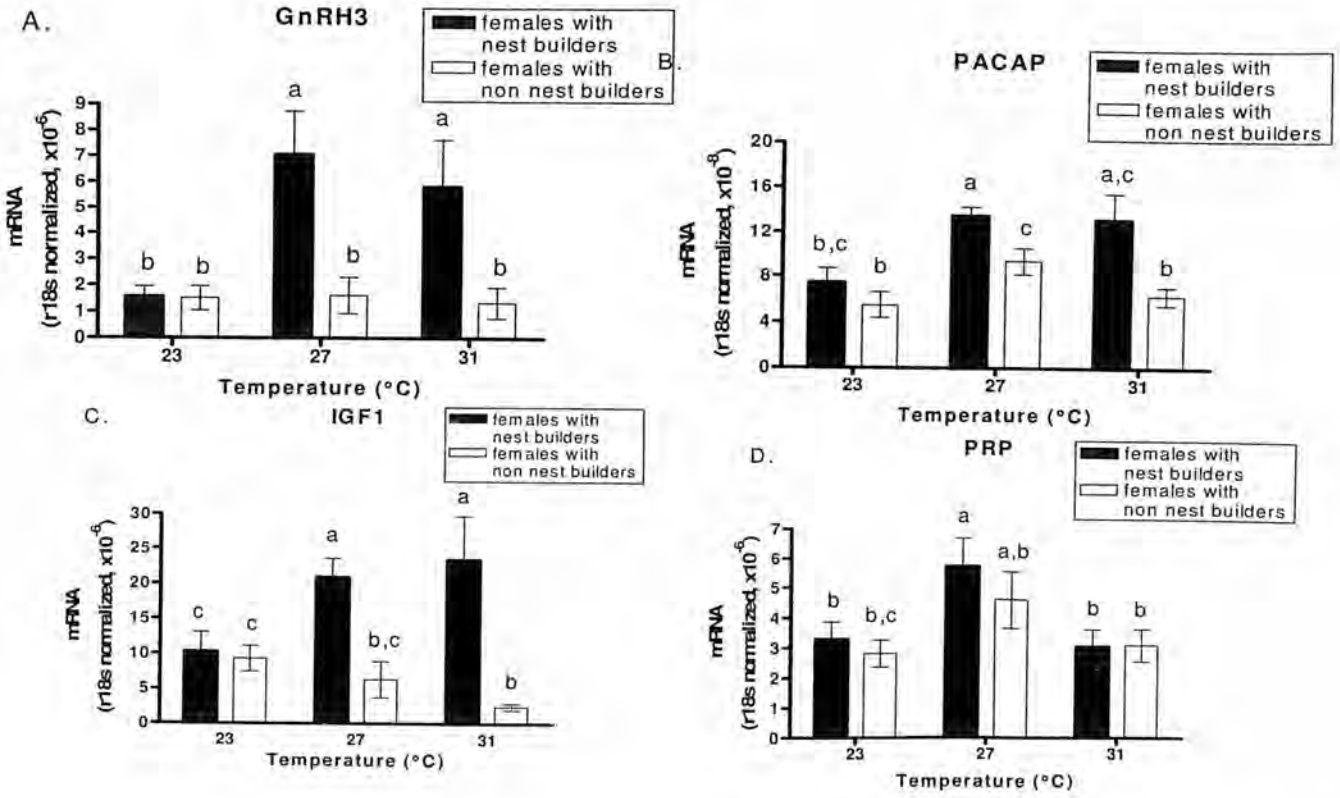
A higher percentage of oocytes in the advanced vitellogenic stage was found in FNRC kept at 27 °C than in those kept at 23 °C or 31 °C ($P<0.05$,Fig. 1a). In contrast, in FRM and FNRM, a higher percentage of oocytes at the FOM stage were observed at 23 °C, than at 27 °C (Fig. 1b). In FNRC kept at 23 °C and 27 °C, as compared to the group at 31 °C, significantly higher mRNA levels of brain GnRH3 were detected, whereas no significant differences in the mRNA levels of brain IGF-1, PACAP and PRP were observed in these females at 23 °C, 27 °C or 31 °C. In FRM, mRNA levels of brain GnRH3, IGF1, PACAP and PRP were higher when kept at 27 °C than at 23 °C (Figs. 2A-D). PRP mRNA levels were also higher at 27 °C than at 31 °C in these fish. On the other hand, mRNA levels of PACAP were greater in FNRM kept at 27 °C than in those fish maintained at 23 °C and 31°C (Fig. 2B); and mRNA levels of IGF-1 were higher in the brains of FNRM kept at 23 °C than in those kept at 31 °C (Fig. 2C).

Fig 1. (A) Percentage of oocytes at the advanced vitellogenic stage in adult FNRC ovaries. (B). Females at the vitellogenic stage were separated after an acclimation period of four days and held at 23 °C, 27 °C or 31 °C in the presence of a mature male for one day (mean±SEM; n=15). Different letters above each bar of the histogram denote a significant difference among the temperatures ($P<0.01$, Student's t-test).



Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

Fig 2. Relative mRNA levels of (A) GnRH3, (B) PACAP, (C) IGF-1 and (D) PRP in brains from FRM or FNRM. Each histogram represents the average of at least five independent measurements (mean±SEM; n=5–9). Different letters above each histogram denote a significant difference among the mRNA levels ($P<0.05$, one way ANOVA, followed by Student Newman–Keuls post-hoc test).



Conclusion:

We propose that in the blue gourami female, vitellogenesis and FOM may be affected by changes in the environmental temperature, through modifications in GnRH3, IGF1, PACAP and PRP gene expression.

References:

[1]DAVID, D., DEGANI, G., 2011. Temperature affects brain and pituitary gene expression related to reproduction and growth in the male blue gouramis, Trichogaster trichopterus. J. Exp. Zool. A Ecol. Genet. Physiol., 313A: 1-11.

[2]DEGANI, G., YOM-DIN S., GOLDBERG, D., JACKSON, K. 2011. cDNA cloning of blue gourami (Trichogaster trichopterus) prolactin and its expression during the gonadal cycles of males and females. J. Endocrinol. Invest., 33: 7-12.

[3]GOLDBERG, D., JACKSON, K., YOM-DIN, S., DEGANI, G. 2004. Growth hormone of Trichogaster trichopterus: cDNA cloning, sequencing and analysis of mRNA expression during oogenesis. J. Aquacult. Tropics, 19(3): 215-229.

[4]JACKSON, K., GOLDBERG, D., OFIR, M., ABRAHAM M., DEGANI G. 1999. Blue gourami (Trichogaster trichopterus) gonadotropic beta subunits (I and II) cDNA sequences and expression during oogenesis. J. Mol. Endocrinol., 23(2): 177-187.

[5]LEVY, G., DEGANI G. 2011. Evidence for a reproduction-related function of pituitary adenylate cyclase-activating polypeptide-related peptide (PRP) in an Anabantidae fish. J. Mol. End., 46: 1-11.

[6]LEVY, G., JACKSON, K., DEGANI, G. 2010. Association between pituitary adenylate cyclase-activating polypeptide and reproduction in the blue gourami. Gen. Comp. Endocrinol., 166: 83-93.

HETEROGENEOUS DISTRIBUTION OF OOCYTES IN THE OVARIES OF *PROCHILODUS LINEATUS*

Hainfellner P., de Souza T.G., Nascimento T. S. R., Freitas G. A., Batlouni S. R.

Aquaculture Center of the São Paulo State University (CAUNESP), Reproduction Laboratory, Via de Acesso Prof. Paulo Donato Castelane, s/n. 14884-900, Jaboticabal, SP, Brazil. Fax +55-16-3209-2615 E-mail: patrick@caunesp.unesp.br

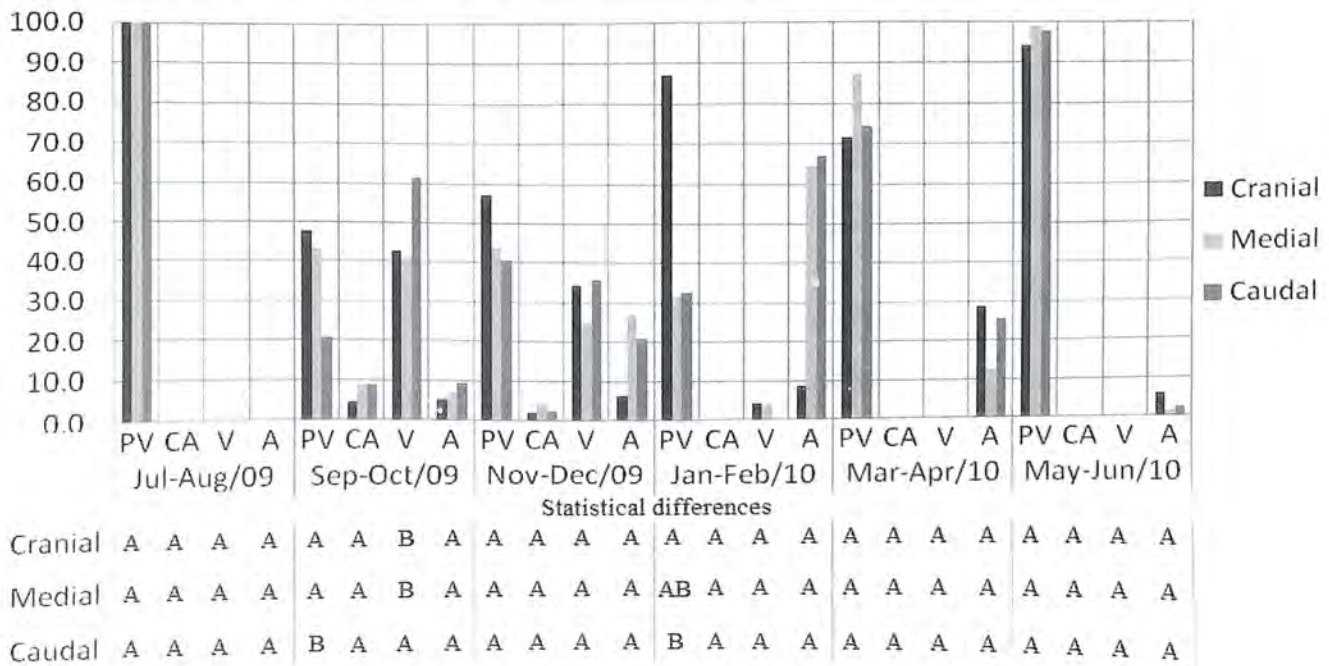
Introduction:

Brazil, despite being the bearer of most species of freshwater fish in the world, due economic, cultural and production knowledge has a production constituted mainly by introduced species, among which are trouts, tilapia and carp fish, while native species are produced on a smaller scale [3]. *Prochilodus lineatus* is of great scientific importance, very popular in fish farms in Brazil, and is a rheophilic species, which reproduce in captivity by induction with hormone [9], which often gives adverse results obtained in spawning induction in rheophilic fish [1][2][4-9]. This study was carried out in order to determine the best way to get a more representative sample from a fish ovary, by microscopic analysis of different portions of ovaries of females throughout gonadal development along the year, thereby establishing a standard method to avoid misleading results.

Methods:

In March 2009, 150 P. lineatus specimens (12 months of age, average length 31.48 + 1.03 cm, and average weight 394.85 + 29.85g) were housed in Aquaculture Center (CAUNESP), São Paulo State University, Jaboticabal, São Paulo, Brazil. The animals were distributed in three earthen ponds (EP) of 50m², of 1.50m average depth, and 18 L/min flow. Initial stocking densities of 1 fish.m-2 (0.27 kg.m-3). Water conditions were monitored during experimental period: pH (7.7±0.45), dissolved oxygen (3.77±1.6mg.L-1), conductivity (69.34±15.94µS.cm-1), transparency (68.3cm), ammonia (129.8±80.8µ.L-1) and temperature (23.22± 3.1°C). Fish were kept in these conditions for a period of 440 days; 360 days of experimental period after 80 days of acclimation. During the experimental period samples were taken every 60 days. Only one EP unit was used in each sampling to avoid excessive management over the specimens, so that breeders were manipulated only every six months. 5 females from EP were randomly selected, sacrificed by a lethal dose of benzocaine(0.15g.L-1) for collection of gonads. All procedures used followed approved guidelines for the ethical treatment of animals and national laws. Experimental protocols were submitted to, and approved

Fig 1. Frequency of the different oocyte types. PV: Pre-vitellogenic; CA: Cortical Alveoli; V: Vitelogenic; A: Atretic; Capital letters show statistical differences between regions.



by, the Animal Ethics and Welfare Committee CEBEA, (Comite de Ética e Bem-Estar Animal) of The Faculdade de Ciências Agrárias e Vetrinárias (FCAV), Unesp, Jaboticabal, SP, Brazil. Ovarian samples (cranial, medial and caudal regions) were collected and fixed in a solution of 2.5% glutaraldehyde for 24 hours. The material was embedded in paraplast, cut into 3-5 μm thick sections, submitted to hematoxylin and eosin staining. Histological sections were utilized to determine the frequency of different oocyte types of each region. All oocytes present in thirty fields (ten fields per region/ 5x magnification) were considered. Previously, the maximum diameter of vitelogenic oocyte was established to determine the section thickness that avoids counting the same oocyte twice. Data were subjected to the test of homogeneity and normality. Statistical differences between values obtained were detected by ANOVA followed by Tukey's test, using the computer program SAS 9.1.

Results:

Analysis of frequency of the different oocyte types (Fig. 1) showed that at the first sampling (Jul-Aug/09), during winter all the regions had the same oocyte types (pre-vitellogenic stage). Two months later (Sep-Oct/09), at thc beginning of vitelogenic phase, data show that caudal region is in a earlier process of maturation compared with the other two regions and therefore, pre-vitellogenic oocyte of cranial region progressively increased up to the end of breeding season. On the third sampling during summer, medial and caudal region begins to show some atretic oocytes, at Jan-Feb/10 medial and caudal regions shows advanced stage of atresia. At the last two samplings data show that all three regions began to have similar aspects.

Conclusion:

The present results showed that during the beginning of vitelogenic phase to the reproductive cycle end, spring and summer, each region has its own profile. Thus, the best way to obtain a sample that truly reflects the state of the ovary is to collect at least three samples (cranial, middle, caudal) and calculates the average.

References:

[1]AGOSTINHO AA, GOMES LC, SUZUKI HI, JÚLIO HF. 2003. Migratory fish from the upper Paraná River Basin, Brazil. In: Carolsfeld, J.; Harvey, B.; Ross, C. & Baer, A. (Eds). World Fisheries Trust. The World Bank and the International Development Research Centre. Victoria; 372p.

[2]BATLOUNI SR, ROMAGOSA E, BORELLA MI. 2006. The reproductive cycle of male catfish, cachara *Pseudoplatystoma fasciatum* (Teleostei, Pimelodidae) revealed by changes of the germinal epithelium. An approach addressed to aquaculture. An. Reprod. Sci., 96: 116-132.

[3]FAO – Fisheries Department.. State of world aquaculture. Rome: Food and Agricultural Organization of the United Nations. 145p.http://www.fao.org 2006

[4]GODINHO HM, ROMAGOSA E, CESTAROLLI MA, NARAHARA MY, FENERICH-VERANI N. 1984. Reprodução induzida de curimbatá, *Prochilodus scrofa*, Steind. 1881 sob condições de cultivo experimental. Rev. Bras. Reprod. Anim., Belo Horizonte, 8 (2): 113-119.

[5]NARAHARA MY, BASILE-MARTINS MA, GODINHO HM, CIPÓLLI MN. 1988. Escala de maturidade, época de reprodução e influência de fatores abióticos sobre o desenvolvimento gonadal de *Rhamdia hillarii* (VALENCIENNES, 1840). Boletim do Instituto de Pesca , São Paulo 15 (2): 201-2111.

[6]ROMAGOSA E. 1998. Desenvolvimento gonadal (morfologia; ultra-estrutura) e indução da reprodução do matrinxã, *Brycon cephalus*, (Günther, 1869) em cativeiro. Vale do Paraíba, São Paulo. Tese de Doutorado. Universidade Federal de São Carlos, 218p.

[7]ROMAGOSA E, PAIVA P, GODINHO HM, STORFER EB. 1988. Desenvolvimento de ovócitos de *Piaractus mesopotamicus* em condições de cultivo intensivo. Ciência e cultura, 40 (1): 60-64.

[8]ROMAGOSA E, PAIVA P, GODINHO HM, GUILHERME MCM. 1990. Fecundidade do pacu, *Piaractus mesopotamicus*, mantido em confinamento, durante o 1° e o 2° período reprodutivo. B. Inst. Pesca; 17: 99-103.

[9]ZANIBONI-FILHO E, WEINGARTNER M. 2007. Induced breeding in migratory fishes. Rev. Bras. Reprod. Anim. Belo Horizonte, 31 (3): 367-373.

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

INFLUENCES OF GUARDING TERRITORY ON REPRODUCTIVE ACTIVITY IN THE MALE DWARF GOURAMI, *COLISA LALIA*

Hayakawa, Y. , Kobayashi, M.
Department of Biology, International Christian University, 3-10-2 Osawa, Mitaka, Tokyo 181-8585, Japan
Fax: +81-422-33-1449, e-mail: hyouichi@nt.icu.ac.jp

Introduction:

Among teleost fishes, alternative mating tactics are commonly observed. Particularly in pair-spawning species with external fertilization, social status is generally divided into two types: the territorial dominant male, which establishes its territory and courts females to perform pair-spawning, and the sneaker, which have no territory of its own but tries to release semen during pair-spawning of other dominant males. In such species, it has been predicted that the sneakers possess relatively larger testes compared to the dominant males in order to overcome sperm competition by increasing number of sperm. Data from many species have proved the prediction [1].The dwarf gourami, *Colisa lalia*, is known to be included among the pair-spawning species [2, 3]. Males establish their territories (bubble nest) under a floating plant to obtain females and to care eggs. However, no research has reported an occurrence of sneaking. During spawning, male *C. lalia* exhibits a clasping behavior where the male bends its body around a female under the nest. Since the testes are situated in the vicinity of the fulcrum which is formed when a male bends its body, male may emit semen by squeezing out semen from the testes by bending its body. In other words, the clasping behavior is necessary for emitting semen, and subordinate males cannot emit semen probably because of lacking situation to perform clasping behavior [3].From this speculation and the fact that spawning is taken place under the bubble nest, we assumed that the reproductive condition concerning sperm production is influenced by whether males establish territories or not. In this study, we examined the influence of guarding territory (nest) on reproductive activity of males.

Methods:

In order to examine the influence of social status on the reproductive ability in males, three males were forced to fight for territories in an aquarium. After three weeks, their testes were removed to calculate gonadosomatic indices (GSI), and were stained with hematoxylin and eosin for microscopy after fixed in paraffin to make serial sections (7μm).

Results:

Reproductive status was divided into three classes: the dominant male which established and kept the territory under the floating nest, the second male which located near the nest and occasionally attacked the dominant male, and the third male which was unwarlike and kept a distance with the other two males. A significant difference was detected in GSI between the initial control (males not involved in any experiments: 0.85±0.10, n=5) and the dominant (1.19±0.07, n=5), between the second (0.81±0.15, n=5) and dominant and between the third (0.62±0.08, n=5) and dominant (Student-Newman-Keuls's test, P=0.007), indicating that the testes of the dominant males enlarged while guarding their territories, and those of males which failed to obtain territory became smaller or did not change. In the testes of all males examined, cysts at various stages of spermatogenesis were observed and the lobule lumen was filled with sperm. However, in the testes of the dominant male, broken vacuous cysts, which represent frequent occurrence of spermiaiton, were conspicuously observed and each lumen was enlarged, compared to other status males including the control group.

Discussion:

After forcing a three-week fight, testes of the dominant males enlarged by guarding territory, whereas testes of males which failed to obtain territory did not, and in particular males situated on the third became smaller. Histological observation revealed that sperm production of the dominant males was more active compared to males of other status, although spermatogenesis was confirmed in all examined males. This means that dominancy physiologically influences on sperm production. Social-status dependent development of testes may reflect the absence of sperm competition due to the lack of sneaking by subordinate males.

Fourcelius (1957) reported that taking-over the dominance between the dominant and the subordinate males frequently occur under natural conditions [2]. Since dominancy affect not only on sperm emission but also on reproductive ability, the way to increase reproductive success in males of *C. lalia* is not by alternative tactics (e.g. sneaking) but by obtaining and keeping territory.

References:

[1]MONTGOMERIE, R., FITZPATRICK, J. L. 2009. Testes, sperm, and sperm competition. In: Reproductive Biology and Phylogeny of Fishes (Agnathans and Bony Fishes) Vol. 8A. (ed. Jamieson, B. G. M), pp 1-53, Science Publishers, Enfield, NH.

[2]FOURCELIUS, S. 1957. Studies of anabantid fishes. I. A quantitative description of the reproductive behavior in territorial species investigated under laboratory conditions with special regard to *Colisa lalia*. Zool. Bidr. Uppsala, 32: 93-597.

[3]HAYAKAWA, Y., KOBAYASHI, M. 2010.Clasping behavior and the asymmetrically latitudinal structure of the testes in the male dwarf gourami *Colisa lalia*. Ichthyol. Res., 57: 40-48.

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

ANNUAL REPRODUCTIVE CYCLE AND SPAWNING BEHAVIOR OF CHAMELEON GOBY, *TRIDENTIGER TRIGONOCEPHALUS* IN KOREA

In Joon Hwang (1), Sang Jun Choi (1), Hyung Bae Kim (2), Hea Ja Baek (1)

(1) Department of Marine Biology, Pukyong National University, Busan 608-737, Korea, Fax: +82-51-6295931, email: hjbaek@pknu.ac.kr

(2) Department of Marine Bio-resources, Gangwon Provincial University, Gangnung 210-804, Korea

Introduction:

Gobiid fish are appropriate species for investigating the effects of endocrine disrupting chemicals with their small size, easy to handle, and strong tolerance [1]. Chameleon goby, *Tridentiger trigonocephalus* is distributed in mud flat, brackish waters of western and southern Korea, China and Japan [2]. At present, there is very little knowledge about its reproductive characteristics. In this study, we investigated the annual reproductive cycle and spawning behavior of chameleon goby.

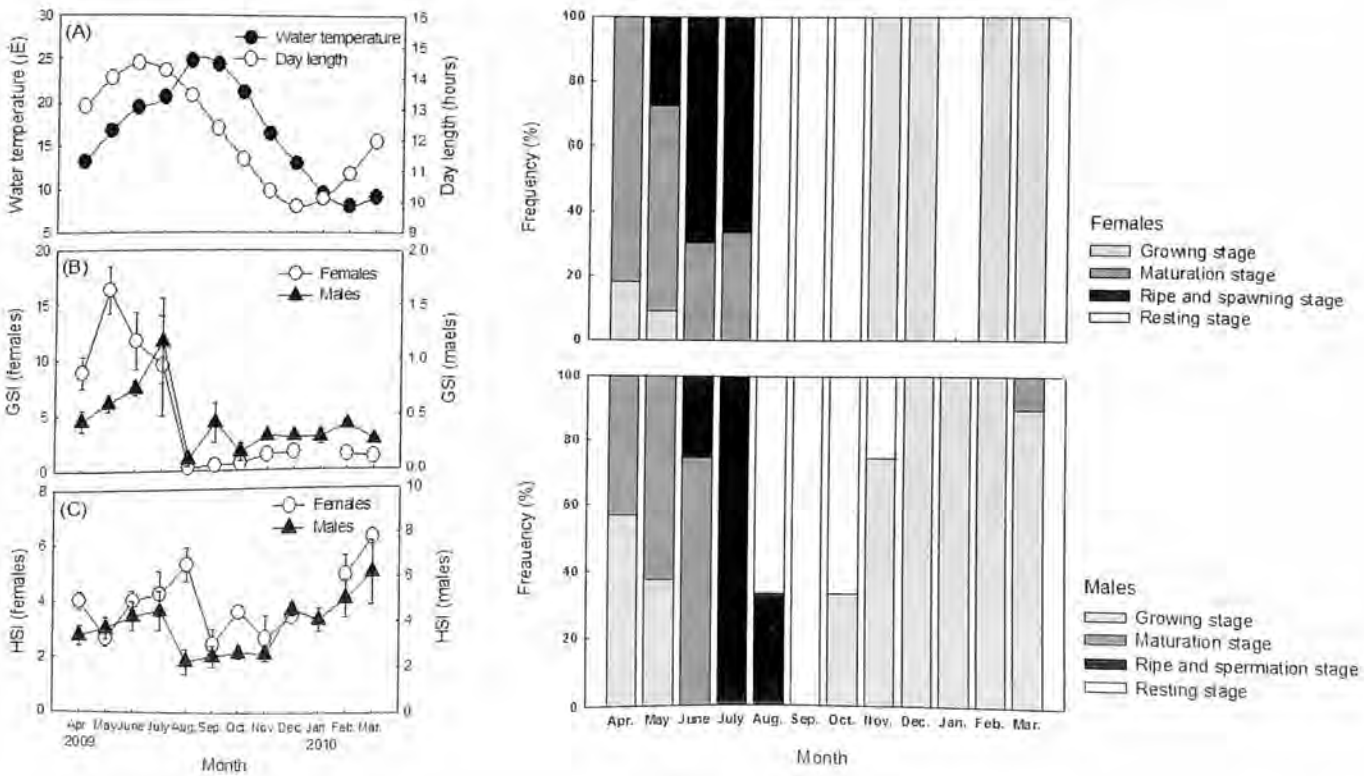
Methods:

In the field investigation, fishes were sampled monthly from the seagrass bed of Dongdae Bay (southern coastal waters of Korea) between April 2009 and March 2010. Gonadosomatic index (GSI) was recorded and gonads were removed and fixed in Bouin's solution and processed for light microscopy. For observation of spawning process from indoor tank, sexually mature fish were held under an artificial light and temperature regime. Fertilized eggs and guarding male were transferred to incubation tanks until hatching.

Results:

GSI of females increased from April and peaked in May (16.31±2.16), then decreased from June and were the lowest value in August (0.34±0.0). GSI of males increased from April and peaked in July (1.17±0.35), then decreased dramatically in August (Fig. 1). In histological observation, the annual reproductive cycle can be divided into four successive stages in females: the growing (November-March), maturing (April-May), ripe and spawning (June-July), and recovery (August-October) stages. Males passed through growing (November-March), maturing (April-June), ripe and

Fig. 1. Monthly changes of water temperature and day length (A), GSI (B), HSI (C) during annual reproductive cycle and



spawning (July-August), and recovery (September-October) stages. In the spawning process from indoor tank, mature males exhibited nuptial coloration (white band in pectoral fin). A male attracted female with courtship behavior (shaking his body), then sexually mature female spawned and attached the eggs in PVC pipe and male fertilized them immediately. After fertilization, male guards the egg mass until hatching (Fig. 2). Approximately 160 hrs after fertilization, hatched larvae were observed. The newly hatched larva were 2.44±0.04 mm in total length (TL), and 38.76±1.15 mm in TL at 100 days post hatching.

Conclusion:

The present study represents fundamental information in reproduction of chameleon goby. The spawning period of chameleon goby appears to be between June to July. In spawning period, males were distinguished well by nuptial coloration. The captive breeding of hatched larva led to the successful production easily and could mature less than a year.

Acknowledgement:

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (No. 2010-0017176).

References:

[1]ROBINSON, C.D., BROWN, E., JOHN, A.C., DAVIES, I.M., MEGGINSON, C., MILLER, C., MOFFAT, C.F. 2007. Bioindicators and reproductive effects of prolonged 17β-oestradiol exposure in a marine fish, the sand goby (*Pomatoschistus minutus*). Aqua. Toxicol., 81: 397-408.

[2]NELSON, J.S. 1984. Fishes of the world. 2nd ed. John Wiley and Sons., New York, 523 pp.

SEASON DEPENDENT EFFECT OF DIETARY LIPIDS IN REPRODUCTION IN THE INDIAN CATFISH *CLARIAS BATRACHUS*

Kaberi Acharia*, B. Lal and T. P. Singh

*Department of Zoology, Kirori Mal College, University of Delhi, Delhi 110007,
Centre of Advanced Study in Zoology, Banaras Hindu University, Varanasi, UP 221005, India.

Introduction:

Quantity and quality of diet influence the reproductive activity of fish. Amongst various dietary constituents lipids have drawn attention to fish reproductive biologists not only because they are the major energy source but can influence the hormone production, thereby gonadal maturation, egg quality and survival. However, the effect of diet is dependent on the reproductive phase of the fish. Moreover, feeding activity as well as the lipid profile of fish also varies with season. Considering these facts we tried to explore the effect of dietary lipids on gonadal recrudescence in two different phases of the female Indian catfish *Clarias batrachus*.

Methods:

Freshwater female catfish, *Clarias batrachus* were subjected to different photothermal conditions and fed with a laboratory prepared basal diet supplemented with linseed oil (C18 n-3 PUFA) or sunflower oil (C18 n-6 PUFA) during early and late postspawning phase of its reproductive cycle. Parameters considered were body weight, ovarian weight, gonadosomatic index, plasma levels of testosterone (T) and oestradiol-17β (E_2). Steroids were measured by radioimmunoassay. In addition, different fractions of lipids like free fatty acids, phospholipids, free and esterified cholesterol, mono, di and triglycerides were also estimated in liver, plasma and ovary by thin layer chromatography. Two way ANOVA followed by Tukey's test was employed to find the significant difference between group means.

Results:

Data showed that linseed oil supplementation to the basal diet was gonadostimulatory whereas sunflower oil was supportive to somatic growth during the late postspawning phase (Table 1). In this phase, gonadal weight was significantly elevated but body weight remained unchanged by linolenic acid feeding, in fish maintained under high temperature. Linoleic acid i.e sunflower oil feeding had an influence on body growth. Under normal temperature none of the dietary oils could bring any effect on the body or gonadal weight. Circulating testosterone was greatly affected by both the dietary lipids. During the early postspawning phase GSI was greatly reduced by either of the fatty acids which was due to the unchanged ovarian weight and enhanced

Table 1. Changes in the body weight (g), ovarian weight (g) and gomadosomatic index (%) in response to linseed oil (18:3 n-3) and sunflower oil (18:2 n-6) feeding in female catfish *Clarias batrachus* during late post-spawning phase. Values are mean (n=8) ± SEM

Parameter	Diet	LPAT	LPHT
Body Weight	A	43.38 ± 1.04	47.41 ± 1.11
	B	44.00 ± 1.45	47.31 ± 1.19
	C	46.08 ± 1.90	53.94 ± 2.16*
Ovarian Weight	A	0.21 ± 0.02	0.40 ± 0.04
	B	0.20 ± 0.023	1.09 ± 0.13*
	C	0.23 ± 0.024	0.38 ± 0.04
GSI	A	0.48 ± 0.041	0.84 ± 0.069
	B	0.46 ± 0.039	2.30 ± 0.21*
	C	0.44 ± 0.032	0.73 ± 0.062
T	A	2.50 ± 0.36	5.30 ± 0.40
	B	2.98 ± 0.38	7.34 ± 0.59*
	C	3.13 ± 0.51	3.62 ± 0.23*
E_2	A	0.46 ± 0.02	3.30 ± 0.30
	B	0.54 ± 0.05	6.44 ± 0.71*
	C	0.57 ± 0.04	3.48 ± 0.45

* = significant from Basal. A = Basal Diet, B = Linseed oil rich diet, C = Sunflower oil rich diet

Table 2. Changes in the body weight (gm), ovarian weight (gm) and gomadosomatic index (%) in response to linseed oil (18:3 n-3) and sunflower oil (18:2 n-6) feeding in female catfish *Clarias batrachus* during early post-spawning phase Values are mean (n=8) ± SEM

Parameter	Diet	NPNT	LPNT
Body Weight	A	34.06 ± 1.26	36.05 ± 1.02
	B	38.99 ± 1.03*	41.97 ± 1.24*
	C	40.70 ± 1.04*	41.99 ± 1.30*
Ovarian Weight	A	0.11 ± 0.01	0.10 ± 0.01
	B	0.09 ± 0.02	1.115 ± 0.02
	C	0.08 ± 0.024	0.117 ± 0.02
GSI	A	0.32 ± 0.02	0.306 ± 0.01
	B	0.23 ± 0.02*	0.295 ± 0.02
	C	0.20 ± 0.02*	0.286 ± 0.02
T	A	0.75 ± 0.082	1.59 ± 0.11
	B	0.829 ± 0.083	1.643 ± 0.08
	C	0.84 ± 0.05	1.614 ± 0.11
E_2	A	0.256 ± 0.02	0.29 ± 0.03
	B	0.304 ± 0.03	0.20 ± 0.02
	C	0.234 ± 0.02	ND

* = significant from Basal. A = Basal Diet, B = Linseed oil rich diet, C = Sunflower oil rich diet ND = Not Detectable

body weight. Plasma levels of T and E_2 were not affected by these diets (Table 2). The concentrations of the estimated lipid fractions (data will be displayed at the time of presentation) were quite correlative with the present findings.

Discussion:

The influence of the dietary lipids on the female *Clarias batrachus* was found to be season dependent. Interestingly, change in the GSI had a correlation with fluctuation in the plasma T and E_2. In addition, temperature played a very crucial role in bringing out the change in these parameters. Our study may have a great significance in controlling the catfish production in aquaculture programme.

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

REPRODUCTIVE PHYSIOLOGY AND AGGRESSIVE BEHAVIOUR OF FEMALES OF THE SOCIAL CICHLID FISH *CICHLASOMA DIMERUS* (PERCIFORMES).

Tubert C., Lo Nostro F. and Pandolfi M.

Departamento de Biodiversidad y Biología Experimental, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Argentina. Ciudad Universitaria. Pabellón II. CABA (C1428EHA).
pandolfi@bg.fcen.uba.ar. Fax: 0054 11 4576 3384.

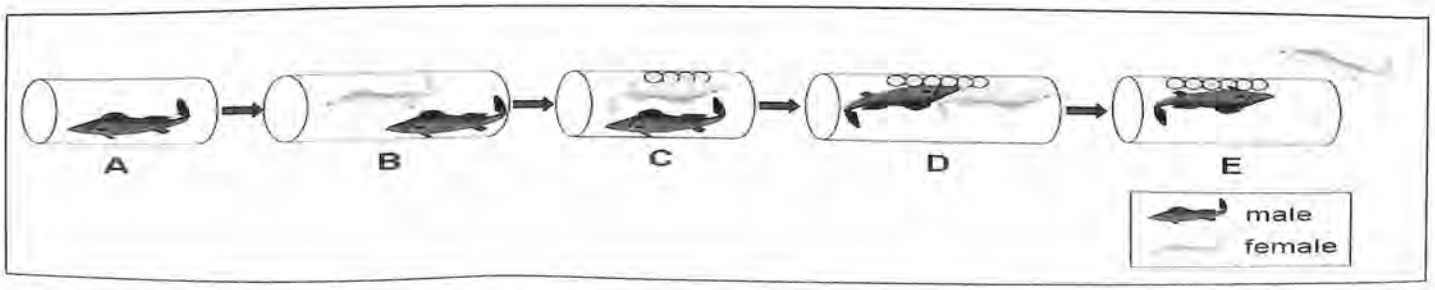
Introduction:

Teleost fish present a great variety of reproductive behaviours, including aggressive interactions among conspecifics and defense of a territory or progeny. The behaviour displayed during the reproductive season is critical for reproductive success. Among vertebrates, gonadotropin-releasing hormone (GnRH) plays a major role in the control of reproduction, regulating the hypothalamus-pituitary-gonad axis. GnRH type 3 (GnRH-3) neurons are located in the olfactory bulbs and project their axons toward the retina and pineal organ, which suggests a relationship with behavioural and physiological responses due to changes in light intensity and photoperiod [1, 2]. GnRH-3 has also been proposed as a potent neuromodulator of reproductive behaviour. Circulating androgens are potent mediators of male and female aggressive behaviour. In fact, several experiments have demonstrated that androgen depletion decreases aggression, whereas androgen supplementation recovers or increases aggression [3, 4, 5]. However, most of these studies were only performed in males. *Cichlasoma dimerus* is a South American freshwater cichlid species that presents social hierarchies, a highly organized breeding activity and biparental care. Territories are progressively established and defended by both male and female members of a pair. The dominant pair aggressively defends the spawning site and displays stereotypical prespawning behaviour. Spawning (egg clutch size of 1500) is followed by a period of parental care when the pair aggressively guards the eggs. Larvae hatch at day 3 post fertilization and remain adhere to the substrate through three pairs of adhesive glands located in the head. After 5 days, larvae swim freely. During this period, the reproductive pair also displays aggressive behaviour against subordinated individuals, protecting the offspring. Aggressive displays include biting, mouth holding, chasing, fin erection. Submissive displays of subordinate fish include escape reactions and fin retraction. Aggressive and submissive behaviour can be associated with differences in body color patterns [6, 7]. The present work characterizes the reproductive physiology and aggressive behaviour of females of the cichlid fish *Cichlasoma dimerus* at different phases during the reproductive stage.

Methods:

Fish were maintained in aquaria with constant temperature (26°C) and a 14:10 h photoperiod. Aggressive behaviour of females throughout the different phases were identified and quantified. For this purpose, 12 females referred to as "local females" were selected, 3 at each different previously established reproductive phase: (1) female with prespawning activity (FP), (2) female with eggs (FE), (3) female with hatched larvae (FHL) and (4) female with swimming larvae (FSL). Another 12 females were taken from established pairs at an early prespawning phase, and referred to as "intruder female". Males were removed from the "local female" tank, and a random "intruder female" was introduced. The number of aggressive interactions (biting and chasing) between "local female" and "intruder female" was recorded during 1 hour and quantified. Body color patterns and shape of dorsal fins were also examined. In another set of experiments, 16

Fig. 2. Serial process of spawning and fertilization of chameleon goby. A, a male nested in the substrate. B, Nested male attracts matured female. C and D, Stimulated female spawned on inner wall of substrate and male immediately fertilized. E, After fertilization, female goes out and male guards the fertilized eggs until hatching.



females at the different established phases were weighed and measured, blood was extracted and circulating androgens (RIA), 17-beta estradiol and cortisol (ECLIA) were measured in plasma. Liver, spleen and gonadosomatic indexes were calculated. Brains were dissected, and GnRH-3 neural populations were studied by IHC and morphometrical analysis of immunoreactive cells (nuclear area, somatic area and optical density). Semiquantification of gonadotropins (beta-FSH and beta-LH) pituitary content was performed by Western blot. Ovaries were processed for histological analysis.

Results:

Females presented a great variation in the studied parameters between the different phases. FP displayed the most aggressive behaviour in terms of number of biting and chasing attacks. This is consistent with the maximum plasmatic androgen levels observed during this stage. FP also showed the highest nuclear and somatic area, and optical density of GnRH-3 neurons. Moreover, FP presented maximum levels of plasmatic 17-beta estradiol and GSIs. Cortisol reached its highest levels in FE. There were no detectable differences in beta-FSH and beta-LH pituitary content between the different phases. At the ovarian level, each phase was characterized by the preponderance of different structures (FP: mature oocytes, FE: post ovulatory follicles, FHL: atresic bodies, FSL: secondary growth oocytes).

Conclusion:

Taken together these results suggest that GnRH-3 and androgens regulate female reproductive physiology and aggressive behaviour in adult *C. dimerus* during the reproductive stage.

References:

[1]DUBOIS E.A., ZANDBERGEN M.A., PEUTE J., GOOS H.J.T. (2002). Evolutionary development of three gonadotropin-releasing hormone (GnRH) systems in vertebrates. Brain Res. Bull., 57: 413-418.

[2]PANDOLFI M., MUÑOZ CUETO J.A., LO NOSTRO F.L., DOWNS J.L. PAZ D.A., MAGGESE M.C., URBANSKI H.F. (2005). The GnRH systems of *Cichlasoma dimerus* (Perciformes, Cichlidae) revisited: a localization and developmental study using antibodies and riboprobes to the GnRH-associated peptidel. Cell Tissue Res., 321: 219-232.

[3]KINDLER P.M., BAHR J.M., PHILIPP D.P.(1991). The effects of exogenous 11-ketotestosterone, testosterone, and cyproterone acetate on prespawning and parental care behaviors of male bluegill. Horm. Behav., 25: 410-423.

[4]FRANCIS R.C., JACOBSON B., WINGFIELD J.C., FERNALD R.D. (1992). Castration lowers aggression but not social dominance in male *Haplochromis burtoni* (Cichlidae). Ethology, 90: 255-347.

[5]HUME J.M., WYNNE-EDWARDS K.E. (2005). Castration reduces male testosterone, estradiol, and territorial aggression, but no paternal behavior in biparental dwarf hamsters (*Phadopus campbelli*). Horm. Behav., 48: 303-310.

[6]PANDOLFI M., CÁNEPA M.M., MEIJIDE F.J., ALONSO F., REY VAZQUEZ G., MAGGESE M.C., VISSIO P.G. (2009). Studies on the reproductive and developmental biology of *Cichlasoma dimerus* (Perciformes, Cichlidae). Biocell 33(1): 1-18.

[7]ALONSO F., CÁNEPA M., MAGGESE M.C., PANDOLFI M. (2011). Reproductive physiology and behavior of the social cichlid fish *Cichlasoma dimerus* under laboratory conditions. Fish Physiol. Biochem., In press.

ANNUAL VARIATIONS OF MATURATION INDUCING STEROID IN TWO CULTURED GENERATIONS OF SENEGALESE SOLE, *SOLEA SENEGALENSIS*.

Bayarri M.J., Guzmán, J.M., Ramos J., Piquer V., Mañanós E.

Instituto de Acuicultura de Torre la Sal (CSIC), 12595-Ribera de Cabanes, Castellón, Spain. Fax: +34-964319509. E-mail: evaristo@iats.csic.es

Introduction:

The Senegalese sole is a highly valuable species in Southern Europe. But, the development of sole aquaculture is limited by its dependence on captures from the wild to constitute broodstock of wild fish, since F1 soles (hatched in captivity) are unable to produce fertilized spawning in captivity. In a previous study, an F2 generation was obtained by hormonal treatment of F1 breeders [1]. These F2 fish were grown to puberty and, spawning performance and plasma profiles of sexual steroids (testosterone (T), estradiol (E2) and 11-ketotestosterone (11-KT)) and vitellogenin (VTG) followed through three consecutive years [2], and compared with previous data on F1 [3] and wild [4] sole breeders. The present study was aimed to add further information by analyzing plasma profiles of the maturation inducing steroid (MIS; 17alfa,20beta-dihidroxi-4-pregnen-3-one) in F1 and F2 broodstock, in an attempt to get comparative data between all three generations (wild, F1, F2) and potentially detect endocrine differences that could be associated with failed reproduction in cultured generations.

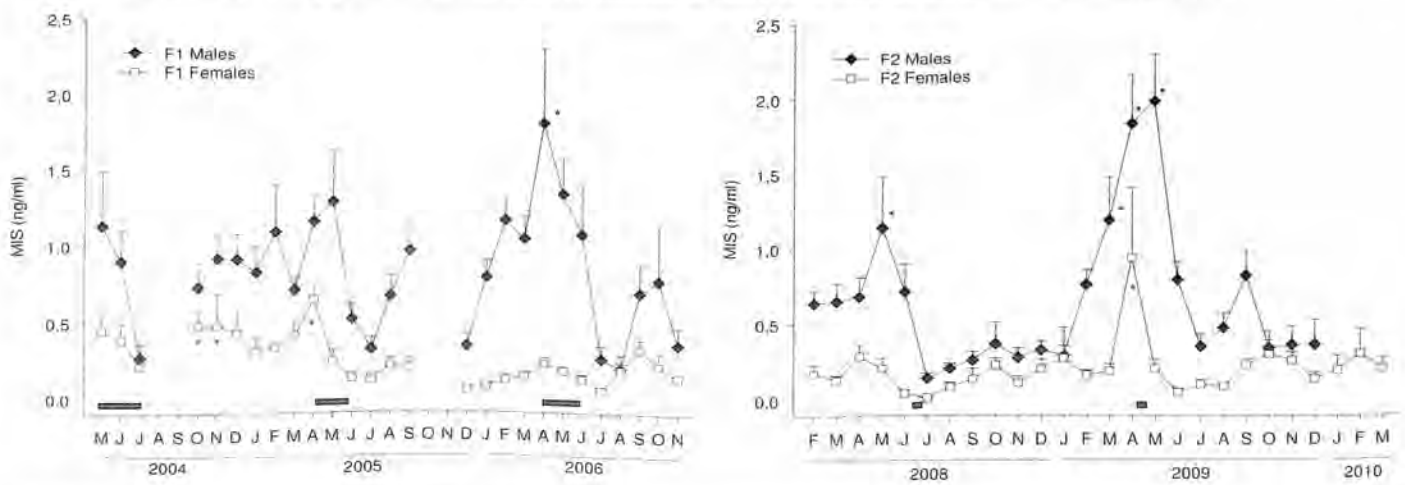
Methods:

Fish were reared at our facilities (40°N, 0°E) under natural photoperiod and temperature. Females and males were sampled monthly from puberty (3 years old) for weight, length and blood through three consecutive years. Plasma levels of MIS were analyzed by using a newly developed ELISA method. Specific antibodies were produced in rabbits (Agrisera AB, Sweeden) against conjugated MIS-CMO, kindly provided by Dr. A. Scott (CEFAS, UK); a commercial acetylcholinesterase tracer (Cayman, USA) was used. The ELISA showed a sensitivity (Bo-2SD) of 40 pg/ml, and intra- and inter-assay coefficients of variation of 2.49 % (n=11) and 9.91 % (n=69), respectively. One wayANOVA ($p<0.05$) was performed for statistical analysis.

Results and Discussion:

Plasma levels of the MIS are shown in figure 1. Two annual peaks of the MIS were detected in F1 females, in spring (major spawning season) and autumn (secondary spawning season). Significant differences from basal levels (minimum detected in December 2005) were only found in October-November 2004 and April 2005, on 4+ year-old fish. Profile and plasma levels in F2 females were similar to those of F1's, with significant peak levels in spring and minor elevations in autumn. Similarly, wild broodstock have also shown maximum MIS levels in January-February, coinciding with the beginning of their spawning period [4]. Previously described annual profiles of T, E2 and VTG in F1 and F2 soles also showed annual peak spring levels, correlated with highest maturational degree of the gonad and beginning

Fig. 1. Plasma levels of MIS in F1 (left) and F2 (right) generations of Senegalese sole during three consecutive years after puberty, when fish were 3, 4 and 5 years old. Significant differences respect minimum values for each sex and generation are represented by *. Spawning periods are indicated by horizontal black bars. Data given as mean ± SEM (n=5-8).



of spawning [2,3]. The spawning period seemed to be shorter in F2 than in F1 broodstock (Fig. 1).

In F1 males, annual profiles of MIS were similar than those observed in F1females, with highest levels in spring and lower elevations in autumn. Peak levels of MIS were observed in April-May of each year, being significantly different from basal levels (August 2006) only during the spring of 2006 (5 years old). F2 males showed a similar profile, with significant peak levels of MIS in May 2008 (3 years old) and March-April-May 2009. The annual profiles of T and 11-KT analysed in previous studies [2, 3] were similar to those obtained for MIS, as occurred in females. In general, levels of MIS were higher in males than in females of both generations, similarly to what has been previously described for wild breeders [4].

Conclusion:

The levels and annual profiles of MIS found in F1 and F2 sole were similar, with major peaks during the main spawning season (spring), and lower elevations during the secondary spawning period (autumn). These profiles were similar to those of T, 11-KT, E2 and VTG previously described for these cultured generations. No fertilized spawning were obtained from F1 or F2 breeders. Previous works by others [4] showed similar hormonal profiles in wild broodstock, which do reproduce normally in captivity. Thus, no differences have been observed between the hormonal profiles of wild, F1 and F2 sole breeders that could be associated to the observed differences in the reproductive performance between wild and cultured sole generations.

Research funded by the Spanish Ministry of Science and Innovation (MICIIN; AGL2006-13777-C03-01) and JACUMAR (2009, III National plan for the cultivation of sole).

References:

[1]GUZMÁN JM, RAMOS J, MYLONAS CC, MAÑANÓS E. 2011. Comparative effects of human chorionic gonadotropin (hCG) and gonadotropin-releasing hormone agonist (GnRHa) treatments on the stimulation of male Senegalese sole (*Solea senegalensis*) reproduction. Aquaculture, 316: 121-128.

[2]BAYARRI MJ, PIQUER V, GUZMÁN JM, RAMOS J, MAÑANÓS E. 2010. F2 generation of Senegalese sole: biometrical, hormonal and spawning monitoring. In: Proceedings of Aquaculture Europe 2010. Porto, Portugal.

[3]GUZMÁN JM, NORBERG B, RAMOS J, MYLONAS CC, MAÑANÓS E. 2008. Vitellogenin, steroid plasma levels and spawning performance of cultured female Senegalese sole (*Solea senegalensis*). Gen.Comp. Endocrinol., 156: 285-297.

[4]GARCÍA-LÓPEZ A, ANGUIS V, COUTO E, CANARIO AVM, CAÑABATE JP, SARASQUETE C, MARTÍNEZ-RODRÍGUEZ G. 2006. Non-invasive assessment of reproductive status and cycle of sex steroid levels in a captive wild broodstock of Senegalese sole *Solea senegalensis* (Kaup). Aquaculture, 254: 583-593.

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

EFFECTS OF PERFUSION OF TESTICULAR FRACTIONS ON BRAIN CATECHOLAMINES IN THE FEMALE CATFISH *HETEROPNEUSTES FOSSILIS*

Mishra S. and Chaube R.

Zoology Section, Mahila Mahavidyalaya, Banaras Hindu University, Varanasi, U.P. - 221005, India
email: chauberadha@rediffmail.com

Introduction:

Fish have highly developed chemosensory and chemical signaling systems as they inhabit aquatic environment devoid of light but rich in dissolved compounds. Four major classes of chemicals have been identified as specific olfactory stimuli namely amino acids, sex steroids, bile acids or salts and prostaglandins. Pheromones are defined as substances which are secreted to the outside by an individual, in which they release specific reaction; for eg; a definite behavior or a developmental process in conspecifics[1]. In many teleost species, pheromones play an important role in various events of reproductionand spawning [7,4]. The gonads of teleosts secrete pheromonally active substances, most likely steroidal glucuronides, which can activate the neuroendocrine axis to release gonadotropin–II. In the African catfish, strong olfactory sensitivity to steroid conjugate fraction has been attributed to glucuronides synthesized by the seminal vesicle [3,2]. It was reported that the testicular homogenate or its, glucuronide fraction [6] and the seminal vesicle fluid or its glucuronide fraction [2] induced either ovulation or behavior effects in zebrafish and African catfish, respectively. The testis of the *Heteropneutes fossilis* seems to synthesize steroid glucuronides like the seminal vesicle and may be responsible for the olfactory sensitivity [5]. Sex steroids have been reported to alter hypothalamic aminergic metabolism and modify gonadotropin secretion. In the present study, female catfish were perfused with testicular fractions in olfactory organ intact and ablated groups and brain catecholamines were measured to study the relationship between steroidal pheromones and catecholamine secretion during spawning phase.

Materials and Methods:

Female catfish *Heteropneustes fossilis* were collected from local fish markets during spawning phase (July - August) and acclimatized in laboratory conditions for 48hr. After acclimatization, intact or olfactory organ-ablated fish were perfused with various testicular (pooled from 20 fish) fractions (crude homogenate, organic fraction, aqueous fraction, aqueous fraction after β glucuronidase treatment and organic fraction after β glucuronidase treatment) for 18hr in a static system at a flow rate of 4ml/hr, individually (n = 5 fish). Each of the fraction was diluted in 70ml dechlorinated water and taken in a common reservoir tank connected to the aquarium tanks containing sham control (intact) or anosmic fish placed individually and perfused continuously at the rate of 4ml/hr. Brain tissues were collected at 18 hr by decapitation and transferred into vials containing 0.2M perchloric acid under dark conditions. The brain samples were homogenized and centrifuged at 10,000 rpm for 15 min at 4°C. The supernatant was collected separately, protected from light and directly used for catecholamines assay by HPLC (Wood and Hall, 2000). The data were expressed as means ± SEM and analysed by one way analysis of variance (ANOVA), followed by Newman-Keuls' test ($p< 0.05$).

Results and Discussion:

In the present study, effects of perfusion of testicular fractions viz., whole homogenate (fraction 1), dichloromethane extract (fraction 2), aqueous extract (fraction 3), deglucuronated aqueous extract (fraction 4) and deglucuronated organic (fraction 5) on brain contents of catecholamines (dopamine-DA, norepinephrine-NE and epinephrine-E) and metabolite (3, 4-DOPAC and Normetanephrine) in female catfish *Heteropneustes fossilis* were studied. The content of brain DA increased in the OE-ablated groups compared to the OE-intact fish. However, fraction 4 decreased the DA level in comparison to other fractions perfused in OE-intact group. The DOPAC level decreased significantly in all ablated groups except the homogenate perfused group. The decrease was higher in aqueous fraction group. The DA turnover index was low in the OE-intact group in comparison to the OE-ablated group. It is the lowest in fraction 2, 3 and 4. The perfusion with various testicular fractions produced significant effects on brain contents of NE and NME. NE content increased with fractions 2, 3, 4 and 5 significantly, though the levels did not vary significantly between the fractions (3 and 4). Ablation of the olfactory organ abolished the stimulatory effect of the perfusion with all the fractions. On the other hand, in the perfused fish, NME content was increased in fractions 1, 2 and 5 and decreased in fraction 3 and 4 in olfactory organ intact fish. In ablated groups there was either no effect or a decrease in NME content with all fractions. The NE turnover index was significantly higher OE-intact group with fraction 2, 3 and 4. The content of epinephrine showed significant

increase when perfused with fractions 1, 2, 3 and 4. However, fraction 5 perfusion did not alter the content significantly in the olfactory organ intact fish. Ablation of the olfactory organ abolished the stimulatory effect of the perfusion with the fractions. The present study signifies that the perfusion with testicular fractions elicited changes in brain catecholamine levels in both sham anosmic (OE-intact) and anosmic (OE-ablated) fish.The changes in the content of DA, DOPAC, NE, NME, E and its turnover index may be attributed to the olfactory organ –mediated pheromonal activity of testicular fractions since such changes were not significant in anosmic fish. The results strongly support the existence of a neuroendocrine pathway involving catecholaminergic system to mediate sex pheromone-mediated changes.

Conclusion:

In conclusion our investigation suggests that testicular pheromones, which on release into water medium may form a chemical communication link by interacting with the olfactory–neuroendocrine-gonadal axis of the conspecifics, by altering central catecholaminergic activity.

References:

[1]KARLSON, P., LUSCHER, M. 1959. "Pheromones" a new term for a class of biologically active substances. Nature, 183:55-56.

[2]RESINK, J. W., VOORTHUIS, P. K., Van den HURK, R., PETERS, R. C., Van OORDT, P. G. W. J. 1989. Steroid glucuronides of the seminal vesicle as olfactory stimuli in African catfish,*Clarias gariepinus*. Aquaculture, 83:153-166.

[3]SCHOONEN, W. G. E. J., LAMBERT, J.G.D. Van OORDT, P. G. W. J. 1986. Quantitative analysis of steroids and steroid glucuronides in the seminal vesicle fluid of the feral spawning and cultured non spawning African catfish *Clarias gariepinus* in ponds. Gen. Comp. Endocrinol., 70: 91-100.

[4]SORENSEN, P. W., BRASH, A. R., GOETZ, F. W., KELLNER, R. G., BOWDIN, L. VRIEZE , L. A. 1995. Origins and functions of F prostaglandins as hormones and pheromones in the goldfish. In Reproductive Physiology of Fish (Goetz, F. W., and Thomas., Eds.). pp 252-254, Fish Symposium 1995, Austin, USA.

[5]THARAKAN, B., 1998. Neuroendocrine control of gonadotropin secretion in female catfish *Heteropneustes fossilis*: Basic mechanisms and involvement of olfactory system. PhD thesis, The Banaras Hindu University, Varanasi, India.

[6]Van den HURK, R., SCHOONEN, W. G. E. J., Van ZOELEN, G. A., LAMBERT, J. G. D. 1987. The biosynthesis of steroid glucuronides in the testis of the Zebrafish, *Brachydanio rerio*, and their pheromonal function as ovulation inducers. Gen. Comp.Endocrinol., 68:179-188.

[7]Van WEERD, J. H., RICHTER, C. J. J. 1991. Pheromones and ovarian development in teleost fish. Comp. Biochem. Physiol., 100:517-527.

EXPRESSION PROFILES OF GTHβ SUBUNIT mRNAs DURING SEASONAL AND SPAWNING CYCLES IN FEMALE CHUB MACKEREL *SCOMBER JAPONICUS* AND *IN VITRO* STEROID POTENCY OF PURIFIED GTHS IN VITELLOGENIC OOCYTES

Nyuji M.[1], **Ohga H.**[1], **Kitano H.**[1], **Selvaraj S.**[1], **Yoneda M.**[2], **Shimizu A.**[2], **Yamaguchi A.**[1] and **Matsuyama M.**[1]

[1]Faculty of Agriculture, Kyushu University, Fukuoka 812-8581, Japan
Tel/Fax: +81 92 642 2888; E-mail rinya_m@agr.kyushu-u.ac.jp
[2]Fisheries Research Agency, Yokohama 236-8648, Japan

Introduction:

The physiological functions of teleost gonadotropins (GtHs), follicle-stimulating hormone (FSH) and luteinizing hormone (LH) are well established in salmonids, which are single spawning fish. In salmonids, FSH regulates vitellogenesis via estradiol-17β (E2) production, whereas LH has a specific role in stimulating final oocyte maturation (FOM) through 17α,20β-dihydroxy-4-pregenen-3-one (17,20β-P) production [1]. In contrast, their roles in multiple spawning fish are still poorly understood. To understand the role of GtHs during ovarian development of female chub mackerel, *Scomber japonicus*, which are multiple spawners, changes in mRNA expression of FSHβ and LHβ were investigated during seasonal and spawning cycles. In addition, the effects of purified chub mackerel GtHs (cmFSH and cmLH) on the steroid production were examined in the vitellogenic oocytes of chub mackerel *in vitro*.

Methods:

Gene expression analysis- Fish were reared in sea pens and sampled during November 2008, March, April and August 2009 to investigate seasonal changes in *fshβ* and *lhβ*. During the spawning season, fish showing multiple spawning in 3-ton concrete tanks were sampled at 1300, 1600, 2000, and 0600 to investigate those changes during the spawning cycle. For analysis of each sample, mRNA was isolated from the pituitary and template cDNA was synthesized. A real-time PCR reaction method was used to assay *fshβ* and *lhβ*. *In vitro experiments*- Fully grown post-vitellogenic and mid-vitellogenic oocytes were separated from the ovary and incubated in the absence or presence of purified cmFSH or cmLH (6, 25, 100, and 200 ng/ml), respectively. After incubation for 18 hr at 20 °C, culture media were frozen and later, E2 and 17,20β-P were measured using ELISA.

Fig. 1. Changes in *fshβ* and *lhβ* in the female chub mackerel pituitary during seasonal and spawning cycles. IM, immature; EV, early vitellogenesis; LV, late vitellegenesis; PS, post-spawning; GVM, germinal vesicle migration; HY, hydration; OV, ovulation; POV, post-ovulation.

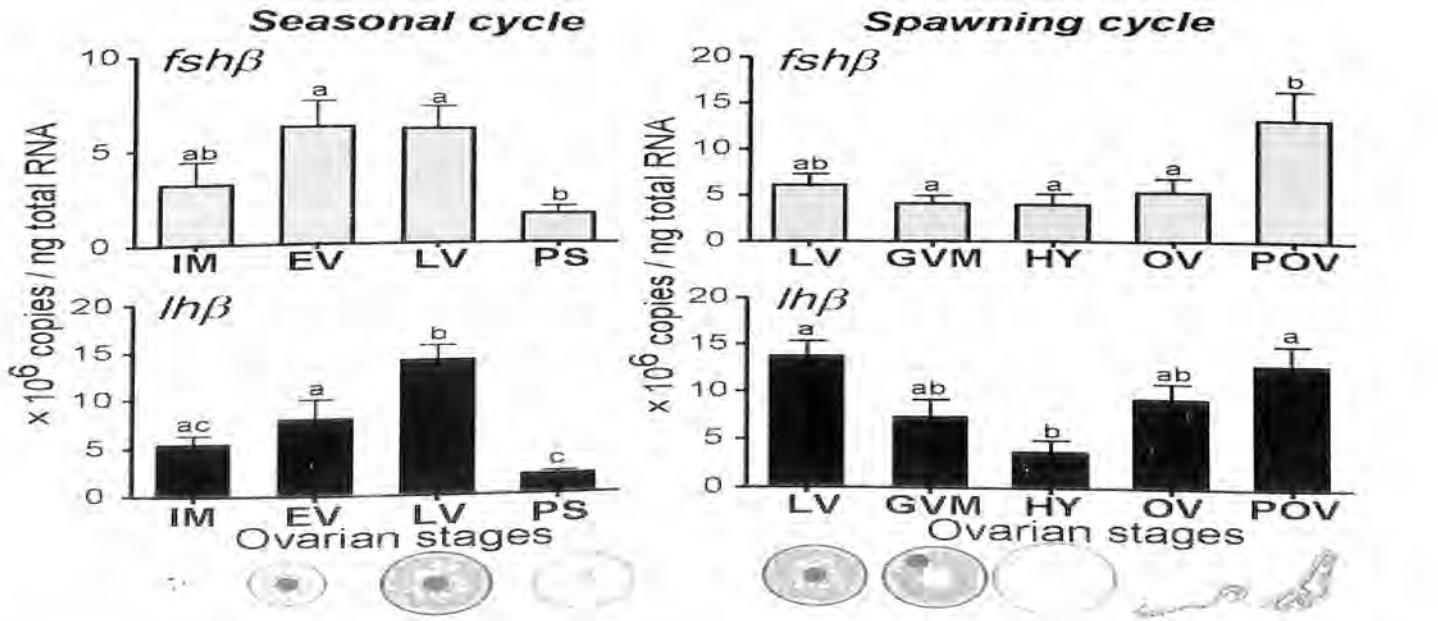
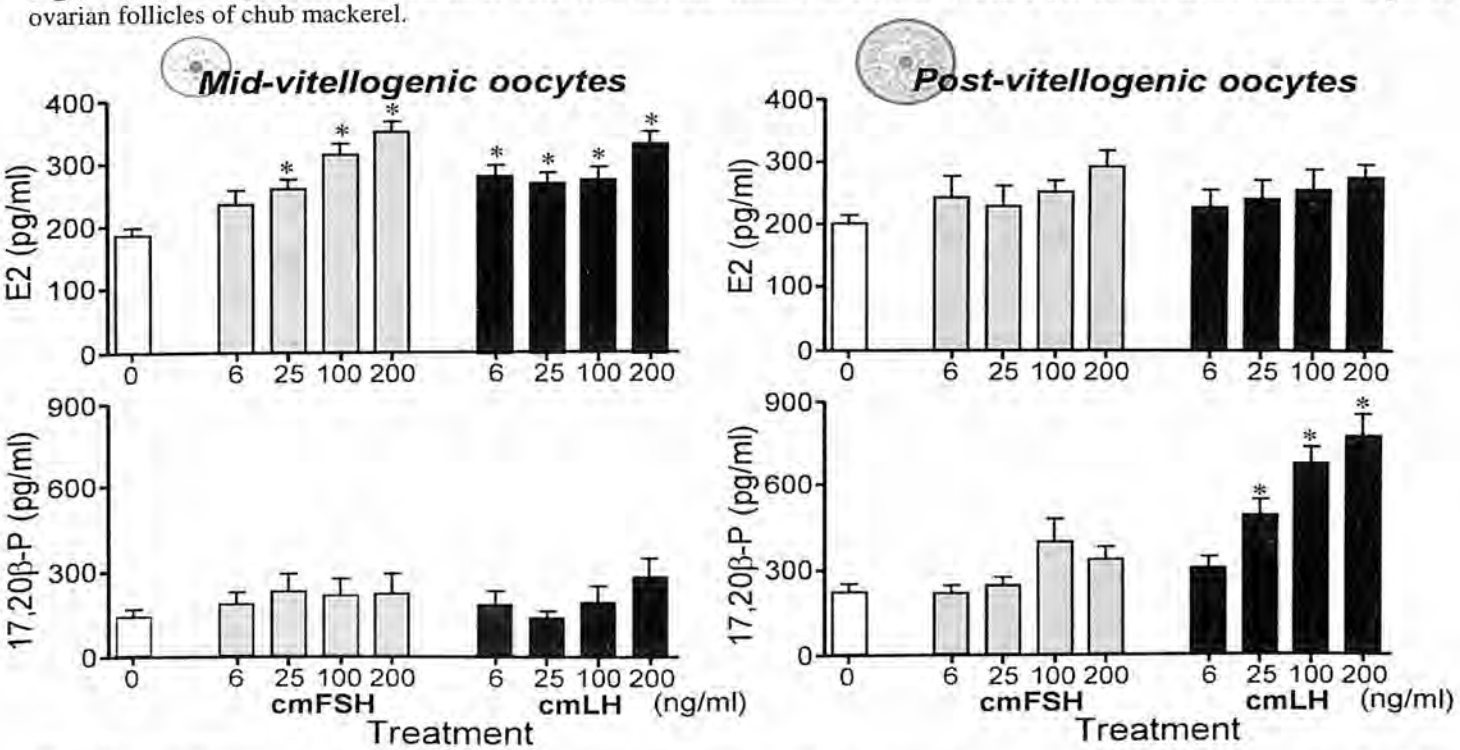


Fig. 2. Effects of purified chub mackerel GtHs (cmFSH and cmLH) on E2 and 17,20β-P production in the different stages of ovarian follicles of chub mackerel.



Results and Discussion:

The levels of *fshβ* remained high during vitellogenesis while *lhβ* increased in association with ovarian development and reached a peak at late vitellogenesis (Fig. 1). Both *fshβ* and *lhβ* levels decreased during the post-spawning period. The *in vitro* cultivation of oocytes demonstrated that cmFSH stimulated E2, but not 17,20β-P production, whereas cmLH stimulated both E2 and 17,20β-P synthesis (Fig. 2). It has been revealed that in ovarian follicles of chub mackerel, when hormonal stimulation was investigated by radiolabeled precursors *in vitro*, E2 was synthesized during vitellogenesis while 17,20β-P was produced during FOM [2]. Therefore, in chub mackerel, both FSH and LH may be involved in the vitellogenic process through the stimulation of E2 production, whereas FOM may be regulated by the LH, regulating the synthesis of 17,20β-P production. Interestingly, during the spawning cycle, high expression of *fshβ* further increased after ovulation (POV, in Fig. 1), suggesting that the FSHβ transcription may be promoted to accelerate the vitellogenesis. Conversely, high expression of *lhβ* declined from germinal vesicle migration (GVM) to hydration (HY) and increased after ovulation (Fig. 1).

Conclusion:

Seasonal expression profiles of GtHβ subunit mRNAs and *in vitro* steroid productions by cmFSH and cmLH suggests that chub mackerel FSH may act on vitellogenesis and LH on both vitellogenesis and FOM. Moreover, changes in *fshβ* and *lhβ* during the spawing cycle indicate that the synthesis of FSH and LH may be differently regulated during FOM, ovulation and spawning in the multiple spawning fish.

References:

[1]PLANAS, J.V., SWANSON, P. 2007. Physiological function of gonadotropins in fish. In: Rocha, M.J., Arukwe, A., Kapoor, B.J. (Eds.), Fish Reproduction. Science Publishers, Enfield, NH: pp. 37–66.

[2]MATSUYAMA, M., SHIRAISHI, T., SUNDARAY, J.K., RAHMAN, M.A., OHTA, K., YAMAGUCHI, A. 2005. Steroidogenesis in ovarian follicles of chub mackerel, *Scomber japonicus*. Zool. Sci., 22: 101–110.

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

'OUT-OF-SEASON' PRODUCTION OF 17,20 -DIHYDROXYPREGN-4-EN-3-ONE CHALLENGES THE DOGMA THAT IT IS SOLELY A 'MATURATION-INDUCING HORMONE' IN FISH

Scott, Alexander P. and Ellis, Tim

Centre for Fisheries, Environment and Aquaculture Research (Cefas), Barrack Road, Weymouth, Dorset, DT4 8UB, UK. e-mail: Alexander.Scott@cefas.co.uk

The two main progestins in teleost fish, 17,20β-dihydroxypregn-4-en-3-one (17,20β-P) and 17,20β,21-trihydroxypregn-4-en-3-one (17,20β,21-P), are widely believed to be solely involved in the very final stages of the reproductive cycle (i.e. final oocyte maturation/ovulation in females and spermiation in males). However, we have discovered two peaks of production (i.e. high levels in blood and water) of these two steroids in male and female roach (*Rutilus rutilus*; a cyprinid), one around the time of spawning in early summer and the other in the late summer and early autumn, when the gonads are at a relatively early stage of development (early vitellogenesis in females and primary spermatocyte formation in males). Although the coincidence of elevated 17,20β-P levels and primary spermatocyte formation in males favours a proposed role in initiation of the first meiotic division [1], there is no such correlation in another cyprinid, the tench *Tinca tinca* (in which a late summer peak of 17,20β-P was also observed; [2]). The secretion of these steroids at times of the year other than spawning highlights how much more there is to learn about the function of progestins in teleosts.

We acknowledge funding from Defra CN division, UK and the participation of others in the UK, Greece and Czech Republic (without whose efforts this study would not have been possible)

References:

[1] MIURA, T., HIGUCHI, M., OZAKI, Y., OHTA, T., MIURA, C., 2006. Progestin is an essential factor for the initiation of the meiosis in spermatogenetic cells of the eel. Proc. Natl. Acad. Sci. U S A., 103: 7333-7338.

[2]ANTONOPOULOU, E., TSIKLIRAS, A. C., KOCOUR, M., ŽLÁBEK, V., FLAJŠHANS, M., GELA, D. PIAČKOVÁ, SCOTT, A. P. (2011). Teleost maturation-inducing hormone, 17,20β-dihydroxypregn-4-en-3-one, peaks after spawning in *Tinca tinca*. Gen. Comp. Endocrinol., 172: 234–242.

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

CIRCANNUAL VARIATIONS IN PLASMA LEVELS OF UNCONJUGATED AND CONJUGATED TESTOSTERONE AND ESTRADIOL-17β IN FEMALE *CIRRHINUS MRIGALA* (Ham.)

Singh[1], H .K. and Singh[2], P. B.

[1]Department of zoology, Tilak Dhari Postgraduate College, Jaunpur-222 002, India
2Ganpat Sahai Postgraduate College, Sultanpur-228 001
Fax: o5362 240170 email: pratap_b_singh@rediffmail.com

Introduction:

Estradiol-17β has been identified in the plasma of many female teleost fishes and its role in the synthesis and secretion of hepatic vitellogenic protein is well established It has been reported that teleost gonadotropin directly stimulated estradiol-17β production by early vitellogenic ovarian follicles of amago salmon, *Oncorhynchus rhodurus*.The present study was conducted to obtain scientific knowledge on the relationship between seasonal pattern of plasma levels of testosterone and estradiol-17β and their conjugated forms (glucuronides and sulfates) in *Cirrhinus mrigala* and also for further studies on the mechanisms controlling steroidogenesis in this species. This is the first investigation in any major carp *Cirrhinus mrigala* (Ham.) showing the relationship between glucuronide and sulfate sex steroid hormones measured by enzyme linked immunoassay (ELISA) during annual reproductive cycle.

Methods:

Adult experimental female fish of *Cirrhinus mrigala* was collected during each phase from a pond cultured by a fish former Banrahia Bagh, Gaura Badshahpur, Jaunpur fish farm fish and was bled by caudal incision. Blood was collected in heparinized glass culture tubes. The blood was centrifuged at 4000 rpm for 15 minutes in a refrigerated centrifuge at 4^0C.The plasma was separated and kept -20^0C till further analysis of conjugated and unconjugated steroids. The ovaries were dissected out and gonadosomatic index (GSI) was calculated as total gonad weight / Body weight × 100 Extraction of unconjugated and conjugated sex steroid hormones was followed as per methods described [1]. Extraction of free, glucuronides and sulfate steroids was assayed for various hormones by ELISA Kit.

Result and Discussion:

Plasma levels of TF were high during prespawning phase and declined its level in the rest of phases. The

Fig; 1 Seasonal profile of Gonadosomatic index (GSI) and testosterone free (TF), testosterone glucuronide (TG), and testosterone sulfate (TS) during different phase of annual reproductive cycle in fresh water female major carp, *Cirrhinus mrigala* (Ham.). Analysis of variance two way (ANOVA-TW); GSI, Phase, F: 65535, P < 0.001, GSI, 99.35, < 0.001, Phase × GSI, F: 65535, < 0.001, Testosterone F: 21.25, P < 0.001, Hormone, F: 142.87, P < 0.001, Phase × Hormone, F: 8.86, P < 0.001

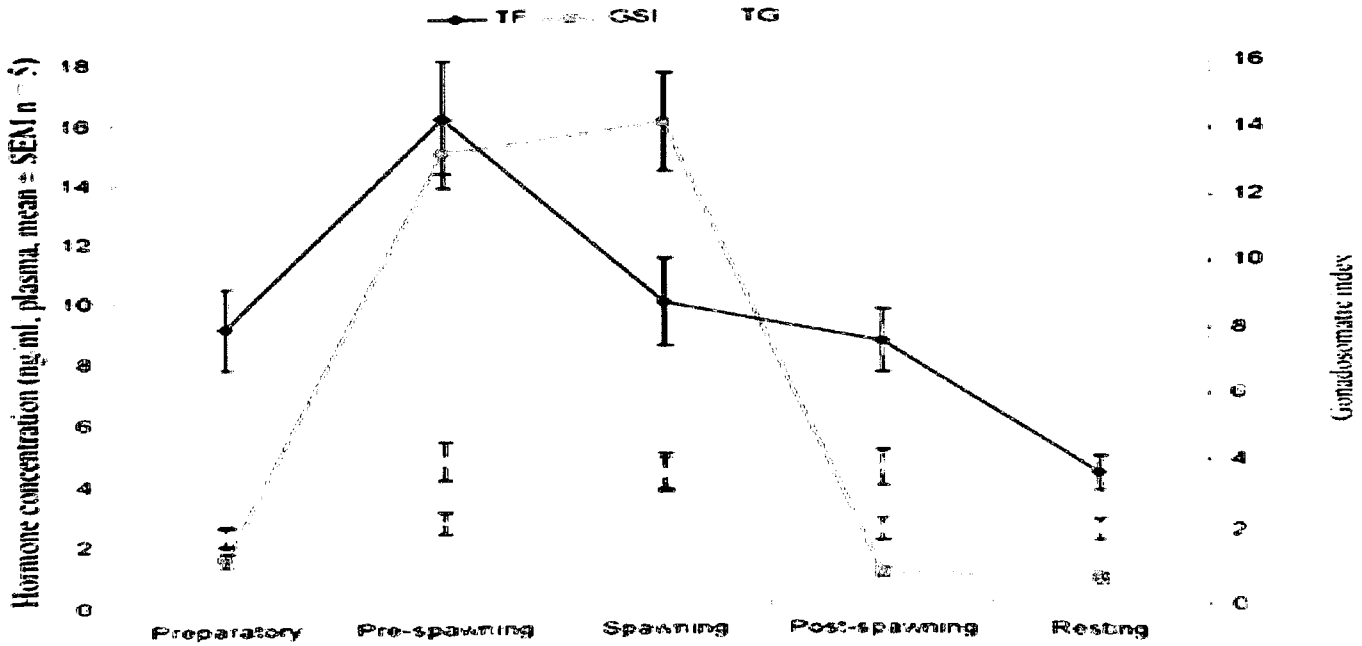
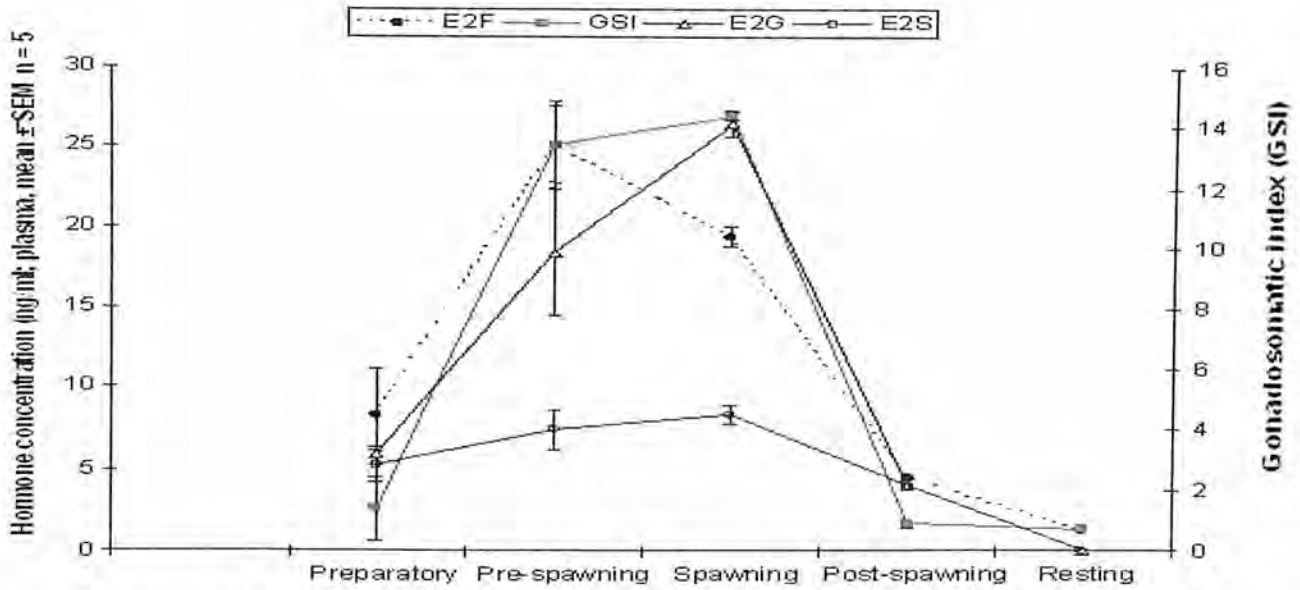


Fig: 2 Seasonal profile of Gonadosomatic index (GSI) and estradiol-17β free (E2F), estradiol-17β glucuronide (E2G) and estradiol-17β sulfate (E2S) during different Phase of annual reproductive cycle in fresh water female major carp, *Cirrhinus mrigala* (Ham.) Analysis of variance two ways (ANOVA-TW); GSI, Phase, F: 65535, P < 0.001, GSI, 99.35. P < 0.001, Phase × GSI: F, 65535 P < 0.001 estradiol-17β Phase F: 42.90, P P < 0.001, Hormone, F: 13.82, P < 0.001, Phase × Hormone, F: 7.45, P < 0.001



level of TG was high during prespawning phase and declined its level in the rest of phases the plasma levels of TS and TG were approximate same during the pre-spawning phase and. Plasma levels of E2F was high during prespawning phase where as E2G was declined during spawning phase . Analysis of variance indicated that gonadosomatic index has correlations with free steroids in this species. Result indicated that production of conjugated sex steroids which play important role in the pheromonal behavior and spawning in the freshwater carp, *Cirrhinus mrigala* (Ham.) during annual reproductive cycle of this species.

Conclusion:

These Results indicated that production of conjugated sex steroids which play important role in the pheromonal behavior and spawning in the freshwater carp, *Cirrhinus mrigala* (Ham.) during annual reproductive cycle of this species as have been reported earlier [2,3].

References:

[1]SINGH, P.B., KIME D.E. 1995. Impact of γ-hexachlorocyclohexane on the *in vitro* production of steroids from the endogenous and exogenous precursors in the spermiating roach, *Rutilus rutilus*. Aquat. Toxicol., 31: 231-240.

[2]SCOTT, A.P., SUMPTER, J.P., STACEY, N. 2010 The role of the maturation – inducing steroid, 17,20β-dihydroxy pregnen-4-en-3-one in male fishes. J. Fish. Biol., 76:183-224.

[3]PAVLIDS, M., GREENWOOD L., SCOTT A.P. 2004: The role of sex steroids on spawning performance and on the free and conjugated sex steroids released in to the water by common dentex (*Dentex dentex*) broodstock.

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

OSMOREGULATORY, METABOLIC AND ENDOCRINE RESPONSES OF AIR-BREATHING FISH (*ANABAS TESTUDINEUS* BLOCH) TO EXOGENOUS ESTRADIOL-17β

Valsa S. Peter, M C Subhash Peter

Department of Zoology, University of Kerala, Kariavattom 695581, Thiruvananthapuram, Kerala, India e-mail: subashpeter@yahoo.com

Introduction:

Estrogens have been shown to affect many physiologic processes in many vertebrates including fishes [1,2]. However, the exact role of estradiol-17β in hydromineral and metabolic regulations in fish is less studied in teleosts particularly in air-breathing fishes. The phylogenetic importance of air-breathing fishes demand a detailed examination of functional role of estradiol-17β as this estrogen has shown more functional diversity in homeothermous vertebrates which are unique with their ability to utilize atmospheric oxygen. Air-breathing as an advanced physiologic adaptation makes these fish more vulnerable and it is probable that these fishes rely on many hormones including estradiol-17β to perform both fundamental and specific functions during their survival in both water and land.

Methods:

The purpose of this study was, therefore, to test the role of estradiol-17β in the regulation of water and mineral balance and metabolic and endocrine status in air-breathing fish, though this sex hormone has specific reproductive functions. To achieve this goal, experiments were designed and the osmotic competence of major osmoregulatory and target organs were studied in the air-breathing fish *Anabas testudineus* Bloch. We quantified Na^+, K^+-ATPase activity in these organs and measured the major electrolytes such as [Na], [K], [Cl], metabolites like glucose and lactate and hormones like E_2, T_3, T_4 and cortisol in the plasma. Appropriate statistical methods (ANOVA with SNK tests) were employed and tested the significance between test and control fish groups.

Results and discussion:

Varied doses of estradiol-17β (E_2) showed dose (2 and 5 μg g^{-1} body wt) and time (24 and 96 h)-dependent actions on the activity of Na^+, K^+- ATPase and the levels of minerals and metabolites. A strong correlation between plasma E_2 and Na^+ K^+- ATPase activity was found in the gills, a major osmoregulatory organ. Lliver and ovary as target organs also responded to E_2 treatment. This suggests that E_2 has a specific osmoregulatory role in this air-breathing fish. The pattern of metabolite and electrolytes in the plasma also responded to E_2 treatment and this point to a coupled action of E_2 with the indices of metabolic and hydromineral regulations. The dynamics of thyroid hormones and cortisol in E_2-treated fish indicates correlations of E_2 with these hormones in the test species.

Conclusion:

Overall, our data provide evidence that estradiol-17β has a fundamental role in and hydromineral and metabolite homeostasis in air-breathing fish. The results also support the hypothesis that the reproductive status of fish contributes to the physiologic performance of fish.

References:

[1]LERNER D.T., BJORNSSON B.T., MCCORMICK S.D. 2007. Environ. Toxicol. Chem., 26, 1433-1440.

[2]MANCERA J.M., SMOLENAARS M., LAIZ-CARRION R., RIO M.P.M.., WENDELAAR BONGA S.E., FLIK G. 2004. Comp. Biochem. Physiol.,139 B:183-191.

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

ENVIRONMENTAL CONTROL OF GONADAL DEVELOPMENT IN THE FEMALE BLACKTIP GROUPER *EPINEPHELUS FASCIATUS*

Yong-Ju Park[1], Hyeong-Cheol Kang[1], Chi-Hoon Lee[1], Young-Bo Song[1], Sang-Woo Hur[1]Hea-Ja Baek[2], Hyung-Bae Kim[3] and Young-Don Lee[1]

[1]Marine and Environmental Research Institute, Jeju National University, 3288 Hamdeok, Jeocheon, Jeju, South Korea, 695-965. Fax:+82-64-782-8281, e-mail:leemri@jejunu.ac.kr
[2]Department of Marine Biology, Pukyong National University, Busan, South Korea
[3]Department of Marine Bio-resources, Gangwon Provincial College, Gangwon, South Korea

Introduction:

Photoperiod and temperature are potent environmental factors in regulating cyclic physiological and behavioural events in temperate fishes. It is not still unclear how fishes utilize environmental changes for their reproductive activity. The aim of the present study was to examine the involvement of photoperiod and temperature in ovarian development of the blacktip grouper *Epinephelus fasciatus*, which has been declined in Jeju Island, Korea, due to overfishing, and to commercial production of the species.

Methods:

Blacktip grouper broodstock was maintained at a 1:1 sex ratio in 500 L circle tanks. During March 2010, the photoperiod and water temperature was adjusted to 12L:12D and 22°C, respectively. In the treatment group, every 3 weeks daylight was increased as follows a 13L:11D and 14L:10D, and control group was maintained at 12L:12D. After 9 weeks water temperature was increased 25°C both treatment and control group. Every 3 weeks fish sampled, and gonads were removed, weighed and fixed for histological analysis.

Results and Discussion:

The GSI (0.5±0.25) and oocytes diameter (60.9±3.4 μm) of the female blacktip gouper under all photoperiod conditions (12L:12D, 13L:11D and 14L:10D) at water temperature of 22°C was maintained at low levels. However, GSI of the fish reared under photoperiod conditions of 14L:10D at water temperature of 25°C increased highly to 4.5±0.45 (Fig. 1A). Moreover, highly developed oocytes at the yolk stages were observed in these conditions (Fig. 1B). In the sevenband grouper, sexual maturation induced at photoperiod of 14L:10D and water temperature of 18.5°C conditions [1]. These results suggested that the long photoperiod of 14L:10D and high water temperature of over 25°C were essential environmental factors for the reproductive activity of the female blacktip grouper.

Conclusion:

This study shows that environmental factors play an important role on the gonadal maturation in female blacktip grouper. A short daylength (12L:12D and 13L:11D) and low temperature (under 25°C) inhibits the oocyte development. However, long daylength (14L:10D) and high water temperature (25°C) conditions strong influence to gonadal maturation of the blacktip grouper. Photoperiodic and temperature variations appear to both plays a crucial role in the control of the blacktip grouper reproduction.

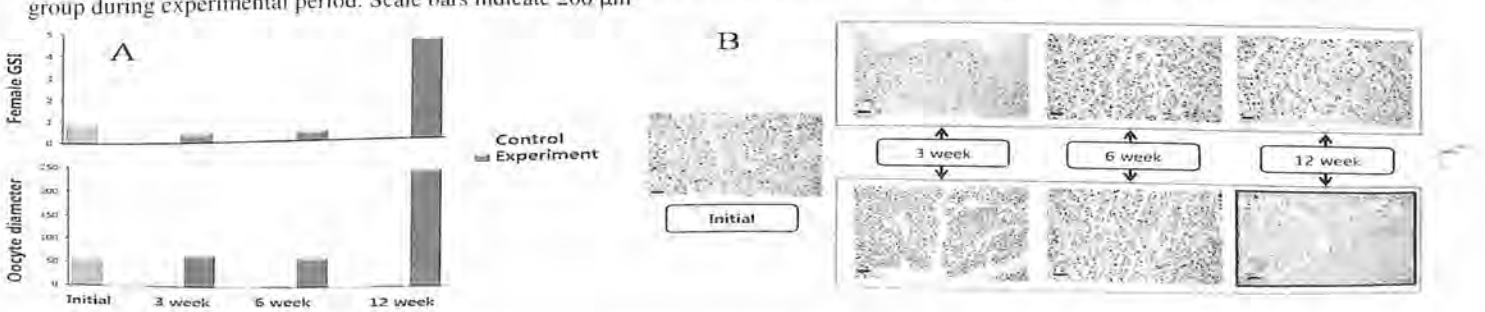
Acknowledgements:

This research was supported by development of the suitable systems for aquaculture of blacktip grouper, *Epinephelus fasciatus*, Ministry for Food, Agriculture, Forestry and Fisheries, Republic of Korea.

References:

[1]TSUCHIHASHI, Y., TAKATORI, Y., KURIYAMA, I., HANYUU, K., TSUJI, M. AND TSUMOTO, K. 2007. Induced spawning of cultured sevenband grouper, *Epinephelus septemfasciatus*, in September by manipulation of water temperature and photoperiod. Aquacul. Sci., 55: 395-402.

Fig. 1. Change of gonadosomatic index (GSI), oocyte diameter and ovarian development of female blacktip grouper in control and treatment group during experimental period. Scale bars indicate 200 μm



Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

EFFECT OF PHOTOPERIOD ON GONADOTROPIN (FSH AND LHβ) REGULATION IN THE DAMSELFISH, *CHROMIS NOTATA*

Yong-Ju Park (1), Gi-Su Song (1), Byeong-Hoon Kim(1), Seung-Hyeon Lee (1), Se-Jae Kim (2), Akihiro Takemura (3), Young-Don Lee (1)

[1]Marine and Environmental Research Institute, Jeju National University, 3288 Hamduk, Jocheon, Jeju Special Self-Governing Province 695-814, Republic of Korea,

[2]Department of Biology, Jeju National University, 66 Daehakno, Jeju Special Self-Governing Province 690-756, Republic of Korea,

[3]Department of Biology, Chemistry and Marine Sciences, Faculty of Science, University of the Ryukyu, Nishihara, Okinawa 903-0213, Japan.

Introduction:

Photoperiodic signals were important factors in regulating rhythmic physiological and behavioural events in teleosts. It is not fully understood how fishes utilize environmental changes in order to exert reproductive activity. The purpose of the present study is to investigate the involvement of photoperiod in gonadal development of the damselfish, *Chromis notata*, inhabiting in Jeju, Korea.

Methods:

The fish were divided into three groups and reared under three different artificial photoperiod regimes [short day photoperiod (LD 10:12), long day photoperiod (LD 14:10), and control group (LD 12:12)] for 60 days at natural water temperatures (range from 19 °C to 26 °C). The gonadosomatic index (GSI) was calculated for each fish. Pieces of the gonads were stained with hematoxylin-eosin for light microscopic observation. The pituitaries were dissected out from the fish brain for analysis of FSHβ and LHβ mRNA expression by using a real-time quantitative RT-PCR.

Results:

The fish rearing under LD 14:10 resulted in a significant increase in the GSI and induction of vitellogenic oocytes or spermatogenesis, whereas the gonads of fish reared under LD 10:14 and LD 12:12 conditions were occupied by the immature oocytes or spermatogonium. Similar tendencies showed in expression changes of FSHβ and LHβ mRNA levels under different photoperiod regimes.

Conclusion:

The GSI and histological observation indicate that LD 14:10 induces advancement of gonadal development. Furthermore similar expression patterns of FSHβ and LHβ mRNA under same photoperiod condition could be explained as a photoperiodic effect on the hypothalamus-hypophysis-gonad axis for the initiation and termination of reproductive activity in certain fish in the temperate region.

Gametogenesis - I (Spermatogenesis)

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

SPERMATOGONIAL STEM CELL BIOLOGY AND NICHE IN FISH

Luiz R. França[1], Samyra M.S.N. Lacerda[1], Rafael H. Nóbrega[1,2], GuilhermeJ.M. Costa[1], Jan Bogerd[2], Rüdiger W. Schulz[2]

[1]Federal University of Minas Gerais, Belo Horizonte, MG, Brazil. 31270-901. [2]Utrecht University, The Netherlands. e-mail: lrfranca@icb.ufmg.br

Fish are the most diverse and numerous group of vertebrates. However, our knowledge on spermatogenesis in fish is still incomplete and limited to a few species used in basic research and/or in aquaculture biotechnology (e.g. catfish, cod, eel, guppy, medaka, salmon, tilapia, trout, and zebrafish). Spermatogenesis is a developmental process during which a small number of diploid spermatogonial stem cells (SSCs) produce a large number of highly differentiated spermatozoa carrying a haploid, recombined genome. The survival and development of germ cells depends on their continuous and close contact to Sertoli cells that support, in a species-specific manner, a limited number of germ cells. Hence, Sertoli cell number determines the magnitude of sperm production for each fish species. Different from mammals, Sertoli cells in fish are capable of proliferating in adult life. Therefore, detailed knowledge about the mechanisms controlling Sertoli cell proliferation and function is crucial for male reproductive fitness. The number of spermatogonial generations is also important for sperm production. Thus, information on the number of mitotic divisions that spermatogonia undergo is essential for analyzing the regulatory mechanisms underlying spermatogonial fate. It seems that the number of spermatogonial generations is phylogenetically determined, particularly in relation to order level. The most undifferentiated spermatogonia, the SSCs, are considered to reside within a specific microenvironment in the testes called "niche", which regulates stem cell properties such as self-renewal, pluripotency, quiescence, their ability to differentiate and, as shown recently in mammals, this unique cell type is able to transdifferentiate in other cell types forming different organs.

Mainly because there are still no specific and reliable morphological or molecular markers for SSCs in vertebrates, spermatogonial transplantation is the only functional approach available to study SSC biology. First established in mammals, using laboratory rodents (rats and mice), syngeneic and xenogeneic germ cells transplantation techniques were established almost two decades ago (1994) by Brinster and colleagues. In fish, this technique was first utilized only one decade later by Yoshizaki and colleagues in Japan using immature fish (salmon and trout) and, more recently, by our research groups in Brazil and in The Netherlands using adult fish (tilapia and zebrafish). Because it allows the production of fertile sperm, germ cell transplantation using fresh, cryopreserved and *in vitro* cultured cells, opens new possibilities for biotechnological investigations, for example, with a high potential for improving the production of commercially valuable fish, generating transgenic animals, or preserving endangered species. However, for the achievement of these important goals, the better characterization and isolation of SSCs are crucial aspects that remain to be investigated. In this regard, we introduced recently the zebrafish (*Danio rerio*) as a new experimental laboratory model for the study of SSCs in vertebrates. Using 5'-bromo-2'-deoxyuridine (BrdU), we identified long term BrdU-retaining germ cells, named type A undifferentiated spermatogonia, as putative stem cells in zebrafish testes. Similar to rodents, these cells were preferentially located near the interstitium, suggesting that the SSC niche might be conserved across vertebrates. This localization was also confirmed by analyzing the topographical distribution of type A undifferentiated spermatogonia in normal, *vasa::egfp* and *fli::egfp* zebrafish testes. In the latter one, the topographical arrangement suggested that the vasculature is important for the SSCs niche, perhaps as a supplier of nutrients, oxygen and/or signaling molecules among other factors. Aiming at isolating and phenotypically characterizing SSCs in a commercially important fish species, we are also investigating potential molecular markers for SSCs in the Nile tilapia (*Oreochromis niloticus*), particularly those already well established in higher vertebrates. The results found in our ongoing studies suggest that GFRα-1, Notch-1, and Oct-4 are also potentially good markers for the SSCs in tilapias, opening for instance the possibility of using these cells as a mean of generating transgenic fish.

Financial support: CNPq, FAPEMIG, Norwegian Research Council, and Utrecht University.

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

SPERMATOZOA OF CHONDROSTEAN FISH SPECIES: STRUCTURE, MOTILITY AND FERTILZING ABILITY

Linhart, O[a], Alavi, S.M.H[a], Psenicka, M[a], Rodina, M[a], Kaspar, V[a], Li, P[a,b], Hulak, M[a], Boryshpolets, S[a], Dzyuba, B[a,c], Gela, D[a], Flajshans, M[a], Peknicova, J[d], Cosson, J[a,e], Ciereszko, A[f]

[a]University of South Bohemia, Faculty of Fisheries and Protection of Waters, Vodnany 389 25, Czech Republic. Fax +42-387774634 e-mail: linhart@vurh.jcu.cz

[b]Key Laboratory of Freshwater Biodiversity Conservation and Utilization, Ministry of Agriculture, Yangtze River Fisheries Research Institute, Chinese Academy of Fishery Science, Wuhan, Hubei, China

[c]Institute for Problems of Cryobiology and Cryomedicine of the National Academy of Sciences of Ukraine, Pereyaslavskaya str., 23, 61015 Kharkov, Ukraine

[d]Institute of Biotechnology AS CR, 142 20 Prague, Czech Republic

[e]Station Marine, CNRS, UMR 7009, 06230 Villefranche sur Mer, France

[f]Institute of Animal Reproduction and Food Research PAS, Tuwima 10, 10-747 Olsztyn, Poland

Sturgeons (Acipenseriformes: Acipenseridae) and paddlefishes (Acipenseriformes: Polyodontidae), the producers of caviar, are the remnant survivors of the once flourishing chondrosteans, dominant fishes of the Permian period. Although *Acipenseriform* fish first appeared in the fossil record approximately 200Mya, they have seemingly not undergone much morphological change since that time. So far, sturgeons have been an interesting object of multiple studies. However, the several aspects of their genetics as well as reproductive physiology remain debated. In this review, we examine the basic aspects of their gamete biology with special emphasis on sperm physiology, ultrastructure and fertilization process.

In general, the certain aspects of sturgeon's reproductive physiology are different from typical teleost fishes. Typically, their gametes differ from those of most fish in that, the sperm posse's acrosomes that undergo exocytosis and filament formation while the eggs possess numerous micropyles located at the animal pole. Basically, the main body of sperm cells, which includes the acrosome, head, and midpiece, is long, cylindrical and radially symmetrical, however an inter-species difference already exists. More specifically, the spermatozoon of majority of sturgeon species is composed of an elongated head with an acrosome and 8-10 posterolateral projections, a cylindrical midpiece with 2-8 mitochondria and two centrioles and a flagellum with the 9+2 structure of axoneme. Furthermore, from two to four endonuclear canals traverse the nucleus from the junction with the acrosome towards the implantation fossa are situated in front of the midpiece. Additionally, the plasma membrane is folded into one or two lateral fins along major part of the flagellum [1,2,3]. Moreover, an interesting feature of sturgeon spermatozoa is presence of an acrosome as well as subsequent acrosomal reaction (a reaction that causes development of a filament on the heads of sturgeon sperm which assists in penetration of the egg). The main function of the sperm acrosome in aquatic species is the lysis of the jelly coat layer, the outer-most layer surrounding the egg, but this does not appear to be the case in sturgeon. However, our experiments showed that the acrosome reaction includes the formation of a spear-like fertilization filament coming from three endonuclear canals and implantation fossa through the acrosome. Moreover, the presence of Ca^{2+} ions in highly alkaline activation media (pH 10) can induce acrosomal reaction as well. Furthermore, our experiments revealed that the acrosome plays two major functions: (a) the appearance of a long fertilization filament transmitting a signal to the egg that initiates the perivitelline space blocking to polyspermy and (b) the opening of posterolateral projections serving like an anchor against release from the micropyle [4].

Spermatozoa of sturgeons and paddlefish are essentially immotile in the seminal plasma due to its high concentrations of K^+ ions. Spermatozoa are immediately activated when they are transferred into swimming medium, usually freshwater or low salt concentration solutions. The all sperm motility parameters (frequency, velocity and wave amplitude) decrease rapidly during the period after activation and the percentage of motile cells also gradually decreases. During the earliest period of motility, spermatozoa of sturgeons and paddlefish move at velocities of 175–250 mms^{-1} and then the forward motility gradually reduces to between 50 and 100 mms^{-1} at 3–6 min after activation. Occasionally, some spermatozoa are motile for up to 9 min [5, 6].

Spermatozoa of chondrostean are haploid or diploid compared to functional diploidy or tetraploidy of each species as revealed by erythrocytes image cytometry and flow cytometry. However, we have also found fertile hexaploid male of Siberian sturgeon, *Acipenser baerii* (a functionally tetraploid species) releasing viable triploid spermatozoa. The subsequent experimental hybridization

with normal females of *A. baerii* demonstrated full fertility of this triploid male.

Acknowlegements:

Present study was financially supported by following grants: CZ.1.05/2.1.00/01.0024, MSM 6007665809, LC06073, IAA608030801, ME10015.

References:

[1]PSENICKA, M., RODINA, M., LINHART, O. 2010. Ultrastructural study on the fertilization process in sturgeon (Acipenser), function of acrosome and prevention of polyspermy. Anim. Reprod. Sci., 117: 147-54.

[2]HATEF, A., ALAVI, SM., NOVEIRI, SB., POORBAGHER, H., ALIPOUR, AR., POURKAZEMI, M., LINHART, O. 2011. Morphology and fine structure of Acipenser persicus (Acipenseridae, Chodrostei) spermatozoon: Inter-species comparison in Acipenseriformes. Anim. Reprod. Sci., 123:81-88.

[3]WEI, Q., LI, P., PSENICKA, M., ALAVI, SM, SHEN, L., LIU, J., PEKNICOVA, J., LINHART, O. 2007. Ultrastructure and morphology of spermatozoa in Chinese sturgeon (Acipenser sinensis Gray 1835) using scanning and transmission electron microscopy. Theriogenology, 67:1269-1278

[4]PSENICKA, M., VANCOVA, M., KOUBEK, P., TESITEL, J., LINHART, O. 2009. Fine structure and morphology of sterlet (*Acipenser ruthenus*, L. 1758) spermatozoa and acrosin localization. Anim. Reprod. Sci., 111: 3-16.

[5]LINHART, O., COSSON, J., MIMS, SD., SHELTON, WL., RODINA, M. 2002. Effects of ions on the motility of fresh and demembranated paddlefish (*Polyodon spathula*) spermatozoa. Reproduction,124: 713-719.

[6]COSSON, J. 2010. Frenetic activation of fish spermatozoa flagella entails short-term motility, portending their precocious decadence. J. Fish. Biol. 76: 240-279.

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

DOES LEPTIN PLAY A ROLE IN EARLY SEXUAL MATURATION IN MALE ATLANTIC SALMON (*SALMO SALAR* L.)?

Trombley S.M.*, Maugars G.°, Kling P.°, Björnsson B. T.° and Schmitz M.°

*Evolutionary Biology Center, Department of organismal biology/Comparative Physiology, Norbyvägen 18A, SE-752 36 Uppsala, Sweden.
Fax: +46184716425 e-mail: susanne.trombley@ebc.uu.se
°Muséum National d'Histoire Naturelle, UMR BOREA UPMC/CNRS 7208, Paris, France
°Department of Zoology/Zoophysiology, University of Gothenburg, Box 463, SE-405 30,Gothenburg, Sweden

Introduction:

Leptin has been established as a signal conveying the energy status of an individual to the reproductive axis affecting puberty onset and fertility in mammals, but the possible role of leptin in reproduction in teleost fish remains largely unexplored. In Atlantic salmon, as in most teleost fish, leptin is mainly synthesized by the liver. The lack of correlation between adiposity stores and leptin plasma levels, suggests that leptin might have a different function in teleost fish during the attainment of reproductive function than that seen in mammals where plasma leptin levels are directly proportional to the amount of adipose tissue. In this study we investigated the impact of food restriction on the prevalence of early maturing males and the pattern of hepatic leptin gene expression, brain leptin receptor gene expression and circulating plasma levels during early sexual maturation in Atlantic salmon parr.

Methods:

One-year old Atlantic salmon parr was given different feeding regimes (full or restricted feeding) during spring and summer and fish were sampled monthly. The temporal gene expression profile as well as the tissue distribution of the two leptin paralogues (lepa1 and lepa2) and the leptin receptor (lepr) was assessed using quantitative real-time PCR during the period of early puberty onset and advancement to detect possible changes in expression due to season, restricted feeding and developmental stage. Plasma leptin levels were measured by a homologous salmonid radioimmunoassay.

Results and Discussion:

Restricted feeding significantly decreased the incidence of early sexual maturation. High mRNA levels of both lepa1 and lepa2 were found in the liver only, but low or very low lepa1 gene expression was found in a few other tissues such as pituitary, gills and heart. Lepr was expressed in all tissues investigated and a differential expression pattern was observed in the testis depending on the reproductive stage. Maturation and restricted feeding increased lepa1 mRNA expression as well as plasma leptin levels, although the change in plasma leptin was not as pronounced. Increased lepa1 gene expression in maturing males could be seen only at later stages during spermatogenesis and not during onset. Lepa2 transcript levels were highest in early spring and declined during early summer in the full fed group. By mid-June, lepa2 levels were very low in both maturing and immature fish, but an up-regulation in maturing males occurred later in the season. A decline in lepa2 transcript levels were also seen in the restricted group but levels started to increase again by June and both immature and maturing fish had at this time higher levels compared to the control. Lepr in the brain was slightly but significantly up-regulated in full fed immature males by early summer, while remaining at a steady expression level in the maturing fish.

No correlation could be found between adiposity and leptin plasma concentration or hepatic lepa1 and lepa2 gene expression at any sampling occasion. Correlation between the two leptin paralogues was found at some, but not all, sampling dates, which indicate that the two genes are differentially regulated.

Conclusion:

Our results show that both lepa1 and lepa2 transcription and plasma leptin levels were increased by restricted feeding. Leptin is therefore unlikely to act as an adiposity signal to the reproductive axis in Atlantic salmon parr. The leptin system seems not to play a role in triggering, or acting as a permissive signal, for the onset of early sexual maturation in male Atlantic salmon, but may rather play a role during energy reallocation in later stages of spermatogenesis.

elevation

Sex steroid ⇒ Leptin ?

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

INTRA-TESTICULAR NITRIC OXIDE REGULATES STEROIDOGENESIS IN FISH

Lal B. and Dubey N.

Department of Zoology, Banaras Hindu University, Varanasi- 221 005, India.
lalbhu@yahoo.co.in

The entire reproductive events of fishes are primarily controlled by hormones secreted from hypothalmo-hypophysial-gonadal axis. Varieties of neuro-peptides and neuro-secretions from the hypothalamus stimulate pituitary to secrete tropic hormones, gonadotropin (GtHs) in particular, which induce the production of sex steroids like estrogens and progestogens in female and androgens in male, regulating ultimately the various reproductive events. In past decade, researches in mammals, however, have provided enough evidences that some factors, produced locally in gonads, influence the reproductive activity greatly. These local factors not only control reproduction directly, but also modulate the action of endocrine hormones delivered to gonads from the hypothalmo-hypophysial axis. Such factors include cytokines, growth factors, regulatory peptides, reactive oxygen and nitrogen species. These factors are synthesized and released by a variety of testicular and ovarian cells; such as Leydig, Sertoli, peritubular cells, macrophages, granulosa cell, thecal cells and germ cells, influencing steroidogenesis, follicular development, germ cell maturation, ovulation and many other reproductive processes. One of such intra-gonadal factor is nitric oxide (NO). NO, a highly versatile gaseous signaling molecule is reported to regulate diverse physiological functions such as secretion of hormones, immune response, reproductive activity, etc. It is produced from L-arginine by catalytic actions of one of the three isoforms of nitric oxide synthase (NOS): neuronal (nNOS), endothelial (eNOS) and inducible (iNOS) in a variety of tissues. NO has been recognized as an important inter- and intra-cellular modulator of testicular steroidogenesis in higher vertebrates with conflicting results.

Moreover, its role in regulation of testicular steroidogenesis in ectothermic vertebrates is non-existent. Understanding the role of NO in Leydig cell steroidogenesis in teleost is highly essential, as the NO is implicated in stress-response and fishes are often subjected to variety of environmental and chemical stressors leading to reproductive dysfunction and gonadal steroidogenesis. Therefore, attempts were made to examine whether testis, particularly Leydig cells and testicular macrophages of a fresh water catfish, *Clarias batrachus* produce NO, if so, does it regulate its steroidogenesis activity? Are Leydig cells sensitive to exogenous/paracrine NO? What are the probable endocrine factors that regulate testicular NO production? Series of *in vitro* experiments performed suggest that fish testis is equipped with NOS system and is capable of producing NO and its production is under endocrine inhibitory control. Cellular sources of NO in the testis of the catfish are numerous like germ cell, Leydig cell as well as macrophages. Expression of NOS exhibits seasonality and depends of reproductive status of fish. Leydig cells are also highly sensitive to chemical as well as biological NO (released by macrophages). NO inhibits testosterone production by Leydig cells *in vitro*.

SPERMATOGENESIS IN FISH UNDER THE INFLUENCE OF VARIOUS FACTORS WITH SPECIAL REFENCE TO INDIAN MAJOR CARPS

Routray, P.*,Verma, D. K.*, Sahu, A. D.*, Purohit, G.K.* and Sundaray, J.K.†

*Aquaculture Production and Environment Division, Central Institute of Freshwater Aquaculture, (I.C.A.R), Kausalyaganga, Bhubaneswar-751002, India.
†Central Institute of Brackishwater Aquaculture, Kakdwip Centre, West Bengal, India.
E-mail: routray30@yahoo.co.in

Introduction:

Spermatogenesis is a dynamic process that follows a definite pattern in nature and ultimately produces haploid spermatozoa.The cellular basis of reproduction in males is the spermatozoon. They develop from testicular germinal epithelium [3] (spermatogonial stem cells), which first proliferate (spermatogonia), then differentiate into spermatocytes that undergo meiosis to become haploid spermatids that differentiate to become spermatozoa (spermiogenesis). The process as a whole is called spermatogenesis and requires a special environment. The gonadal function changes during annual reproductive cycles in male fish. During proliferation of diploid germ cells, the spermatogonia repopulate the testis lobules by mitotic cell division. Though spermatogenesis is a natural dynamic process, it has been modulated and manipulated by various factors to get maximum viable sperms for aquaculture practice. Environment and chemicals of natural and synthetic origin can interact with the endocrine system and alter the male germ cell generation and proliferation/inhibition in fish. In the present investigative review, an attempt has been made to ascertainvarious factors that influence the spermatogonial proliferation and maturation of fish with a special reference to Indian major carps (*Catla catla, Labeo rohita, Cirrhinus mrigala and Labeo calbasu).*

Methods:

Various maturity stages of fish (Indian major carps) were sampled to estimate their testis somatic index (TSI) at different times of the year. The immature fish were subjected to several external factors (environment) and some chemical inducers such as hormones and other inducing agents to estimate the semen quantity and quality and assess the spermatogonial proliferation due to these and also the regulation of spermatogenesis annually. A comparative evaluation of spermatogenic cycles with other species has also been done.

Results and Discussion:

Fish species inhabiting diverse ecological niche offers an enormous challenge to generalize their reproductive characters that includes theirsexual dimorphism, spermatogenic cycle (*in vivo and in vitro*), spermatozoon structure and the external inducers that modulate the spermatogenesis in fish with particular reference to Indian major carps (IMCs).The testes in

Table 1. A comparative account of gonadal (spermatogenic cycle) in different fishes

Species	Spermatogenesis	Inducement	Reference
Catfish, *H. fossilis*	Annual gonadal cycle	Natural cycle	[9]
Rainbow trout, *S. gairdneri*	Twice in year (Autumn and summer)	Natural cycle	[1]
Rainbow trout, *S. gairdneri*	Advanced maturation and spawning by 6-12 weeks at 9°C	Photo period manipulation	[12]
Tilapia	Testicular activity restricted to autumn and winter, and testes are in resting phase from July-September in equatorial zone	Natural photoperiod	[4]
Indian major carps	Increasing gonadal recrudescence; males attain maturity earlier than females	Photo period manipulation	[10]
Indian major carps	Advancement of sexual maturation and off-season spawning of IMCs	Photothermal manipulation	[7]
Zebra fish *Danio rerio*	Combined duration of meiotic and spermiogenic phases is very short in this species and lasts approximately 6 days	Natural cycle	[5]
C. fasciata	Spermatogenicphase 6 days	Natural cycle	[8]
Black molly, *P. sphenops*	Spermatogenic phase 21days	Natural cycle	[2]
IMCs & L.calbasu	Spermatogenic phase 15-21 days	GnRH based inducement	Present study

most teleost reported including that of the IMCs are bilobed, with spermatogonia dispersed throughout the gametogenic epithelium of the seminiferous tubules.The sexual dimorphism is clear in species during the reproductive season (perennial spawners in nature) however, some are very inconspicuous during non-spawning season (IMCs) [11, 6]. The spermatogenic cycle varies among the fish species (Table 1) and also the sperm density (40 to 53,000 millions/ml). With the advancements in reproductive biology of fishes, it is now possible that spermatogenesis can be modulated and manipulated by various factors to get maximum viable sperms for aquaculture practice.

References:

[1]BILLARD, R., BRETON,B. 1978. Rhythms of reproduction in teleost fish. In Rhythmic activity of fishes, edited by J.E. Thorpe. London, Academic Press, pp. 31-53.

[2]FELICE, DE DA., RASH E. M. 1969. Chronology of spermatogenesis and spermiogenesis in poeciliid fishes. J. Exp. Zool., 171:191–208.

[3]GRIER, H. J., TAYLOR, R. G. 1998. Testicular maturation and regression in the common snook. J. Fish Biol., 53:512-542.

[4]HYDER, M., 1970 Gonadal and reproductive patterns in *Tilapia leucosticta* (Teleostei: Cichlidae) in an equatorial lake. Lake Naivasha, Kenya. J. Zool., 162:179-95

[5]MARCELO, C., LEAL, EDSON R., CARDOSO, RAFAEL H. NO´BREGA., SERGIO, R., BATLOUNI, JAN BOGERD., LUIZ R. FRANC., SCHULZ,RU¨DIGER, W. 2009. Histological and Stereological Evaluation of Zebrafish (*Danio rerio*) spermatogenesis with an emphasis on spermatogonial generations. Biol. Reprod., 81: 177–187

[6]ROUTRAY, P., VERMA, D. K., SARKAR, S. K., SARANGI, N. 2009. Recent advances in carp seed production and milt cryopreservation. Fish Physiol. Biochem., 33: 413-427.

[7]SARKAR, S. K.,SAHA, A.,DASGUPTA, S., NANDI, S.,VERMA, D. K.,ROUTRAY, P., DEVARAJ, C.,MOHANTY, J.,SARANGI, N., EKNATH, A. E., AYYAPPAN, S. 2010.Photothermal manipulation of reproduction in Indian major carp: a step forward for off-season breeding and seed production. Curr. Sci., 99: 960-964.

[8]SINHA, G. M., MONDAL, S.K., MIDYA,T., GHOSAL, S.K. 1979. Chronology of meiosis and spermiogenesis in a fresh-water teleost fish, *Colisa fasciata* (Bloch and Schneider). Z. Mikrosk.- Anat. Forsch., 9z3:442–448.

[9]SUNDARARAJ, B.I., VASAL, S. 1976. Photoperiod and temperature control in the regulation of reproduction in the female catfish, *Heteropneustes fossilis*. J. Fish Res. Board Can., 33: 959-73.

[10]VERGHESE, P. 1975. Internal rhythm of sexual cycle in a carp *Cirrhinusreba* (Ham.) under artificial conditions of darkness. J. Inland Fish. Soc. India, 7: 182-8.

[11]VERMA, D. K., ROUTRAY, P., NANDA, P.K., SARANGI, N. 2009.Seasonal variation in semen characteristics and biochemical composition of seminal plasma of mrigal, *Cirrhinus mrigala* (Ham.).Asian Fisher. Sci., 22: 429-443.

[12]WHITEHEAD, C., BROMAGE, N. R., FORSTER, J. R. M., MATTY, A. J. 1978.The effects of alterations in photoperiod on ovarian developmental and spawning time in the rainbow trout (*Salmo gairdneri*). Ann. Biol. Anim. Biochim. Biophys., 18:1035-43.

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

RELAXIN-3 AND RELAXIN/INSULIN-LIKE FAMILY PEPTIDE RECEPTOR 3 IN RAINBOW TROUT: SITES OF GENE EXPRESSION AND CHANGES IN MESSENGER RNA LEVELS DURING SPERMATOGENESIS IN TESTES.

Kusakabe M.[1], Takei Y.[1], Luckenbach J.A.[2]

[1]Atmosphere and Ocean Research Institute, The University of Tokyo, Kashiwa, Japan.
[2]Northwest Fisheries Science Center, NOAA Fisheries, Seattle, WA, USA.

Background:

Relaxin is thought primarily to be a hormone that regulates pregnancy and reproduction in mammals. Recent studies have demonstrated the presence of six members of the relaxin family: Relaxin-1/2, -3, and insulin-like peptides-3, -4, -5 and -6. Relaxin-3 has recently been identified as a novel member of the insulin/relaxin family from human genomic databases [1]. In teleosts, genomic database searches revealed the existence of multiple relaxin-3-like genes [2, 3]. Relaxin-3 genes have been identified in the brain of eel and zebrafish [4, 5, 6], and were recently reported in the ovary of zebrafish and coho salmon [7, 8], suggesting that relaxin-3 like genes may play a role in oogenesis. However, little attention has been paid to the functions of relaxin-3 for male reproductive systems in teleosts. In order to explore the possibility of testicular functions of relaxin-3, we isolated cDNAs encoding relaxin-3 and relaxin/insulin-like family peptide receptor 3 from rainbow trout (*Oncorhynchus mykiss*). Sites of gene expression and seasonal changes in testicular relaxin-3 transcripts were investigated.

Methods:

Complimentary DNAs encoding rainbow trout relaxin-3 (rtRLN3) and relaxin/insulin-like family peptide receptor 3 (rtRXFP3) were identified from the brain. Sites of rtRLN3 and rtRXFP3 gene expression were examined by RT-PCR and *in situ* hybridization. Changes in levels of rtRLN3 and rtRXFP3 mRNA in testes were determined by quantitative real-time PCR throughout the reproductive cycle of male rainbow trout.

Results: Rainbow trout RLN3 transcripts were abundant in brain and found at lower levels in gonads. *In situ* hybridization analysis showed that the rtRLN3 transcripts were localized in the middle-posterior region of the brain, though the rtRLN3 transcripts were undetectable in the testes. The quantitative real-time PCR analysis showed that the levels of rtRLN3 transcripts were low during early spermatogenesis (January to February) and significantly increased 20-40 fold ($p<0.001$) during mid-spermatogenesis (March to May) just prior to significant increases in plasma 11-ketotestosterone [9]. The transcript levels then decreased in June prior to spawning ($p=0.0002$) and were maintained at low levels through the post-spawning phase (November). Rainbow trout RXFP3 transcripts were abundant in brain and immature gonads. The levels of rtRXFP3 transcripts were significantly elevated during early spermatogenesis in January ($p=0.0007$) and the post-spawning phase in November ($p=0.0049$). However, transcript levels were maintained at low levels through the spermatogenesis phase (February to October).

Conclusion:

Identification of the sites of rtRLN3 and rtRXFP3 gene expression provides a foundation for exploring the function of relaxin-3 in teleosts. Although the brain showed the highest transcript levels of rtRLN3 and rtRXFP3, the functions of relaxin-3 in brain still remain unclear. The seasonal changes in rtRLN3 transcripts in testes demonstrated that rtRLN3 gene expression was elevated prior to the significant increase of plasma 11-ketotestosterone [9]. Thus, the relaxin system may play a role in regulation of sex steroid synthesis during the early phase of gametogenesis [7]. However, there was no apparent correlation between the pattern of rtRLN3 and rtRXFP3 gene expression. Further experiments will be necessary to determine the precise functions of the relaxin system in male teleosts.

References:

[1]BATHGATE R.A., SAMUEL C.S., BURAZIN T.C., LAYFIELD S., CLAASZ A.A., REYTOMAS I.G., DAWSON N.F., ZHAO C., BOND C., SUMMERS R.J., PARRY L.J., WADE J.D., TREGEAR G.W. 2002. Human relaxin gene 3 (H3) and the equivalent mouse relaxin (M3) gene. Novel members of the relaxin peptide family. J. Biol. Chem., 277:1148–1157.

[2]WILKINSON T.N., BATHGATE R.A.D. 2007. The evolution of the relaxin peptide family and their receptors. Adv. Exp. Med. Biol., 612:1-13.

[3]WILKINSON T.N., SPEED T.P., TREGEAR G.W., BATHGATE R.A.D. 2005. Evolution of the relaxin-like peptide family. BMC Evol. Biol., 5:14-31.

[4]HU G., KUSAKABE M., TAKEIY. 2011. Localization of diversified relaxin gene transcripts in the brain of eels. Gen. Comp. Endocrinol., in press.

[5]DONIZETTI A., GROSSI M., PARIANTE P., D'ANIELLO E., IZZO G., MINUCCI S., ANIELLO F. 2008. Two neuron clusters in the stem of

postembryonic zebrafish brain specifically express relaxin-3 gene: first evidence of nucleus incertus in fish. Dev. Dynam., 237:3864-3869.

[6]DONIZETTI A., FIENGO M., MINUCCI S., ANIELLO F. 2009. Duplicated zebrafish relaxin-3 shows a different expression pattern from that of the co-orthologue gene, Dev. Growth Differ., 51:715-722.

[7]WILSON B.C., BURNETT D., RAPPAPORT R., PARRY L.J., FLETCHER E.K. 2009. Relaxin-3 and RXFP3 expression, and steroidogenic actions in the ovary of teleost fish. Comp. Biochem. Physiol., A 153:69-74.

[8]LUCKENBACH J.A., GOETZ F.W., SWANSON P. 2008. Expressed sequence tags (ESTs) of follicle/interstitial cell enriched ovarian tissue from previtellogenic coho salmon. Cybium, 32 *suppl*:141-144.

[9]KUSAKABE M, NAKAMURA I, EVANS J, SWANSON P, YOUNG G. 2006. Changes in mRNAs encoding steroidogenic acute regulatory protein, steroidogenic enzymes and receptors for gonadotropins during spermatogenesis in rainbow trout testes. J. Endocrinol., 189:541-554

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

PATHWAYS USED BY ANDROGENS OR FSH TO REGULATE TESTIS MATURATION

Sambroni E.[1] Rolland A.[2] Goupil AS.[1] Lareyre J.J.[1] Le Gac F.[1],

[1]INRA UR 1037, SCRIBE, [2]INSERM U625, 35042 Campus de Beaulieu, Rennes, France
Fax : +33223485020 email : florence.legac@rennes.inra.fr

Introduction:

In fish, gonadotropin hormones and sex steroids are known to trigger the initiation of testicular puberty and to promote final maturation of the gametes. In a series of studies, the molecular mechanisms involved were investigated through identifying androgen and FSH/LH modulated gene expression in the trout testis.

Methods:

A search for androgen dependent genes was initiated in pre-pubertal rainbow trout treated with physiological doses of androgens. Groups of seven to ten immature male trout were treated with testosterone (T) or 11-ketotestosterone (11KT) by implantation for 7-14 days. The treatment induced large increases in androgen plasma levels but these remained within the physiological range for adult male trout. FSH-regulated genes were also identified from trout testicular explants in early stages of development (I-II: spermatogonia only, III: initiation of meiosis) cultured in the presence of salmonid FSH at 500ng/ml. RNA was extracted from all testicular samples. In transcriptome studies using trout cDNA chips, the transcript accumulation for 9000 probes, corresponding to ~6000 non-redundant genes was measured. A limited number of candidate genes were also confirmed by *in situ* hybridisation and qPCR.

Results and discussion:

1) Up or down regulations of specific mRNA levels were observed after the androgen treatments or in response to LH /FSH. Early changes appeared to affect gene expression mainly in the somatic compartment, where receptors for these hormones have been described in fish. For many of these genes, significant developmental expression patterns during the annual maturation of the trout testis were observed. Some changes also corresponded well with the natural increases in circulating hormone levels during the reproductive cycle, reinforcing the idea that the hormone induced changes that we describe are *physiologically* relevant.

2) Conversely to mammals, FSH and LH had similar effects on one third of gonadotropin regulated transcripts. These genes are candidate targets for FSH physiological action during the pubertal transition since only FSH is detected at these stages of testis development. Interestingly, we also found that about 80 candidates appeared regulated exclusively or much more efficiently by FSH (rather than by LH) which should help to clarify the respective roles of the 2 gonadotropins in fish.

3) Data mining of the candidates revealed that hormones affected somatic genes involved in germ cell sustaining proliferative activity or evading growth suppressors, or differentiation.

- In prepubertal testis FSH and/or androgens affected the expression of several transcripts coding for paracrine factors of somatic origin, like GSDF, AMH, inhibin, ,Follistatin, BMP7, which are all potentially involved in germ cell proliferation or differentiation
- In addition to modifying gene expression in supporting somatic cells, androgen treatment resulted in a shift in germ cell gene expression profiles, with a decrease in several genes preferentially expressed in spermatogonia (like *noc2l*) and an increase in transcripts with a meiotic/post meiotic profile (like *morn3, rsph3, bty*). This reinforces the involvement of androgens in the transition from spermatogonia towards more differentiated germ cells expression profiles.
- Two transcripts encoding **G2/mitotic-specific cyclins** were up regulated by FSH/LH; one, annotated ccnb3 is known as involved in early meiosis in mouse; the second one, annotated *ccnb1* and expressed in late germ cells responded better to FSH, with a stronger effect in stage III (in contrast the G1/S-specific cyclin-E1 was found down regulated by the gonadotropins).
- Gonadotropins up regulated some germinal or somatic genes encoding intracellular factors known to take part, directly or indirectly, in germ cell fate, for example *nanos3* (FSH and LH), *dmrt1* (FSH and LH) or *sox8* (LH) and also affected genes encoding factors involved in other developmental processes like apoptosis (*casp8, faf1* and *clu1*) or testicular fluid homeostasis (iron/ion transporters *slc30a1, fth1*).
- FSH regulated several transcripts encoding proteins involved in microtubule and cytoskeleton rearrangement (calponin2 *cnn2*, desmin *des*, cytokeratins *krt18, krt8*, microtubule-associated protein *mapre1*) and proteins of the extracellular matrix (collagen *col1a1, col1a2*, proteases *mmp19, mmp9* and protease inhibitors, *timp2*) important for cell adhesion and migration and cell junctions.

4) The steroidogenic pathway was of course affected by the hormone treatments: the gonadotropins were generally stimulatory (*star, hsd3b1*), while *in vivo* treatment with high levels of androgens were inhibitory

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

(indirectly, through an FSH inhibition, or directly, via a short loop feed-back on steroidogenic enzymes).

5) Furthermore, a specific group of the regulated transcripts probably take part in the morpho-functional changes in the testis and sperm duct during spermiation in trout. We found that sex steroids possibly control these genes involved in the regulation of water exchanges (*aqp1, aqp4, vtl*) and in proton and potassium regulation in seminiferous tubules (*cahz, vtl, slc26a4, atp1b1, lgil*), and are therefore relevant concerning sperm maturation and excretion.

Conclusions:

We provide meaningful information on a large set of transcripts implicated in the testicular somatic cell response to gonadotropin and/or androgens. These include growth factors, extra-cellular matrix components and intracellular pathways which are potentially involved in the mechanisms by which reproductive hormones direct germ cell renewal or differentiation at puberty, then sperm maturation and excretion at spawning time in adult fish.

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

USE OF SOMATIC GENE TRANSFER FOR STUDYING GONADOTROPIN ACTIONS ON SPERMATOGENESIS IN EUROPEAN SEA BASS (*DICENTRARCHUS LABRAX*).

Mazón M.J., Zanuy S., Carrillo M., Gómez A.

Department of Fish Physiology and Biotechnology, Instituto de Acuicultura de Torre la Sal (IATS), Consejo Superior de Investigaciones Científicas (CSIC), Ribera de Cabanes 12595, Castellón, Spain. e-mail: ana@iats.csic.es. Fax: +34 964319509

Introduction:

The follicle-stimulating hormone (FSH) and the luteinizing hormone (LH) play central roles in vertebrate reproduction. These gonadotropins act by binding and activating specific receptors (FSHR and LHR) located in certain cell types of the fish gonads, and their action begins a signalling cascade controlling different steps of gametogenesis and steroidogenesis. Although the structural duality of these hormones is well defined, their individual roles are not clear in most fish species. Furthermore, promiscuous hormone-receptor interactions have been found in some species complicating our understanding of fish gonadotropin functions.

The availability and use of recombinant gonadotropins offers a unique tool for studying gonadotropin actions in a given species. One step further is the direct administration of the gonadotropin coding genes to the fish. This technique known as somatic gene transfer comprises a number of different approaches that aim to introduce a gene into an adult tissue. A broad range of delivery systems has been tested in mammals, where the application of this technique is mainly focused on gene therapy [1]. In fish, its development and improvement has been mainly directed to DNA vaccination. However, its application as a method for *in vivo* delivery of gene products into fish blood has hardly been explored [2].

We have developed a series of tools to study and control gonadotropin actions in the European sea bass. This species is a good model due to the already existing knowledge on its reproductive patterns and the availability of other tools for physiological studies. In addition, it is one of the most cultivated marine teleosts in the Mediterranean area.

Methods:

Animals: Mature adult male sea bass (3 years old) and prepubertal (1 year old) male sea bass. *Plasmids*: pCMVtk-Lacz and pcDNA3 (Invitrogen) were used as control plasmids. The gonadotropin expression plasmids, pcDNA3-scLH and pcDNA3-scFSH, contained sea bass single-chain (sc) gonadotropin cDNAs [3]. *Injections*: Intramuscular injection of the different plasmids was performed with an insulin syringe rostro-ventral to the dorsal fin. Uptake of the DNA by the muscle cells was potentiated by electroporation pulses. *Hormonal analysis*: Blood samples were taken through the study to evaluate FSH, LH [4] and 11-ketotestosterone [5] levels. *Histology*: Gonad sections were stained and testis developmental stages were classified according to [6]. BrdU incorporation was used to evaluate cell proliferation. *Sperm assessment*: Weekly samples of sperm were taken from the adult males to evaluate sperm volume and density. Gene *expression analysis*: Gonad samples from the injected animals taken at different intervals were used for RNA extraction. Variations in mRNA levels for different sea bass genes were measured by qPCR using Taqman chemistry and a specific standard curve for each gene.

Results and Discussion:

The availability of the genes coding for the sea bass gonadotropin subunits has enabled us to generate appropriate expression DNA constructs and recombinant gonadotropins in different eukaryotic expression systems [3], including the production of single-chain gonadotropins. All these recombinant hormones are functionally active, but their *in vivo* stability showed remarkable differences depending on the system used. Thus, somatic gene transfer has been evaluated as an alternative method for *in vivo* delivery of gonadotropins into fish blood. Intramuscular injections of the expression plasmids coding for a single-chain LH or FSH in sea bass produced a significant increase of the corresponding gonadotropins in the plasma. The effect of plasmid injection on gonadotropin plasma levels lasted longer than direct delivery of the recombinant hormone.

To test the functionality of the plasmid-derived gonadotropins, mature adult male sea bass were injected with the plasmid pcDNA3-scLH and we observed that the derived scLH was able to increase the production of sperm in the injected animals. On the other hand, immature yearling male sea bass were injected with pcDNA-scFSH to evaluate the functional effects of scFSH. The produced recombinant hormone was able to induce spermatogenesis in immature animals, as evaluated by histological and cell proliferation analysis. Besides, the expression of some germ cell markers, such as *scp3* or *piwi*-like, or potential target genes, such as the *LHR* was also assessed.

As a next step sequential injections of pcDNA-scFSH and/or pcDNA-scLH were performed in immature

yearling males to analyze the different and synergic actions of these hormones on spermatogenesis. After histological examination and qPCR expression analysis we can conclude that FSH but not LH is able to trigger spermatogenesis in sea bass, while LH potentiates the actions of FSH allowing a further progression of the spermatogenic process.

Conclusion:

The injection of the gonadotropin subunit genes into sea bass muscle produced functional hormones and has been used for basic studies on sea bass, revealing the differential actions of FSH and LH in spermatogenesis. Therefore, this approach represents a powerful tool for basic studies on gene function and a promising procedure for biotechnological applications.

Research supported by MICINN (AGL2008-02937), GV (ACOMP/2010/083 and PROMETEO/2010/003) and EU (LIFECYCLE, FP7-222719-1). MJ.Mazónreceived a FPI fellowship from the Spanish MICINN.

References:

[1]DUAN D (Ed.) 2010 Muscle Gene Therapy. 1st. Edition. Springer.

[2]LORENZEN, N., CUPIT, P.M., EINER-JENSEN, K., LORENZEN, E., AHRENS, P., SECOMBES, C.J., CUNNINGHAM, C. 2000. Immunoprophylaxis in fish by injection of mouse antibody genes. Nat. Biotechnol., 18: 1177-1180

[3]MOLES, G., ZANUY, S., MUÑOZ, I., CRESPO, B., MARTINEZ, I., MAÑANOS, E., GOMEZ, A. 2011. Receptor Specificity and Functional Comparison of Recombinant Sea Bass (*Dicentrarchus labrax*) Gonadotropins (Fsh and Lh) Produced in Different Host Systems. Biol. Reprod., 84: 1171–1181

[4]MATEOS, J., MAÑANOS, E., SWANSON, P., CARRILLO, M., ZANUY, S. 2006. Purification of luteinizing hormone (LH) in the sea bass (*Dicentrarchus labrax*) and development of *a specific immunoassa*y. Cien. Mar., 32: 271-283.

[5]RODRÍGUEZ, L., BEGTASHI, I., ZANUY, S., CARRILLO, M. 2005. Long-term exposure to continuous light inhibits precocity in European male sea bass (*Dicentrarchus labrax*): hormonal aspects. Gen. Comp. Endocrinol. 140: 116–125.

[6]BEGTASHI, I., RODRÍGUEZ, L., MOLES, G., ZANUY, S., CARRILLO, M. 2004. Long-term exposure to continuous light inhibits precocity in juvenile male European sea bass (*Dicentrarchus labrax*). I. Morphological aspects. Aquaculture, 241:539-559

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

THE EFFECTS OF STOCKING METHOD ON THE GONAD DEVELOPMENT OF ANDROGEN TREATED FALSE CLOWNFISH, *AMPHIPRION OCELLARIS*

Abduh, M.Y.[1], Abol-Munafi, A.B.[1], Ambak, M.A.[2], Norazmi-Lokman, N.H.[2]

[1]*Institute of Tropical Aquaculture, Universiti Malaysia Terengganu, 21030 Kuala Terengganu, Malaysia.*
[2]*Faculty of Agrotechnology and Food Science, Universiti Malaysia Terengganu, 21030 Kuala Terengganu, Malaysia.*
email: abduhyazed@yahoo.com

Introduction:

The false clownfish (*Amphiprion ocellaris*) is the most commonly kept clownfish in aquaria. *A. ocellaris* is protandrous hemaphrodite, meaning that all individuals develop first into males and then possibly into females later in life with socially structured reproductive behavior [1]. The demand for *A. ocellaris* as ornamental fish has increased but due to deterioration of its natural habitat and the effects of over-fishing, their numbers have drastically decreased. To replenish its natural population, attempts are underway to raise this popular anemonefish in artificial conditions for mass production. One of the current methods for the mass production of clownfish is to directly produce male and female broodstock using hormone treatment. Among the reproductive steroids, 17α -methyltestosterone is the most commonly used androgen to masculinize fishes and it has been tested in more than 25 species [5]. Several methods of steroid administration are available but immersion and oral treatments are preferred due to their efficiency [2]. Therefore, this study was conducted to study the effect of stocking methods (pairing and non-pairing) on the hormone treated fish.

Methods:

Twenty false clownfish juveniles (*A. ocellaris*) (3.5cm-5.5cm total length) were immersed in 1ppm of 17α-methyltestosterone (MT) for 15 days and then kept for 2 months. At the end of the treatmant, 10 of them were separated into 10 different aquariums while the rest were paired and put into 5 aquaria. Sampling was done twice, 1 and 2 months post hormone treatment for gonad histology where 3 fish and 2 pairs were taken from the first and second group, respectively.

Results and Discussion:

At the end of the 15-day hormone treatment, huge amount of testicular cells can be found inside the gonad of all treated fish. After 1 month post-exposure to 15 days of MT, the ovotestes from the non-paired fish (α) and the smaller from the paired fish (β), possessed all stages of spermatogenesis. Meanwhile, the bigger fish from the pair (γ) have no testicular cells in its ovotestes (Figure 1). The ovotestes were consisting mainly of pre-vitellogenic oocytes and some vitellogenic oocytes. After 2 months of rearing post-treatment, fish γ ovotestes possessed mostly developed ovarian cells. It becomes more aggressive and much bigger than their pair (fish β) which still has some testicular cells (Figure 2). It is the same with study done on *A. ocellaris* [4] and the sex changing *A. melanopus* [3]. This may suggest that when paired, the more aggressive and much bigger fish might change sex to female which is the same condition with *A. ocellaris* in wild. Meanwhile fish α still possessed some testicular tissue though the amount started to decrease. A study on *A. bicinctus* [6] shows that, the gonads of *A. bicinctus*, which are held alone, contained mainly growing ovarian tissues. The development and maturation of testicular cells without a female to mate with would be a waste of resources [3].

Figure 1: Gonadal cross section (40X10) of *A. ocellaris* juvenile 1 month post treatment with MT showing (A) gonad of (α) fish, (B) gonad of (β) fish and (C) gonad of (γ) fish. SG, spermatogonia; SC, spermatocytes; ST, spermatid, SZ, spermatozoa, PVO, pre-vitellogenic oocytes, VO, vitellogenic oocytes.

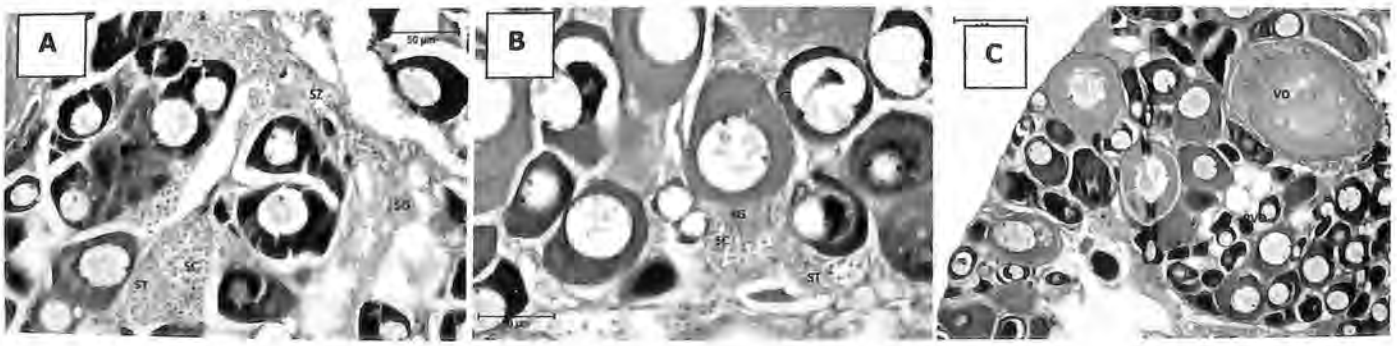
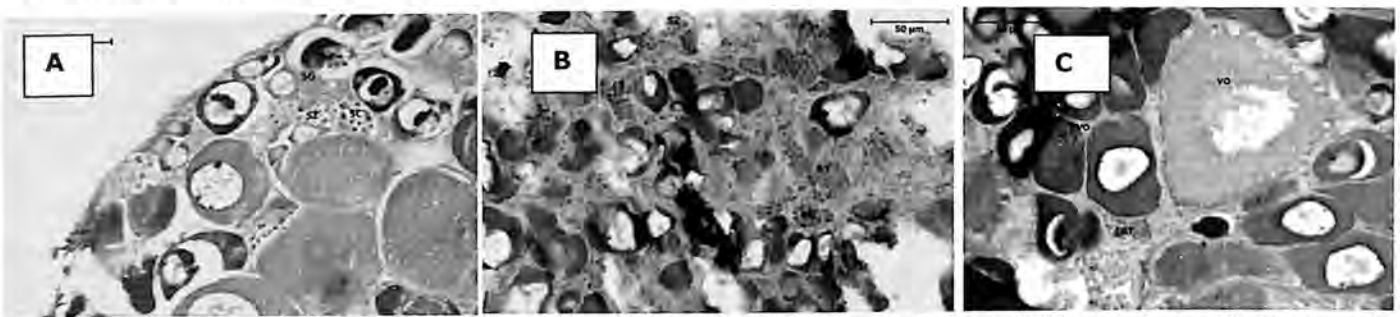


Fig 2: Gonadal cross section (40X10) of *A. ocellaris* juvenile 2 month post treatment with MT showing (A) gonad of (α) fish, (B) gonad of (β) fish and (C) gonad of(γ) fish. SG, spermatogonia; SC, spermatocytes; ST spermatid, SZ, spermatozoa, PVO, pre-vitellogenic oocytes, VO, vitellogenic oocytes.



Conclusion:

Even though MT treated fish can change sex into male, but they still can change into female since the social structure influence their sex determination mechanism. It is also suggested that, in order to maximize the effect of masculinization on *A. ocellaris*, the fish should be paired directly with a matured female. By directly pairing them, it is expected that the treated fish will become an active matured male.

References:

[1]ALLEN, G.R. 1974. The Anemonefishes. 2nd ed. The classification and biology. T.F.H Publication,Inc. USA. 352 p.

[2] DONALDSON E.M. and DEVLIN R.H. 1996. Uses of biotechnology to enhance production. In: Principal of Salmonids Culture (ed by W. Pennel & B. Barton). Pp. 969-1020.

[3]GODWIN J.R. 1994. Behavioural aspects of protandrous sex change in the anemonefish, *Amphiprion melanopus,* and endocrine correlates. Anim. Behav., 48: 551-567.

[4]IWATA E., NAGAI Y., HYOUDOU M., SASAKI H. 2008. Social environment and sex differentiation in the false clown anemonefish, *Amphiprion ocellaris*. Zool. Sci., 25: 123-128.

[5]PANDIAN T.J. and SHEELA S.G. 1995. Hormone induction of sex reversal in fish. Aquaculture, 138: 1-22.

[6]SHAPIRO D.Y. 1992. Plasticity of gonadal development and protandry in fish. J. Exp. Zool., 261: 194-2003.

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

STEROIDOGENESIS DYNAMICS DURING MATURATION IN THE MALE EUROPEAN EEL

Huertas, M.*, Hubbard, P.C.* and Canário, A.V.M.*

*Centro de Ciências do Mar, Universidade do Algarve, Campus de Gambelas, 8005-139 Faro, Portugal. Fax: +351 289 800 069 e-mail: mhuertas@ualg.pt

Introduction:

The European eel (*Anguilla anguilla*) is a synchronous species that, in captivity, only matures after several weeks of hormone treatment. To date, a few studies have investigated common sex steroid levels in male eels after weekly plasma sampling [1,2]. However, the involvement of novel sex steroids in maturation is unknown [3]. Moreover, the measured plasma concentrations of sex steroids differs between eel studies, probably because the sampling time after human chorionic gonadotropin (hCG) differs. In order to understand steroidogenesis in male eels our objectives were to determine steroid production, including novel steroids, before and after hCG injection, and, at the same time, to measure steroid release to the water. Concentrations of cortisol and sex steroids (common and novel) in plasma, mucus and water before and after hCG injection at prespermiating stage (5 weeks, testis stage III-IV) and spermiating stage (10 weeks treatment, testis stage VI) were investigated, as were plasma steroid binding properties.

Methods:

Male eels (N=50, mean weight = 80g) were injected weekly with 3 $IU.g^{-1}$ hCG. At weeks 5 (prespermiating males, PSM) and 10 (spermiating males, SM) six fish were removed from the tank at time 0, 6h, 12h, 24h, 48h and 72h after hCG injection, each male was placed in a separate tank with clean water (10 l) for 2h and blood, mucus and water samples were taken and the fish returned to the main tank. Before and at the end of experiment 6 males were euthanized to obtain bile fluid. Water samples were passed through C18 sep-pack and eluted with methanol. Steroid extraction from water, mucus and blood, and quantification of testosterone (T), 11-ketotestosterone (11KT), androstenedione (AD), 17,20β-progesterone (17-20βP), 11-deoxycortisol (S) and cortisol (F) by radioimmunoassay (RIA) were done according to Huertas *et al.* [1]. All steroid measurements were validated for recovery, specificity and identity following the guidelines of Scott *et al.* [4]. To characterize affinity, capacity and specificity for sex steroids of eel samples was followed Scott *et al.* [5].

Results and Discussion:

Cortisol remained constant at all times at all maturation stages, suggesting that stress associated with sampling was minimal.

Prespemiating males (PSM-: Plasma concentrations and release rates of free T followed similar dynamics with a peak 12h after hCG injection. Plasma free 11KT showed a significant decrease after injection and free AD showed a peak at 12h, whereas release rates of both steroids remained unchanged. 17-20βP and S had low plasma concentrations but high release rates, with a peak of sulphated form at 24h.

Spermiating male (SM- Plasma concentrations of all free steroids measured remained constant but significantly higher than pre-spermiating levels. Mean plasma concentrations were: F; 20 $ng.ml^{-1}$, 11KT; 17 $ng.ml^{-1}$, T; 11$ng.ml^{-1}$ and AD, 17-20βP and S were <2 $ng.ml^{-1}$. Release rates of 11KT and S decreased after hCG injection, whereas free AD and sulphated 17-20βP reached a peak 12h after injection. Eel steroid binding protein (SBP) showed a high affinity for T and moderate capacity (K_d = 0.4 nM, B_{max} = 41nM). Other steroids were less effective ligands: T (100%)> S (69%)>AD (44%)> 17, 20βP (43%) > 11KT (19%)> F (3%).Concentrations of all sex steroids in mucus were for all steroids and for all eel stages<2 $ng.ml^{-1}$, indicating no steroid production or storage in skin. Concentrations of all sex steroids in bile fluid were all below 2 $ng.ml^{-1}$ prior to induction of maturation. In SM all bile concentrations of free steroids ranged between 20-40 $ng.ml^{-1}$ except AD (200 $ng.ml^{-1}$). Conjugated steroids were present in markedly higher concentrations: T; 250 $ng.ml^{-1}$, 11DOC; 336 $ng.ml^{-1}$, AD; 650 $ng.ml^{-1}$, 17-20βP; 2000 $ng.ml^{-1}$ and 11KT; 4000 $ng.ml^{-1}$. The values were similar for sulphated and glucoronidated forms.The fact that changes in release rates are most evident in SM could be related to a saturation of plasma binding

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

capacity by testosterone, the steroid with the highest affinity for SBP. Thus T is mainly stored in plasma in high quantities and metabolized in bile or/and released to the water in lesser amounts than the other steroids. To maintain high concentrations of 11KT in plasma (with 80% less affinity for SBP), its production would have to be 10-100 times higher than T; this is reflected in the amount of metabolized 11KT in bile and released to the water. T seems to be an important androgen in the whole spermatogenesis process since 11KT should be a key steroid in the firs steps of sex maturation.The other steroids (AD, S, and 17,20βP) are poorly retained in plasma and are therefore released to the water or metabolized. AD could be a precursor for the androgens T and 11KT. 17,20βP is a maturing inducing steroid that is produced by the testes and rapidly metabolized and stored in bile. The role of S in sex maturation could be related with sperm hydration and motility.

Conclusion:

Peak production of sex steroids occurs at different times after hCG injection. Production of 17,20βP and S may be as important as that of the androgens, but are only easily detected in water and bile. Together 17,20βP, S and AD may plays important roles in sexual maturation of male eels.

References:

[1]HUERTAS, M., SCOTT, A.P., HUBBARD, P.C., CANARIO, A.V.M., CERDA, J. 2006. Sexually mature European eels (*Anguilla anguilla* L.) stimulate gonadal development of neighbouring males: Possible involvement of chemical communication. Gen. Comp. Endocrinol., 147: 304-313.

[2]PEÑARANDA, D.S., PÉREZ, L., GALLEGO, V., JOVER, M., TVEITEN, H., BALOCHE, S., DUFOUR, S., ASTURIANO, J.F. 2010. Molecular and physiological study of the artificial maturation process in European eel males: From brain to testis. Gen. Comp. Endocrinol., 166: 160-171.

[3]ECKSTEIN, B., COHEN, S., HILGE, V. 1982. Steroid-production in testicular tissue of the European eel. Endocrinology, 110: 916-919.

[4]SCOTT, A.P., HIRSCHENHAUSER, K., BENDER, N., OLIVEIRA, R., EARLEY, R.L., SEBIRE, M., ELLIS, T., PAVLIDIS, M., HUBBARD, P.C., HUERTAS, M., CANARIO, A. 2008. Non-invasive measurement of steroids in fish-holding water: important considerations when applying the procedure to behaviour studies. Behaviour, 145: 1307-1328.

[5]SCOTT, A.P., PINILLOS, M.L., HUERTAS, M. 2005. The rate of uptake of sex steroids fromwater by *Tinca tinca* is influenced by their affinity for sex steroid binding protein in plasma. J. Fish Biol. ,67: 182-200.

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

ENRICHMENT OF SPERMATOGONIAL STEM CELL BY USING SIDE POPULATION IN RAINBOW TROUT

Hayashi M.*, Iwasaki Y.°, Nagasaka Y.° and Yoshizaki G.°

°Department of Marine Biosciences, Tokyo University of Marine Science and Technology, 4-5-7 Konan, Minato-ku, Tokyo 108-8477, Japan
Fax: +81 35463 0558 email: goro@kaiyodai.ac.jp
Beckman Coulter Japan, Tokyo, Japan

Background:

Spermatogonial stem cells (SSCs) provide the foundation for spermatogenesis. Using the rainbow trout (*Oncorhynchus mykiss*), we recently established a novel germ cell transplantation technique to identify SSCs, based on their biological function [1]. A portion of type A spermatogonia (A-SG) transplanted into the peritoneal cavity of the recipient embryo migrated toward and colonized within the recipient embryonic gonad, then produced a large number of functional sperm for several years. These results demonstrated the colonized A-SG were SSCs. These colonizable SSCs can be retrospectively identified after transplantation analysis; however, there is no available technique to prospectively enrich these SSCs before transplantation. In this study, we established a novel technique for SSCs enrichment by using side population (SP).

Methods:

As the method for enriching SSCs, we focused on the SP. SP cells are identified as those cells that are less stained by Hoechest 33342 dye (H33342) as detected by fluorescence activated cell sorting (FACS) analysis [2]. SP cells are firstly identified from bone marrow, and enrich the hematopoietic stem cells. However, it remains controversial whether SSCs are enriched in SP cells; some groups successfully enriched SSCs in SP cells [3-6] but not others [7, 8] in mouse. In this study, we optimized the staining conditions: concentrations of H33342, temperatures, and incubation times, to determine whether SP cells are observed among A-SG in rainbow trout. Then, to determine whether the SSCs were enriched in SP cells, we analyzed the colonizing activity of the SP cells within the recipient gonad.

Results:

SP cells were detected among A-SG by staining with 5μg/ml H33342 for 10 hours at 16°C. To analyze whether these SP cells have the higher colonizing activity, about 150 isolated SP cells or non-SP cells were transplanted into the peritoneal cavity of the recipient embryos. SP cells were colonized within the gonad in 46.6% of transplanted recipient embryos (N=84), whereas non-SP cells were colonized only in 7% of recipient embryos (N=59). These results showed the colonization efficiency was significantly increased by the transplantation of SP cells compared with non-SP cells.

Conclusion:

In this study, we showed the SSCs were enriched in the SP cells in fish. This is very useful technique to isolate the population highly enriched SSCs without SSC-Specific surface markers. SSCs enriched with SP will provide us powerful tools to biochemically and molecularly characterize the SSCs. From an applicational point of view, this technique will increase the efficiency of surrogate broodstock technology.

References:

[1]OKUTSU, T., SUZUKI, K., TAKEUCHI, Y., TAKEUCHI, T., YOSHIZAKI, G. 2006. Testicular germ cells can colonize sexually undifferentiated embryonic gonad and produce functional eggs in fish. Proc. Natl. Acad. Sci. USA, 103: 2725-2729.

[2]GOODELL, M.A., BROSE, K., PARADIS, G., CONNER, A.S., MULLIGAN, R.C. 1996. Isolation and functional properties of murine hematopoietic stem cells that are replicating in vitro. J. Exp. Med. 183: 1797-1806.

[3]FALCIATORI, I., BORSELLINO, G., HALIASSOS, N., BOITANI, C., CORALLINI, S., BATTISTINI, L., BERNARDI, G., STEFANINI, M., VICINI, E. 2004. Identification and enrichment of spermatogonial stem cells displaying side-population phenotype in immature mouse testis. FASEB. J., 18: 376-378.

[4]LASSALLE, B., BASTOS, H., LOUIS, J.P., RIOU, L., TESTART, J., DUTRILLAUX, B., FOUCHET, P., ALLEMAND, I. 2004. 'Side population' cells in adult mouse testis express Bcrp1 gene and are enriched in spermatogonia and germinal stem cells. Development, 131: 479-487.

[5]TAKUBO, K., OHMURA, M., AZUMA, M., NAGAMATSU, G., YAMADA, W., ARAI, F., HIRANO, A., SUDA, T. 2008. Stem cell defects in ATM-deficient undifferentiated spermatogonia through DNA damage-induced cell-cycle arrest. Cell Stem Cell, 2: 170-182.

[6]BARROCA, V., LASSALLE, B., COUREUIL, M., LOUIS, J.P., LE PAGE F., TESTART, J., ALLEMAND, I., RIOU, L., FOUCHET, P. 2009.

Mouse differentiating spermatogonia can generate germinal stem cells in vivo. Nat. Cell Biol., 11: 190-196.

[7]KUBOTA, H., AVARBOCK, M.R., BRINSTER, R.L. 2003. Spermatogonial stem cells share some, but not all, phenotypic and functional characteristics with other stem cells. Proc. Natl. Acad. Sci., USA, 100: 6487-6492.

ANOSMIA IMPAIRS REPRODUCTION IN THE MOZAMBIQUE TILAPIA

Huertas, M., Pereira, D., Silva, S. Canário, A.V.M. and Hubbard, P.C.

Centro de Ciências do Mar, Universidade do Algarve, Campus de Gambelas, 8005-139 Faro, Portugal.
Fax: +351-289800051 e-mail: mhuertas@ualg.pt

Introduction:

Chemical signals released to the water (i.e. pheromones) can trigger physiological responses that influence reproduction in fish. In the Mozambique tilapia (*Oreochromis mossambicus*), males can discriminate between olfactory stimuli released by pre- and post-ovulated females [1] and increase their urination rate in the presence of pre-ovulatory females [2]. This suggests that female urine contains a pheromonal message directed at males. It is not known, however, whether olfactory input from females is necessary for maintenance of reproductive capacity in males. The current study therefore investigated the effects of anosmia on behaviour, steroid production and gametogenesis in males.

Methods:

Firstly, three groups of 14 size-matched males were established: control, anosmic (olfactory epithelia removed) and sham-operated. After 7 days urine and plasma were collected. Plasma levels of testosterone (T), 11-ketotestosterone (11KT), 17,20β-P and cortisol were measured by radioimmunoassay and olfactory potency of the urine was assessed by the electro-olfactogram. On day 15 fish were killed and the testes fixed for histology. Secondly, three groups of eight males were established: control, anosmic and sham-operated. Each male was kept in a family tank with three or four females; behaviour of the male was assessed and a urine sample taken each week for four weeks. After the final urine sample, plasma samples were taken, the fish were killed and the testes fixed.

Results and Discussion:

Spermatogenesis- The GSI was 1.00 ± 0.05 % (sham) and 0.60 ± 0.07 % (anosmic) after four weeks ($P < 0.001$). Testes from anosmic fish appeared different to the naked eye from those from shams; greyish and rigid rather than white and flexible. Histology showed significant regression of spermatogenesis in anosmic fish. At day 15 anosmic males had a reduction of the number of testicular lobules/ unit area and fewer Sertoli cells and spermatogonia A and B in the posterior testis. At day 28 germinal cell counts per lobule also decreased in anosmic fish, and were more dramatic in the posterior half testicle. Lobules from anosmic fish were filled with a fibrous matrix and germ cells appeared to detach from the lobule walls. Leydig cells in anosmic fish at day 28 were more numerous than in controls, possibly linked with an increase of 17,20βP concentrations in this group.

Steroid production- Plasma cortisol increased significantly in anosmic fish (from 20.9 ± 3.8 in control fish to 40.8 ± 5.2 ng/ml) at day 7. However, these values are low compared with those of stressed tilapia (106 ± 1 ng/ml [3]) and may be a specific response to anosmia. After 28 days, plasma cortisol was similar to controls and sham-operated. The concentrations of T and 11-KT decreased at day 7 in anosmic and sham, being lower in anosmic (T from 4.4 ± 0.3 ng/ml in control fish to 2.7 ± 0.4 ng/ml and 11KT from 5.5 ± 0.6 in control fish to 2.3 ± 0.5 ng/ml). The decrease of androgens in shams may be related to a slight loss of olfactory sensitivity due to the operation. This slight decline, however, had no further effect on spermatogenesis (see above). Cortisol, T and 11KT after 28 days were not different from controls. However, 17,20β-P glucuronide was significant higher in anosmic fish at day 28 (from 0.4 ± 0.03 to 0.6 ± 0.08 ng/ml), which could be related with the inhibition of androgen production (linked to a possible gonadotrophin insufficiency) and accumulation of progestin.

Behaviour- After one week, aggressive behaviour increased significantly in anosmic males but returned to control values after day 14. Nesting behaviour was absent in anosmic males until day 14 and thereafter remained lower than controls. Increased aggression in anosmic males is indicative of conspecifics odour-mediated aggression inhibition [4].

Olfactory potency of urine: Urine from anosmic fish was less potent than sham-operated fish at day 7, but recovered after 28 days. This reduction in potency may be linked to odorants originated from the testis.

Conclusion:

The current study shows that olfaction and reproductive physiology are strongly linked in the Mozambique tilapia. In the absence of olfactory input, androgen synthesis is reduced, the olfactory potency of the urine is lower, and males become more aggressive. In the long term, testicular function is gravely affected. We suggest that chemical cues from the females and / or odorants from other males are important for normal reproductive physiology in the males.

References:

[1]MIRANDA, A., ALMEIDA, O.G., HUBBARD, P.C., BARATA, E.N., CANÁRIO, A.V.M. (2005). Olfactory discrimination of female reproductive status by male tilapia (*Oreochromis mossambicus*). J. Exp. Biol., 208: 2037-2043.

[2]ALMEIDA, O.G., MIRANDA, A., FRADE, P., HUBBARD, P.C., BARATA, E.N., CANARIO, A.V.M. (2005). Urine as a social signal in the Mozambique tilapia (*Oreochromis mossambicus*). Chem. Senses, 30: I309-i310.

[3]FOO, J.T.W., LAM, T.J. (1993). Serum cortisol response to handling stress and the effect of cortisol implantation on testosterone level in the tilapia, *Oreochromis mossambicus*. Aquaculture, 115, 145-158.

[4]BARATA, E.N., HUBBARD, P.C., ALMEIDA, O.G., MIRANDA, A., CANARIO, A.V.M. (2007). Male urine signals social rank in the Mozambique tilapia (*Oreochromis mossambicus*). BMC Biol., 5.

POSSIBLE MEDIATORS OF GROWTH HORMONE ACTION ON TESTICULAR TESTOSTERONE PRODUCTION IN THE ASIAN CATFISH, *CLARIAS BATRACHUS*

Dubey N. and Lal B.

Department of Zoology,Banaras Hindu Univ.,Varanasi-221005, India. lalbhu@yahoo.co.in

Introduction:

In recent past, GH has been recognized as co-gonadotropin in mammals [1] because of its potential role in regulation of reproductive physiology including steroidogenesis. GH is reported to regulate gonadal steroidogenesis in fishes too [2-3]. However, these studies on fishes are performed either *in vivo* or by taking testicular tissue *in vitro*, which do not provide any idea about the mode and mechanism of GH action on gonadal steroidogenesis and also about the receptor-coupled downstream signaling system involved in GH action. In the present study, therefore, efforts were made to determine the mode and mechanism of GH action on the testicular steroidogenesis in *C. batrachus.*

Methods:

Leydig cells were isolated from the adult freshwater catfish, *C. batrachus* as per the methods described elsewhere [3]. Two milliliter of homogenous suspension of the Leydig cells (1.6×10^6 cells/ml of M-199 medium) were plated in 24 well culture plates and treated with GH & IGF-I alone as well as in combinations with different compounds & IGF-I antiserum as the per tables 1-5, separately and were incubated for 24 hrs in the CO_2 incubator at 25^0C. After treatments, Leydig cells free medium was taken out for testosterone (T) and IGF-I estimation using ELISA and RIA methods [3,5], respectively. Data were expressed as Mean±SE in ng/ml and analyzed employing ANOVA followed by Newman-Keuls' Multiple range test at α level 0.05. Means with similar scripts in the tables1-5 do not differ statistically.

Results:

GH and IGF-I alone as well as in combination increased testosterone production by Leydig cells *in vitro*, however, addition of actinomycin D (AcD) @ 2μg/ml, cycloheximide (Cylx) @ 2μg/ml, and anti-barramundi IGF-I abolished the GH-induced T production. GH also elevated IGF-I production considerably by Leydig cells. Treatment of Leydig cells with adenylyl cyclase inhibitor (SQ 22,536), PKA inhibitor (H-89) and tyrosine kinase inhibitor (lavendustin A) significantly abolished the GH-stimulated IGF-I production by Leydig cells.

Discussion:

Stimulation of testosterone production by GH is in concurrence to the earlier reports [2], where GH is shown to increase testosterone and 11-KT production by killifish and trout testis. It has been shown that GH binds to its specific GH receptors in the testis of mature trout [2]. Present findings also suggest that GH action on testosterone production requires transcription of certain gene and its ultimate protein product, which appears to be the IGF-I here, as the addition of anti-barramundi IGF-I to the GH-incubated Leydig cells eliminated the GH-induced testosterone production. GH-stimulated IGF-I secretion by the isolated Leydig cells in a

Table 1. Effect of GH and IGF-I alone as well as along with AcD, Cylx and 200x diluted anti-IGF-I on *in vitro* testosterone production by Leydig cells of *C. batrachus.*

	Con-trol	GH 10ng/ml	GH+ AcD	GH+ Cylx	GH+IGF-I	GH+anti-IGF-I	IGF-I 10ng/ml
Testo-sterone	7.8 ±0.6	12.71 ±0.74	3.77±0.25**a**	4.29 ±0.26**a**	15.97 ±0.83	4.51 ±0.55**a**	10.50 ±0.62

Table 2. Effect of GH on *in vitro* IGF-I production by Leydig cells of *C. batrachus.*

	Control	GH (1ng/ml)	GH (10ng/ml)	GH (100ng/ml)
IGF-I	3.96 ±0.86	8.76±0.68	16.04±1.67**a**	14.56±1.65**a**

Table 3. Effect of GH alone and along with SQ 22536 on *in vitro* IGF-I production by Leydig cells of *C. batrachus.*

	Control	GH (1ng/ml)	GH+SQ22536 (1ng ml,each)	GH (10ng /ml)	GH+SQ22536 (10ng/ml,each)	GH (100ng/ml)	GH+SQ22536(100 ng/ml,each)
IFG-I	4.32±0.4	7.88±1.3	2.71±0.33	15.35±0.75**a**	0.88±0.29**b**	12.94±1.09**a**	1.04±0.38**b**

Table 4. Effect of GH alone and along with H-89 on *in vitro* IGF-I production by Leydig cells of *C. batrachus*

	Control	GH (1ng/ml)	GH+H89 (1ng/ml,each)	GH (10 ng/ml)	GH+H89(10 ng/ml,each)	GH(100ng /ml	GH+H89 (100 ng/ml,each)
IGF-I	4.13±.31	9.25±0.89	1.69±0.16**b**	15.53±1.04**a**	0.92±0.14**b**	13.96±0.90**a**	1.48± 0.17**b**

Table 5. Effect of GH alone and along with lavendustin A (LA) on *in vitro* IGF-I production by Leydig cells of *C. batrachus*

	Control	GH (1ng/ml)	GH+LA (1ng/ml each)	GH (10ng/ml	GH+ LA (10 ng/ml, each)	GH(100ng /ml, each)	GH+LA(100 ng/ml,each)
IGF-I	4.13±.31	9.25±0.89	1.69±0.16**b**	15.53±1.04**a**	0.92±0.14**b**	13.96±0.90**a**	1.48± 0.17**b**

dose dependent manner substantiates this notion further. Thus, it is likely that GH might have stimulated Leydig cell steroidogenesis in the present study by augmenting IGF-I production by Leydig cells. Suppression of GH-induced IGF-I production by Leydig cells of the catfish after the treatments with SQ 22,536, H-89 and lavendustin A provides convincing evidence that cAMP/PKA, and TK signaling system are involved in GH action. The receptor-coupled downstream intracellular signaling system, which mediate the effect of GH on IGF-I production by Leydig cells is not yet known.

Nevertheless, some report on intracellular signaling system involved in signaling systems involved in GH action on other tissues like ovary, liver, etc., are available, which indirectly support our findings.

Makarevich and Sirotkin [4] have reported that GH involves cAMP/PKA and/or TK dependent pathways in releasing IGF-I from the porcine granulosa cells.

Conclusion:

Results clearly suggest that the steroidogenic action of GH is indirect through genomic pathway and is mediated through the Leydig cells produced–IGF-I. GH appears to employ cAMP/PKA and tyrosine kinase signaling pathways to induce IGF-I production, as the adenylyl cyclase inhibitor, PKA blocker and tyrosine inhibitor abolished the GH-induced IGF-I production by Leydig cells.

References:

[1]CHILD, G.V. 2000.Growth hormone cells as co-gonadotropes: partners in the regulation of the reproductive system. Trends Endocrinol. Met., 5: 168-175.

[2]LeGAC, F., BLAISE, O., FOSTIER, A., LeBAIL, P.Y., LOIR, M., MOUROT, B. AND WEIL, C. 1993. Growth hormone (GH) and reproduction: a review. Fish Physiol. Biochem., 11:219-232.

[3]DUBEY, N. AND LAL, B. 2008. Nitric oxide: An autocrine regulator of Leydig cell steroidogenesis in the Asian catfish, *Clarias batrachus*. Gen. Comp. Endocrinol., 158: 161-167.

[4]MAKAREVICH, A.V., SIROTKIN, A.V. 2000. Presumptive mediators of growth hormone action on insulin like growth factor-I release by porcine ovarian granulosa cells. Biol. Sig. Recept., 9: 248-254.

[5]SINGH, A.K. AND LAL, B. 2008. Seasonal and circadian time dependent dual action of GH on somatic growth and ovarian development in the catfish, *Clarias batrachus:* Role of temperature. Gen. Comp. Endocrinol., 158: 161-167.

Gametogenesis - II (Folliculogenesis)

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

WATER HOMEOSTASIS IN THE TELEOST OOCYTE: NEW INSIGHTS INTO THE ROLE AND MOLECULAR REGULATION OF AQUAPORINS

Cerdà J.*, Zapater C. *, Chauvigné F.*, Finn R.N.*,3

*Laboratory of Institute of Research and Technology in Food and Agriculture (IRTA)-Institute of Marine Sciences (CSIC), Passeig marítim 37-49, 08003 Barcelona, Spain.
Fax: +34-932309555. E-mail: joan.cerda@irta.cat
*Department of Biology, University of Bergen, and [3]Institute of Marine Research, Bergen, Norway

The pre-ovulatory hydration of the oocyte of marine teleosts that produce pelagic (buoyant) eggs is a unique process among vertebrates. Water influx occurs concomitantly with meiosis resumption (oocyte maturation), and results in a massive expansion and dilution of the egg cytoplasm. This mechanism is critical for the development of viable gametes as it assures a water reservoir when eggs are released into the hyperosmotic seawater, and facilitates egg buoyancy and dispersal. While a number of studies have identified the major inorganic and organic osmolytes that create the intracellular osmotic driving force for oocyte hydration, water influx was thought to occur passively. However, the discovery of molecular water channels (aquaporins) in all kingdoms of life recently led to the investigation of the role of these membrane proteins during teleost oocyte hydration.

Initial studies in the gilthead seabream uncovered a novel water channel (Aqp1o, now named Aqp1b) that mediates water permeation and resultant swelling of the oocytes. Further functional, genomic and phylogenetic analyses revealed that Aqp1b belongs to a teleost-specific subfamily of water-selective aquaporins, which likely evolved by tandem duplication of a common ancestor. This gene is highly expressed in the ovary of marine and catadromous teleosts that produce hydrated eggs, including the Atlantic halibut, which spawns one of the largest pelagic eggs known. In this species, we recently obtained the first unequivocal functional evidence for the essential role of Aqp1b during oocyte hydration. Interestingly, however, *aqp1b* transcripts are also highly accumulated in the ovary of some freshwater species, such as the stinging catfish, in which oocytes partially hydrate during meiotic maturation, suggesting that Aqp1b may also play a role in the oocyte of teleosts producing non-buoyant (benthic) eggs.

Using the seabream as the experimental model, we noted that Aqp1b is highly regulated during oogenesis. Our new data suggest that transcriptional activation of the seabream *aqp1b* promoter in oogonia and primary growth oocytes may be dependent on Sry-related high mobility group [HMG]-box (*sox*) genes as well as on a nuclear progestin receptor. However, Aqp1b protein synthesis is not observed until cortical alveoli form and vitellogenesis commences. Subsequently Aqp1b-containing vesicles are transported towards the oocyte cortex. Structural analyses have revealed that the cytoplasmic tail of Aqp1b is highly divergent in teleosts, but retains specific motifs that regulate vesicular trafficking and temporal insertion in the oocyte plasma membrane during hydration. These processes appear to involve alternative mechanisms of phosphorylation and/or dephosphorylation of specific C-terminal residues. Evidence for the rapid neofunctionalization of the C-terminus is found in the Atlantic halibut, which causes *ex vivo* loss of function of Aqp1b when expressed in amphibian oocytes, but not in zebrafish or native oocytes.

These findings are revealing that the Aqp1b-mediated mechanism of oocyte hydration in marine teleosts is a conserved, but divergently regulated process based on the interplay between osmolyte generation and the controlled synthesis and insertion of Aqp1b at the oocyte surface. The discovery of Aqp1b thus provides important new insight into the physiological role of water homeostasis in the reproductive biology of teleosts.

cAMP.
FSH

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

PARACRINE AND AUTOCRINE COMMUNICATION NETWORK IN THE ZEBRAFISH OVARIAN FOLLICLE

Ge W.

School of Life Sciences and Centre for Cell and Developmental Biology, The Chinese University of Hong Kong, Shatin, N.T., Hong Kong, China
Email: weige@cuhk.edu.hk; Fax: 852-2603-5646

Introduction:

Gonadotrophic hormones (FSH and LH) play pivotal roles in controlling folliculogenesis and steroidogenesis in vertebrate ovary. However, their actions in the ovary depend on a sophisticated local communication network within the follicle that involves various growth factors.

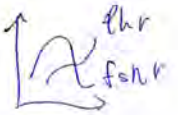
Methods:

We have used a variety of techniques in our research to characterize the intrafollicular communication network in the zebrafish ovary, including real-time qPCR, primary culture of follicle cells, follicle incubation, in situ hybridization, recombinant protein production, Western blot analysis, etc.

Results and Discussion:

We have characterized several important growth factor families within the follicle of zebrafish, including EGF, activin-inhibin-follistatin, Kit/Kit ligand, GDF9/BMP families and the mini axis of PACAP-GH-IGF. These factors are likely involved in bidirectional communication between the oocyte and follicle cells. As a potential mediator of gonadotropin actions, activin subunits (*inhbaa* and *inhbb*) are exclusively expressed in the follicle cells whereas its receptors are abundantly expressed in the oocyte. In contrast, EGF and BMP families are mostly expressed in the oocyte, but their receptors (EGFR and BMPRII) are exclusively located in the follicle cells. The Kit/Kit ligand system is unique in that the system consists of two ligands (*kitlga* and *kitlgb*) and two receptors (*kita* and *kitb*). The distribution of Kits/Kit ligands in the follicle and the evidence for receptor specificity suggest that Kitlga-Kita and Kitlgb-Kitb may represent two paracrine regulatory pathways within the follicle that mediate reciprocal communications between the two compartments. Our functional studies have demonstrated extensive interactions among these signaling pathways in the follicle. We have recently characterized inhibin, a natural activin antagonist, in the zebrafish ovary. Similar to activin subunits, inhibin alpha subunit (*inha*) is exclusively expressed in the follicle cells and its expression level surges at the full-grown stage prior to oocyte maturation, which has led us to hypothesize that the preovulatory surge of inhibin production may serve as an ovarian messenger to signal the pituitary for final maturation, and inhibin may act by antagonizing the effects of activin on FSH and LH biosynthesis in the pituitary. The expression of *inha* is likely subject to the regulation by oocyte-derived factors such as BMPs (supported by RGC grants to WG).

BMP → Inhα



Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

A NOVEL CLASS OF OVARIAN LIPOPROTEIN RECEPTOR IN CUTTHROAT TROUT: MOLECULAR CLONING AND EXPRESSION ANALYSIS

Hiramatsu N.*, Luo W.*, Mizuta H.*, Todo T.*, Reading B.J., Sullivan C.V.** and Hara A.***

*Faculty of Fisheries, Hokkaido University, 3-1-1 Minato, Hakodate, Hokkaido, 041-8611, Japan. Tel&Fax: +81-138-40-8878; email: naoshi@fish.hokudai.ac.jp

**Department of Biology, North Carolina State University, Raleigh, North Carolina 27695-7617, USA

Introduction:

Teleost eggs contain a substantial yolk mass, which serves as a protein- and lipid-rich nutritional source for embryonic development and larval growth. A large portion of the yolk mass is derived from multiple types of vitellogenins (Vgs), which are incorporated into the oocytes from the maternal circulation *via* endocytosis mediated by Vg receptors (VgRs) [1, 2]. A member of the low-density lipoprotein receptor (LDLR) gene family, which contains a single ligand binding (LB) domain consisting of 8 LB repeats has previously been identified and designated as the VgR. This VgR also has been termed 'lipoprotein receptor with 8 ligand repeats' (LR8) and 'very low-density lipoprotein receptor' (VLDLR). Our recent findings, however, have revealed multiplicity in ovarian membrane proteins that specifically bind Vg in perciforms, indicating that Vg is possibly incorporated by other oocyte LDLR family receptors [3]. With the exception of this classical LR8-type VgR, no prior characterization has been performed on other ovarian Vg-binding receptors in terms of their structures, expression profiles, or ligand specificities. As an initial step to understanding the physiological significance of multiple ovarian receptors during teleost yolk formation, we aimed to clone and characterize a novel LDLR family receptor (LR) from the ovary of cutthroat trout (*Oncorhynchus clarki*).

Methods:

A full-length cDNA encoding a novel LR was cloned from previtellogenic ovary of cutthroat trout by RT-PCR and TA cloning using degenerate PCR primers designed based on nucleotide sequences of a novel ovary LR in striped bass (*Morone saxatilis*) and white perch (*M. americana*) (Reading and Sullivan, *unpublished data*) and other mammalian LDLR family genes. The full-length sequence was obtained by 3' and 5' rapid amplification of cDNA ends (RACE). Expression levels of the LR mRNA in female cutthroat trout were determined using quantitative real-time reverse transcription PCR assay (qRT-PCR). *In situ* hybridization was performed by a routine method using specific probes labeled with digoxigenin.

Results and Discussion:

The cDNA cloned in this study contained a complete coding sequence (4,500 bp), encoding a protein with an expected mass of ~163 kDa (1,500 amino acid residues). The deduced amino acid sequence of this cDNA clone included several domains that are conserved in sequences of LDLR gene family members, including (from the N-terminus): an N-terminal LB domain consisting of 13 LDLR class-A LB repeats, an epidermal growth factor (EGF) precursor homology domain (A, B, C and D), five LDLR class B repeats flanked by EGF repeats A and B, three LDLR class B repeats flanked by EGF repeats B and C, a C-terminal LB domain consisting of one LDLR class-A LB repeat flanked by EGF repeats C and D, a transmembrane domain, and a cytoplasmic domain. A phylogenetic analysis placed this LR sequence into a new LR cluster consisting of several unclassified LR sequences predicted from genomes of various animals, and which included the novel ovary LR of *Morone* species ([3]; Reading and Sullivan, *unpublished data*). This novel receptor cluster (designated herein as LRX+1 class, where X = number of N-terminal LB repeats) was more similar to the insect VgR cluster than to the clusters of vertebrate LR7s (LDLR), LR8s (VgR, VLDLR), and LDLR-related proteins (LRPs). Expression of cutthroat trout LRX+1 (CtLRX+1) mRNA was exclusively observed in the ovary when various tissues were examined by qRT-PCR. The CtLRX+1 mRNA expression was high in the previtellogenic ovaries and gradually decreased during the vitellogenic phase, followed by a slight increase in ovaries at ovarian follicle maturation and in the post-ovulatory follicles. *In-situ* hybridization revealed an intense localization of CtLRX+1 mRNA expression in the cytoplasm of previtellogenic oocytes, while no detectable signal was observed in vitellogenic oocytes.

Conclusion:

In summary, a full-length cDNA encoding a novel ovarian LR was cloned from the ovary of cutthroat trout. Structural analyses suggest that this sequence is a new class of LR in the LDLR gene family (tentatively called LRX+1 class) and should be designated as cutthroat trout LR13+1 (CtLR13+1). The CtLR13+1 might function as a VgR, since the patterns of distribution in somatic and ovarian tissues, as well as the patterns of expression during oogenesis, were found to be similar to those of VgR in fishes. The present study provides further evidence for a system of yolk formation mediated by multiple ovarian receptors in teleosts.

Acknowledgements:

This study was supported by JSPS KAKENHI (#22380103: Grant-in-Aid for Scientific Research B; #19380106 & #19780143: Grant-in-Aid for Young Scientists B).

References:

[1]HIRAMATSU, N., CHAPMAN, R.W., LINDZEY J.K., HAYNES, M.R., SULLIVAN, C.V. 2004. Molecular characterization and expression of vitellogenin receptor from white perch (*Morone americana*). Biol. Reprod., 70(6): 1720-1730.

[2]HIRAMATSU, N., HARA, A., HIRAMATSU, K., FUKADA, H., WEBER, G.M., DENSLOW, N.D., SULLIVAN, C.V. 2002. Vitellogenin-derived yolk proteins of white perch, *Morone americana*: purification, characterization, and vitellogenin-receptor binding. Biol. Reprod., 67(2): 655-667.

[3]READING, B.J., HIRAMATSU, N. AND SULLIVAN, C.V. 2011. Disparate binding of three types of vitellogenin to multiple forms of vitellogenin receptor in white perch (*Morone americana*). Biol . Reprod., 84(2): 392-399.

DIFFERENTIAL EXPRESSION OF VITELLOGENIN GENES (VGA AND VGB) BY HEPATOCYTES OF THE INDIAN FRESHWATER MURREL, *CHANNA PUNCTATUS*, ON EXPOSURE TO ESTRADIOL

Sehgal Neeta, Rawat V. S., Rani K.V. and **Phartyal R.**
Department of Zoology, University of Delhi, Delhi 110 007, India
email: neetasehgal@mailcity.com

Introduction:

The egg-yolk of fishes is derived from a blood-borne, female-specific precursor, vitellogenin (Vg), which is synthesized in the liver under estrogenic influence during periods of reproductive activity. This protein is modified in the liver, secreted into blood and transported to the ovary where it is incorporated by the growing oocytes via specific receptor-mediated endocytosis. More than one form of vitellogenin (VgA, VgB and VgC) has been reported in teleosts. We have reported earlier that murrel Vg exists as three charge isomers in the blood [1]. In the present study we have shown the existence of VgA and VgB in this fish comparable to other teleosts and confirm the presence of both the proteins at gene level. Further VgA and VgB genes are expressed in liver when fishes are exposed to estrogen under *in vivo* condition whereas only Vg B is expressed by cultured hepatocytes.

Methods:

Fish were injected with E_2, plasma was collected in the presence of proteolytic inhibitors and processed for isolating Vg by gel filtration chromatography on Ultrogel AcA-34 followed by FPLC on a strong anion exchanger (UNO Q). One of the FPLC purified Vg isomer was subjected to LCMS/MS for molecular identification. Peptide sequences were matched with fish Vg sequences database. Amino acid sequences and nucleotide sequences were aligned using Clustal W software. cDNA was amplified using primers for VgA and VgB, and the amplicons were sequenced which were subjected to bioinformatical analysis. Estradiol-treated fish were bled and Vg was estimated by ELISA; total RNA was extracted from liver, gill, kidney and ovary, and processed for amplification of Vg gene by RT-PCR. Hepatocytes were isolated and cultured. Monolayer of cultured hepatocytes was exposed to estradiol; Vg was quantified in the culture medium, total RNA was extracted from all the treated hepatocytes and processed for amplification of Vg gene.

Results and Discussion:

In order to assess the purity of Vg and ti isolate charge isomers of Vg fractionated by gel filtration chromatography on Ultrogel AcA 34, specific fractions were further subjected to FPLC on a strong anion exchanger, which separated the proteins on charge differences. The Vg fraction resolved into two distinct peaks eluting at 0.3 M and 0.32 M NaCl suggesting that variants of vitellogenin have different charge composition. The fraction eluting at 0.3 M NaCl was electrophoresed under denaturing conditions, digested with trypsin and processed for LC-MS/MS. The sequences of peptide fragments of 'β' isomer of Vg matched significantly with amino acid sequences of VgB and Vg precursor derived from Melanogrammus aegelefinus, Oryzias latipes, Cyprinus carpio, Oreochromis aureus, Oncorhynchus mykiss, Pimphales promelas, Sillago japonica and Fundulus heteroclitus. The presence of VgA was also indicated with peptide fragment of VgA from *Melanogrammus aegelefinus, Oncorhynchus mykiss* and *Sillago japonica.* VgA derived yolk proteins are cleaved into free amino acids and play distinct roles with respect to oocyte maturation and embryonic nutrition. Free amino acids appear to increase the buoyancy and support early embryonic development whereas a major part of VgB remained as a large peptide and is probably used as an important nutrient source at later stages of embryonic development [2]. *Channa punctatus* lays pelagic eggs the precondition for which is the presence of VgA.

First strand cDNA was synthesized by reverse transcriptase. Primers were designed from the aligned sequences for VgA and VgB (Table 1). These primers were used to amplify single strand cDNA (Fig. 1) to study expression patterns of VgA and VgB genes when murrel hepatocytes were exposed to estradiol in vivo or in vitro conditions. This study suggests that E_2 up-regulates levels of Vg in plasma or in culture medium in a dose dependent manner. The VgA and VgB mRNA levels in the liver of E_2 treated murrel was analyzed by RT-PCR showed a

Table 1: Primer sequences for amplification of Vitellogenin A and Vitellogenin B

mRNA	Primer	Primer sequence
VgA	Forward primer	5' TGAGGAACATTGCAAAGAAGG 3'
	Reverse primer	5' ATTCCCTCAGTTCTCACTCC 3'
VgB	Forward primer	5' AGTGAGAACAGAGGGAATCC 3'
	Reverse primer	5' TGTTCACGCCCATGACAGC 3'
	Forward primer	5' ATGTCCAAGCTACTGTGACG 3'
	Reverse primer	5' TCGCCAACTTGAATCTCAACC 3'

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

Fig. 1: Agarose (1%) gel electrophoresis of purified PCR products of FA1RA1 primers for Vg A, FB1RB1 and FB2RB2 primers for Vg B.

Lane 1: DNA ruler

Lane 2: FA1RA1 PCR product

Lane 3: FB1RB1 PCR product

Lane 4: FB2RB2 PCR product

pattern of expression which was also dose dependent. Cultured hepatocytes expresses only VgB gene.

Conclusion:

These results suggest that most of the peptide sequences of purified isomer of murrel matches with VgB while a few sequences also match with VgA of fishes. We conclude that both VgA and VgB genes are present in the genome of *Channa punctatus*. The investigation also suggests that *in vitro* system cannot be entirely translated into *in vivo* system.

References:

[1]SEHGAL, N., GOSWAMI S.V. 2005. Vitellogenin exists as charge isomers in the Indian freshwater murrel, *Channa punctatus* (Bloch). Gen. Comp. Endocrinol., 141: 12-21.

[2]HIRAMATSU, N., MATSUBARA, T., FUJITA, T., SULLIVAN, C.V., HARA, A. 2006. Multiple piscine vitellogenins: Biomarkers of fish exposure to estrogenic endocrine disruptors in aquatic environments. Mar. Biol., 149: 35-47.

DISTINCT LOCALIZATION OF CYCLIC AMP DEPENDENT PROTEIN KINASE CATALYTIC SUBUNIT (PKAC) DURING MEIOTIC MATURATION IN PERCH (*ANABAS TESTUDINEUS*) OOCYTE: PKA INHIBITORS MIMIC MIH ACTION

Pragya Paramita Khan [a], Suman Dasgupta[b], Samir Bhattacharya[b*], Sudipta Maitra[a*]

[a] Laboratory of Reproductive and Developmental Physiology, Department of Zoology, Visva-Bharati University, Santiniketan, India

[b] Molecular Endocrinology Laboratory, Department of Zoology, Visva-Bharati University, Santiniketan, India

Telephone: +91 3463 261268; Fax: +91 3463 261176

E-mail: bhattacharyasa@gmail.com; sudipta.maitra@visva-bharati.ac.in

Intracellular localization of signaling molecules play key role in controlling cell cycle regulation. Here we report that during meiotic cell cycle in perch oocyte, dynamics of PKAc localization could be critical in PKA inhibition allowing G2-M transition. In immature oocyte with high PKA activity, PKAc was located at the central nuclear region; however it gradually shifted its locations towards the cortical ooplasm when maturation was induced by MIH. Thermo-stable PKA inhibitor (PKI) or H89 induced PKA inhibition resulted re-initiation of meiosis in absence of MIH which showed similar pattern of PKAc localization. Mos was absent in immature oocyte though *c-mos* mRNA was detected. Inhibition of PKA either by MIH or PKI/H89 allowed expression of Mos much before MPF activation and GVBD. Mos could be detected at the animal pole by MIH, interestingly substitution of MIH by PKI/H89 showed similar pattern of intra-oocyte localization. That PKA is related in suppressing Mos was further evident from forced increase of cAMP that prevented Mos accumulation. MIH action on Myt1and Cdc25 to achieve G2-M transition was mimicked by PKI/H89. Our findings focus a new dimension of PKA regulation during oogenesis and sub-cellular localization of PKAc could be instrumental in cell cycle progression.

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

LIPID METABOLISM DURING EARLY OOGENESIS – WHAT CAN WE DEDUCE FROM TRANSCRIPTOME ANALYSIS AND WHAT HAVE ANDROGENS GOT TO DO WITH IT?

Lokman P. M.*, Divers S. L.*, Ozaki Y.* and **Black M. A.°**

*Department of Zoology, University of Otago, PO Box 56, Dunedin 9054, New Zealand. Fax +64-3-479 5846 e-mail: mark.lokman@otago.ac.nz

°Department of Biochemistry, University of Otago, Dunedin 9054, New Zealand

Introduction:

Fat content and composition have been correlated with egg quality in a suite of teleosts. The eggs primarily store triacylglycerides, phospholipids, steryl esters and wax esters, and the relative importance of each of these lipid classes is species-dependent [see 3]. The enzyme lipoprotein lipase (LPL) has been implicated in lipid uptake in several fish species; in the eel, LPL activity in the ovary increases from early vitellogenesis (EV), peaks at midvitellogenesis and decreases by the time the oocyte is fully grown [1]. LPL may be responsible for over 70% of total triacylglyceride (TAG) uptake [1], and uptake strongly hinges on TAG being offered in association with lipoproteins [2]. There is further robust evidence that 11-ketotestosterone (11KT) can greatly enhance lipid uptake, at least in the eel, in part by increasing LPL gene expression [1]. It thus appears that 11KT controls at least some of the phenotypic changes associated with progression of previtellogenic (PV) to early vitellogenic stages, but "global effects" on the ovary remain to be clarified. The use of Next Generation Sequencing (NGS) platforms is ideally suited for this task. Therefore, in the present study, we subjected ovarian tissue from PV and EV fish to trans-criptomic analyses to obtain insights into the importance of key pathways. We compared our findings with those on 11KT-treated fish to assess what part of the differences between PV and EV may be androgen-mediated. Lastly, to validate the transcriptome data, we conducted an in-house PCR array on ~25 genes involved in fatty acid metabolism in the eel ovary and compared the outcome with the transcriptome results.

Methods:

Wild PV and EV eels (*Anguilla australis*) were caught and sampled within a few h after capture. Ovarian tissue was collected, snap-frozen and stored at -70 °C until use in routine quantitative PCR (qPCR) analysis. In a subsequent year, ovarian tissue from one EV eel was collected for NGS sequencing. Another cohort of PV eels was collected and divided between two groups – one group received slow-release 11KT implants, the other vehicle. After two weeks, fish were euthanized and ovarian tissue collected and stored. Tissue from one control and one 11KT-treated fish was processed for NGS and tissue from remaining animals was used for qPCR analysis. Sequencing was conducted on an Illumina HiSeq 2000 by a sequencing contractor (BGI, China) following double-stranded DNA synthesis from total RNA template. Sequence information from all three analyses (5 Gb) was used to construct contigs and relative transcript abundance was subsequently estimated from RKPM (reads per kilobase of sequence per million reads) data provided by the sequencing contractor. We arbitrarily accepted a two-fold difference in RKPM value as significant and a 50%-99% increase (33-49% decrease) as a trend, provided the sum of the RKPM values was greater than 4 (i.e., evidence for a substantial number of reads).

Results and Discussion:

Using PCR array, we did not identify any difference between wild EV (gonadosomatic index, GSI = 3%) and PV eels (GSI = 0.2%) for some of the target genes associated with fatty acid metabolism (ongoing). However, a notable effect was seen for mRNA levels of monoacylglycerol (MAG) lipase, which were increased in EV eels compared to PV eels. Similarly, MAG lipase reads were more abundant in an EV ovarian transcriptome than in that from an experimental PV control (used as a proxy for a wild control). Transcript levels of a further four enzymes increased in EV compared to PV ovaries, and these enzymes were all associated with either the hydrolysis of acylglycerides or their synthesis – it is conceivable that the hydrolysis of acylglycerides by lipoprotein lipase, diacyl glycerol lipase and MAG lipase assists with the uptake of fatty acids for subsequent re-esterification and storage into acylglycerides and/or cholesterol esters (increased sterol O-acyltransferase 1 mRNA levels). Concurrently, mRNA levels of adipose triglyceride lipase and enzymes involved in fatty acid oxidation or phospholipid metabolism were reduced (two enzymes), or tended to be reduced (six enzymes). By comparison, 11KT treatment increased, or tended to increase mRNA levels for several of the enzymes involved in acylglyceride metabolism (c.f. PV vs EV), but the reductions in transcript abundance for genes involved in phospholipid metabolism (c.f., PV vs EV) were not evident.

Conclusion:

Our transcriptome data give tentative insights into the changes that occur in the growing eel oocyte as it accumulates lipid during oogenesis. Thus, the oocyte appears to activate a number of genes associated with

acylglyceride metabolism whilst seemingly reducing transcript abundance of several genes implicated in phospholipid metabolism, findings that can be partly induced by 11KT. As a result, the oocyte may be expected to stockpile neutral lipids, an observation in keeping with the presence of putative neutral lipid stores (lipid droplets) in mature eel eggs. We note that our transcriptome data stem from single observations, and assume here that the three fish used for the transcriptome analysis are representative for their respective groups – to ensure this is indeed the case, we are currently validating the transcriptome findings by in-house PCR array.

References:

[1] DIVERS, S.L., M^{c}QUILLAN, H.J., MATSUBARA, H., TODO, T., LOKMAN, P.M. 2010. Effects of reproductive stage and 11-ketotestosterone on LPL mRNA levels in the ovary of the shortfinned eel. J. Lipid Res., 51: 3250-3258.

[2] ENDO, T., TODO, T., LOKMAN, P.M., KUDO, H., IJIRI, S., ADACHI, S., YAMAUCHI, K. 2011. Androgens and very low density lipoprotein are essential for the growth of previtellogenic oocytes from Japanese eel, *Anguilla japonica*, in vitro. Biol. Reprod., 84: 816-825.

[3] WIEGAND, M.D. 1996. Composition, accumulation and utilization of yolk lipids in teleost fish. Rev. Fish Biol. Fisher., 6: 259-286.

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

GHRELIN AND KISSPEPTIN: TWO NOVEL ENDOCRINE MODULATORS OF OOCYTE MATURATION IN FISH

Shepperd E, Kerzman K and Unniappan S.,

Laboratory of Integrative Neuroendocrinology, Department of Biology, York University, Toronto, Ontario M3J 1P3, Canada. Fax: 1-416-736-5698; E-Mail: suraju@yorku.ca

Introduction:

Ghrelin is an orexigenic hormone that has been proposed to link the hypothalamo-pituitary-gonadal axis, thus integrating energy balance and reproduction in fish. Ghrelin stimulates luteinizing hormone secretion in fish. However, the role of ghrelin on gonadal physiology remains poorly understood. Similarly, kisspeptin is another recently discovered reproductive hormone and appears to have many important regulatory roles in mammals and non-mammalian vertebrates. Kisspeptin and its receptor have been found to play a role in regulating reproduction by acting locally in the gonads of mammals. Kisspeptin has been identified in several fishes and it was found to regulate reproduction by influencing the hypothalamic and pituitary derived reproductive hormones. However, whether kisspeptin has a direct role in regulating gonadal functions is yet to be determined. We hypothesized that both ghrelin and kisspeptin acts directly on the ovary of fish to regulate oocyte maturation. Our specific objectives were to characterize the presence of ghrelin, ghrelin receptor, kisspeptin and kisspeptin receptor in the ovary of goldfish and zebrafish, and to study the direct effects of ghrelin and kisspeptin on oocyte maturation in zebrafish.

Methods:

We used RT-PCR and fluorescence immunohistochemistry to detect the expression of mRNAs or proteins in the ovary, respectively. Quantitative PCR was used for assessing mRNA abundance in tissues. In vitro oocyte maturation assays on freshly collected oocytes from zebrafish were used to test the effects of ghrelin and kisspeptin on oocyte maturation. All primers for PCRs were designed based on native mRNA sequences. Mammalian antibodies were used for immunolocalization of ghrelin, kisspeptin and its receptors.

Results and Discussion:

We found ghrelin and ghrelin receptor mRNA expression in the ovaries of goldfish and zebrafish. Ghrelin receptor mRNA expression in the ovary was relatively lower during the sexually mature stages of goldfish. Ghrelin-like and ghrelin-receptor like immunoreactvity was found in the follicle cells in paraffin embedded cross sections of goldfish ovary. These results suggest that the ghrelin system is present in fish ovary and this peptide could contribute to the regulation of ovarian physiology. In agreement with this, we found that the incubation with native synthetic ghrelin at 50ng/mL and 100ng/mL concentrations significantly inhibited zebrafish basal oocyte maturation in vitro at 24 hours post-incubation. Ghrelin also inhibited maturation inducing hormone (MIH) stimulated oocyte maturation. This result indicates that ghrelin has an inhibitory role in zebrafish oocyte maturation. We also found kisspeptin and its receptor expressed in goldfish and zebrafish ovaries, suggesting its role in oocyte maturation. As expected, zebrafish synthetic kisspeptin-10 (zfKP-10) at 10 ng/mL and 100 ng/mL acted directly on oocytes to induce its maturation in vitro. In addition, zfKP-10 significantly increased luteinizing hormone receptor, follicle stimulating hormone receptor and 20beta-hydroxysteroid dehydrogenase mRNA expression in the oocytes. These results clearly indicate a direct role for kisspeptin in inducing oocyte maturation in zebrafish. It appears that kisspeptin affects local regulators of gonadal physiology to elicit its functions.

Conclusions:

Overall, our results provide several lines of supportive data for the presence of ghrelin and kisspetin systems in the ovaries of both zebrafish and goldfish. The presence of ghrelin and kisspeptin receptors in oocytes clearly suggest direct actions of both peptides on the gonadal tissue. While ghrelin has an inhibitory role on zebrafish oocyte maturation *in vitro,* kisspeptin has a prominent stimulatory role. It was also found that kisspeptin affects oocyte maturation by influencing the local effectors of ovarian functions. Collectively, our results for the first time indicate a direct role for ghrelin and kisspetin in the ovarian physiology of fish.

HORMONAL REGULATION OF *AQUAPORIN-1ab* IN *HETEROPNEUSTES FOSSILIS* OOCYTES *IN VITRO*

Acharjee A*. Chaube R°., Joy K. P*., Cerdà J+.

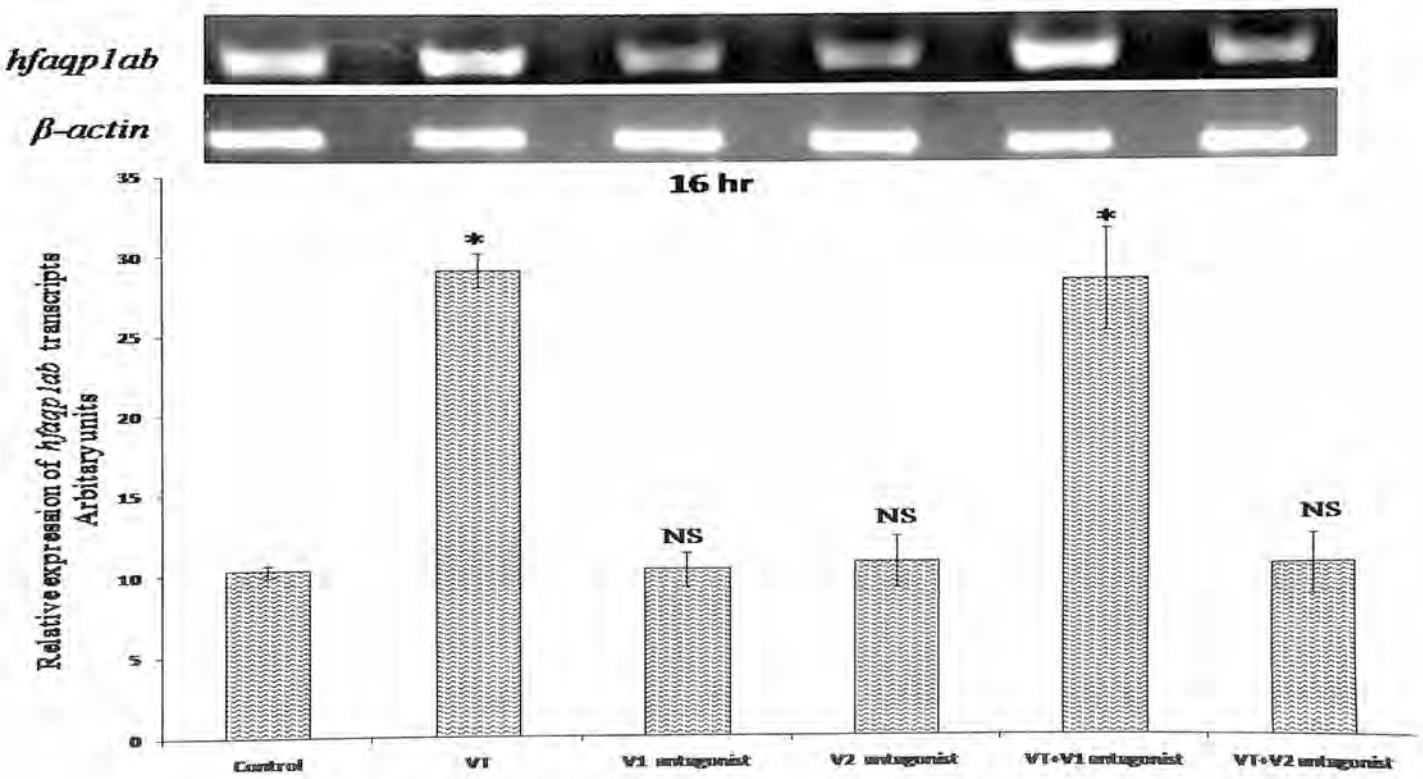
*Department of Zoology, Banaras Hindu University, Varanasi 221005, India
°Zoology Section, MMV, Banaras Hindu University, Varanasi 221005, India.
+IRTA-Institut de Ciències del Mar, CSIC, 08003 Barcelona, Spain.
Email chauberadha@rediffmail.com, kpjoy@bhu.ac.in, joan.cerda@irta.cat.

Introduction:

In teleosts, the maturing oocytes undergo swelling and a significant increase in volume due to water uptake prior to ovulation. This physiological process called oocyte hydration was first described in marine teleosts. Aquaporins (AQPs) are selective water or water and solute transporting membrane channels that have been remarkably conserved throughout evolutionary history [1]. In mammals, 13 AQP isoforms (AQP0-AQP12) have been identified [4]. The functions and physiological actions of AQPs have received extensive attention in humans and some other mammals. However, in non mammalian vertebrates such as fish, much less is known about AQP distribution, function and regulation [2]. The oocytes of the freshwater catfish *Heteropneustes fossilis* hydrate during hormone-induced meiotic maturation [5]. Recently, we cloned and characterized an ortholog of the teleost aquaporin-1ab in catfish (HfAqp1ab), whose transcripts were detected exclusively in ovary and brain, and followed a seasonal expression pattern [3]. In ovarian follicles, *hfaqp1ab* expression was stimulated during vasotocin (VT) and hCG-induced final oocyte maturation and hydration. In the present investigation, the response of VT was compared to that of 17α, 20β-hydroxy-4-pregnen-3-one (17,20βP, a maturation-inducing steroid in the catfish), and the type of VT receptor involved was investigated.

Methods:

Acclimatized catfish were sacrificed in the late prespawning phase by decapitation and ovaries were removed, weighed, and transferred into sterile culture plates containing incubation medium alone or with optimized doses of 17,20βP (1µg/ml), VT (Arg-8-oxytocin) acetate salt (100nM), and arginine-vasopressin



Expression of *hfaqp1ab* in oocyte after16 hours of incubation. The relative densitometric values of the PCR product bands from four gels are presented as mean ± SEM and analyzed by one way ANOVA followed by Tukeys' test (P<0.05)

V1 receptor antagonist deamino Pen[1], O-Me-Try[2], Arg[8] (10^{-6}M) and V2 receptor antagonist 1-adamantaneacetyl-O-Et-D-Try[2],Val[4],Abu[6], Arg[8,9] (10^{-6}M). The oocyte incubation protocol was that described by Singh and Joy [5]. Oocytes were sampled at 8, 16 and 24 h and processed for *hfaqp1ab* gene expression by semi-quantitative RT-PCR.

Results and Discussion:

The expression of *hfaqp1ab* increased when the ovarian follicles were incubated with VT or 17,20βP in relation to the control groups. The expression was higher after 16 h of incubation with VT and resulted in higher transcript levels than with 17,20βP. At 24 h, the expression decreased to the control levels. However, when the follicles were incubated with both VT and 17,20βP no synergistic effect was found, rather the expression was low and inverse with the time period, suggesting an early saturation of the transcriptional process by the combined stimulus. The V1 type receptor antagonist did not inhibit the VT-induced *hfaqp1ab* gene expression, rather this was enhanced at 8 h or was unchanged at 16 and 24 h. (See Fig) In contrast, co-incubation of VT with the V2 receptor antagonist inhibited the induction of *hfaqp1ab* expression by VT, suggesting that the V2 type receptor may be involved in the mechanism of VT-mediated oocyte hydration. Thus, the V2 receptor may control the expression of HfAqp1ab during oocyte hydration, similarly as it occurs for the vasopressin-dependent mammalian AQP2 which is regulated via the V2 type receptor. This finding strengthens the hypothesis that although *hfaqp1ab* belongs structurally to the AQP1 subfamily of water channels, it shows functional similarities with amphibian AQP-h2 and Japanese quail and mammalian AQP2 [3]. The V1 type receptor, which activates the IP3-PKC pathway, may not be directly involved in oocyte hydration, or the stimulation of *hfaqp1ab* gene expression may be consequential to the suppression of the IP3-PKC pathway triggering some yet unknown mechanism.

Conclusion:

These present results indicate that VT stimulates *hfaqp1ab* gene expression in catfish ovarian follicles through the V2 receptor type, being more effective than 17,20βP.

References:

[1]CARBREY, J.M., AGRE, P., 2009. Discovery of the aquaporins and development of the field in aquaporins. In: Beitz, E. (Ed.), Handbook of Experimental Pharmacology, vol. 190. Springer-Verlag, Berlin-Heidelberg, pp. 3–28.

[2]CERDÀ, J., FINN R.N. 2010. Piscine aquaporins: an overview of recent advances. J. Exp. Zool., 313A: 623–650.

[3]CHAUBE, R. , CHAUVIGNÉ, F.,TINGAUD-SEQUEIRA, A., JOY, K.P., ACHARJEE A., SINGH, V., CERDÀ, J., 2011 Molecular and functional characterization of catfish *Heteropneustes fossilis* aquaporin-1b: Changes in expression during ovarian development and hormone-induced follicular maturation. Gen. Comp. Endocrinol., 170: 162–171.

[4]KING L. S., KOZONO D., AGRE, P. 2004. From structure to disease: the evolving tale of aquaporin biology. Nat. Rev. Mol. Cell Biol., 5: 687–698. .

[5]SINGH, V., JOY, K.P., 2010. An involvement of vasotocin in oocyte hydration in the catfish *Heteropneustes fossilis*: a comparison with effects of isotocin and hCG. Gen. Comp. Endocrinol.,166: 504–512.

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

EFFECTS OF DIETARY LIPID LEVELS ON SEXUAL MATURATION AND FECUNDITY IN FEMALE ATLANTIC COD (*GADUS MORHUA*)

Bogevik, A.S.*, Karlsen, Ø., Andersson, E., Thorsen, A., Hamre, K.[a], Rosenlund, G.[b] & Norberg, B.

Institute of Marine Research, 5392 Austevoll, Norway.
[a]National Institute for Nutrition and Seafood Research (NIFES), Bergen, Norway
[b]Skretting Aquaculture Research Centre, Stavanger, Norway
*Corresponding author andre.bogevik@imr.no

Introduction:

Atlantic cod (*Gadus morhua*) are determinate spawners where the standing stock of advanced or vitellogenic oocytes present prior to spawning equals the number of egg spawned, defined as the potential fecundity [1]. Maturation, time of spawning and spawning success are, however, influenced by the food availability and body condition. Skipped spawning may occur if the availability of food is low. The reserves and availability of protein and lipid are thus important for gonadal maturation in cod. Increased dietary lipid levels have previously also been shown to increase gonad size at the time of spawning [2]. However, the biological mechanism and endocrine control of this regulation are not known. An experiment was therefore started prior to maturation in first-time spawning Atlantic cod to investigate the effect of dietary fat levels on endocrine regulation and potential fecundity throughout maturation and the spawning season.

Methods:

Eighteen hundred yearling Atlantic cod (local breeding strain; Institute of Marine Research, Austevoll Research Station, Norway) were distributed in equal numbers in six net pens in June 2009. The fish were fed in triplicate with either a low fat (LF; 130 g kg^{-1}) or a high fat (HF; 200 g kg^{-1}) diet produced by Skretting ARC (Stavanger, Norway). Length, weight, liver somatic index (HSI) and gonadosomatic index (GSI) were recorded, and blood plasma and ovaries were sampled from 15 females at the start in June and from 5 females from each net pens once a month from September 2009 until May 2010. Blood plasma and ovarian tissue were stored at -80 °C prior to analysis of plasma sex steroids and vitellogenin, and ovarian gene expression of StAR and P450aromatase. Histological samples of ovaries were stored on 3.6% buffered formaldehyde (29.5 mM $NaH_2PO_2 \cdot H_2O$ and 460 mM $Na_2HPO \cdot H_2O$) for at least 14 days before measurements of egg size and calculation of potential fecundity according to Thorsen *et al.* [3].

Results and discussion:

The fish were in good condition at the start of the experiment in June, with a condition factor at 1.1 and a liver somatic index (HSI) at 9.6% of the body weight. This was a good indication that the fish would mature and spawn in the following season. The fish were fed daily to satiation with the respective diets. Although the LF diet had a lower energy level compared with commercial diets normally used, the incidence of maturation was close to 100%, most likely due to the overall availability of food and the natural light condition [2]. The fish grew from 310 g in June 2009 to 1336 g in February 2010, at the start of the spawning season. There were some minor, although significant, weight differences between the dietary treatments in October and December 2009, and January 2010, with a higher mean sampling weight in the HF group compared to the LF group (one-way ANOVA, $P<0.01$). This was followed by a loss of body weight in the spawning season to an average weight at 1044 g in April 2010. The same trends were also seen for condition factor and HSI, with increasing condition up to spawning and thereafter a drop (especially in the LF group), and minor differences between the dietary treatments. Fish fed the HF diet had a higher HSI compared to the LF fed fish through the spawning season, fluctuating from 12 to 11%, while the LF group had a drop from 11 to 9%. This indicates that the depleted lipid reserves in the LF group were compensated by reduction in liver size (increased catabolism). Recruitment to a new spawning season could be seen through increased gonadosomatic index (GSI), occurrence of previtellogenic oocytes and steroidogenesis. Presence of increasing plasma testosterone levels in October in the present experiment, was a strong indication of maturation. Testosterone is converted to estradiol-17β (E2), which is released and transported to the liver where it induces vitellogenin (VTG) production. The testosterone levels in blood plasma showed a gradual increase throughout the autumn 2009 with highest levels in December, followed by a drop in January 2010. The plasma levels of E2 increased gradually until February, where the highest levels were recorded. The HF group showed significantly higher mean E2 levels (4.0 and 15.5 ng E2 mL^{-1} plasma) than the LF group (2.5 and 9.3 ng E2 mL^{-1} plasma) in December 2009 and February 2010, respectively. Plasma VTG levels started to increase in October, with the steepest increase from November (2 mg mL^{-1}) to December (4-5 mg mL^{-1}). Dietary effects were only seen in December, where significantly higher

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

VTG levels were found in the fish fed the HF diet compared to fish fed the LF diet. The increasing levels of steroid hormones were followed by increasing GSI from September 2009, with a high peak at spawning in February. GSIs were higher in the HF group than in the LF group in the months of October 2009 to January 2010, although only significantly different in December 2009 (one-way ANOVA, $P<0.03$).

Conclusion:

In conclusion, an increased level of dietary lipid given to Atlantic cod prior to and during maturation stimulated steroidogenesis and increased the recruitment of eggs, resulting in higher fecundity. Individual data for calculation of potential fecundity will be presented on the conference.

References:

[1]Kjesbu, O.S., Klungsoyr, J., Kryvi, H., Witthames, P.R., Walker, M.G., 1991. Fecundity, atresia, and egg size of captive Atlantic cod (*Gadus morhua*) in relation to proximate body-composition Can. J. Fish. Aquat. Sci., 48: 2333-2343.

[2]Karlsen, Ø., Hemre, G.I., Tveit, K., Rosenlund, G., 2006. Effect of varying levels of macro-nutrients and continuous light on growth, energy deposits and maturation in farmed Atlantic cod (*Gadus morhua* L.). Aquaculture, 255: 242-254.

[3]Thorsen, A., Kjesbu, O.S., 2001. A rapid method for estimation of oocyte size and potential fecundity in Atlantic cod using a computer-aided particle analysis system. J. Sea Res., 46: 295-308.

EFFECTS OF α-METHYLPARATYROSINE, A CATECHOLAMINE INHIBITOR ON OVARIAN STEROID HORMONE LEVELS IN THE CATFISH *HETEROPNEUSTES FOSSILIS*

Singh V., Chaube R.*, Chourasia T. K., and Joy K. P.#

#Department of Zoology, Banaras Hindu University, Varanasi-221005, India. e-mail: kpjoy@bhu.ac.in
*Zoology Section, Mahila Mahavidyalaya, Banaras Hindu University, Varanasi-221005, India. e-mail: chauberadha@rediffmail.com

Introduction:

In higher vertebrates like mammals, the ovary elicits high catecholaminergic activity that originates either from extrinsic sympathetic innervation or intrinsic catecholaminergic system [1]. Ovarian innervation by the autonomic nervous system (ANS) has been described in a few teleosts and the innervations pattern shows considerable species variation [2]. Recent, investigation from our laboratory has demonstrated that the catfish ovary is innervated by seven pairs of nerves, originating from the paired sympathetic chain lying dorsal to the posterior kidney. Further we demonstrated tyrosine hydroxylase (TH, the rate limiting enzyme in catecholamine synthesis) and catecholamine seasonal activities in the ovary of the catfish during the annual reproductive cycle [3, 4] implying a functional role in ovarian gametogenesis and ovulation. Previous studies have shown that steroid hormone secretion is regulated by catecholamines [5]. However, investigations on the direct role of catecholamines in regulating ovarian steroid hormone levels are lacking. Since surgical denervation of the ovary, like in mammals is difficult. A pharmacological blocker to inhibit catecholaminergic activity was employed to investigate the role of catecholamines in ovarian function. The methylated derivative of tyrosine, α-methyl paratyrosine (α-MPT) is a competitive inhibitor of TH. This drug has been widely used as a pharmacological tool to investigate the CA metabolism [6]. In the present study, post vitellogenic follicles were incubated in vitro with α-MPT and steroid hormone levels were measured.

Materials and Methods:

The study was conducted during preparatory (March) and prespawning (June) phases. The acclimatized fish were sampled for the collection of ovaries, weighed and transferred into a sterile petri dish containing freshly cooled incubation medium. The ovary pieces (about 350 mg each) were rinsed and transferred to culture plates containing 5ml medium each with α-MPT (250μg/ml), L-DOPA (1μg/ml) and hCG (20IU/ml) alone or in co-incubation. In co-incubation groups, ovaries were preincubated with α-MPT (250μg/ml) for 3 hr and then co-incubated with L-DOPA (1μg/ml), hCG (20IU/ml) or L-DOPA+ hCG (1μg/ml+ 20IU/ml) at 22°C for 12 or 24 hr. All incubations were done in triplicate. The medium was changed after every 4 hr and collected group-wise. After completion of the incubation, the tissues along with the incubation medium were collected separately and processed for steroid measurement by HPLC/ELISA, as described by Singh and Joy, [7]. Control groups (plain medium and medium containing vehicle) were set up in parallel. Data were presented as mean ± SEM and analysed by two-way ANOVA, followed by Newman Keuls' test.

Results and Discussion:

In vitro incubations of ovarian pieces with α-MPT, L-DOPA and hCG, alone or in co-incubation, produced an overall significant effect on ovarian steroids. In the preparatory phase, the incubation with α-MPT decreased significantly both E_2 and P_4 in a time-dependent manner. The incubations with L-DOPA and hCG significantly increased both E_2 and P_4 in a duration- dependent manner, and the effect was higher in the hCG group. In co-incubation studies (α-MPT + L-DOPA, α-MPT + hCG and α-MPT+L-DOPA + hCG), L-DOPA or hCG reversed the inhibitory effect of α-MPT and restored the levels to that of the control or even higher but lower than that of L-DOPA or hCG per se. In the preparatory phase 17-P and17, 20β- dihydroxy-progesterone were not detectable. The incubations with L-DOPA and hCG decreased E_2 significantly, the inhibition was higher at 24 hr. The co-incubations with α-MPT reduced significantly the inhibitory effect of L-DOPA or hCG, and the response was higher in the 12 hr groups. α-MPT decreased P_4, 17-P and 17, 20β-dihydroxy-progesterone levels in a time-dependent manner. The incubations with L-DOPA and hCG increased significantly P_4, 17-P and 17, 20β-DP in a time- dependent manner. In the co-incubation groups (α-MPT + L-DOPA, α-MPT + hCG and α-MPT + L-DOPA + hCG), the inhibitory effect of α-MPT was reversed and progestin levels increased significantly but lower than that of the L-DOPA and hCG groups. The increase was higher in the α-MPT + L-DOPA + hCG group.

Conclusion:

Our investigation suggests that α-MPT and L-DOPA modulated in vitro ovarian steroid hormone production, influencing gametogenesis and ovulation and maturation of oocytes.

References:

[1]OJEDA, S., DISSEN, G.A., 1998. Ovarian innervation. In: Knobil, E., Neill, J.D. (Eds.),

Encyclopedia of Reproduction, Vol 3, Academic Press, California, pp. 583-589.

[2]OHSHIMO, S., UEMATSU, K. 1996. Histological study on the autonomic innervtion of the stomach and ovaries of the Nile tilapia *Oreochromis niloticus*. Fish. Sci., 62: 196-203.

[3]CHOURASIA, T.K., CHAUBE, R., SINGH, V., JOY, K.P., 2010. Annual and periovulatory changes in tyrosine hydroxylase activity in the ovary of the catfish *Heteropneustes fossilis*. Gen. Comp. Endocrinol., 166: 111–116.

[4]SINGH, V., CHAUBE, R., CHOURASIA, T. K., JOY, K. P., 2010. Temporal and periovulatory changes in ovarian catecholamines in the catfish *Heteropneustes fossilis*. Gen. Comp. Endocrinol., 168: 46-54. 5

[5]CHAUBE R, JOY KP. 2002. Effects of ovariectomy and oestradiol-17beta replacement on brain tyrosine hydroxylase in the catfish *Heteropneustes fossilis:* changes in invivo activity and kinetic parameters. J Endocrinol., 175:329-42.

[6]SENTHILKUMARAN, B., JOY, K.P., 1995. Changes in hypothalamic catecholamines, dopamine-beta-hydroxylase and phenylanolamine-N-methyltranserase in the catfish *Heteropneustes fossilis* in relation to season, raised photoperiod and temperature, ovariectomy and estradiol-17β replacement. Gen. Comp. Endocrinol., 97: 121-134.

[7]SINGH, V., JOY, K. P., 2009. Relative in vitro seasonal effects of vasotocin and isotocin on ovarian steroid hormone levels in the catfish *Heteropneustes fossilis*. Gen. Comp. Endocrinol., 162: 257-264.

ROLE OF CATECHOLESTROGENS ON IN VITRO PROSTAGLANDIN SECRETION IN OVARIAN FOLLICLES OF THE CATFISH *HETEROPNEUSTES FOSSILIS*

Chourasia T. K., and Joy K.P.

Department of Zoology, Banaras Hindu University, Varanasi-211005, India
email: tkchourasia@gmail.com; kpjoybhu@gmail.com

Introduction:

In teleosts, prostaglandins (PGs) have been implicated with oocyte final maturation, steroidogenesis and sexual behaviors. Its synthesis is modulated by gonadotropin, progestins or other steroids [1]. Catecholestrogens are estrogen metabolites, which have been implicated in reproductive functions such gonadotropin release, ovarian follicular steroidogenesis, prostaglandin synthesis, parturition, and embryo implantation [2]. Recently, catecholestrogens and estrogen-hydroxylases were characterized in the Indian catfish ovary with seasonal and periovulatory changes [3, 4]. Hydroxyestrogens have been shown to stimulate final oocyte maturation and ovulation by stimulating the secretion of the maturation-inducing steroids 17, 20β-dihydroxy-pregne-3-one production [5]. A direct role of catecholestrogens on PG production was demonstrated in higher animals, 2-hydroxyE_2 stimulated PGF production more than E_2 [6]. Present study demonstrates the role of 2-hydroxyE_2 on ovarian PG secretion in the catfish. Additionally, the pattern of secretion of the two PGs (PGE_2 and $PGF_{2\alpha}$) was monitored during the annual reproductive cycle of the catfish and during hCG-induced oocyte final maturation and ovulation.

Methods:

Five adult female fish (40-50g) each from preparatory, prespawning, spawning, postspawning and resting phases were sacrificed and ovaries were sampled. In the spawning phase, catfish (40-60g) was injected with 100 IU hCG/fish intraperitoneally or with an equal volume of vehicle (0.7 % NaCl) and sampled at 0, 8, 16 and 24 hr post injection for extraction and quantification of prostaglandins. In the prespawning phase, about 200 mg ovary pieces in triplicate from each fish (n = 3) were incubated with 5 ml of incubation medium containing 1, 5 or 10 IU hCG/ml or 1, 10, 100 and 1000nM of E_2 or catecholestrogens (2-hydroxyE_2 and 2-methoxyE_2) for 0, 8, 16 and 24 hr. In the second study, ovary pieces were incubated with 5 ml medium with or without 10nM, 100nM or 1μM each of phentolamine (α-adrenoceptor blocker), propranolol (β-adrenoceptor blocker), or tamoxifen (estrogen receptor blocker) alone or in combination with 1μM 2-hydroxyE_2. Control groups (plain incubation medium containing the vehicle) were run in parallel. After each 4 hr, the incubation medium was changed and collected, and replenished with fresh medium containing the respective hormone concentrations. The tissue and media were processed for extraction of PGs using a solid phase extraction method and quantified by HPLC method, as described by Jayadeep et al [7].

Results and Discussion:

The levels of both PGE_2 and $PGF_{2\alpha}$ increased significantly during ovarian recrudescence and peaked in the spawning phase. In vivo and in vitro, hCG stimulated PG production with peak secretion at 16 hr coinciding with oocyte final maturation and ovulation. The catfish ovary possesses the ability to convert estrogens into hydroxyestrogens and further into methoxyestrogens [3]. A concentration- or duration-dependent increase in PGs was noticed when follicles were incubated with E_2, 2-hydroxyE_2 and 2-methoxyE_2. 2-hydroxyE_2 was more potent than the other steroids. Phentolamine and propranolol (α-and β-antagonists, respectively) did not produce any significant change on basal PG levels but the incubation with tamoxifen lowered the PG levels. The pre-incubation of the follicles with tamoxifen, phentolamine and propranolol resulted in the inhibition of the stimulatory effect of 2-hydroxyE_2 on PGE_2 and $PGF_{2\alpha}$. Phentolamine (α-adrenergic blocker) was more effective, followed by tamoxifen and propranolol (β-adrenergic blocker) in inhibiting the 2-hydroxyE_2 effect.

Conclusion:

In conclusion both $PGF_{2\alpha}$ and PGE_2 showed significant seasonal variation and periovulatory changes. Catecholestrogens increased the PG secretion more than E_2. The 2-hydroxyE_2-induced stimulation appeared to be modulated through estrogen and catecholamine receptors.

References:

[1]GOETZ, F., BERNDTSON, A., RANJAN, M. 1991. Ovulation: Mediators at the ovarian level. In: Vertebrate Endocrinology: Fundamentals and Biomedical Implications, (Pang P., Schreibman M. (Eds.)), pp. 127-203, Academic Press, San Diego, California.

[2]SPICER, L.J., HAMMOND, J.M. 1989. Regulation of ovarian function by catecholestrogens: current concepts. J. Steroid Biochem., 33, 489-501.

[3]MISHRA, A., JOY, K. P. 2006. HPLC-electrochemical detection of ovarian estradiol-17β and cateholestrogens in the catfish *Heteropneustes fossilis*: seasonal and periovulatory changes. Gen. Comp. Endocrinol., 145, 84-91.

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

[4]CHOURASIA, T.K., JOY, K.P. 2010. Seasonal variation in tissue estrogen-2/4-hydroxylases (EH) and in vitro effects of steroids on ovarian EH activity in the catfish *Heteropneustes fossilis*. Steroids, 75, 1097-1105.

[5]MISHRA, A., JOY, K. P. 2006. Effects of gonadotrophin in vivo and 2-hydroxyestradiol-17β in vitro on follicular steroid hormone profile associated with oocyte maturation in the catfish *Heteropneustes fossilis*. J. Endocrinol., 189, 341-353.

[6]KELLY, R.W., ABEL, M.H., KENNEDY, R.L., TAYLOR, P.L. 1983. Effects of catechol estrogens on the synthesis and metabolism of prostaglandins. In:, Catechol Estrogens, (Merriam, G.R., Lipsett, M.B. (Eds.)), pp. 203-214, Raven Press, New York

[7]VAN ROLLINS, M., HO, S.H.K., GREENWALD, J.E., ALEXANDER, M., DORMMAN, N.J., WONG L.K. HORROCKS L.A., 1980. Complete separation by high performance liquid chromatography of metabolites of arachidonic acid from incubation with human and rabbit platelets. Prostaglandins, 20, 571-575.

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

POSSIBLE MEDIATORY ROLE OF TUMOR NECROSIS FACTOR ALPHA (TNF ALPHA) IN TROUT PREOVULATORY FOLLICLES: EFFECTS ON PREPARATORY EVENTS LEADING TO OVULATION

Crespo D. and Planas J. V.

Department of Physiology, School of Biology, University of Barcelona and Institute of Biomedicine of the University of Barcelona (IBUB), Diagonal 645 08028 Barcelona, Spain
Fax: +34-93-4110358 email: dcrespo@ub.edu

Introduction:

Ovulation, which is triggered by a surge of luteinizing hormone (LH), is a complex process leading to the release of the mature oocyte from the ovarian follicle. Several preparatory events must occur prior to the expulsion of the oocyte including follicle contraction, follicle separation and follicle rupture. In line with the notion that ovulation is an inflammatory-like process in mammals, we studied the mechanisms involved in the control of ovulation during the preovulatory period in a teleost fish, the brown trout (*Salmo trutta*). Specifically, we have tested the hypothesis that the pro-inflammatory cytokine tumor necrosis factor alpha (TNF alpha) could affect ovarian function and act as mediator of the effects of LH in preovulatory follicles.

Methods:

For the follicle contraction experiments, preovulatory follicles punctured using a 25-gauge hypodermic needle were incubated in Hank´s balanced salt solution (HBSS) containing 0.2% BSA (HBSS-BSA) in 6 cm culture dishes (10 follicles/4 ml) in the presence of the test compounds for 16 h at 15°C under shaking conditions (100 rpm). Follicle contraction was determined by measuring the weight of the 10 follicles in each replicate after incubation, as previously described and validated [1, 2]. Since contraction results in the expulsion of yolk through the puncture site, a decrease in follicle weight indicates an increase in follicle contraction. We also removed and stored at -20°C the culture medium at the termination of the incubation period to determine by enzyme immunoassay the *in vitro* prostaglandin (prostaglandin F2 alpha, PGF2 alpha) production. To collect ovarian tissue for RNA extraction and gelatinase/collagenase activity determination, preovulatory follicles from each of a total of three-five females were incubated (20 follicles/5 ml in triplicate) in HBSS-BSA in the absence or presence of test compounds, at 15°C for 16 h with gentle shaking (100 rpm). At the end of the incubation follicles were removed, flash frozen in liquid nitrogen and stored at -80°C until assayed.

Results and Discussion:

We examined the *in vitro* effects of coho salmon LH (sLH) on trout preovulatory ovarian follicles and showed that sLH significantly increased follicle contraction and that this effect was blocked by indomethacin (a prostaglandin synthesis inhibitor) and TAPI-1 (an inhibitor of TNF alpha-converting enzyme or TACE/ADAM17, that blocks TNF alpha secretion). Furthermore, sLH treatment increased the expression of *Tnf alpha*, *Tace/Adam17* and prostaglandin synthase 1 and 2 (*Cox-1* and *Cox-2*) in the trout follicle, as well as the production of PGF2 alpha into the culture medium. Interestingly, sLH increased gelatinase/collagenase activity in the trout follicle. We also used a salmonid-specific microarray platform (GEO GPL10706) and observed that sLH induced the expression of genes known to be involved in inflammation, proteolysis and tissue remodeling. In order to further study the possible involvement of TNF alpha as a mediator of sLH in the trout ovary, we incubated trout preovulatory follicles in the presence of recombinant trout TNF alpha (rtTNF alpha). First, rtTNF alpha caused a significant increase in follicle contraction and indomethacin blocked this effect, suggesting a possible involvement of prostaglandins in rtTNF alpha action. Second, rtTNF alpha stimulated the expression of *Cox-1* and *Cox-2*. Third, rtTNF alpha stimulated the *in vitro* production of PGF2 alpha. Fourth, rtTNF alpha stimulated gelatinase/collagenase activity. Interestingly, PGF2 alpha directly stimulated follicle contraction, confirming the results from other studies, as well as gelatinase/collagenase activity.

Conclusion:

In view of these results we propose that TNF alpha may be a potential mediator of the effects of LH in the ovulatory process in trout probably through its stimulation of the production of PGF2 alpha. Specifically, TNF alpha could have an important role in the biomechanics of follicle weakening, ovarian rupture and oocyte expulsion during ovulation in teleosts.

References:

[1]GOETZ, F.W., BRADLEY, J.A. 1994. Stimulation of in vitro ovulation and contraction of brook trout (*Salvelinus fontinalis*) follicles by adrenaline through alpha-adrenoreceptors. J. Reprod. Fertil., 100(2): 381-385.

[2]HSU, S.Y., GOETZ, F.W. 1992. The effects of E and F prostaglandins on ovarian cAMP production and follicular contraction in the brook trout (*Salvelinus fontinalis*). Gen. Comp. Endocrinol., 88(3): 434-443.

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

PATTERN OF OOCYTE GROWTH, PLASMATIC VITELLOGENIN, STEROID AND CORTISOL CONCENTRATIONS OF THE PACIFIC RED SNAPPER (*LUTJANUS PERU*)

Dumas S.[1], Ramírez Luna S.[1]*, Mañanos E.[2], Pintos-Terán P.[3], Vázquez Bouccard C.[4], Tort, L.[3], Rodríguez Jaramillo C.[4]

[1] Centro Interdisciplinario de Ciencias marinas, Ave. IPN s/n La Paz, B.C.S., CP 23096 Mexico; [2] Instituto de Acuacultura de Torre de la Sal C/ Ribera de Cabanes, sn. Cabanes. Castellón. E-12595, Spain ; [3] Centro de Investigaciones Biológicas del Noroeste, Mar Bermejo 195, Col. Playa Palo de Santa Rita, La Paz, BCS CP. 23096, Mexico; [4] Departamento de Biología Celular y Fisiología. Facultad de Ciencias. Universidad Autónoma de Barcelona. 08193. Cerdany-ola, Barcelona, Spain

Introduction:

Pacific red snapper is distributed from Baja California (Mexico) to Peru. It inhabits offshore waters to at least 90 m depth (Allen and Robertson, 1994). In Mexico, the Pacific red snapper is an important catch in artisanal fisheries. Reproductive activity has been evaluated at different localities in Mexico (Reyna-Trujillo, 1993; Santamaria-Miranda, 1998; Lucano-Ramirez, 2001) but little is known about its reproductive physiology.

Methods:

Females were sampled monthly from April to September by hook and line in the Bahía de la Paz, BCS, Mex. Blood samples were taken immediately after capture by caudal or cardiac puncture and centrifuged at 1500 g at 4°C. Plasma was stored with PMSF 1 mM at -80°C (Mañanós et al., 1994). Gonads were kept in Davidson solution for 24-48 h and then transferred to 70 % ethanol and processed by conventional histological methods. Gonadal maturation was assessed by evaluating the proportion of oocytes at different developmental stages. Plasma vitellogenin (VTG) levels were analysed using a newly developed specific ELISA for L. peru VTG (Ramirez-Luna, unpublished results). Plasma levels of estradiol (E2) and testosterone (T) were determined by ELISA and cortisol (C) by radioinmuno assay.

Results:

The gonadosomatic index (GSI), T, and VTG levels were significantly high in July and correlated with vitellogenic and postvitellogenic (GVB) females. In May, July, and September, 50 % of the females were in the vitellogenic stage while August and September were characterized by the presence of vitellogenic and postvitellogenic females. In these months, hydrated oocytes and post ovulatory follicles (POF) were also observed. The levels of E2 and cortisol in September were significantly higher than that in April and May.

Conclusion:

These observations definitely characterized the Pacific red snapper as a multiple spawner.

Vol. 4 No.,S 8 (August 2011) ISSN: 0974- 6846

LIPID STOCK-PILING IN THE GROWING OOCYTE – ARE ANDROGENS DRIVING LIPID DELIVERY TO SUSTAIN THE FUTURE EMBRYO OF THE EEL, *ANGUILLA AUSTRALIS*?

Forbes, E.L. and Lokman, P.M

Department of Zoology, University of Otago, 340 Great King Street, Dunedin 9016, New Zealand. Fax +64-34797584, e-mail: forer069@student.otago.ac.nz

Introduction:

As a prerequisite to producing viable healthy offspring, female fish need to provision their eggs with yolk proteins and lipids. Relatively little is known about the mechanisms that regulate lipid accumulation, despite key roles for lipids in (marine) teleosts in the context of egg quality. The liver is known to first package lipids in lipoprotein complexes made up chiefly of triacylglycerides (TAGs) and apo(lipo)proteins, especially apolipoprotein-B (apo-B). The inherently hydrophobic lipids can accordingly be easily transported through the blood stream for uptake elsewhere in the body. Lipid uptake is greatly dependent on activity of the enzyme lipoprotein lipase (LPL) – this enzyme, associated among others with adipose tissue, can catalyze the cleavage of free fatty acids from TAGs contained within very low density lipoproteins (VLDL) [1]. We recently identified a key role for the androgen 11-ketotestosterone (11-KT) in increasing LPL mRNA levels in the eel ovary [2]. Furthermore, incubation of eel ovarian tissue in the presence of VLDL and 11-KT resulted in histologically-detectable increases in accumulated lipids in the cytoplasm. Are the androgen-mediated increases in lipid accumulation associated with increases in lipid packagers, the apoproteins? To address this question, we artificially elevated the serum level of 11-KT in female eels in a time course experiment and measured hepatic transcript abundance of genes involved in lipid packaging, namely apob and microsomal triglyceride transfer protein, mttp. We also evaluated the effects of 11-KT treatment on expression of the VLDL receptor (vldlr) in the ovary.

Methods:

Experimental fish - Seventy eight eels were captured in fyke nets in Lake Ellesmere (South Island, New Zealand) and transported to Dunedin (Department of Zoology, University of Otago) where they were held in aquaria (6 eels / 200 L) at ambient autumn photoperiod and temperature for one week and allowed to acclimate. All eels, with the exception of six initial controls, were anesthetised (0.15% benzocaine) and implanted intraperitoneally with slow release pellets containing either cholesterol (placebo controls) or 1 mg of 11-KT. Eels were then separated into the treatment groups and placed back into aquaria until terminal sampling at 3 hrs, 9 hrs, 27 hrs, 81 hrs, 243 hrs and 729 hrs after implantation. At each sampling, six controls and six androgen-treated fish were euthanised (0.30% benzocaine). During sampling, length and total fish weight, as well as liver and ovary weights, were recorded, blood samples collected, and fragments of the liver and ovary were snap-frozen. Wild fish – twenty four eels in the previtellogenic (PV) or early vitellogenic (EV) stage were captured in fyke nets in Lake Ellesmere and immediately euthanised. Sampling techniques and tissue collection was identical to that for experimental fish. Analytical techniques - apob, mttp and vldlr transcript abundance were quantified using real-time PCR and serum 11-KT levels were measured using radioimmunoassay.

Results and Discussion:

Significant increases in hepatosomatic (60%) and gonadosomatic indices (300%) were seen in both 11-KT-treated and wild-caught migratory eels compared to their respective controls. The levels of circulating 11-KT in the serum were successfully raised to levels (150-200 ng/ml) crudely comparable to those found in wild caught-migratory animals (50-80 ng/ml) and maintained throughout the duration of the experiment. Changes in *apob* and *mttp* mRNA levels between control and 11-KT treated fish were evident by 3 h of treatment; 11-KT-treated fish had significantly higher transcript abundances for both genes, reflecting the differences seen between wild-caught feeding PV and migratory EV eels, respectively. In contrast, 11-KT treatment had no effect on *vldlr* mRNA levels, although there were significant differences between PV (8.64) and EV eels (14.30).

Conclusion:

This study demonstrates that 11-KT has the ability, at least in part, to increase the hepatic packaging of lipid into lipoproteins by increasing *apob* and *mttp* mRNA levels. This response to androgen is further reinforced by a notable increase in liver weight. However, 11-KT did not affect *vldlr* transcript abundance, suggesting that increases in accumulated lipids are probably not driven by increases in uptake of VLDL and its associated TAGs; instead, it reinforces the importance of LPL as a major mediator of such uptake.

References:

[1]ECKEL, R.H. 1989. Lipoprotein lipase. A multifunctional enzyme relevant to common metabolic diseases. N. Eng. J. Med., 322(7).

[2]DIVERS, S.L., MCQUILLAN, H.J., MATSUBARA, H., TODO, T., LOKMAN, P.M. 2010. Effects of reproductive stage and 11-ketotestosterone on LPL mRNA levels in the ovary of the shortfinned eel. J. Lipid Res., 51.

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

ROLE OF KISSPEPTIN ON GONADAL MATURATION OF STRIPED MURREL, *CHANNA STRIATUS*

Francis T., Rajagopalsamy C.B.T., Jeyakumar N., Venkatasamy M. and Archana Devi C.

Department of Fisheries Biology& Resource Management, Fisheries College and Research Institute, Thoothukudi, 628008, India.
Fax: 0461 2340574, Email: t_franciz2000@yahoo.com

Introduction:

The use of kisspeptin for gonadal maturation and breeding of fish is more important in aquaculture industry. To make an alternative of other inducing agents, standardization of protocol for the use of kisspeptin is very important. Kisspeptin, the peptide products of the kiss- I gene were identified in 2001 as natural ligands orphan G protein coupled receptor, GPRSH. They include, among others, metastin and kisspeptin – 10. In the last two years, kisspeptin have been demonstrated as very potent stimulators of the gonadotropic axis in a number of species and through different routes of administration. In addition, the Kiss – 1 /GPR 4 system has been proven as an essential gatekeeper of GnRH neurons involved in their activation at puperty and their regulation by gonadal steroids. The role of kisspeptin on gonadal maturation of Striped Murrel, *Channa Striatus* was carried out in the present study.

Methods:

Striped murrel, *Channa striatus* with body weight ranging from 750 to 850 g were collected from culture pond/rivers and then stocked in circular cement tanks (4m in length, 2 m in diameter and 1.5m in length). The fishes were maintained at ambient photoperiod (12L: 12D) and temperature that fluctuated from 29 and 32^0 C and fed with cooked chicken meat. The male and female fish were stocked in separate tanks. After Acclamatization, the male and female fish were injected with kisspeptin -10 at the concentration of 0.01 and 0.05 µg/gram body weight respectively. Before injection, the fish were anaesthetised one by one in a 0.1% solution of benzocaine. Following anesthesia the male and female fish were injected with kisspeptin at different concentration (0.01 µg/gram and 0.05 µg/gram body weight). Monthly sampling of gonad from injected and control fish were carried out to assess the matur ation. Levels of testosterone in male and estradiol in female fish serum were measured following enzyme linked immunosorbent assay kit.

Result and discussion:

Gonadal development, Gonadosomatic Index (GSI), histology and level of serum steroid hormones (testosterone and estradiol) were observed in captive striped Murrel (*Channa striatus,* Bloch) injected with kisspeptin for the period of 4 months after injection. Kisspeptin induced a significant increase in the GSI of male and female fish. Histological examination of the gonads of kisspeptin injected fish showed that numerous oogonia were present in the ovary after one month of kisspeptin injection which continued to develop into vitellogenic oocytes in the fourth month. Higher level of testosterone and estradiol was observed from the kisspeptin injected fish compare to the control fish. At the end of fourth month, the level of testosterone in the treated male fish was higher than the control fish. The estradiol level was high in treated female fish compare to control fish. Among the two concentration of kisspeptin tested, the concentration of 0.05 µg/gram body weight influenced the high level of steroid hormone in both male and female. Sethu selvaraj et al.(1), studied the influence of kisspeptin on male and female chub mackerel *Scomber japonicas.*In human and non-human primate, human chorionic gonadotropin injection stimulated Kiss1 expression in the ovary (2). These findings in mammals suggested the possibilities of local role of kisspeptins in the control of ovulation process. The present study revealed the influence of kisspeptin on the gonadal development in murrel, *Channa striatus.*The results of the present work can be used as a reference study for controlled breeding and reproduction of stripped Murrel, *Channa striatus.*

Table 1.Influence of Kisspeptin on the level of testosterone and estradiol in the male and female fish of *Channa striatus*

	Male*			Female**		
Duration	**Control**	**T1**	**T2**	**Control**	**T1**	**T2**
I month	0.25±0.005	0.23±0.003	0.25±0.003	689±13.22	680±16.59	726±13.56
II month	0.38±0.076	0.58±0.011	0.61±0.012	962±18.24	1013±19.23	3541±60.82
III month	0.6±0.012	0.72±0.014	1.0±0.018	1820±32.64	3604±71.23	5780±92.61
IV month	0.85±0.017	1.12±0.022	1.5±0.029	2450±41.22	3852±69.04	7040±92.36
* Not Significant T1- Kisspeptine (0.01µg/g body wt.) T2- Kisspeptine (0.05µg/g body wt.)				** P<0.01 T1- Kisspeptine (0.01µg/g body wt.) T2- Kisspeptine (0.05µg/g body wt.)		

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

Conclusion:

The results confirm that kisspeptin played a major role in influencing the steroid hormone level in male and female *Channa striatus*. Among the two concentration tried, 0.05 µg/gram body weight influenced higher level of steroid hormones in both male and female fish. Further experiments should be carried out to confirm the correct dosage for influencing the gonadal maturation of murrel, *channa striatus*. It could be possible to develop a new kisspeptin based hormone for influencing gonadal maturation and breeding of finfishes.

References:

[1] Sethu Selvaraj , Hajime Kitano , Yoichiro Fujinaga , Hirofumi Ohga , Michio Yoneda, Akihiko Yamaguchi , Akio Shimizu , Michiya Matsuyama. , 2010. Molecular characterization, tissue distribution, and mRNA expression profiles of two Kiss genes in the adult male and female chub mackerel (Scomber japonicus) during different gonadal stages. General and Comparative Endocrinology 169 (2010) 28–38.

[2]Gaytán, F., Gaytán, M., Castellano, J.M., Romero, M., Roa, J., Aparicio, B., Garrido, N.,Sánchez-Criado, J.E., Millar, R.P., Pellicer, A., Fraser, H.M., Tena- Sempere, M.,2009. KiSS-1 in the mammalian ovary: distribution of kisspeptin in human and marmoset and alterations in KiSS-1 mRNA levels in a rat model of ovulatory dysfunction. Am. J. Physiol. Endocrinol. Metab. 296, E520–E531.

EXPRESSION OF MIS RECEPTORS IN THE OOCYTE OF INDIAN MAJOR CARP, *CIRRHINUS MRIGALA*

Anitha R., Gokulakrishnan S., Magesh K.M., Saravanan N. and Inbaraj R.M.

Endocrinology Unit, Department of Zoology, Madras Christian College, Tambaram, Chennai-600 059. India. *email: inbarajmoses2004@yahoo.com

Introduction:

Many actions of steroids are too rapid to be readily explained be the classical genomic mechanism of steroid action mediated by activation of nuclear steroid receptors. The receptors mediating these rapid steroid actions have been studied extensively in many laboratories over the past 30 years. The binding moieties with the characteristics of progestin membrane receptors have been demonstrated in fish and amphibian oocytes and some other vertebrate tissues. Maturation-inducing steroid (MIS) receptors are potential intermediaries in meiotic maturation of oocytes. 17α,20β-dihydroxy-4-pregnen-3-one (17α,20β-DHP) has been identified as the MIS in many teleosts, and induces oocytes to enter into final meiotic maturation leading to ovulation. The MIS receptors are membrane progestin receptors (mPR) and are responsible to mediate rapid non-genomic progestin action. The mPRs are mainly consisting of three forms such as mPRα, mPRβ and mPRγ. An attempt has been made to identify the receptors encoding gene in an Indian major carp, *Cirrhinus mrigala*.

Methods:

The immature and mature oocytes of *C. mrigala* were collected during the month of May (vitellogenic stage) and August (gravid stage) in RNA later, and total RNA was extracted using guanidine thiocyanate method. The extracted RNA was reverse transcribed to cDNA by M-MuLV, RT-PCR kit (Medox). Specific primers (Sigma, USA) were constructed for mPRα, mPRβ and mPRγ and applied to catch the specific gene. The primers are mPRα - sense 'CTGTCCTGTACGGGCTG', and antisense 'CTCCTGCTTGTCTTCTAGATACGC', mPRβ sense 'ACTGGTTTCCCCGTCTACCT', and antisense 'GTACAGGACAGCCAGGCCAGGA', mPRγ sense 'AACTCCTCGGATCCCAAAC', and antisense 'TGTGATAGCACAGCCGAGAC'. The PCR products were visualized by gel electrophoresis using ethidium bromide. The PCR amplified product was quantified and sequenced (Genei, India).

Results and Discussion:

The mPRα does not show any difference between the vitellogenic and gravid stage of oocytes (lane 1 and 2) and the mPRβ has the band intensity difference between the two stages (lane 3 and 4) whereas mPRγ could not be identified in the gravid stage (lane 6) in reference with the 100bp DNA marker (lane 7). The results confirm the possible expression of membrane progestin receptors mPRα and mPRβ in the matured oocytes and mPRγ gene expression in the mid vitellogenic stage of *C. mrigala*. This is the first demonstration of MIS receptor gene expression in the oocytes of *C. mrigala*.

In previous studies Yukinori et al. [1] reported that in channel catfish mPRα transcripts gradually increased during oocyte growth, mPRβ varied slightly throughout the reproductive cycle whereas in zebrafish mPRβ level increased during the follicular development stage. In sea trout, Zhu et al. [2] reported that mPRα was expressed in the plasma membrane, mPRβ brain and oocyte, and mPRγ was expressed in the oocyte as well as kidney. The present result also suggests that the mPRγ is not playing any role in the final maturation, however mPRα and mPRβ transcription is seasonally varied with maturity of oocytes. The partially sequenced genes (mPRα-979bp, mPRβ-981bp and mPRγ-521bp) were used to construct phylograms which indicate that *C. mrigala* is closely related to *Carassius auratus* and *Danio rerio* in comparison with other teleosts.

Fig. 1. The transcripts of mPRα, β and γ of vitellogenic (lane 1, 3 and 5) and gravid (lane 2, 4 and 6) oocytes of *C. mrigala*.

Acknowledgement:

The authors thank FIST Lab, Endocrinology Unit, Department of Zoology, Madras Christian College, Tambaram, Chennai-600059. India.

References:

[1]Kazeto.Y, Goto-Kazeto.R, Thomas P, Trant J. 2005. Molecular characterization of three forms of putative membrane –bound progestin receptors and their tissue

distribution in channel catfish (*Ictalurus punctatus*). J. Mol. Endo., 34: 781-791

[2]Zhu, Y., Rice, C.D., Pang, Y., Pace, M., Thomas, P. 2003. Cloning, Expression and characterization of a membrane progestin receptor and evidence it is an intermediary in meiotic maturation of fish oocytes. Proc. Natl. Acad. Sci. USA, 100: 2231-2236

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

FUNCTION OF GONADOTROPINS IN ASYNCHRONOUS DEVELOPMENT OF OVARIAN FOLLICLES IN THE WRASSE *PSEUDOLABRUS SIEBOLDI*

Kitano H.[1], **Takeshita M.**[1], **Lee J.M.**[2], **Kusakabe T.**[2], **Yamaguchi A.**[1], and **Matsuyama M.**[1]

[1]Faculty of Agriculture, Kyushu University, Fukuoka 812-8581, Japan
Tel/Fax: +81 92 642 2888; E-mail: h-kitano@agr.kyushu-u.ac.jp
[2]Graduate School of Bioresource and Bioenvironmental Sciences, Kyushu University, Fukuoka 812-8581, Japan

Introduction:

Asynchronous development of ovarian follicles, the simultaneous progress of vitellogenesis and oocyte maturation in the ovary, is a rational reproductive strategy for achieving multiple spawning episodes in one reproductive season. The bambooleaf wrasse *Pseudolabrus sieboldi* is a typical daily spawner in which different developmental stages of ovarian follicles are found in the ovary for daily egg production. We previously reported that the expressions of both FSHβ and LHβ mRNA show diurnal fluctuation in the female pituitary, suggesting that ovarian follicles are likely to be exposed to both FSH and LH regardless of their developmental status [1]. Furthermore, we found that the mRNA of the receptors for FSH (FSHR) and LH (LHR) are highly expressed in the vitellogenic and postvitellogenic follicles, respectively [2]. In the present study, the recombinant gonadotropins of bambooleaf wasse were produced by baculovirus in silkworm *Bombyx mori* larvae. To reveal the mode of action of FSH and LH in asynchronous development of ovarian follicles, the steroidogenic potencies of the recombinant gonadotropins in ovarian follicles at different developmental stages were analyzed by in vitro bioassay. Moreover, the potency to induce final oocyte maturation was also evaluated.

Methods:

The single chain recombinant bambooleaf wrasse FSH (r-bwFSH) and LH (r-bwLH) were constructed by fusion of the nucleotide sequences encoding their β subunits and the GtHα subunit using spacer sequence in which two N-glycosylation sites contained, respectively. They were expressed in the silkworm system [3] and purified using affinity column. The vitellogenic or postvitellogenic ovarian follicles were isolated from mature females (n = 5) using stainless mesh filters, and 80 follicles of them were incubated in 1 ml **Leibovitz's** L-15 medium with (1-1000 ng/ml) or without r-bwFSH or r-bwLH, respectively. The concentration of Estradiol-17β (E2), 17,20β-dihydroxy-4-pregnen-3-one (17,20β-P) and 17,20β,21-trihydroxy-4-pregnen-3-one (20β-S) in the culture media were determined by ELISA. These steroids of interest were selected on the basis of the steroidogenic pathways in the ovarian follicles during vitellogenesis and final oocyte maturation [4]. The percentage of germinal vesicle breakdown (GVBD) was

Fig. 1. In vitro effects of r-bwFSH and r-bwLH on E2 synthesis in the vitellogenic and postvitellogenic follicles. Asterisks represent significant differences from the control.

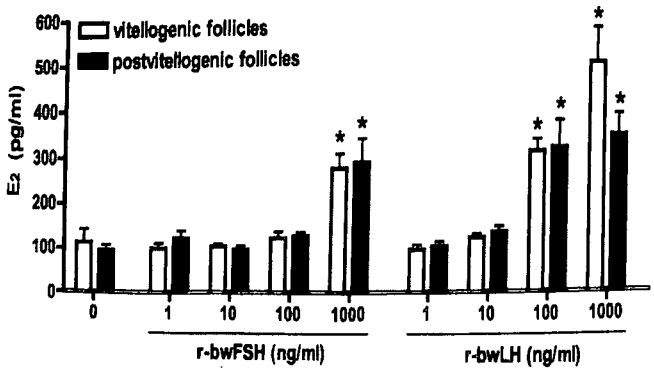


Fig. 2. In vitro effects of r-bwFSH and r-bwLH on 20β-S synthesis in the vitellogenic and postvitellogenic follicles. Asterisks represent significant differences from the control.

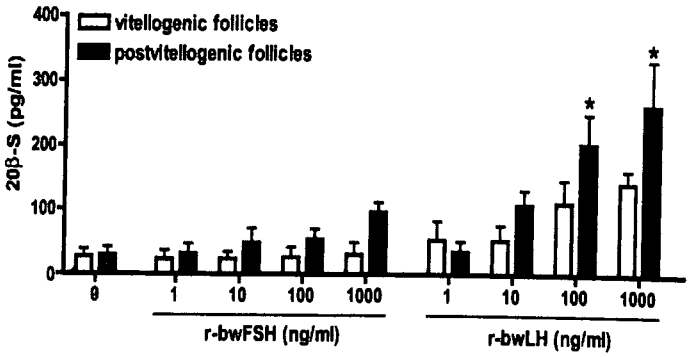
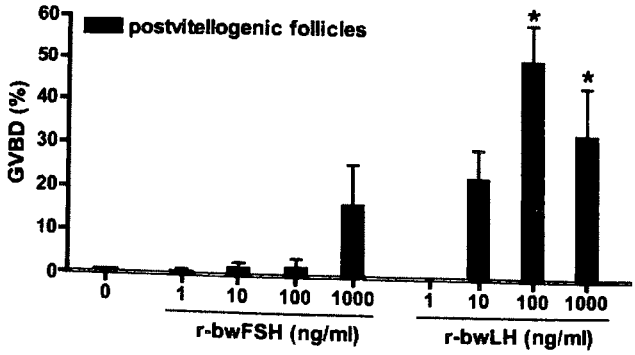


Fig. 3. In vitro effects of r-bwFSH and r-bwLH on GVBD in the postvitellogenic follicles. Asterisks represent significant differences from the control.



calculated to evaluate the potency of r-bwFSH or r-bwLH on oocyte maturation.

Results and Discussion:

E2 synthesis was induced in the vitellogenic and postvitellogenic follicles by both r-bwFSH and r-bwLH, respectively (Fig. 1). 20β-S synthesis and GVBD were specifically induced in postvitellogenic follicles by r-bwLH in a dose dependent manner (Fig. 2 and 3). Interestingly, 17,20β-P, which is a major maturation-inducing hormone (MIH) in many teleosts, was not induced by r-bwFSH and r-bwLH, indicating that principal MIH in the present species is 20β-S. These results suggest that the vitellogenesis is induced by both FSH and LH. On the other hand, oocyte maturation is evoked by LH only in the bambooleaf wrasse. Furthermore, the present results imply that the simultaneous advance of vitellogenesis and oocyte maturation in the ovary of bambooleaf wrasse is defined by the follicular stage-selective receptivity of two gonadotropins. Since the FSHR mRNA is highly expressed in vitellogenic follicles [2], E2 synthesis is suggested to be regulated by FSH and cross-reactive LH via FSHR. On the other hand, the LHR mRNA is elevated in postvitellogenic follicles [2], oocyte maturation followed by MIH synthesis is likely to be facilitated specifically by LH via abundant LHR in postvitellogenic follicles.

Conclusion:

In the bambooleaf wrasse, E2 synthesis was induced in vitellogenic and postvitellogenic ovarian follicles by both r-bwFSH and r-bwLH. On the other hand, 20β-S synthesis and GVBD were specifically induced in the postvitellogenic follicles by r-bwLH. Here we propose that the asynchronous development of ovarian follicles in the bambooleaf wrasse is regulated by 1) co-regulation of vitellogenesis by FSH and LH via FSHR, and 2) the postvitellogenic stage-selective induction of the oocyte maturation by LH via LHR.

References:

[1]OHTA, K., MINE, T., YAMAGUCHI, A., MATSUYAMA, M. 2008. Sexually dimorphic expression of pituitary glycoprotein hormones in a sex-changing fish (*Pseudolabrus sieboldi*). J. Exp. Zool., 309A: 534-541.

[2]KITANO, H., IRIE, S., OHTA, K., YAMAGUCHI, A., MATSUYAMA, M. 2008. Regulation of asynchronous development of ovarian follicles by the expression levels of two gonadotropin receptors in a daily spawning teleost, a bambooleaf wrasse, *Pseudolabrus sieboldi*. Proc. 5th World Fisheries Congress: 3a-11.

[3] KAWAKAMI, M., LEE, J.M., MON, H., KUBO, Y., BANNO, Y., KAWAGUCHI, Y., MAENAKA, K., PARK E.Y., KOGA, K., KUSAKABE, T. 2008. Efficient protein expression in *Bombyx mori* larvae of the strain d17 highly sensitive to *B. mori* nucleopolyhedrovirus. Mol. Biotech., 40: 180-185.

[4]OHTA, K., MINE, T., YAMAGUCHI, A., MATSUYAMA, M. 2001. Steroidogenic pathway to estradiol-17β synthesis in the ovarian follicles of the protogynous wrasse, *Pseudolabrus sieboldi*. Zool. Sci., 18: 937-945

ESTRADIOL INDUCED MECHANISMS REGULATING VITELLOGENIN (VG) AND CHORIOGENIN SYNTHESIS AND EXPRESSION OF VG IN THE CULTURED HEPATOCYTES OF INDIAN FRESHWATER MURREL *CHANNA PUNCTATUS*

Kumari Vandana Rani and Neeta Sehgal

Fish Endocrinology Laboratory, Department of Zoology, University of Delhi, Delhi 110007, India.

Introduction:

Vitellogenin (Vg) and Choriogenin (Chg) are female-specific proteins, synthesized in the hepatocytes of teleosts. Several *in vivo* studies have shown that it is difficult to distinguish the primary effects of a compound from those induced secondarily because liver functions are under the influence of multiple endogenous factors. Therefore, *in vitro* hepatocyte systems present a better experimental model to investigate mechanism(s) by which Vg and chg are synthesized. Primary cultures of fish hepatocytes have been used to study hepatic structure and function, viz. endocrine regulation, Vg synthesis, hormone receptor expression, and toxicology. The Vg and Chg induction in cultured fish hepatocytes is clearly influenced by culture conditions (medium composition, temperature etc.) and culture system (hepatocyte monolayers, aggregates, liver slices etc.). The main advantages of the hepatocyte Vg and Chg assay are considered its ability to detect effects of estrogenic metabolites, since hepatocytes *in vitro* remain metabolically competent, and its ability to detect both estrogenic and anti-estrogenic effects. The aim of the present study was to understand the induction of Vg & Chg by estradiol (E_2) and expression of Vg genes.

Methods:

Hepatocytes were isolated following the method of Rani *et al.*[1]. Hepatocytes were exposed to various doses of E_2, optimum dose was selected and used for rest of experiments. Vg and Chg concentrations were determined in culture medium using standardized ELISAs [2] with few modifications. Cells exposed to a constant dose of E_2 (500nM) in the presence of actinomycin D (3 and 6µg/ml) or cycloheximide (10 µg/ml). After 48 hrs media were collected from all the groups and processed for Vg and Chg estimation. Statistical Significance was calculated in between different doses of same treated groups by Mann-Whitney Wilcoxon test/student t-test; significance is given as $p<0.005$. Vg was extracted from the medium by PAGE and cut band was characterized by LC-MS/MS peptide mapping. Total RNA was isolated by TRIzol method from treated and non treated hepatocytes and processed for expression of Vg gene(s) by reverse transcription PCR (RT-PCR). E_2 exposed hepatocytes were fixed and processed for localization of Vg and Chg in cytoplasm by immunocytochemistry and for ultrastructural analysis by TEM.

Fig. 2: Effect of Actinomycin D and Cycloheximide on vitellogenin and choriogenin levels in hepatocyte culture medium. Freshly isolated cells were maintained for 48 hrs for monolayer formation. Thereafter they are treated with cycloheximide (B) & different dose (3 and 6µg/ml) of Actinomycin D (A) alone or in combination with E_2 and media was collected after 48 hrs and processed for ELISA. Values are expressed as Mean± SEM.

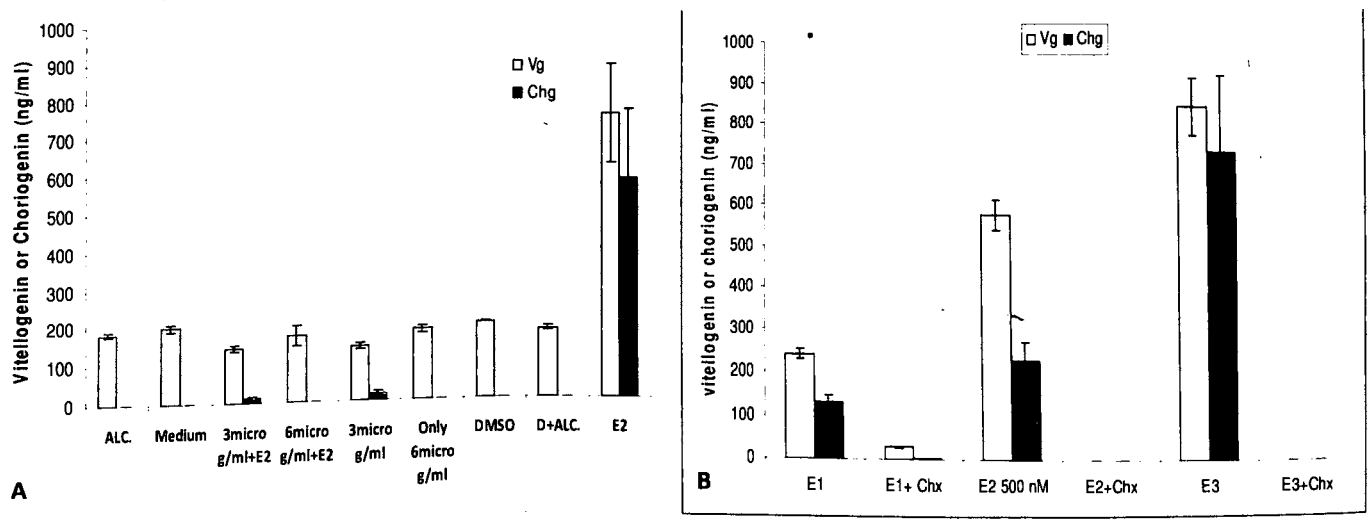
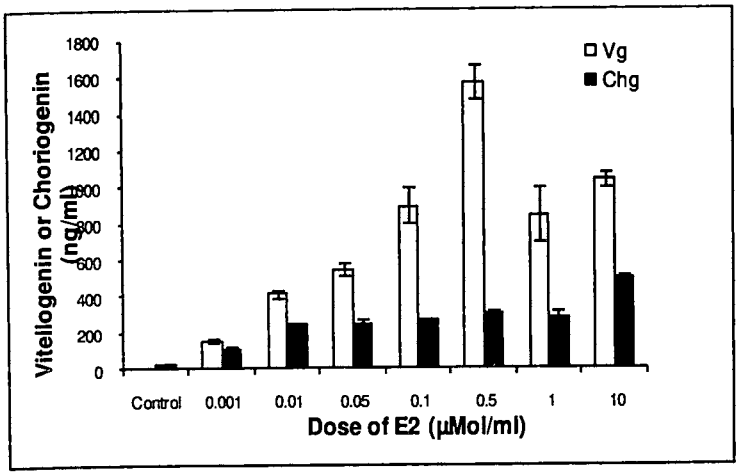


Fig.1 : Vitellogenin& Choriogenin levels in the culture medium following treatment of hepatocytes with various doses of estradiol (E_2) for 48 h and vitellogenin was estimated in the medium by ELISA.Values are expressed as Mean± SEM.



Results:

The ELISA estimations in medium collected from E_2 treated cells showed the presence of Vg and Chg (Fig. 1). Actinomycin D and cycloheximide, inhibitors of protein synthesis, significantly reduced E_2-induced Vg and Chg (Fig 2A,B). MALDI LC-MS/MS analysis revealed that the hepatocytes secreted the Vg B form in the medium. This was further confirmed by the homology search of the PCR amplified Vg gene from the hepatocytes RNA. The electron microscopy also showed distinguished difference in the hepatocyte structure in the treated and non-treated cells. Immunocytochemical localization of induced Vg showed the color production in the cell cytoplasm.

Discussion:

It has been established through this study that E_2 exposed cells synthesize Vg. Inhibition of Vg induction by Actinomycin D and Cycloheximide evidently suggest involvement of genomic pathway in Vg expression. Vg and Chg, exhibits various forms in teleosts, viz Vg A, Vg B and Vg C and Chg H and Chg L. LC-MS/MS and RT-PCR sequence analysis revealed similarities of synthesized cultured Vg with Vg B. Extensive increase in number of mitochondria, enlargement of Golgi apparatus, proliferation of ER arranged in parallel arrays and whorls are some of the characteristic features of E_2 stimulated hepatocytes which were evident in this study.

References

[1] RANI K. V., SEHGAL N., GOSWAMI S.V., OM PRAKASH. 2010. Relative potencies of Natural estrogens on vitellogenin and choriogenin levels in the Indian freshwater spotted snakehead, *Channa punctata*: *Invivo* and *invitro* studies. Fish Physiol. Biochem., 36: 587-595.

[2]OM PRAKASH, GOSWAMI S.V., SEHGAL N. 2007. Establishment of ELISA for murrel vitellogenin and choriogenin, as biomarkers of potential endocrine disruption. Comp. Biochem. Physiol., C 146(4):540–551.

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

EXPRESSION, TRANSCRIPTIONAL REGULATION AND POSSIBLE ROLES OF INSULIN-LIKE GROWTH FACTORS IN GONADAL STEROIDOGENESIS IN TILAPIA

Li M.H., Wang H., Gu Y., Yang S.J., Sun Y.L., Zhou L.Y., Wang D.S.

Key Laboratory of Freshwater Fish Reproduction and Development (Ministry of Education), Key Laboratory of Aquatic Science of Chongqing, School of Life Science, Southwest University, Chongqing, 400715, China.
Fax: +86-2368253005, E-mail: wdeshou@swu.edu.cn

Introduction:

The insulin-like growth factors (igfs), igf-1, igf-2 and a novel igf termed igf-3, are implicated in a wide variety of physiological roles in teleost gonadal development. Sexual differentiation occurs along with cell proliferation and tissue growth, suggesting that growth factors are involved in these physiological processes, particularly igfs, which are recognized as exerting certain actions on the gonadal development, sex determination and differentiation.

Methods:

Animal- All genetic female (XX) and male (XY) tilapia (*Oreochromis niloticus*) were obtained by artificial fertilization of eggs from normal females (XX) with sperms from either sex-reversed males (XX) or super males (YY), respectively. Tilapias were reared in 0.5-ton tanks with re-circulating aerated fresh water at 26°C under natural photoperiod. *Real-time PCR assay-* Gonads from 10, 20, 30, 40, 50, 60 and 70 days after hatching (dah) were dissected. Total RNA was extracted and cDNA was prepared for the real-time PCR. *In situ hybridization-*Probes of both sense and antisense digoxigenin (DIG)-labeled RNA strands of *igf-1*, *igf-2* and *igf-3* were transcribed *in vitro*, using the RNA labeling kit. *In situ* hybridization was carried out as follows: sections were deparaffinized, hydrated and treated with proteinase K and then hybridized with the probe at 60°C for 16~22hrs. The hybridization signals were detected using alkaline phosphatase-conjugated anti-DIG antibody and NBT/BCIP. *Cell culture, transient transfections and luciferase assays-* HEK293 cells were maintained in DMEM at 37°C. Cells were transfected with the following plasmids: 1) 0.5μg of the *igf-1* (or *igf-2, or igf-3*) promoter cloned into the pGL3-basic luciferase reporter vector; 2) 0.01-0.25μg of pcDNA3.1 expression plasmid, containing the cDNAs encoding tilapia *Ad4BP/SF-1*, *Foxl2*, *Dmrt1*, *Dax1*, *Dax2*, *CREBt* and *CREBo*; 3) pRL-TK, at 100ng/well. Firefly luciferase and *Renilla* luciferase readings were obtained using the Dual-Luciferase Reporter Assay System 48 hrs after transfection. *In vitro incubation of ovary fragments with Igf-3 recombinant protein-* The culture medium consisted of 1:1 (v:v) mixture of DMEM and Ham's F-12 medium. The ovaries of tilapia were dissected out and placed in a dish containing 5 ml medium. Each ovary was dispersed into small fragments and transferred into the 24-well plate. After 12 hrs incubation, the igf-3 recombinant protein was added and 24 hrs later, the medium was centrifuged and the sediments were collected. Total RNA was isolated from the sediments using TRIzol Reagent.

Results:

Real-time PCR analysis revealed a similar expression pattern of *igf-1* and *igf-3* in gonad, higher in ovary than testis between 10 to 40 dah, followed by a lower expression than testis from 50 to 70 dah, and ultimately with a peak at 70 dah. Obviously higher level of *igf-2* expression was found in the ovary than testis from 10 to 30 dah, while sharply decreased to similar expression levels in both testis and ovary from 40 to 70 dah. *In situ* hybridization using gonads from 10 to 180 dah demonstrated that both *igf-1* and *igf-2* mRNAs were detected in the primordial germ cells and somatic cells at 10 dah in both XX and XY gonads. *igf-1* and *igf-2* mRNAs were also detected at 30 dah in female germ cells, but not in male germ cells, in which both signal were detected from as late as 50 dah. Later, *igf-1* and *igf-2* were detected in spermatogonia and spermatocytes as well as in Leydig cells of the testis and in the interstitial cells of the ovary about 180 dah. In contrast, *igf-3* was found to be expressed only in the somatic cells and later in granulosa cells of the ovary, and in the interstitial cells of the testis. Promoter analysis using luciferase assays in HEK293 cells revealed that the *igf-3* gene expression was directly activated by Ad4BP/SF-1, and further enhanced by Foxl2, Dax1 and Dax2. In contrast *igf-2* gene transcription was repressed by Ad4BP/SF-1 and Foxl2 but unaffected by Dax1 and Dax2. Promoter activity of *igf-1* was activated by CREBt and CREBo but was not influenced by Ad4BP/SF-1. All *igfs* promoter activities were repressed by Dmrt1. Finally, Ad4BP/SF-1 activated *igf-3* transcriptional expression was enhanced, *igf-2* repressed while *igf-1* not influenced with simultaneous treatment of forskolin. *In vitro* incubation of ovary fragments were performed and igf-3 recombinant protein was added to the culture medium after 12hrs. The fragments were collected, total RNA was isolated and Real-time PCR was performed to examine the expression levels of various transcription factors and steroidogenic enzymes. *Foxl2*, *Ad4BP/SF-1*, *Cyp19a1a*, *3beta-HSD-I*, *3beta-HSD-II*, *P450scc* and *P450C17*, were up-regulated in a time- and dose-

dependent manners, while *20beta-HSD* was not influenced by igf-3 treatment.

Conclusion:

Taken together, these data indicated that 1) *igf-1*, *igf-2* and *igf-3* showed different expression patterns during gonad development; 2) igf-1 and igf-3 might be involved in early ovarian differentiation and later testis development; 3) Their transcriptional mechanisms were distinct from each other and were controlled by a various transcription factors in gonad; 4) The gonad specific novel igf-3 may be the principle growth factor involved in fish ovarian differentiation because of its ability to regulate the expression of *Foxl2, Ad4BP/SF-1* and steroidogenic enzyme genes, and in turn, its expression was tightly regulated by Foxl2, Dmrt1 and Ad4BP/SF-1, the key transcription factors involved in fish sex determination and differentiation.

DIFFERENTIAL REGULATION OF GONADOTROPIN RECEPTORS (*fshr* AND *lhcgr*) BY ESTRADIOL IN THE ZEBRAFISH OVARY INVOLVES NUCLEAR RECEPTORS THAT ARE LIKELY LOCATED ON THE PLASMA MEMBRANE

Liu K.C. and Ge W.

School of Life Sciences, The Chinese University of Hong Kong, Shatin, New Territories, Hong Kong, China
Fax: +852-2603-5646; E-mail: weige@cuhk.edu.hk

Introduction:

Follicle-stimulating hormone (FSH) and luteinizing hormone (LH) are gonadotropins that control all major events of gonadal function, including folliculogenesis, steroidogenesis and ovulation in females. FSH and LH signal through their cognate receptors, FSH receptor (FSHR) and LH/choriogonadotropin receptor (LHCGR), respectively, across vertebrates. Compared to the information in mammals, very little is known about these receptors in fish, especially the mechanisms that control their expression. Using zebrafish as the model, we have demonstrated that although both *fshr* and *lhcgr* increase their expression during follicle growth and maturation in the ovary, they exhibit significant temporal difference with *lhcgr* expression lagging behind that of *fshr* [1]. This raises an interesting question: how are these two gonadotropin receptors differentially controlled during folliculogenesis? We, therefore, have initiated this study to investigate the expression control of *fshr* and *lhcgr* in the zebrafish ovary.

Methods:

A primary follicle cell culture was used in the present study to investigate the regulation of *fshr* and *lhcgr* by various hormones and their action mechanisms. The expression levels of *fshr* and *lhcgr* were assessed by extraction of total RNA from the treated cells followed by reverse transcription and real-time qPCR. To elucidate the signaling mechanism, we collected the total protein from the treated cells to examine the phosphorylation level of certain signaling molecules with Western blot.

Results:

Estradiol (E2), but not testosterone (T), significantly and differentially up-regulated the expression of *fshr* and *lhcgr* within 1 hour. Although E2 stimulated the expression of both *fshr* and *lhcgr*, its effect on the steady-state level of *lhcgr* mRNA was much higher (> 10-fold up-regulation) than that on *fshr* expression (~ 0.5-fold increase). This regulatory effect could be mimicked by E2 conjugated to bovine serum albumin (E2-BSA). Additionally, nuclear estrogen receptor (ER) antagonists (ICI 182,780) and transcription inhibitor (actinomycin D) abolished this E2-induced up-regulation while MEK inhibitor (U0126) greatly hindered the action of E2. Further immunoblot evidence confirmed that E2 rapidly induced phosphorylation of MAPK in less than 20 minutes and the activation of p38 MAPK could enhance the E2 action.

Conclusion:

We have provided the first evidence that E2 acts as a potent endocrine hormone involved in the differential expression of *fshr* and *lhcgr* in the zebrafish ovary. The regulation occurs at the transcription level via the nuclear ERs; however, these receptors appear to be located on the plasma membrane. This study has provided evidence for a novel action mechanism of E2 in the ovary of teleosts and the distinct mechanisms controlling *lhcgr* expression in mammals and teleosts.

Reference:

[1]KWOK H.F., SO W.K., WANG Y., GE W. 2005. Zebrafish gonadotropins and their receptors: I. Cloning and characterization of zebrafish follicle-stimulating hormone and luteinizing hormone receptors – evidence for their distinct functions in follicle development. Biol. Reprod., 72:1370-1381.

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

MOLECULAR CLONING, CHARACTERIZATION AND EXPRESSION PROFILES OF THREE ESTROGEN RECEPTORS IN PROTOGYNOUS HERMAPHRODITIC ORANGE-SPOTTED GROUPER (*EPINEPHELUS COIOIDES*)

Chen H. [1*], Zhang Y. [1*], Li S. [1], Lin M. [1], Shi Y.[1], Sang Q. [1], Liu M.[2,3], Zhang H.[4], Lu D. [1], Meng Z. [1], Liu X. [1§], Lin H. [1,5§]

[1]State Key Laboratory of Biocontrol, Institute of Aquatic Economic Animals, and the Guangdong Province Key Laboratory for Aquatic Economic Animals, School of Life Sciences, Sun Yat-Sen University, Guangzhou 510275, Guangdong, China.

[2] The Swire Institute of Marine Science and The Division of Ecology & Biodiversity, The School of Biological Sciences, The University of Hong Kong, Pokfulam Road, Hong Kong.

[3]Key State Laboratory of Marine Environmental Science, College of Oceanography and Environmental Science, Xiamen University, Xiamen 361005, Fujian, China.

[4]Guangdong Daya Bay Fisheries Development Center, Huizhou 516081, Guangdong, China.

[5]College of Ocean, Hainan University, Haikou 570228, Hainan, China.

[§]Corresponding authors: Xiaochun Liu and Haoran Lin

E-mail addresses: lsslxc@mail.sysu.edu.cn (Xiaochun Liu); lsslhr@mail.sysu.edu.cn (Haoran Lin)

Tel: +86-20-84112511; +86-20-84113791

Fax: +86-20-84113717

Estrogen plays key roles in vertebrate reproductive system via estrogen receptors (ERs) as mediating pathways. In the present study, three full-length ERs cDNA sequences were isolated from a protogynous teleost, the orange-spotted grouper (*Epinephelus coioides*), and were 2235bp for gERα, 1967bp for gERβ1 and 2158bp for gERβ2, respectively. Phylogenetic and amino acid alignment analyses showed that each gER was clustered in the corresponding taxonomic groups of the perciformes and exhibited high evolutionary conservation in functional domains. RT-PCR revealed that gERs expressed at different levels in all the obtained tissues. gERα highly expressed in mature ovaries, gERβ1 mainly expressed in immature ovaries and gERβ2 varied greatly during ovarian development. During female to male sex reversal induced by 17α-methyltestosterone (MT) implantation, gERα decreased gradually, gERβ1 increased gradually, and gERβ2 decreased firstly and recovered subsequently in male stage. The present study speculated the potential roles of gERs during female maturation and female to male sex reversal induced by MT in the protogynous grouper *E. coioides*.

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

A study on yolk proteolysis and oocyte hydration in freshwater fish *Clarias gariepinus*

Luni S. and **Sehgal N.**

Department of Zoology, University of Delhi, Delhi, India
Email:neetasehgal@mailcity.com

Introduction:

The process of oogenesis in fish involves oocyte growth, maturation, hydration and ovulation. Full grown oocytes respond to the maturation inducing steroid (MIS) secreted by the follicle cells during resumption of meiosis. During this process, besides breakdown of germinal vesicle, morphological and volume changes also occur in oocyte. The changes in oocyte volume range from slight in most fresh water and euryhaline species to several fold in marine species. High increase in oocyte volume (3.1-8.4 fold) is associated with marine species that produces pelagic eggs while lower oocyte volume increase (1-3 fold) in species that produce benthic eggs. The studies suggest that increase in oocyte volume is due to increase in osmolality which occurs either due to proteolysis of yolk protein or due to increase in inorganic ions, leading to an increase in uptake of water by the developing oocytes. In freshwater fish species a limited oocyte volume increase was reported during the preovulatory period in rainbow trout, *Barbus tetrazona* and *Rasbora trilineata*, while others like *Cichlasoma nigrofasciatum*, *Carassius auratus*, *Gymnocorymbus ternetzi* and *Coregonus lavaretus* (Greeley et al., 1986) do not show any increase in volume.

Methods:

Gravid female catfish *Clarias gariepinus* of similar size were divided into groups and injected with GnRH. At different time points (0 hr, 9 hr, 13hr and 17 hr) fishes in each group were decapitated and ovaries excised. Cluster of 20-30 eggs were taken as a sample and wet wt. and dry wt. measured for gravimetric analysis. SDS PAGE of yolk proteins extracted at different time points was performed to analyze proteolysis pattern.

Results and Discussion:

Clarias gariepinus females ovulate eggs after 9-13 hrs of GnRH injection. A significant increase in oocyte water content was observed at 9 hr 13 hr and 17 hr (Fig. 1a) but the maximum increase was just before the ovulation. A significant change is observed in protein content of the ovulated oocyte from the vitellogenic oocytes (Fig. 1b).To analyse the proteolysis of yolk protein, electrophoresis was performed. An electrophoretic shift (Fig. 2a, 2b) was observed in a major protein band of vitellogenic and ovulated oocytes indicating proteolysis of yolk proteins.

Fig. 1(a) Hydration in oocytes at different time points

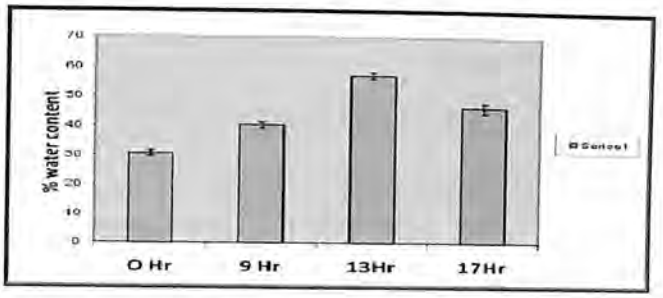


Fig. 1(b) Protein content in Vitellogenic after GnRH injection and Ovulated oocytes

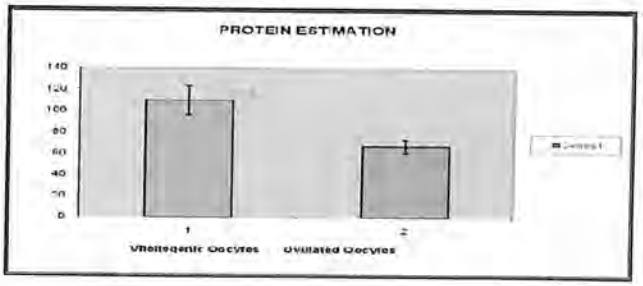


Fig. 2(b) SDS PAGE: Electrophoretic profile in of yolkproteins of Vitellogenic (VgO) and Ovulated (OvuO) oocytes. Marker (M)

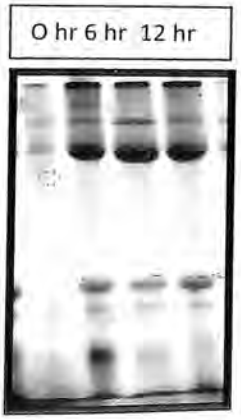
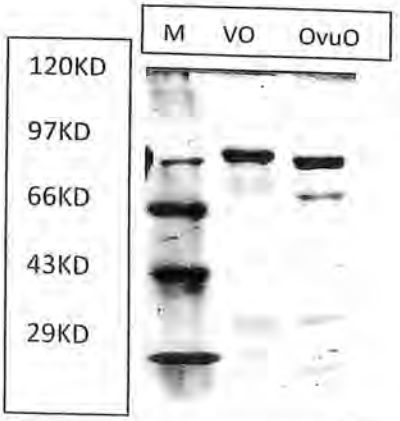


Fig. 2(a) SDS PAGE: Yolk proteolysis pattern during ovulation at different time points



Conclusion:

In *Clarias gariepinus* oocytes at maturation and during ovulation show a significant increase in water content. The increase in water content might be a result of increase in the osmolality of oocytes due to increase in the free amino acids liberated as a result of yolk protein proteolysis as evident by the electrophoretic pattern.

References:

[1]BABIN, P.J., CARNEVALI, O., LUBZENS, E. SCHNEIDER, W.J. 2007. Molecular aspects of oocyte vitellogenesis in fish. *In:* "The Fish Oocyte: From Basic Studies to Biotechnological Applications" (P.J. BABIN, J. CERDÀ AND E. LUBZENS, eds.), pp. 39-76, Springer, Netherlands.

[2]FABRA, M., RALDÚA, D., BOZZO, M.G., DEEN, P.M.T., LUBZENS, E. AND CERDÀ, J. 2006. Yolk proteolysis and aquaporin-1o play essential roles to regulate fish oocyte hydration during meiosis resumption. Dev. Biol., 295: 250-262.

[3]MILLA, S., JALABERT, B., RIME, H., PRUNET, P. AND BOBE, J. 2006. Hydration of rainbow trout oocyte during meiotic maturation and *in vitro* regulation by 17,20_-dihydroxy-4-pregnen-3-one and cortisol. J. Exp. Biol., 209: 1147-1156.

FOLLICLE STIMULATING HORMONE ACTIVATES THE STEROIDOGENIC PROCESS VIA cAMP/PKA and MAP-KINASE PATHWAYS IN THE OVARY OF EUROPEAN SEA BASS (*DICENTRARCHUS LABRAX*)

Mazón M.J., Gómez A., Zanuy S.

Department of Fish Physiology and Biotechnology, *Institute of Aquaculture of Torre de la Sal(IATS), Spanish National Research Council (CSIC),*
Torre la Sal s/n, Ribera de Cabanes, 12595 Castellón, Spain.
e-mail: mazon@iats.csic.es

Introduction:

In fish the onset and consequent stimulation of the gametogenic process is produced by the gonadotropic hormones, follicle-stimulating hormone (FSH) and luteinizing hormone (LH). FSH action is accomplished via the interaction with its specific receptor (FSHR) which is a transmembrane G-protein-coupled receptor localized in the granulosa cells. The mainstream pathway used by FSHR to transduce the signal is the cAMP/PKA route; however, alternative routes have been identified, such as the mitogen-activated protein kinase (MAPK). The extracellular signal-regulated kinases (ERKs) belong to the family of MAPKs and are activated by MEK1 and MEK2, being probably the last one the crosstalk between this signaling pathway and the cAMP/PKA route. Results concerning ERK implication in steroidogenesis and ERK activation through cAMP/PKA are still contradictory. In fish, information on the involvement of MAPK in the steroidogenic process activated by gonadotropins is scarce. The present work aimed to clarify the signaling pathways used by FSH to induce steroid synthesis in sea bass.

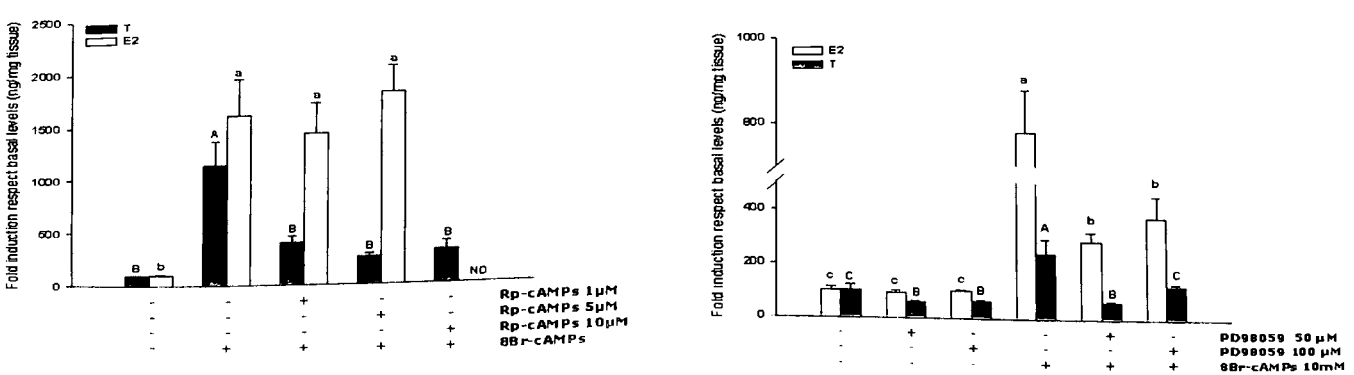
Methods:

Sea bass ovary culture was performed as previously described [1]. Approximately 20 mg of gonadal tissue were pre-incubated with 0.25 ml of sea bass Ringer solution (SBR) in 48-well plates for 30 min under shaking conditions with different doses of the cAMP/PKA inhibitor Rp-cAMPs, or the MAPK inhibitor PD98059. Next, tissues were stimulated with sea bass recombinant FSH (scFSH) [2] or the cAMP/PKA activator 8Br-cAMPs. After 20 hours, culture medium was collected and levels of estradiol (E2) and testosterone (T) were analyzed using specific immunoassays [2, 3]. Tissue was collected and frozen for RNA extraction with Trizol and quantitative real-time PCR was performed after the cDNA synthesis. Changes in mRNA transcripts for *StAR* (steroidogenic acute regulatory protein), *CYP19A* (aromatase), and *3β-hydroxysteroid-dehydrogenase* (*3βHSD*) were evaluated and corrected using ribosomal protein L-13a as a housekeeping gene.

Results and Discussion:

E2 increased after treatment with scFSH or 8Br-cAMPs, while the increase of T was only relevant under 8Br-cAMPs stimulation. In the presence of Rp-cAMPs an important decrease in T levels was observed, however in the case of E2, the reduction was detected only in scFSH-treated ovaries, but not in the ones stimulated with 8Br-cAMPs. Regarding gene expression, a strong up-regulation in the levels of *StAR* and *CYP19A* were observed after the treatments with scFSH and 8Br-cAMPs. In the case of the scFSH stimulated culture, blocking the cAMP/PKA route generated a faint reduction in the transcript levels of *StAR*, with no effects on *CYP19A* expression. In 8Br-cAMPs treatments, the presence of Rp-cAMPs provoked a strong down-regulation, in a dose-dependent manner, of both, *StAR*

Fig.1. Effect of the cAMP analogue 8Br-cAMPs on the ovarian synthesis of E2 and T in the presence of cAMP/PKA inhibitor (A) or MAPK inhibitor (B)



and *CYP19A* transcripts. Rp-cAMPs did not reduce the up-regulated levels of *3βHSD* generated by both, scFSH and 8Br-cAMPs.

The MAPK inhibitor PD98059, either alone or in the presence of FSH and 8Br-cAMPs, was able to reduce T levels, having minor effect on E2 secretion. PD98059 blocked the increase of mRNA levels of *StAR*, *CYP19A* and *3βHSD* generated by scFSH or 8Br-cAMPs stimulation.

Conclusion:

The present study shows the involvement of the cAMP/PKA route on sea bass steroidogenesis, in agreement with findings in other species. By blocking the cAMP/PKA pathway we determined that FSH uses this pathway to up-regulate transcripts of some steroidogenic enzymes such as *StAR* and *CYP19A*, therefore increasing the synthesis of E2 and T. In line with other species [4], MAPKs also participates in the regulation of steroidogenic enzyme activity. Blocking the MAPK route shows that this pathway regulates steroidogenic enzyme transcripts in a different way than cAMP/PKA. Besides, the activity of MAPK seems to be independent of the PKA route, as has been confirmed in other species [5].Research supported by MICINN (AGL2008-02937) and GV (ACOMP/2010/083 and PROMETEO/2010/003). MJ. Mazón received a FPI fellowship from the Spanish MICINN.

References:

[1]MOLÉS, G., GÓMEZ, A., ROCHA, A., CARRILLO, M., AND ZANUY, S. 2008. Purification and characterization of follicle-stimulating hormone from pituitary glands of sea bass (*Dicentrarchus labrax*). Gen. Comp. Endocrinol. 158: 68-75.

[2]MOLÉS, G., ZANUY, S, MUÑÓZ, I., CRESPO, B., MARTÍNEZ, I., MAÑANOS, E., GÓMEZ, A. 2011. Receptor specificity and functional comparison of recombinant sea bass (*Dicentrarchus labrax*) gonadotropins (Fsh and Lh) produced in different host systems. Biol. Reprod., 84: 1171–1181.

[3]RODRÍGUEZ, l., BEGTASHI, I., ZANUY, S., CARRILLO, M. 2000. Development and validation of an enzyme immunoassay for testosterone: Effects of photoperiod on plasma testosterone levels and gonadal development in male sea bass (*Dicentrarchus labrax*, L.) at puberty. Fish Physiol. Biochem., 23:141-150.

[4]PODEROSO, C., CONVERSO, D.P., MALOBERTI, P., DUARTE, A., NEUMAN, I., GALLIS, S., CORNEJO, F., PAZ, C., CARRERAS, M.C., PODEROSO, J., PODESTÁ, E.J.2008. A mitochondrial kinase complex is essential to mediate an ERK1/2-dependent phosphorylation of a key regulatory protein in steroid biosynthesis. Plos ONE e1443:1-11.

[5]BORLAND, G., SMITH, B.O., YARWOOD, S.J.2009. EPAC proteins transduce diverse cellular actions of cAMP. Brit. J. Pharmacol., 158:70-86.

Research supported by MICINN (AGL2008-02937) and GV (ACOMP/2010/083 and PROMETEO/2010/003). MJ. Mazón received a FPI fellowship from the Spanish MICINN.

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

MOLECULAR CLONING AND LOCALIZATION OF TWO CLASSICAL OVARIAN LIPOPROTEIN RECEPTORS IN CUTTHROAT TROUT *ONCORHYNCHUS CLARKI*

Mizuta H.,[1] Hiramatsu N.,[1] Todo T.,[1] Ito Y.,[1] Gen K.,[2] Kazeto Y.,[2] Sullivan C.V.,[3] Reading B.J.[3] and Hara A.[1]

[1] Graduate School of Fisheries Sciences, Hokkaido University, 3-1-1, Minato, Hakodate, Hokkaido, 041-8611, Japan (Fax: +81-138-40-8878 e-mail: sp2654@world.ocn.ne.jp)

[2] Inland Station, National Research Institute of Aquaculture, Fisheries Research Agency, Tamaki, Mie 519-0423, Japan

[3] Department of Zoology, College of Agriculture and Life Science, North Carolina State University, Raleigh, North Carolina 27695-7617

Introduction:

Eggs of teleost fishes contain a substantial yolk mass, which serves as a nutrition source (e.g., proteins and lipids) for embryonic development and larval growth. Various types of ovarian lipoprotein receptors, which belong to the low-density lipoprotein receptor (LDLR) gene family, may be involved in the process of ovarian yolk formation. In teleosts, an ovarian lipoprotein receptor that binds to and regulates uptake of, a major yolk precursor, vitellogenin (vitellogenin receptor, VgR), has been extensively characterized [1, 2, 3]. However, no additional receptors of the LDLR family have been characterized in the ovary of teleosts. In order to understand the physiological significance of multiple ovarian receptors during teleost yolk formation, we aimed to confirm the expression and localization of two classical lipoprotein receptors (i.e., VgR and LDLR) in developing ovarian follicles of cutthroat trout, *Oncorhynchus clarki*.

Methods:

Molecular cloning of cDNAs encoding VgR and LDLR was performed by RT-PCR and TA-cloning using trout ovary cDNA as template. Recombinant receptor proteins targeting the ligand binding domains of each receptor were prepared for use as antigens to produce specific polyclonal antibodies (a-rVgR and a-rLDLR). Expressed recombinant receptor proteins were purified, emulsified with Freund's complete adjuvant, and injected into rabbits. Using these antibodies, both receptor proteins were detected by Western blot analyses of ovarian membrane preparations, as well as by immunohistochemistry of ovarian follicles. Localization of each corresponding *vgr* and *ldlr* mRNA was confirmed by *in situ* hybridization.

Results and discussion:

Two cDNAs were isolated and each appeared to encode either full-length VgR (2529 bp) or LDLR (2625 bp) orthogues in the trout; predicted masses of their translated products were ~93 kDa and ~96 kDa, respectively. Phylogenetic analysis placed the trout VgR and LDLR into two separate branches: the trout VgR peptide sequence clustered with other vertebrate lipoprotein receptors with 8 ligand repeats (LR8 type receptors; i.e., VgR or very low density lipoprotein receptor, VLDLR), while the trout LDLR sequence clustered with other vertebrate LR7 receptors (i.e., LDLR). The a-rVgR and a-rLDLR used in Western blot analyses of ovarian membrane preparations detected major bands with apparent masses of ~105 kDa and ~230 kDa, respectively. Immunohistochemistry using these antibodies revealed that the receptors were uniformly distributed throughout the ooplasm of early perinucleolus stage oocytes. Both receptors appear to migrate toward the oocyte periphery and become localized near the oocyte membrane during the oil droplet stage. In addition, *in situ* hybridization using antisense probes for *vgr* and *ldlr* revealed a strong signal, uniformly distributed in the ooplasm of perinucleolus stage oocytes, which became barely detectable in lipidic and vitellogenic stage oocytes.

Conclusion:

The present study confirmed the patterns of expression and localization of two classical ovarian lipoprotein receptor genes and proteins (i.e., VgR and LDLR) throughout oogenesis of cutthroat trout. Characterization of LDLR was conducted for the first time in teleosts and patterns of LDLR expression and localization appeared similar to those observed for VgR, indicating possible functional similarities in terms of ovarian yolk formation *via* receptor-mediated endocytosis of lipoproteins (e.g., vitellogenin and/or other plasma lipoproteins). The results also support a "translocation-dependent" model for initiation of ovarian receptor-mediated endocytosis. Although *vgr* and *ldlr* transcripts and their protein products are expressed during early oogenesis (i.e., perinucleolus stage), the VgR and LDLR likely begin to function only after they localize near the oocyte membrane during the later stages of oogenesis that are characterized by active endocytosis (e.g., lipidic and vitellogenic stages).

Acknowledgment: This study was supported by JSPS KAKENHI (#22380103: Grant-in-Aid for Scientific

Research B; #19380106 & #19780143: Grant-in-Aid for Young Scientists B). We also acknowledge to Dr. Hideaki Kudo for his help in the immunohistochemical analyses.

References:

[1]HIRAMATSU, N., CHAPMAN, R.W., LINDZEY J.K., HAYNES, M.R., SULLIVAN, C.V. 2004. Molecular characterization and expression of vitellogenin receptor from white perch (*Morone americana*). Biol. Reprod., 70(6): 1720-1730.

[2]HIRAMATSU, N., HARA, A., HIRAMATSU, K., FUKADA, H., WEBER, G.M., DENSLOW, N.D., SULLIVAN, C.V. 2002. Vitellogenin-derived yolk proteins of white perch, *Morone americana*: purification, characterization, and vitellogenin-receptor binding. Biol. Reprod., 67(2): 655-667.

[3]READING, B.J., HIRAMATSU, N., SULLIVAN, C.V. 2011. Disparate binding of three types of vitellogenin to multiple forms of vitellogenin receptor in white perch (*Morone americana*). Biol .Reprod., 84(2): 392-399.

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

MOLECULAR CHARACTERIZATION AND EXPRESSION ANALYSIS OF ESTROGEN RECEPTOR AND VITELLOGENINS IN INSHORE HAGFISH (*EPTATRETUS BURGERI*)

Nishimiya O.[1], **Kunihiro Y.**[1], **HiramatsuN.**[1,2], **InagawaH.**[3], **Todo T.**[1], **Matsubara T.**[4], **Reading B.J.**[2], **Sullivan C.V.**[2] and **Hara A.**[1]

[1]Graduate School of Fisheries Sciences, Hokkaido University, Minato 3-1-1, Hakodate, Hokkaido, 041-8611, Japan. Fax: +81-138-40-8878 email: o.nishimiya@fish.hokudai.ac.jp

[2]Department of Biology, North Carolina State University, Raleigh, NC 27695-7617, USA

[3]Department of Applied Aquabiology, National Fisheries University, Shimonoseki, Yamaguchi 759-6595, Japan.

[4]South Ehime Fisheries Research Center, Ehime University, Funakoshi 1289-12, Ainan-cho, Minami Uwa, Ehime 798-4292, Japan

Introduction:

Vitellogenin (Vg), the major precursor of egg yolk in oviparous vertebrates, is typically synthesized by the liver of maturing females in response to estradiol-17β (E2). E2 induces Vg gene expression through interaction with one or more estrogen receptors (ERs) and E2/ER complexes bind to specific estrogen response elements (EREs) in the promoter regions of Vg genes [1]. Although the molecular mechanisms underlying E2-dependent Vg gene expression have been well described in teleosts and tetrapods, little is known about such mechanisms in jawless hagfish, modern representatives of the most primitive vertebrates. In this regard, we have confirmed the duality Vg (ihVg) gene transcripts (*ihvg1* and *ihvg2*) and the corresponding protein products of inshore hagfish (Kunihiro Y., Hiramatsu N., and Hara A, *unpublished*).The objectives of the present study were to: (1) clone and characterize the cDNA encoding inshore hagfish ER (ihER); (2) analyze the hepatic mRNA expression of *ihvg1*, *ihvg2* and *iher* in hagfish captured from the wild; and (3)observe such gene expressions following E2-administration.

Methods:

Inshore hagfish were caught near the Futaoi Island at Shimonoseki city, Japan. A partial cDNA encoding the C-terminal part of a putative ihER was cloned from gravid female liver by rapid amplification of cDNA ends. Serum E2 levels were quantified using Estradiol EIA Kit and hepatic mRNA expression of *ihvg1*, *ihvg2* and *iher* were quantified by a quantitative real-time reverse-transcription PCR (qRT-PCR) assay. For the estrogen-administration experiment, male and immature hagfish were implanted with a subcutaneous silastic tube containing E2 dissolved in a solution of 50% dimethyl sulfoxide (DMSO) in castor oil (10 or 100 µg / individual). Control hagfish were implanted with 50% DMSO/castor oil. Blood and liver tissues were sampled at 0 (initial), 14 and 28 days after implantation and serum E2 levels, as well as hepatic*ihvg1*, *ihvg2* and *iher* mRNA levels, were quantified as described above.

Results and Discussion:

The obtained partial cDNA (1,685base pair) encoded a polypeptide(439 amino acids, AA) with several structural features typical of vertebrate ERs, including nuclear receptor-characteristic domains C (partial), D, E, and F. Two conserved motifs, the zinc-finger motif (ZnF_C4 domain) in domain C (i.e., the DNA binding domain) and the HOLI motif in domain E (i.e., the ligand binding domain; LBD), also were identified in the ihER protein encoded by this cDNA. The deduced AA sequence of ihER was 62% identical to that of sea lamprey ER (*Petromyzon marinus*; slER). Percent identity between ihER and slER was high in the LBD (79.5%), whereas it was <49% when compared with other vertebrate ERs. Phylogenetic analysis placed ihER and slER into a single clade, located at a position prior to the vertebrate ERα and ERβ divergence. In wild-caught inshore hagfish, hepatic *ihvg1* and *ihvg2*mRNA levels exhibited a positive correlation with gonadosomatic index (GSI), however they did not show any significant correlation with hepatic *iher* mRNA levels nor serum E2 levels. In the estrogen-administration experiment, serum E2 levels in male and immature hagfish treated with 100µg E2 (415~3,415pg/ml) were significantly higher than that in control and wild hagfish including vitellogenic females (19~327pg/ml). Expression levels of both *ihvg1* and *ihvg2* mRNAs appeared to increase in response to E2 implantation, however induced levels were approximately 15 times lower than that in wild-caught vitellogenic females (GSI>0.5). Furthermore, no differences were observed for *iher* mRNA expression between all non-implanted wild-caught hagfish and E2-administeredhagfish. These results indicate that E2 and ihER may not be the only factors that induce ihVg production in inshore hagfish, although we have confirmed that each of the *ihvg* promoter sequences possess several ERE-like domains and ERE half-sites (Nishimiya *et al.*, *unpublished*).

Conclusions:

In this study, a putative ER was cloned and structurally characterized in inshore hagfish. Essential motifs for Vg gene expression were identified in the

promoter sequences of *ihvg1* and *ihvg2*, however changes in levels of ihER and E2 exhibited relation to neither reproductive status nor gender. Furthermore, expression levels of *ihvg1* and *ihvg2* following E2 induction were much lower than that found in naturally vitellogenic females. These findings indicate that the molecular mechanism(s) of Vg gene expression in inshore hagfish might be regulated by other factors in addition to E2, unlike the typical induction mechanism of vitellogenesis in other oviparous vertebrates.

Acknowledgment: This study was supported by JSPS KAKENHI (#22380103: Grant-in-Aid for Scientific Research B).

References:

[1]WAHLI W.1988. Evolution and expression of vitellogenin genes. Trends Genet., 8:227-32.

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

TEMPERATURE AND TEMPORAL MODULATED, DOSE DEPENDENT RESPONSE OF ESTRADIOL-17β ON THE INDUCTION OF VITELLOGENIN AND CHORIOGENIN IN *CHANNA PUNCTATUS*

Om Prakash†, Goswami, S.V.††., Sehgal, N.††

† Department of Zoology, Sri Venkateswara College, University of Delhi, Delhi-110021
†† Department of Zoology, University of Delhi, Delhi-110007

Introduction:

Vitellogenin (Vg) and Choriogenin (Chg) are the precursor proteins of egg-yolk and egg-envelope proteins. Vg & Chg are synthesized in liver in primarily in liver under the influence of circulating estrogen hormone and are deposited in and around the egg respectively. Vg & Chg are influenced by various hormones and environmental factors. Present study has been focused to understand the influence of environmental factors like photoperiod, temperature, estrogen hormones and support these findings through experimentation.

Methods:

The steroid solutions were prepared in 50% alcohol and the fishes were injected intramuscularly using 1-ml tuberculin syringe fitted with 26-gauge needle for all the studies. The control fishes in all the experiments were injected intramuscularly with 50% alcohol. Fishes were collected for 1 year on weekly basis, plasma samples were collected, for estimation of by homologous ELISAs and GSI was calculated. Experiments were carried out to see the dose, time and temperature dependent induced effect of estradiol-17β (E_2) on Vg and Chg synthesis. All the data were analyzed by student t-test using the Sigma plot software. Control groups were compared with experimental groups for both the proteins, Vg and Chg.

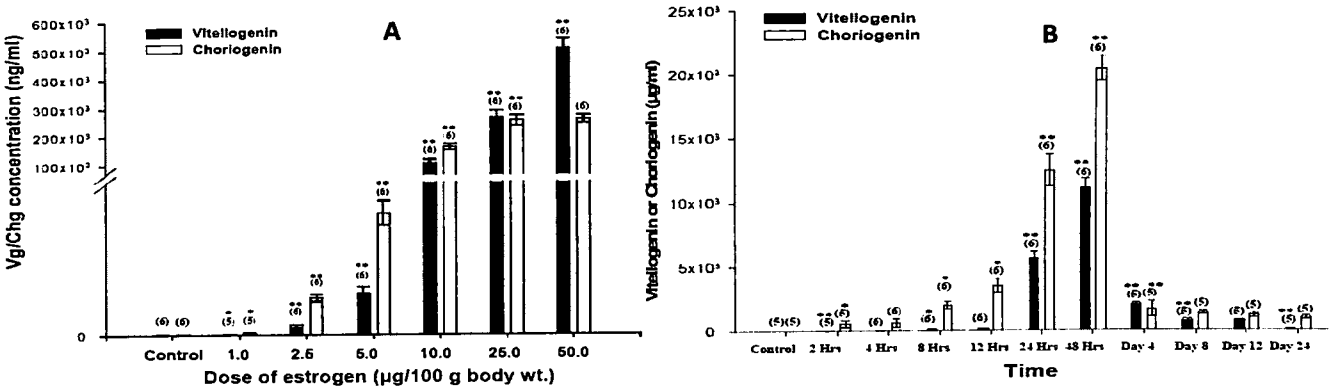
Results:

Annual studies and *in vivo* experiments revealed that concentration of Chg in the plasma was always higher that Vg at low doses (up to 10 µg/ml/100g body wt) and at low temperature (15°C), whereas Vg levels were high at doses higher than 25 µg/ml/100g body wt) and higher temperature (Fig 1A, Fig 2). Temporal study revealed that plasma levels of Chg were higher at low dose of E_2 (Fig1B) and at higher (data not shown).

Discussion:

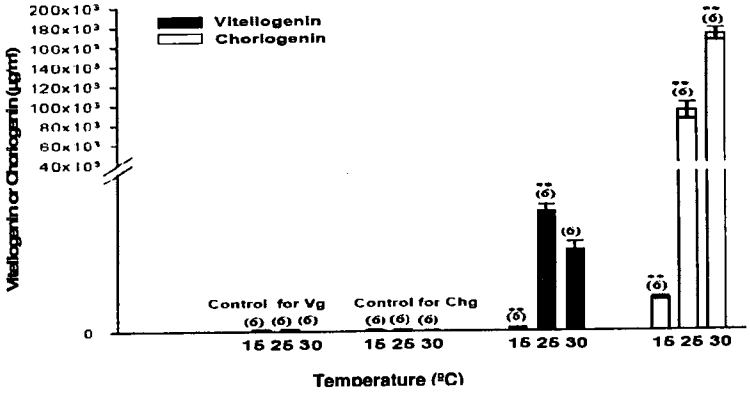
An analysis of the annual reproductive cycle of the female murrel, *C. punctatus* in the context of seasonal variations in temperature and photoperiod reveals that ovarian growth is initiated during Feb-Apr when both photoperiod and temperature are showing gradual but perceptible increase. Thereafter, gonads enlarge rapidly due to yolk deposition in response to higher temperature and longer photoperiod in nature. Maximal gonad size is attained in June and maintained till the next set of environmental cues, monsoon rains, which trigger meiotic maturation, ovulation and spawning of the post-vitellogenic oocytes. Following spawning, ovaries remain regressed throughout winter season. The prevailing cool temperature and decreasing photoperiod prevent the further development of the gonads immediately after the completion of the first. The importance of ambient temperature in promoting ovarian recrudescence is evident in the present study. Investigation on the annual reproductive cycle of the murrel reveals that, Chg was detected in the blood earlier in the year than Vg, suggesting that the egg-chorion is formed very early during oogenesis. Lower temperature and shorter photoperiod favour synthesis of Chg. A physiological significance of this observation can be that egg-chorion has to be formed before active incorporation of heterosynthetic Vg during vitellogenesis.

Fig 1: Effect of E_2 on induction of Vg & Chg; A=different doses, B= temporal response



In the present study, a direct relationship was observed between the dose of E_2 administered and plasma levels of these two proteins. This study further reveals that Chg synthesis is more sensitive to E_2 than Vg. At lower dosages of E_2, levels of Chg in the plasma were higher than those of than Vg. This observation may explain why Chg appears earlier and at higher concentrations than Vg during the annual reproductive cycle of the murrel. The studies suggest that lower temperatures and lower dose of E_2 promote Chg synthesis, whereas higher temperatures and higher dose of E_2 favour Vg production. This assumption is supported by the results of our investigation on the annual reproductive cycle of the murrel.

Fig 2: Effect of E_2 on induction of Vg and Chg at different



References:

[1]ARUKWE, A. 2008. Fish estrogenic pathways: Chemical disruption and related biomarkers. Rocha, M.J., A. Arukwe, B.G. Kapoor, Fish reproduction. Science Publishers, New Hampshire, pp. 471-514.

[2]OM PRAKASH, S.V. GOSWAMI, N. SEHGAL. 2007. Establishment of ELISA for murrel vitellogenin and choriogenin, as biomarkers of potential endocrine disruption. Comp. Biochem. Physiol.C. 146(4):540-551.

EVIDENCE FOR A POSSIBLE MEDIATORY ROLE OF TUMOR NECROSIS FACTOR α ON LUTEINIZING HORMONE-INDUCED OOCYTE MATURATION IN TROUT

Planas J. V., Crespo D.

Department of Physiology, School of Biology, University of Barcelona and Institute of Biomedicine of the University of Barcelona (IBUB), Diagonal 645 08028 Barcelona, Spain

Fax: +34-93-4110358 email: jplanas@ub.edu

Introduction:

In fish, like in other vertebrates, luteinizing hormone (LH) is an essential hormone for the completion of oocyte maturation. In salmonid fish (i.e., salmon and trout), oocyte maturation is induced by LH through its stimulation of the production of the maturation-inducing steroid, 17α,20β-dihydroxy-4-pregnen-3-one (17,20β-P). Specifically, LH stimulates the production of 17,20β-P through the production of its steroidal precursor 17α-hydroxyprogesterone (17-OHP) as well as the expression and activity of 20β-hydroxysteroid dehydrogenase (20β-HSD), the enzyme responsible for the conversion of 17-OHP to 17,20β-P. In mammals, several factors have been reported to modulate the effects of LH on oocyte maturation. For example, a strong body of evidence suggests that tumor necrosis factor α (TNFα) could play multiple physiological roles in the control of ovarian function. In the present study, we have investigated the possible involvement of TNFα in the regulation of oocyte maturation in the brown trout (*Salmo trutta*).

Methods:

After dissection, brown trout ovaries were placed in Hank´s balanced salt solution (HBSS) and individual ovarian follicles were manually separated with forceps from each ovary on ice, as previously described [1]. For the *in vitro* induction of GVBD, brown trout follicles at the preGVBD stage were incubated in HBSS containing 0.2% BSA (HBSS-BSA). Ten follicles were placed in each well of a 6-well culture plate containing 4 ml of HBSS-BSA in the absence or presence of the test compounds for 48 h at 15°C with shaking. At the termination of the incubation period the culture medium was removed and stored at -20°C to determine the *in vitro* production of 17α-hydroxyprogesterone (17-OHP) and 17α,20β-dihydroxy-4-pregnen-3-one (17,20β-P). To collect ovarian tissue for RNA extraction, preGVBD follicles from each of a total of three females were incubated (20 follicles/5 ml in triplicate) in HBSS-BSA in the absence or presence of test compounds, at 15°C for 48 h with shaking. At the end of the incubation period, the ovarian follicles were removed. Also, theca and granulosa layers were manually dissected from ovarian follicles after treatment with the test compounds, as previously described [2]. Subsequently, ovarian tissues were flash frozen in liquid nitrogen and stored at -80°C until assayed.

Results and Discussion:

Our results show that *in vitro* treatment of brown trout preovulatory follicles with coho salmon LH (sLH) significantly increased oocyte maturation, as assessed by germinal vesicle breakdown (GVBD), and this effect was blocked by TAPI-1 (an inhibitor of TNFα converting enzyme or TACE/ADAM17). Furthermore, treatment of trout preovulatory follicles with sLH increased the expression of *Tnfα*. Interestingly, recombinant trout TNFα (rtTNFα) significantly increased the number of follicles undergoing GVBD. Our results also show that the stimulatory effects of rtTNFα on oocyte maturation were the result of the direct involvement of rtTNFα in stimulating the production of 17,20β-P as evidenced by the complete blockage of sLH-stimulated production of both 17-OHP and 17,20β-P by TAPI-1 and by the direct effects of rtTNFα in stimulating the production of 17,20β-P and the expression of *20β-hsd*. Interestingly, sLH and rtTNFα also increased the ovarian expression of the LH receptor (*Lh-r*).

Conclusion:

These results strongly suggest that TNFα may contribute to the regulation of oocyte maturation by LH in trout.

References:

[1]PLANAS, J.V., GOETZ, F.W., SWANSON, P. 1997 Stimulation of brook trout ovarian steroidogenesis by gonadotropins I and II is mediated by the cyclic adenosine 3',5'-monophosphate/protein kinase A pathway, Biol. Reprod., 57: 647-654.

[2]PLANAS, J.V., ATHOS, J., GOETZ, F.W., SWANSON, P. 2000. Regulation of ovarian steroidogenesis in vitro by follicle-stimulating hormone and luteinizing hormone during sexual maturation in salmonid fish, Biol. Reprod., 62: 1262-1269.

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

GROWTH DIFFERENTIATION FACTOR 9 AND BONE MORPHOGENETIC PROTEIN 15 mRNA AND PROTEIN: CELLULAR LOCALIZATION AND DEVELOPMENTAL EXPRESSION IN THE OVARY OF EUROPEAN SEA BASS

García-López, Á.[1], Sánchez-Amaya, M.I.[1], Halm, S.[1], Astola, A.[2], Prat, F[1].

[1]Instituto de Ciencias Marinas de Andalucía (CSIC), República Saharaui 2, 11510 Puerto Real, Cádiz, Spain, f.prat@csic.es, +34 956834701.

[2]Dept. Bioquímica y Biología Molecular, Facultad de Ciencias, Universidad de Cádiz, 11510 Puerto Real, Cádiz, Spain.

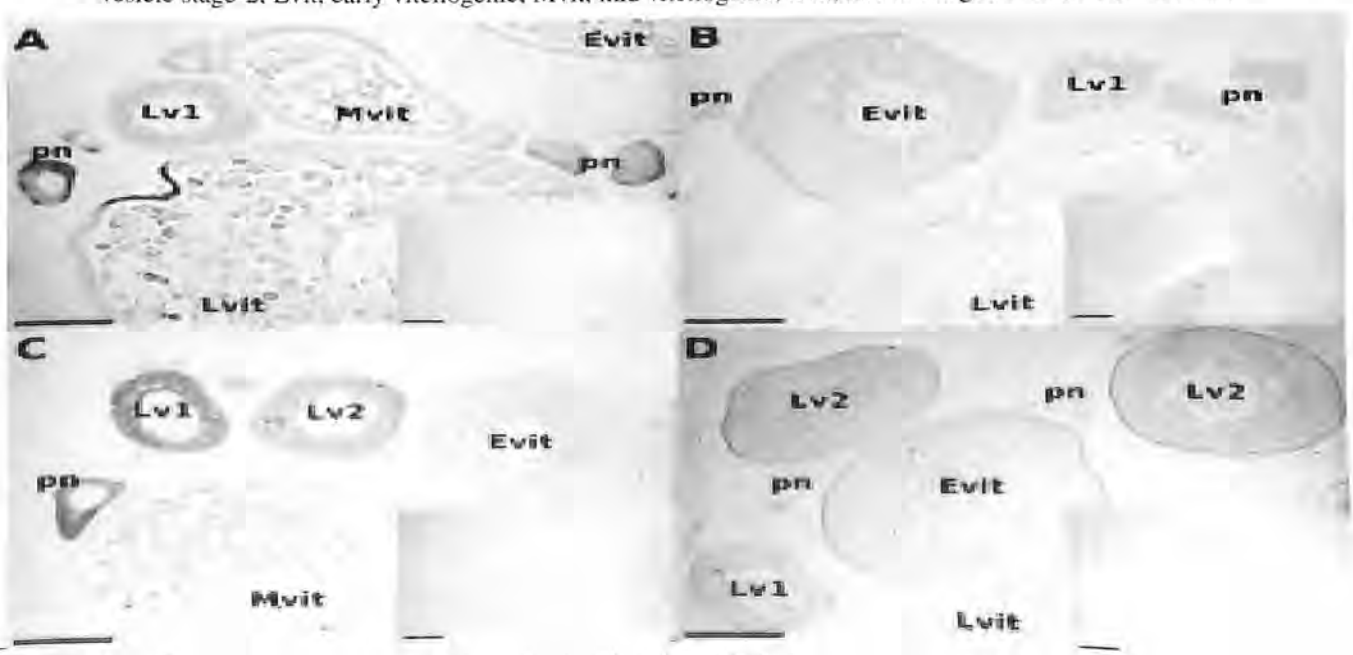
Introduction:

The two oocyte-secreted, transforming growth factor-beta superfamily members, growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15) have crucial roles in early follicular growth in mammals [1]: Gene knockouts or animals with inactivating mutations in the *gdf9* or *bmp15* genes are infertile with oocytes arrested at the early follicular stages. Recently, we cloned both genes in the European sea bass and expression studies showed high levels of *gdf9* and *bmp15* expression in ovaries containing exclusively pre-vitellogenic oocytes [2] suggesting a function in fish similar to what has been described for mammals. Here we present data about the cellular localization of the *gdf9* and *bmp15* mRNA and their respective proteins together with expression levels of *gdf9*/Gdf9 and *bmp15*/Bmp15 in follicles/oocytes at different developmental stages and protein expression in whole ovaries during the annual reproductive cycle of sea bass.

Methods:

In situ hybridization (ISH) was performed in ovaries of adult European sea bass using *gdf9 and bmp15* DIG-labeled riboprobes encoding the mature peptides. Immunohistochemistry (IHC) was carried out using species-specific Gdf9 antiserum (dilution: 1/2000) and Bmp15 antiserum (dilution: 1/500). Antisera were raised in rabbits using recombinant Gdf9 and Bmp15 mature proteins produced in *E. coli* BL21(DE3) cells as antigens. Expression levels of *gdf9* and *bmp15* mRNA in isolated follicles/oocytes was performed by qrtPCR, using specific primers, SYBR Green dye and cDNA retro-transcribed from total RNA as template. Total RNA was extracted from ovarian follicles collected from vitellogenic ovaries and classified into 5 stages according to size and cytoplasm appearance: perinucleolar/primary growth stage (pn/Pg), lipid vesicles stage (Lv), early vitellogenic (Evit), mid vitellogenic (Mvit), and late vitellogenic (Lvit) oocytes. Protein expression analysis was performed by Western blot analysis using protein extracts collected during total RNA extraction and Gdf9 and Bmp15 antisera at 1/6000 and 1/20000 dilutions, respectively. Protein expression in whole ovaries was also analyzed by Western blot using protein extracts of ovaries collected monthly

Fig. 1. Cellular localization of *gdf9*/Gdf9 (A,B) and *bmp15*/Bmp15 (C,D) mRNAs and proteins by *in situ* hybridization and immunohistochemistry. Insets correspond to negative controls. pn, perinucleolar stage; Lv1, lipid vesicle stage-1; Lv2, lipid vesicle stage-2; Evit, early vitellogenic; Mvit, mid vitellogenic; Lvit, late vitellogenic. Scale bars: 100 um.



during a reproductive cycle (n=5 per month).

Results:

ISH showed intense signals for *gdf9* and *bmp15* in the cytoplasm of the oocytes at the perinucleolar stage that decreased steadily until the early vitellogenic stage (Fig. 1A,C). IHC revealed an abundant Gdf9 presence in the cytoplasm of oocytes from perinucleolar to early vitellogenic stages (Fig. 1B). In contrast, signal for Bmp15 was absent in oocytes at the perinucleolar stage,

November (beginning of vitellogenesis) to April (spawning/postspawning period) (Fig. 3).

Conclusion:

In situ hybridization and the analyses of *gdf9* and *bmp15* transcripts in isolated oocytes confirm previous results obtained in the European sea bass [2], and indicate that the mRNAs are synthesized by the oocyte. The simultaneous expression of *gdf9* and Gdf9 furthermore suggests an important role for Gdf9 during

Fig. 2. Expression levels of *gdf9*/Gdf9 (left) and *bmp15*/Bmp15 (right) mRNA and protein in isolated follicle/oocytes. Pn/Pg, perinucleolar/primary growth; LV, lipid vesicles; Evit, early vitellogenic; Mvit, mid vitellogenic; Lvit, late vitellogenic.

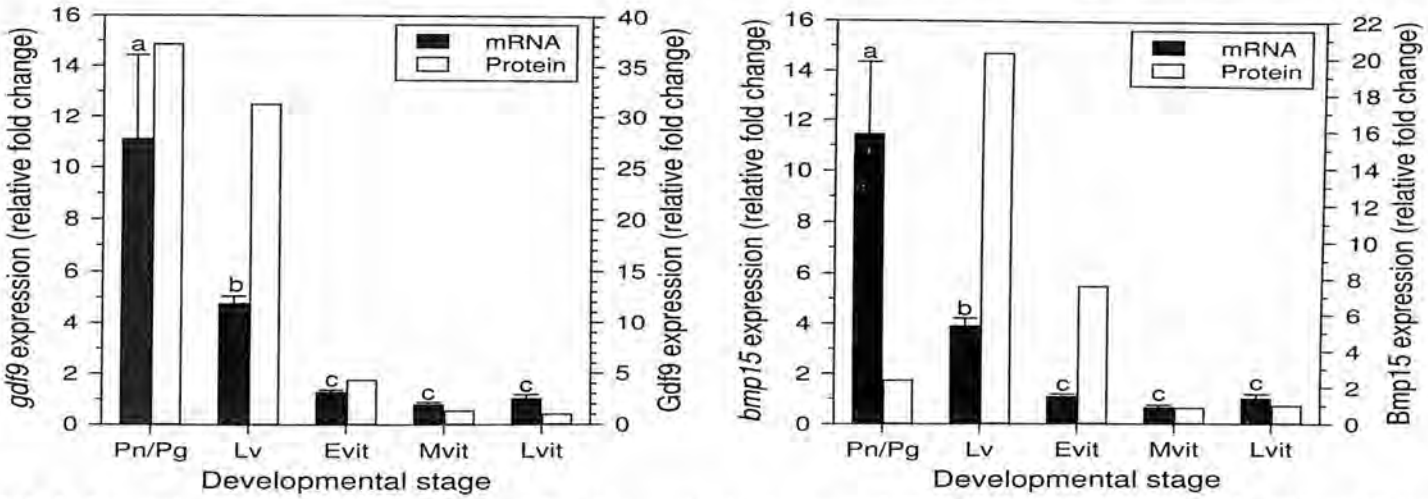
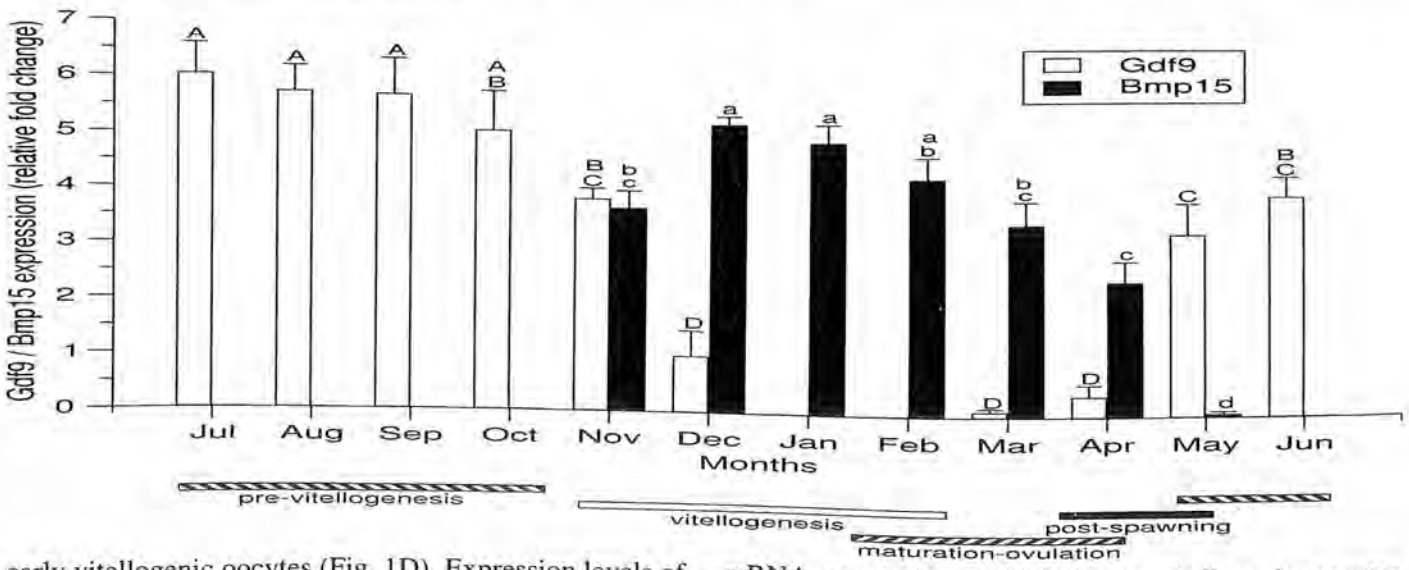


Fig. 3. Seasonal expression levels of Gdf9 and Bmp15 proteins in the ovary during the first reproductive cycle. Different letters indicate significant differences ($p < 0.05$).



early vitellogenic oocytes (Fig. 1D). Expression levels of *gdf9*/Gdf9 and *bmp15*/Bmp15 in isolated follicles/oocytes confirmed the results obtained by ISH and IHC (Fig. 2). During the reproductive cycle, expression levels of Gdf9 were high during pre-vitellogenesis while Bmp15 was only detected from

mRNA expression is high in pre-vitellogenic oocytes and during pre-vitellogenesis of the reproductive cycle [2], high levels of Bmp15 protein are found from late pre-vitellogenesis and the beginning of vitellogenesis, suggesting a role for Bmp15 at later stages of development, maybe preventing premature oocyte maturation as it has been suggested in zebrafish [3].

Funded by the former Spanish Ministry of Education and Science. Grant AGL2007-61192/ACU to F.P. and CSIC-JAE program contract to Á.G.-L.

References:

[1]JUENGEL, J.L., McNATTY, K.P. 2005. The role of proteins of the transforming growth factor-beta superfamily in the intraovarian regulation of follicular development. Hum. Reprod. Update 11: 144-161.

[2]HALM, S., IBAÑEZ, A.J., TYLER, C.R., PRAT, F. 2008. Molecular characterisation of growth differentiation factor 9 (*gdf9*) and bone morphogenetic protein 15 (*bmp15*) and their patterns of gene expression during the ovarian reproductive cycle in the European sea bass. Mol. Cell. Endocrinol., 291: 95-103.

[3]CLELLAND, E.S., TAN, Q., BALOFSKY, A., LACIVITA, R., PENG, C. 2007. Inhibition of premature oocyte maturation: A role for bone morphogenetic protein 15 in zebrafish ovarian follicles. Endocrinology, 148: 5451-5458.

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

REPRODUCTIVE HORMONE PROFILE AND TISSUE ARCHITECTURE OF GONAD IN MATURED GREY MULLET (*MUGIL CEPHALUS*) CAUGHT FROM THE INSHORE WATERS OF KOVALAM, EAST COAST OF INDIA

Prem Kumar, A.R.T. Arasu, M. Kailasam, J.K. Sundaray,* R. Subburaj, G. Thiagarajan, S. Elangeswaran

Fish Culture Division; Central Institute of Brackishwater Aquaculture, RA Puram, 75-Santhome High Road, Chennai-28, India

*Kakdwip Research Centre of CIBA, Kakdwip, 24 Paraganas (South), West Bengal-743347

IndiaGrey mullet (*Mugil cephalus*) is one of the very important marine teleosts suitable for farming in marine, brackishwater and freshwater eco-systems. It is extensively farmed in tropical and sub-tropical conditions. The artificial propagation of grey mullet though has been practised in some countries and in many places it depends upon the natural recruitment. The major constraint in the captive maturation and spawning of grey mullet is a lack of adequate knowledge on the hormonal profile of different stages of maturation to understand the conditions for intervening and administer exogenous hormone to accelerate ovulation and spawning. In order to understand the profile of the reproductive hormone, an attempt was made to analyze the important reproductive hormones of grey mullet female fish caught from the wild. Blood sample was collected from a matured female (1.4 kg size) caught from the inshore catch from Kovalam during the month of November 2010. Serum was separated and the reproductive hormones like testosterone, 17β- estradiol and progesterone following chemiluminescence method. Gonado-somatic index (GSI), egg size and histoarchitecture of the ovary were also analyzed. Results of the analysis showed that in the matured female the mean egg size is of 600± 40 µm (GSI-13.62). The total testosterone was 39.81 mg/dl, 17β- estradiol was 29.4 mg/dl and the progesterone level was 0.0015 mg/dl. The histoarchitecture showed the central location of germinal vesicle, prominent distribution of yolk granules in cytoplasm and movement of cortical alveoli towards oocyte membrane indicating the late maturing stage of oocytes in the analyzed fish. The observation of the present investigation revealed that fishes having oocyte around 600 µm is in advanced stage of maturity, and fishes with this type of oocytes could be induced to spawn under captivity with the administration of gonadotropin hormone.

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

EXPRESSION OF GENES INVOLVED IN OOCYTE LIPIDATION IN CUTTHROAT TROUT, *ONCORHYNCHUS CLARKI*

Ryu Y.-W.[1], Tanaka R.[1], Kasahara A.[1], Saito K.[1], Kanno K.[1], Ito Y.[1], Hiramatsu N.[1], Todo T.[1], Sullivan C. V.[2] and Hara A.[1]

[1]Faculty of Fisheries Sciences, Hokkaido University, Hakodate, Hokkaido 041-8611, Japan. Fax: +81-0138-40-5529
email: mruni@fish.hokudai.ac.jp
[2]Department of Zoology, North Carolina State University, Raleigh, NC 27695-7617

Introduction:

In salmonid fishes, as in many other teleosts, high amounts of neutral lipids (NLs) are accumulated in ooplasm lipid droplets during oocyte growth; they are later utilized as an energy resource by developing embryos and larvae. However, little is known about the origin of such lipids and the mechanisms underlying their accumulation into oocytes. Triacylglycerol (TAG)-rich lipoproteins, such as very low-density lipoprotein (VLDL), are considered to be the primary carriers of the NLs to the cell [1]. Lipoprotein lipase (LPL) is an enzyme engaged in hydrolysis of TAG from VLDL and chylomicron, and the free fatty acids (FAs) released from TAG are transported into the cell through their receptors and transporters [2]. Thus, we have proposed a model for the oocyte lipidation as follows; VLDL are metabolized by LPL outside of the oocyte and resulting FAs then enter oocytes as a source for *de novo* biosynthesis of NLs. In order to verify this model, we cloned cDNAs encoding factors considered to be involved in oocyte lipidation, and analyzed their expression in ovary of the cutthroat trout, *Oncorhynchus clarki*.

Methods:

cDNAs encoding two kinds of lipase, LPL and endothelial lipase (EL), two membrane FA receptors (e.g., FA translocases), cluster of differentiation 36 (CD36) and scavenger receptor class B1 (SR-B1), and intercellular FA transporters, FA binding proteins (FABPs), were isolated from the cutthroat trout ovary using a polymerase chain reaction (PCR) based cloning strategy. Expression of these mRNAs in various tissues and in ovarian follicles was analyzed using simple reverse transcription-PCR (RT-PCR), real-time quantitative RT-PCR and *in situ* hybridization (ISH).

Results and Discussion:

Two types of cDNAs were obtained for both LPL (LPL1 and LPL2) and EL (EL1 and EL2). Both types of LPL mRNA were highly expressed in lipid storage tissues (e.g. adipose tissue, muscle and ovary) and predominantly expressed in granulosa cells of ovarian follicles. Ovarian LPL1 mRNA levels showed a remarkable peak in April (oocyte lipid droplet stage) and then decreased to low values sustained until November (mid-vitellogenesis) and followed by a small peak in LPL1 gene expression in December (late vitellogenesis). LPL2 mRNA levels did not show pronounced changes. In contrast, both ELs were highly expressed in ovary, and their expression was mainly observed in oocytes. Levels of both EL mRNAs were high in early vitellogenesis and otherwise sustained at low values. ISH analysis showed that both LPL mRNAs were strongly expressed in ovarian somatic cells, especially granulosa cells in pre-vitellogenic follicles, whereas expression of both ELs was restricted to oocytes. These results suggest that, in cutthroat trout, VLDL is metabolized by the action of LPLs in the granulosa cell layer to generate free FAs to be incorporated into growing oocytes. It has been shown that EL has significantly higher phospholipase activity than triglyceride activity [3] and, thus, the ELs may act as phospholipases for metabolizing polar lipids of vitellogenin-derived yolk proteins in trout oocytes.

A partial CD36 cDNA and a full length SR-BI cDNA were cloned from the trout ovary. CD36 mRNA was widely expressed in various tissues including ovary and, in ovarian follicles, its expression was found in both somatic cells and oocytes. SR-BI mRNA was highly expressed in ovary; the transcripts were highly expressed in granulosa cells and moderately expressed in oocytes. The expression of CD36 and SR-BI genes in oocytes suggests that they play important roles in uptake of FAs into oocytes.

Among 7 subtypes of FABP (FABP1, 2, 3, 6, 7, 10, 11) known to be present in teleost fishes [4], we found that FABP1, 3, 7 and 11 were expressed in ovary. In RT-PCR analysis of vitellogenic ovarian follicles, mRNAs of FABP1, 3 and 11 were expressed in both somatic cells and oocytes, whereas FABP7 mRNA was expressed very weakly and only in oocytes. ISH analyses using pre-vitellogenic ovary showed that FABP1 mRNA was strongly expressed in both somatic cells and oocytes, whereas FABP3 mRNA was weakly expressed only in somatic cells. Expression of FABP11 mRNA was strong in somatic cells but weak in oocytes. However, strong expression of FABP11 mRNA was found in the ooplasm of atretic follicles. These results suggest that FABP1 and

FABP11 are involved in FA transportation in the ooplasm of pre-vitellogenic ovarian follicles.

Conclusion:

In summary, we have cloned cDNAs encoding factors likely to be involved in oocyte lipidation (lipid droplet formation) from the ovary of cutthroat trout, and analyzed their expression in ovarian follicles. Results of the present study update our proposed model for oocyte lipidation, as follows: 1) VLDL is metabolized by the action of LPLs in the granulosa cell layer to generate free FAs, 2) the FAs are incorporated into growing oocytes through CD36 and/or SR-BI, 3) the FAs are then transferred to endoplasmic reticulum (ER) by FABP1 and/or FABP11 in ooplasm, and 4) NLs are synthesized from the FAs and lipid droplets are formed in the oocyte ER [5].

References:

[1]WIEGAND, M.D. 1996. Composition, accumulation and utilization of yolk lipids in teleost fish. Rev. Fish Biol. Fish., 6: 259-286.

[2]van der VUSSE, G.J., van BILSEN, M., GLATZ, J.F.C., HASSELBAINK, D.M., LUIKEN, J.J.F.P. 2002. Critical steps in cellular fatty acid uptake and utilization. Mol.Cell. Biochem., 239: 9-15.

[3]RADER, D.J., JAYE, M. 2000. Endotherial lipase: a new member of the trigleceride lipase gene family. Curr. Opin. Lipidol., 11: 141-147.

[4]AGULLERIO, M.J., ANDRÉ M., MORAIS, S., CERDÁ, J., BABIN P.J. 2007. High transcript level of fatty acid-binding protein 11 but not of very low-density lipoprotein receptor is correlated to ovarian follicle atresia in a teleost fish (*Solea senegalensis*). Biol. Reprod., 77: 504-516.

[5]WALTHER, T.C., FARESE JR, R.V. 2009. The life of lipid droplets. Biochim. Biophys. Acta 1791: 459-466.

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

PREVITELLOGENIC OOCYTE GROWTH IN A TELEOST FISH, *ANGUILLA AUSTRALIS*: CROSSTALK BETWEEN INTRAOVARIAN FACTORS AND GROWTH AXIS

Shoae A., Setiawan A. N., Lokman P. M.

Department of Zoology, University of Otago, 340 Great King Street, Dunedin 9016, New Zealand. Fax +64-34797584, e-mail: ashoae@yahoo.com

Introduction:

It is well-established that oocyte growth and follicular development in vertebrates involves communication between the germ cell and its surrounding follicular layers. This communication is believed to be controlled by a combination of intraovarian factors, such as growth factors and sex steroids, and extraovarian factors, such as growth hormone (GH), insulin-like growth factor-1 (IGF-1) and follicle-stimulating hormone (FSH). While a substantial number of publications has focused on regulatory factors of advanced stages of oogenesis (i.e. vitellogenesis and final oocyte maturation), less attention has been paid to early stages of oocyte growth (previtellogenesis) in teleost fish. In this study, we addressed the relationship among intraovarian mRNA levels of *igf-1*, growth differentiation factor-9 and bone morphogenetic protein-15 (*gdf-9* and *bmp-15*), growth hormone receptor-1 (*ghr-1*), insulin-like growth factor type-1 receptor (*igf-1r*) and serum levels of sex steroids (estradiol-17β, E_2 and 11-ketotestosterone, 11-KT). We further included a suite of extraovarian factors (pituitary *gh* and hepatic *igf-1* mRNA abundance) in our analyses in order to tease out the factors that are likely to play the most prominent role during previtellogenesis in the shortfinned eel, *Anguilla australis.*

Methods:

Immature wild shortfinned eels of different size classes were caught from Lake Ellesmere, South Island, New Zealand during spring and autumn of 2008/09. The *fish were euthanized, weighed and a piece of ovary, liver* and pituitary removed for histological and molecular analyses. Blood samples were processed to enable measurement of E_2 and 11-KT by radioimmunoassay. The target genes were partially cloned and subjected to real-time qPCR assay. On the basis of histological observations of ovarian tissues, the eels were subdivided between chromatin nucleolus (CN), perinucleolus (PN) and oil droplet (OD) stages of previtellogenesis. Using SPSS-16, steroid and transcript abundance means for the three previtellogenic stages (two-way ANOVA with season and stage of oocyte development as fixed factors) were compared and correlation coefficients calculated.

Results and discussion:

Oocyte size significantly increased during previtellogenesis (Fig-1A). Furthermore, we observed significant increases in serum 11-KT levels (Fig-1B) and mRNA abundance of ovarian *gdf-9, bmp-15, ghr-1 and igf-1r* with advancing previtellogenesis in wild eels (Fig-2A-D). These results point to the involvement of metabolic factors (GH-IGF-1 axis) during previtellogenic oocyte growth. Our previous *in vitro* experiments similarly identified a stimulatory role of 11-KT and IGF-1 on previtellogenic oocyte growth in eel [1]. Although an effect of 11-KT on Gdf-9 expression was not previously apparent in eel [2], we detected a strong correlation between oocyte diameter and *gdf-9*, *bmp-15* and 11-KT ($r=0.66$, $r=0.49$ and $r=0.68$, $P<0.01$, respectively). No relationship was found between intraovarian and hepatic *igf-1* mRNA levels. Likewise, pituitary *gh* and *fsh* did not significantly correlate with oocyte diameter; however, oocyte diameter *did* show a correlation with body weight ($r=0.83$, $P<0.01$).

Fig. 1: Oocyte diameter (A) and serum level of 11-KT (B) of New Zealand shortfinned eel during different stages of previtellogenesis in autumn (closed bars) and spring (open bars) of the 2008/09 austral summer; values are mean ± SEM. See text for abbreviations.

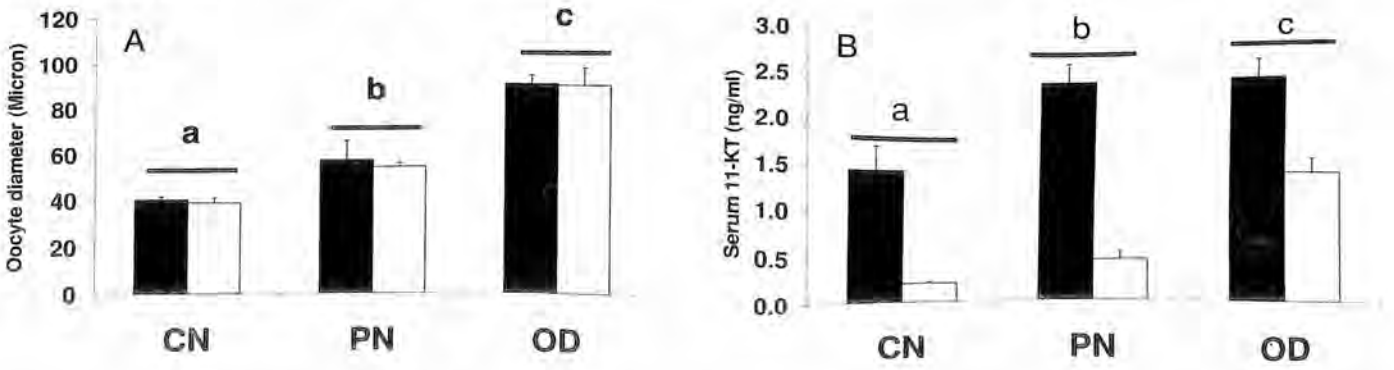
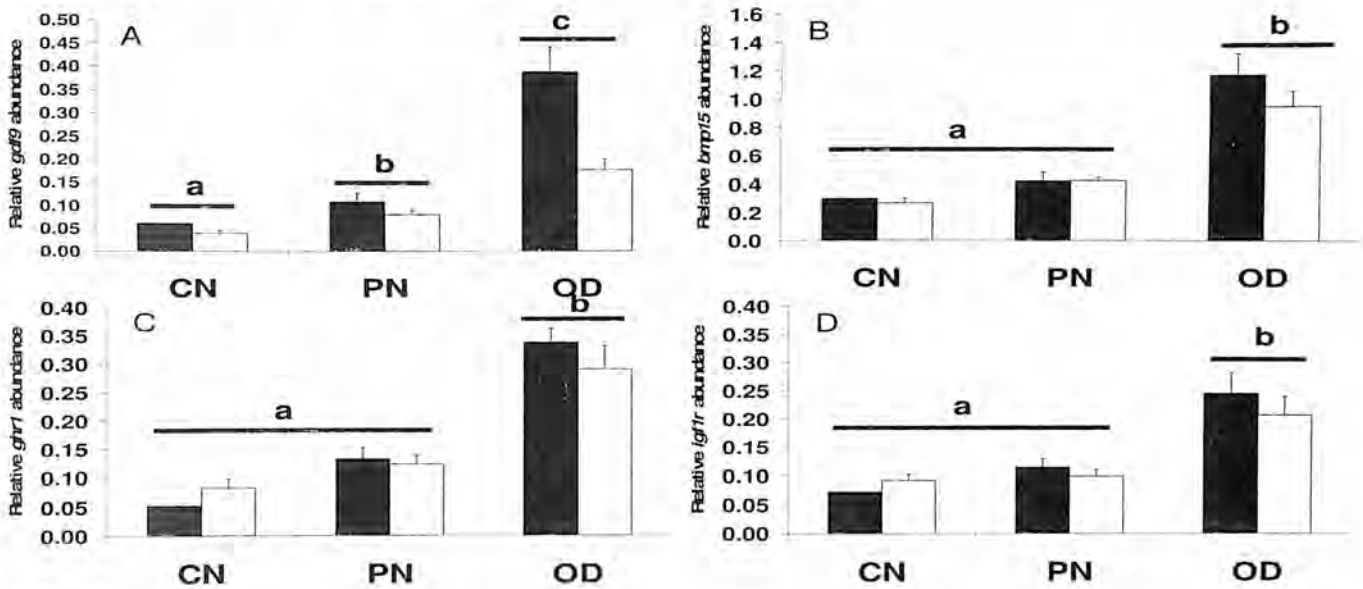


Fig.2: Relative mRNA abundance of *gdf-9* (A), *bmp-15* (B), *ghr-1* (C) and *igf-1r* (D) of New Zealand shortfinned eel during different stages of previtellogenesis in autumn (closed bars) and spring (open bars) of the 2008/09 austral summer, values are mean ± SEM. See text for abbreviations.



Conclusion:

The results of this study show a strong association between oocyte growth and mRNA levels of ovarian-derived factors, such as *gdf-9* and *bmp-15*, and 11-KT. In addition, the increasing abundances of transcripts from genes that are components of the growth axis (IGF-1R and GHR) may reflect increasing ovarian sensitivity to metabolic factors with advancing stages of early oocyte growth in teleost fish.

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

KNOCKDOWN OF ZEBRAFISH DMC1 CAUSES GAMATOGENESIS ABNORMAL

Xiaojuan Cui, Ji Chen, Wei Hu, Zuoyan Zhu

State Key Laboratory of Freshwater Ecology and Biotechnology, Institute of Hydrobiology, Chinese Academy of Sciences,Wuhan 430072,China

E-mail address: huwei@ihb.ac.cn (wei Hu); cuixiaojuan0228@163.com (xiaojuan Cui)

In mouse, dmc1 gene plays a key role in pairing and synapsis of homologs, which is essential step for the formation of haploid gametes. In order to investigate the fuction of dmc1 in zebrafish meiosis, we firstly analyzed its expression patterns in zebrafish. We found that dmc1 did not express in embryogenesis and juvenile, while in adult, dmc1 was predominantly expressed in testis and ovary.

To knockdown the dmc1 exprssion, we secondly designed microRNA(miRNA) vectors, and transferred them respectively into zebrafish fertilized eggs with microinjection. Then in situ cell death assay was carried out in the adult gonad of P0 germ-line chimeras. In the negative control group, gametogenesis progressed normally; but in the test group, the apoptosis signal was easily observed where the exogenous DNAs intergrated. Even in the female gonad, where exogenous DNAs expressed, no oocyte developped at stage II-IV. Thus, we presume that knockdown of dmc1 in zebrafish causes gametogenesis abnormal, even cell death.

Acknowledgments:

This work was financially supported by "863" high Technology Project, the National Natural Science Foundation of China, and the Knowledge Innovation Project of Chinese Academy of Sciences.

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

IDENTIFICATION AND CHARACTERIZATION OF A NOVEL 20BETA-HSD IN THE NILE TILAPIA

Zhou L.Y.[1,2], **Wang D.S.**[1], **Nagahama Y.**[2]

[1]Key Laboratory of Freshwater Fish Reproduction and Development (Ministry of Education), Key Laboratory of Aquatic Science of Chongqing, School of Life Science, Southwest University, Chongqing, 400715, China
Fax:+86-02368253005 E-mail: wdeshou@swu.edu.cn
[2] Eheme University, Funakoshi, Ainan, Ehime, 798-4292, Japan

Introduction:

Steroidogenic shift from estrogen (E2) production during oocyte growth to 17alpha, 20beta-DP production in the oocytes final maturation stage is a prerequisite during reproductive cycle in teleosts. The dramatic increase in the capacity of postvitellogenic follicles to produce 17alpha, 20beta-DP is correlated with the down-regulation of E2 producing steroidogenic enzymes, i.e. P450c17-I and aromatase, up-regulation of P450c17-II and carbonyl reductase/20beta-hydroxysteroid dehydrogenase. Vast investigation has been carried out in fish steroidogenesis, however, the molecular mechanism of steroidogenic shift controlling the reproductive cycle is far from clear. In our present studies, a novel 20beta-HSD (named as 20beta-HSD-II), which showed high expression in the follicular cells during oocyte maturation stages, was characterized in tilapia.

Methods:

Animals- All genetic female (XX) and male (XY) tilapia (*Oreochromis niloticus*) were obtained by artificial fertilization of eggs from normal females (XX) with sperm from either sex-reversed males (XX) or super males (YY), respectively. Tilapias were reared in 0.5-ton tanks with re-circulating aerated fresh water at 26^{0}C under natural photoperiod until use. *Molecular cloning-* Partial sequence of tilapia 20beta-HSD-II was obtained from the EST database. Subsequently four primers were designed to amplify a full-length cDNA sequence by 5'-rapid amplication of cDNA ends (RACE) and 3'-RACE. Finally, two gene specific primers were designed to amplify the full cDNA sequence. *RT-PCR-* Total RNA was extracted, cDNA was synthesized, and RT-PCR was carried out to check the expression levels of tilapia 20beta-HSD-II in various tissues. Positive and negative controls were set up with plasmid DNA and water, respectively. A fragment of β-actin was amplified (as internal control) from tilapia to test the quality of the cDNAs used in the PCR. *In situ hybridization-* The whole bodies of XX and XY fry at 2, 5, 11, 20, 60 day after hatching and gonads of adult tilapia were fixed in 4% paraformaldehyde in 0.85XPBS at 4°C to check the expression of 20β-HSD -I and -II in the gonad by ISH. Similarly, gonads from regularly spawning fish were sampled and fixed at d 1, 3, 5, 8, 10, 12, and 14 of the spawning cycle. Probes of sense and antisense digoxigenin-labeled RNA strands were transcribed in vitro with an RNA labeling kit from plasmid DNA containing ORFs of tilapia 20beta-HSD, 20beta-HSD-II.

Results and discussion:

The predicted amino acid sequences of 20beta-HSD-II showed the highest homology to tilapia and medaka 20beta-HSD-I (80.2% and 78.9%, respectively), but relatively low similarity to 20beta-HSD-I of human (65.5%) and chicken (65.8%). Tissue distribution by RT-PCR showed that 20beta-HSD-II was expressed in various tissues including gill, liver, intestine, kidney, muscle, ovary and testis. *In situ* hybridization (ISH) analysis demonstrated that 20beta-HSD-II was expressed in the follicular cells and cytoplasm of oocyte, as well as in the interstitial cells in the testis. Additionally, the expression of 20beta-HSD-II was also detected in both the epithelial cells of intestine and interrenal cells of the head kidney. However, the expression of 20beta-HSD-I was only detected in the early vitellogenic oocytes. No expression of 20beta-HSD-I could be detected in the testis, intestine and head kidney by ISH.

Interestingly, two type of 20beta-HSDs displayed distinct spatial and temporal expression profiles during the reproductive cycle. 20beta-HSD-I was exclusively expressed in the previtellogenic ooctyes and no expression was detected in the follicular layer cells. Interestingly, the expression of 20beta-HSD-II was dominantly expressed in the both theca cells and granulosa cells from 4 day after spawning to 12 day after spawning and was restricted to theca cells in degenerated follicular cells after ovulation. The expression profiles suggested that 20beta-HSD-II, but not 20beta-HSD-I, might be involved in the production of 17alpha, 20beta-DP.

Ontogenic expression by ISH showed that 20beta-HSD-I was only detected in germ cells in the XX gonads and no expression was found in the XY gonads. Importantly, the expression of 20beta-HSD-II in both somatic cells and germ cells was initiated from 5dah in XX gonads, while it was delayed to 20dah in the XY gonads. The ontogenic expression pattern revealed that 20beta-HSD-II might be responsible for 17alpha, 20beta

production which is required for the initiation of meiosis in fish during early development stages.

Conclusion:

A novel 20beta-HSD (20beta-HSD-II), which showed the highest homology with tilapia 20beta-HSD-I, was cloned from the Nile tilapia. The spatial and temporal expression profile of 20beta-HSD-I and –II during reproductive cycle strongly suggested that 20beta-HSD-II, but not 20beta-HSD-I, might be involved in the biosynthesis 17alpha, 20beta-DP in tilapia.

Folliculogenesis (Continued..), Spawning, Fertilization and Early Embryogenesis

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

UNDERSTANDING THE FUNCTION OF FOLLICLE-STIMULATING HORMONE PRIOR TO ONSET OF VITELLOGENESIS

Luckenbach J.A.*, Yamamoto Y.°, Guzman J.M.*, Swanson P.*

*Northwest Fisheries Science Center, National Oceanic and Atmospheric Administration, 2725 Montlake Blvd East, Seattle, Washington 98112, USA
Fax: +1-206-860-3467 email: adam.luckenbach@noaa.gov
°School of Aquatic and Fishery Sciences, University of Washington, Seattle, Washington 98195, USA

Introduction:

Follicle-stimulating hormone (FSH), produced primarily by the pituitary, is thought to play an important role in regulating growth of the ovarian follicle in fishes. However, little is known about FSH function at the level of the ovary during early stages of oocyte growth, prior to vitellogenesis [1,2]. Initially we identified ovarian genes in coho salmon (*Oncorhynchus kisutch*) that were differentially expressed during the transition from primary to early secondary growth, a period when FSH levels begin to increase in the blood [3]. Using a candidate gene approach we then identified several FSH-regulated ovarian genes in vitro during early secondary oocyte growth, which included the gonadotropin receptors, specific steroidogenesis-related mRNAs, and cell survival, growth and differentiation factors [4]. Recently we used a global genomics approach known as suppression subtractive hybridization (SSH) to identify additional genes and cellular processes influenced by FSH during early secondary oocyte growth. Since we anticipated that many FSH-regulated genes would be localized to the follicle cells surrounding the oocyte, SSHs were conducted on mRNA from follicle/interstitial cell enriched ovary preparations [5] as well as whole follicles. Time-course and FSH concentration-response experiments were conducted to characterize the responsiveness of genes to FSH, and a steroid inhibitor experiment was conducted to determine whether steroids produced by the ovary in response to FSH mediate its actions on ovarian gene expression.

Methods:

All experiments utilized mid to late cortical alveolus stage ovarian follicles from 2-year old coho salmon produced and reared in captivity. Clusters of ovarian follicles were incubated in L-15 medium containing coho salmon FSH (treatment) or in blank medium (control) in time-course, concentration-response, and steroidogenesis-inhibitor experiments. Incubation times ranged from 0-72 h and FSH concentrations ranged from 5-500 ng of hormone per ml of medium. The steroidogenesis inhibitor tested was aminoglutethimide, which inhibits P450 aromatase and P450 side-chain cleavage activity. After incubations, media samples were collected for testosterone (T) and estradiol-17 beta (E2) measurements, ovarian total RNA was isolated, and transcript levels were determined by quantitative RT-PCR. Messenger RNA was further isolated from follicles cultured for 36h to construct four SSH libraries comparing FSH-treated and control follicles. A total of 2,016 clones from the SSHs were sequenced and 1,774 sequences greater than 50 nucleotides were assembled and analyzed to determine their identity. In situ hybridizations were performed with cortical alveolus stage follicles using gene-specific probes labeled with digoxigenin.

Results and Discussion:

FSH treatment consistently increased ovarian production of T and E2 in a concentration-dependent manner. A variety of ovarian genes putatively regulated by FSH were revealed by SSH. Quantitative PCR validations showed that although some false positives existed, a number of FSH-regulated genes had been revealed. For example, a *connexin* gene (*cx34.3*) likely involved in cell-to-cell communication was upregulated 16-fold by FSH, a cell survival factor, *clusterin 1* (*clu1*), was upregulated 4-fold by FSH, and two genes likely involved in cell differentiation and tissue remodeling, *fibronectin* (*fn*) and *decorin* (*dcn*), were upregulated 3- and 1.5-fold, respectively. In the SSH libraries for genes suppressed by FSH treatment, *connective tissue growth factor* (*ctgf*), a gene that decreases in mammalian follicles as granulosa cells mature [6], was down-regulated 3-fold and *proliferating cell nuclear antigen* (*pcna*) was down-regulated 2-fold by FSH. The majority of the FSH-regulated genes identified were found in the follicle/interstitial cell enriched tissue libraries suggesting that many genes regulated by FSH originate from the somatic cells of the ovary and not the oocyte. In support of this, we localized *cx34.3* transcripts exclusively to salmon granulosa cells. Additional transcripts are currently being localized with ISH. Lastly, our initial investigation into mechanisms of FSH actions showed that FSH actions may be mediated by steroids, as effects of FSH on *cx34.3*, *fn*, and *ctgf* were completely blocked by the steroidogenesis inhibitor, or FSH may act through non-steroid pathways as seen for genes like *steroidogenic acute regulatory protein* (*star*) and *clu1*.

Conclusion:

As evident by the rapid and significant increases in T and E2 production and changes in specific steroidogenesis-related genes, FSH clearly regulates steroidogenesis during early secondary oocyte growth in coho salmon. This work further demonstrates that FSH regulates genes likely involved in cell communication, differentiation and maturation, and tissue remodeling. Actions of FSH on ovarian gene expression appear to be via steroid and non-steroid pathways. Together these findings suggest that FSH and steroids may induce differentiation of follicle cells surrounding the growing oocyte and remodeling of the ovarian follicle structure in preparation for onset of vitellogenesis. The recent development of coho salmon gonadotropin analogs will facilitate upcoming in vivo studies of FSH function.

References:

[1]PATIÑO, R. AND SULLIVAN, C.V. 2002. Ovarian follicle growth, maturation, and ovulation in teleost fish. *Fish Physiol Biochem* 26: 57-70.

[2]LUBZENS, E., YOUNG, G., BOBE, J. AND CERDÀ, J. 2010. Oogenesis in teleosts: how fish eggs are formed. *Gen Comp Endocrinol* 165: 367-389.

[3]LUCKENBACH, J.A., ILIEV, D.B., GOETZ, F.W. AND SWANSON, P. 2008. Identification of differentially expressed ovarian genes during primary and early secondary oocyte growth in coho salmon, *Oncorhynchus kisutch. Reprod Biol Endocrinol* 6: 2.

[4]LUCKENBACH, J.A., DICKEY, J.T. AND SWANSON, P. 2011. Follicle-stimulating hormone regulation of ovarian transcripts for steroidogenesis-related proteins and cell survival, growth and differentiation factors in vitro during early secondary oocyte growth in coho salmon. *Gen Comp Endocrinol* 171: 52-63.

[5]LUCKENBACH, J.A., GOETZ, F.W. AND SWANSON, P. 2008. Expressed sequence tags (ESTs) of follicle/interstitial cell enriched tissue from previtellogenic coho salmon. *Cybium* 32 (Suppl. 2): 142-144.

[6]HARLOW, C.R., DAVIDSON, L., BURNS, K.H., YAN, C., MATZUK, M.M. AND HILLIER, S.G. 2002. FSH and TGF-beta superfamily members regulate granulosa cell connective tissue growth factor gene expression in vitro and in vivo. *Endocrinology* 143: 3316-3325.

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

REGULATION OF DEVELOPMENT OF LATE PRIMARY AND EARLY SECONDARY OVARIAN FOLLICLES OF COHO SALMON BY SEX STEROIDS

Forsgren K.L[(1)], Swanson P.[(2)], Young G.[(1)]

[(1)] School of Aquatic and Fishery Sciences, University of Washington, Seattle, WA 98195, USA
Fax: +1-206-685-4674 email: grahamy@u.washington.edu
[(2)] Northwest Fisheries Science Center, NOAA Fisheries, Seattle, WA 98112, USA

Introduction:

Relatively few studies have examined the endocrine control of pre-vitellogenic ovarian follicle development in teleosts. Follicles of hypophysectomized teleosts can develop to the late perinucleolar/early cortical alveolus stage in the absence of the pituitary, suggesting that pituitary gonadotropins are not mandatory for completion of primary development of the follicle [1]. However, several lines of evidence suggest that sex steroids regulate growth of pre-vitellogenic follicles. Estradiol-17β (E2) stimulated development of cortical alveolus stage ovarian follicles in hypophysectomized goldfish, and several in vivo and in vitro studies on anguillid eels provide strong evidence for a role of 11-ketotesterone (11-KT) in the regulation of primary ovarian follicle development [2]. Since female anguillid eels display uniquely high blood levels of 11-KT at the start of the migration to oceanic spawning sites [2],it is not known if androgenic control of primary ovarian follicle development is a general phenomenon. In this study, we addressed the hypotheses that: 1) Androgens regulate primary ovarian follicle development; 2) E2 stimulates development of cortical alveolus stage follicles; and 3) plasma steroid levels reflect the roles of androgens and estrogen during primary and early secondary ovarian follicle development.

Methods:

In vitro experiments: Ovaries containing follicles at the late (Balbiani bodies absent) perinucleolar and early alveolus stages were removed from one year-old coho salmon. Ovarian fragments were cultured for 7, 14 and 21 days without or with 0.03-30 ng/ml 11-KT, E2 or testosterone (T), fixed, and processed for histology. The circumference of oocytes sectioned through the center of the nucleus was measured, and oocyte volume was calculated. Abundance of cortical alveoli was quantified. Further experiments examined whether androgen (flutamide) or estrogen (tamoxifen) receptor antagonists inhibited the growth response to steroids, and a specific aromatase inhibitor (exemestane) was used to determine if the effects of T were partially due to its aromatization to E2. *In vivo experiments:* Females with ovaries containing late perinucleolar or early cortical alveolus stage oocytes were injected with sustained release implants containing either 11-KT or E2, and sampled after 10 and 20 days. Effects on follicle size were determined as above, and plasma 11-KT and E2 levels were measured by immunoassay.

Plasma sex steroid levels during ovarian follicle development: Females were sampled at six stages: chromatin nucleolar, early and late perinucleolar, early and late cortical alveolus, and lipid droplet. Plasma 11-KT, T and E2 levels were measured by immunoassay.

Results and Discussion:

In vitro experiments: Low concentrations of 11-KT had significant growth-promoting effects on late perinucleolar follicles in vitro, with maximum size reached by 7 days of culture. Flutamide inhibited this growth promoting effect. T, although causing a significant increase in volume of late perinucleolar follicles, was less effective that 11-KT. Blocking aromatization to E2 enhanced the growth promoting effect of T. E2 had no effect on growth of perinucleolar follicles but by 21 days, E2-treated follicles displayed a few cortical alveoli. At the early cortical alveolus stage, 11-KT increased follicle volume but there was no effect on the abundance of cortical alveoli. E2 strongly promoted growth and stimulated synthesis of cortical alveoli, actions that were inhibited by tamoxifen. T had modest growth-promoting effects and stimulated a moderate increase in cortical alveoli abundance. These results indicate that completion of primary follicle growth is under androgenic control, while early secondary growth (cortical alveolus stage) is predominantly under the control of E2.

In vivo experiments: Modest increases in plasma 11-KT resulted in a significant increase in volume of late perinucleolar follicles by 10 days and in contrast to in vitro treatment, induced the appearance of a few cortical alveoli. E2 had no observable effect by day 10, but by day 20 induced increases in volume similar to those seem with 11-KT, and was also more effective than 11-KT in stimulating cortical alveoli formation. By contrast, in females with ovaries that contained early cortical alveolus stage follicles at the time of implantation, E2 was much more effective than 11-KT in stimulating follicle growth, and strikingly, the ooplasm of follicles from E2-treated fish was filled with cortical alveoli. These results are generally consistent with those from in vivo experiments; but with more overlap between the

stage-specific effects of steroids, likely due to steroids actions on the brain and/or pituitary.*Plasma sex steroid levels during ovarian follicle development:* Plasma levels of T were constant during follicle development, aside from a decrease at the early cortical alveolus stage. E2 levels, consistent with previous studies [4] became elevated at the early cortical alveolus stage. Although we used 11-KT in experimental studies as a non-aromatizable androgen without assumptions about levels in vivo and its potential role in normal development, 11-KT levels increased significantly at the late perinucleolar stage, suggesting that this steroid participates in the regulation of primary growth of follicles.

Conclusion:

These studies demonstrate the stage-specific effects of androgens and E2 on development of late primary and early secondary ovarian follicles of coho salmon. The former appears to be under predominantly androgenic control while the latter appears to be largely due to the effects of E2. (This study was supported by grant IOS-0949765 from NSF).

References:

[1]LUBZENS, E., YOUNG, G., BOBE, J., CERDÀ J. 2010. Oogenesis in teleosts: how fish eggs are formed. Gen. Comp. Endocrinol., 165: 367-389.

[2]KHOO, K.H. 1979. The histochemistry and endocrine control of vitellogenesis in goldfish ovaries. Can. J. Zool., 57: 617-626.

[3]LOKMAN, P.M., GEORGE, K.N., DIVERS, S.L., ALGIE, M., YOUNG G. 2007. 11-Ketotestosterone and IGF-I increase the size of previtellogenic oocytes from shortfinned eel, *Anguilla australis*, in vitro. Reproduction, 133: 955-967.

[4]CAMPBELL, B., DICKEY, J., BECKMAN,B., YOUNG, G., PIERCE, A., FUKADA, H., SWANSON, P. 2006. Previtellogenic oocyte growth in salmon: relationships among body growth, plasma insulin-like growth factor-1, estradiol-17β, follicle-stimulating hormone and expression of ovarian genes for insulin-like growth factors, steroidogenic-acute regulatory protein and receptors for gonadotropins, growth hormone, and somatolactin. Biol. Reprod., 75: 34-44.

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

NME FAMILY AND DETERMINATION OF EGG QUALITY, NEW INSIGHTS FROM THE ZEBRAFISH *DANIO RERIO*

Desvignes T.*°, Fauvel C.°, Fostier A.*, Bobe J.*
*Sex differentiation and oogenesis group, INRA, SCRIBE UR 1037, Rennes F-35042, France, email: Thomas.Desvignes@rennes.inra.fr
°Biology of Marine Organisms, IFREMER, Palavas-les-flots F-34250, France

Background:

Early development of fish relies on maternal gene products (i.e. mRNA and proteins) stored into the oocyte during oogenesis as the genome of the embryo does not start to be transcriptionally active until the MZT (Maternal-to-Zygotic Transition) that occurs during the Mid-Blastula Transition (MBT). A previous genomic analysis of fish eggs pointed out the Nme family as being linked to the developmental potential of the egg in Atlantic sea bass (*Dicentrarchus labrax*) [1]. The Nme family is known to be involved in key cellular processes such as cell proliferation and differentiation. It has also been extensively studied in relationship with the metastatic potential of tumors in cancer research [2]. In contrast, very little is known about the role of the Nme family during early development of non-mammalian vertebrate species despite its known importance in *Drosophila* development. The aim of the present work was thus (i) to characterize the Nme gene family in fish, (ii) to study the expression of *nme* genes during oogenesis and early development, and (iii) to characterize its participation in the molecular mechanisms that define the developmental potential of the egg.

Methods:

After an extensive characterization of the evolutionary history of the Nme gene family in metazoans with special attention for the vertebrates and teleosts, the ten members of the gene family were studied in a panel of eight zebrafish (*Danio rerio*) tissues by real time PCR (QPCR). Transcripts showing a predominant ovarian expression were subsequently monitored throughout oogenesis and early development by QPCR and *in situ* hybridization.Whenever possible,

Fig.1. Tissue expression of *nme* genes in zebrafish.

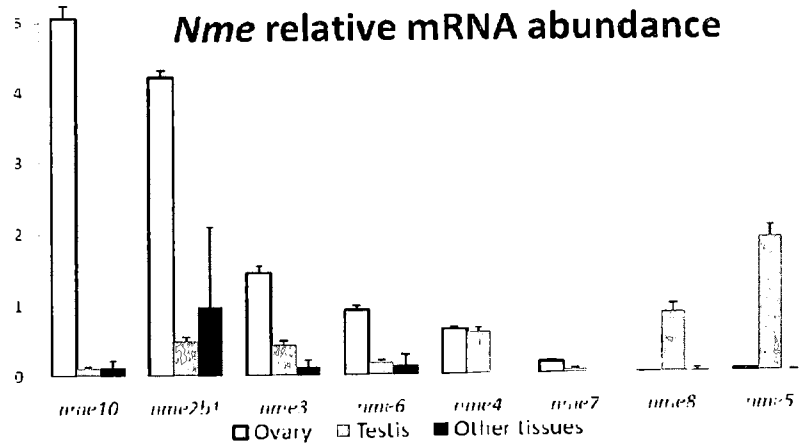
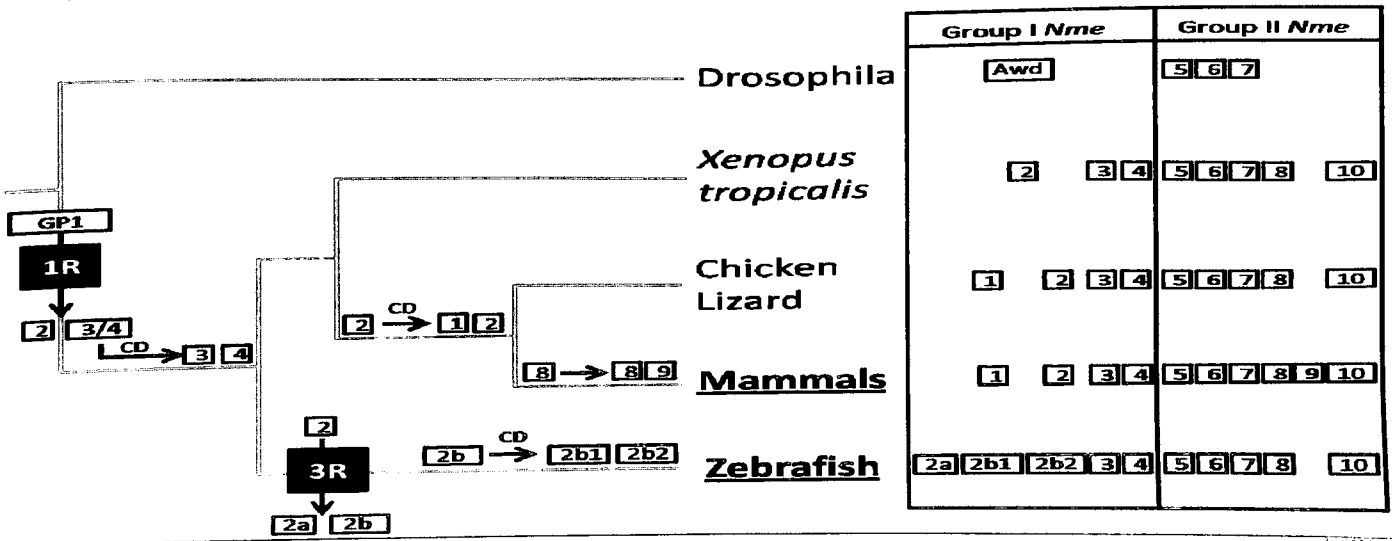


Fig.2. Nme gene family evolutionary history. 1R and 3R refer to the first and third round of whole genome duplication respectively. CD stands for Cis-duplication of the corresponding gene. Redrawn from Desvignes *et al* 2009 and 2010.



the corresponding protein products were studied by Western Blot and immunostaining. Finally, knock-down experiments directed against maternally-inherited *nme* transcripts were carried out using injection of antisense morpholino oligos (MOs) into the embryo at 1-cell stage in order to study the functional implication of the corresponding proteins in embryonic development.

Results and Discussion:

First, the evolutionary characterization of the Nme family revealed that among the 10 *nme* genes, 7 are common to all vertebrates while the 3 othergenes underwent independent duplication events in the teleost and tetrapods radiations (Fig. 1) [3,4].

Interestingly, the tissue expression survey revealed that the 10 *nme* genes are expressed in the zebrafish gonads (Fig. 2).Whereas *nme5* and *nme8* are testis-predominant, in agreement with existing data in mammals [2], most of the 8 other genes are predominantly expressed in the ovary [3]. Moreover, oogenesis and early development expression survey showed that 3 *nme* genes are maternally-inherited as mRNAs in the zebrafish egg [5]. Another *nme* gene, highly expressed during the first steps of oogenesis is likely to be maternally-inherited in the egg as a protein. Finally, knock-down experiments using MOs led to lower embryonic survival during the first 24h of development, important morphological defects, and decreased hatching rates in injected embryos compared to embryos injected with a negative control MO at the same concentration.

Conclusion:

In addition to the already documented implication of Nme genes in key cellular processes for cell proliferation and differentiation, the present study clearly demonstrates a predominant ovarian expression of several *nme* genes for the first time in any vertebrate species. Together the maternal profiles of several *nme* genes and the results of the knock down study strongly suggest an important role for oocyte developmental competence in fish. Given the ancient origin of the family in metazoans, a significant role of the Nme family in reproductive process can also be anticipated in the teleost lineage.

References:

[1]CRESPEL A, RIME H, FRABOULET E, BOBE J, FAUVEL C. 2008. Egg quality in domesticated and wild seabass (*Dicentrarchus labrax*): A proteomic analysis. Cybium, *32(2) suppl.*: *205.*

[2]BOISSAN M, DABERNAT S, PEUCHANT E, SCHLATTNER U, LASCU I, LACOMBE ML. 2009.The mammalian Nm23/NDPK family: from metastasis control to cilia movement. Mol. Cell Biochem., 329: 51-62.

[3]DESVIGNES T, PONTAROTTI P, FAUVEL C, BOBE J. 2009.Nme protein family evolutionary history, a vertebrate perspective. BMC. Evol. Biol., 9: 256.

[4]DESVIGNES T, PONTAROTTI P, BOBE J. 2010.*Nme*gene family evolutionary history reveals pre-metazoan origins and high conservation between humans and the sea anemone, *Nematostella vectensis*. PLoS ONE, 5: e15506.

[5]DESVIGNES T, FAUVEL C, BOBE J. 2011. The *nme* gene family in zebrafish oogenesis and early development. N-S Arch Pharmacol, in press.

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

ARACHIDONIC ACID EFFECTS ON REPRODUCTIVE PERFORMANCE, FRY QUALITY AND PGE_2 LEVEL IN SILVERSIDE *CHIROSTOMA ESTOR*

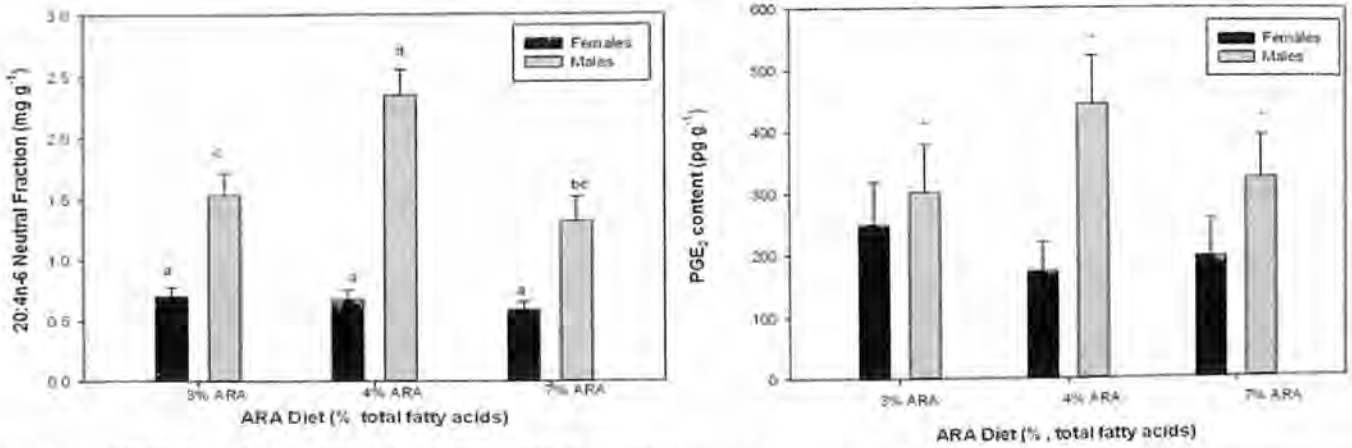
Palacios M. E.[*]-Salgado, G. R. L.[*]- Racotta, D. I.[*]- Guerrero, T. D.[*]-Fonseca, M. J[*]. [**]- Rodríguez, J. C.[*]- Treviño, C. L[*]- Martínez, P. C. A.[**]- Campos, M. A.[***]

[*]Lab. Metabolismo Lípidos. Centro de Investigaciones Biológicas del Noroeste (CIBNOR). Mar Bermejo195. Col. Playa Palo de Santa Rita. La Paz, Baja California Sur 23090, México. Fax (+52) 6121253625. epalacio@cibnor.mx.
[**]IIAF. UMSNH. Av. San Juanito Itzicuaro S/N. Col. San Juanito Itzicuaro. Morelia, Michoacán, México.
[***]Facultad de Biología. UMSNH. Ciudad Universitaria. Morelia, Michoacán, México.

Introduction:

Arachidonic acid (ARA, 20:4n-6) is the precursor of prostaglandins type 2, such as PGE_2 and PGF_{2a}, which modulate reproduction, ovulation and spawning in vertebrates [1]. In a previous study we proposed that low ARA levels in spawns of cultured silverside *Chirostoma estor* (2% vs 8% in wild, total fatty acids) could be associated to poor spawn quality [2]. The silverside (*C. estor*) is a freshwater endemic fish from Patzcuaro Lake in Michoacan, Mexico. Aquaculture development has been implemented partially to replenish wildstock; however, there is limited knowledge about its reproductive physiology. In the present study, silverside broodstock was fed with adult artemia (*Artemia franciscana*) enriched with ARA (3%, 4% and 7%, total fatty acids) during 10 weeks in order to evaluate dietary ARA effects upon reproductive performance, spawn quality, PGE_2 level and fatty acid composition in gonads

Fig.1. ARA content (mg g^{-1}) in neutral lipids of gonadal tissue broodstock (left) and PGE_2 level in gonads of both sexes fed with different ARA level during 10 weeks (right). Statistical differences ($P \leq 0.05$) between means are shown by different letters for ARA content (two ways ANOVA), and with an (asterisk)?? in PGE_2 to compare among males and females (t-test)



differences ($P \leq 0.05$) between means are shown with an asterisk.

Variable	3% ARA (Control)		4% ARA		7% ARA	
Sex	♀	♂	♀	♂	♀	♂
Hepatosomatic Index (%)	1.74±0.12*	0.98±0.08	1.65±0.08*	0.95±0.08	1.83±0.17*	0.97±0.07
GonadosomaticIndex (%)	5.61±1.16*	2.09±0.27	3.69±0.82*	2.09±0.27	3.99±0.96*	2.74±0.24
Total produced eggs	16,068	-	13,876	-	23,487*	-
Egg diameter (µm)	1077.±1.7	-	1085±1.3	-	1085±1.2*	-
Oil droplet size (μm^2)	175±7.2	-	169±6.8	-	186±7.2	
Larvae length (mm)	4.8±0.05	-	5.0±0.03*	-	4.8±0.06	
Sperm Count (10^9 cells ml^{-1})	-	1.31±0.09	-	2.02±0.28	-	2.39±0.57
Total Sperm count index (IZ)	-	0.18±0.04	-	0.21±0.04	-	0.18±0.03
Total Testicular index (IT)	-	0.44±0.07	-	0.39±0.07	-	0.43±0.06

of silverside broodstock (*C. estor*) under culture conditions.

Methods:

Farmed two-years-old adult silversides (36.2±1.0g) were fed with different dietary ARA level (3, 4 and 7%). Each experimental group consisted in three replicates with 21 fish (1♀:2♂) maintained under culture conditions during 10 weeks. Adult fish were fed daily with a mixed commercial pellet diet (2:1) and adult artemia (*A. franciscana*) enriched during 18 hours with ARA (3, 4 and 7%) using 1% of fish weight (grams). Presence or absence of spawns was recorded daily in order to evaluate quality of spawns in females and fatty acids composition of eggs and gonadal tissues of both sexes from neutral and polar lipids by gas chromatography according with Palacios *et al.*, 2007 [2]. At day 70, we obtained sperm samples of males in order to evaluate sperm count (cel ml^{-1}) and samples of gonads of both sexes for biochemical and histological analysis. Finally, we evaluated PGE_2 content (pg mg^{-1}) using a commercial assay of competitive enzymatic.

Results and Discussion:

Dietary ARA level did not affect survival or growth of broodstock, but fish fed 7% ARA had higher lipid accumulation in gonads than fish fed low ARA level (3% and 4%). Females fed high ARA level (7%) had larger spawns. Dietary ARA level did not affect sperm count in males (cells ml^{-1}). Spawners fed high ARA level (7%) had better production parameters (fertilization, hatching, lipid droplet size and egg diameter). No differences for PGE_2 content in gonads of males (373±35 pg g^{-1}) or females (245±34 pg g^{-1}) were found in relation to ARA levels in diet (Table 1).

We found differences in ARA content (mg g^{-1}) in the neutral lipid fraction from gonads of both sexes, which was affected by dietary ARA level (Fig. 1). ARA content was stable in the phospholipids from gonads of both sexes.

Conclusion:

These results indicate that silverside broodstock could benefit from an ARA-enriched diet during the reproductive period, since it can reverse some negative effects of culture condition on spawn quality. Nevertheless, it is necessary to evaluate specific ARA requirement of both sexes, taking into account the omega 3/omega 6 ratio, and its effects on synthesis of other eicosanoids.

References:

[1]MERCURE, F., VAN DER KRAAK, G. 1996. Mechanisms of action of free arachidonic acid on ovarian steroid production in the goldfish. Gen. Comp. Endocrinol., 102 (1): 130.140.

[2]ALACIOS, E., RACOTTA, I., APARICIO, B., ARJONA, O., MARTÍNEZ, P. C. A. 2007. Lipid classes and fatty acids during embryogenesis of captive and wild silverside (*Chirostoma estor estor*) from Pátzcuaro Lake. Fish Physiol. Biochem., 33: 81-91.

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

A NOVEL FUNCTION OF KISS

Hodne K[1,2,3], Weltzien FA[1,2], Okubo K[3]

1. Norwegian School of Veterinary Science, Department of Basic Science and Aquatic Medicine, Weltzien Lab, PO Box 8146 Dep, 0033, Oslo, Norway, e-mail: kjetil.hodne@nvh.no, fax: +47 22597310
2. University of Oslo, Department of Molecular Biosciences, Oslo, Norway
3. University of Tokyo, Department of Aquatic Bioscience, Tokyo, Japan

Background:

First discovered as a metastasis suppressor in breast cancer and melanoma cell lines, kisspeptin with its receptor has now emerged as one of the key players in regulating reproduction through the brain-pituitary-gonadal (BPG) -axis both in mammals and in fish. In addition, recent studies in mammals also suggest that the kiss system(s) may have several additional functions both in the CNS and in peripheral organs. However, besides its probable involvement in teleost reproduction, knowledge about the possible pleiotropic function of kiss is still lacking in teleosts.

Methods:

In the present study we have investigated the involvement of kiss during embryonic development in medaka (*Oryzias latipes*), using qPCR, in addition to GripNA and Morpholino knockdown combined with whole mount *in situ* hybridization.

Results and Discussion:

QPCR results suggest that the two *kiss* ligands in addition to *kissr1* are maternally prepared. *Kissr1* expression declines until 2-somite stage but is still detectable throughout embryonic development. Both *kiss1* and *kiss2* ligands have similar expression profiles with a steady increase in expression from gastrulation to 2-somite stage followed by a decline and then a stable but low expression from around 35-somite stage. *Kissr2* expression, on the other hand, is detectable only from gastrulation onwards with a sharp increase in expression starting around 22-somite stage. Based on this early expression, we hypothesized that kiss may have important roles during early embryogenesis. To investigate this, a series of knockdown assays were made and validated for both kiss ligands and receptors. Interestingly, moderate doses of *kiss1* gripNA knockdown (0.2 mM) resulted in early death, before gastrulation, while we observed no effect using even higher gripNA doses (1.9 mM) for *kiss2* knockdown. Reducing the *kiss1* gripNA concentration to 0.05 mM made the embryos surpass gastrulation. However, profound morphological malformation to the brain region was observed, in particular to the forebrain region including impaired eye development. These observations were consistent with splice site knockdown of *kiss 1* and *kiss2* using Morpholino. In addition, we obtained comparable results knocking down the two kiss receptors. Moderate doses of *kissr1* gripNA (0.2 mM) knockdown caused early death, before gastrulation, while the embryos died subsequently to gastrulation with *kissr2* gripNA (0.5 mM). Reducing the gripNA concentration made the embryos, as following *kiss1* knockdown, surpass gastrulation but with severe morphological malformation to the brain region, including loss of eyes and forebrain deformities. Co-injection of 5`UTR modified *kiss1* with 0.1mM *kiss1* GripNA, a concentration inducing more severe phenotype like lack of body development, rescued the morphant phenotype. To further investigate these morphological alterations after knockdown, we carried out whole mount *in situ* hybridization using the two marker genes *dlx2* and *six3*. Compared to wild type embryos, we observed clear differences in expression pattern for both marker genes following knockdown. The observed differences were most pronounced from day 3 post fertilization onwards.

Conclusion:

We have investigated the possible function of the kiss systems during embryogenesis using medaka as a model. Our results suggest a novel function of kiss during embryogenesis. Based on the knockdown studies we suggest that kiss play a pivotal role for survival and proper development, in particular concerning forebrain and eye development.

GOLDFISH EMBRYO DEVELOPMENT AFTER SOMATIC CELL NUCLEAR TRANSFER IN NON-ENUCLEATED OOCYTES: FIRST MITOSIS PROFILE, PLOIDY STATUS, AND GENETIC CONFORMITY

Labbe C.*, Depince A., Chenais N., Marandel L., LeBail P-Y.

INRA, Cell Cryopreservation and Fish Reconstruction, UR 1037 SCRIBE, Campus de Beaulieu, F-35000 Rennes, France. *E-mail: Catherine.Labbe@rennes.inra.fr Fax +33 (0)2 23 48 50 20

Introduction:

Nuclear transfer has the potential to become one strategy for fish genetic resources management, by allowing fish reconstruction from cryopreserved somatic cells. Survival rates after nuclear transfer are still low however [5], both because of biological and technical constraints. Among them, the difficulty to enucleate the recipient oocyte in fish, together with the need to alleviate some of the alterations associated with the enucleation methods, led several authors to inject the donor nucleus into non-enucleated oocytes [1,2,4]. Strikingly, diploid clones were produced which exhibited genetic characteristics of the donor fish. The group of Wakamatsu demonstrated moreover that diploidized activated eggs were a good template for the development of somatic cell nuclear transplants [6], although the mechanism underlying a putative spontaneous erasure of the maternal genome remains to be explored. In a previous work, we developed a nuclear transfer method in goldfish where the recipient oocytes were at the metaphase II stage (non-activated) [3]. As also proposed by [5] and in several works in mammals, a metaphase II recipient should improve the reprogramming of the injected nucleus, by allowing better cell cycle synchronization between the donor and the recipient.

In the present work, we explored the development of embryos reconstructed after somatic cell nuclear transfer into non-enucleated non-activated oocytes in goldfish (*Carassius auratus*). We assessed whether such recipient could still allow spontaneous erasure of the maternal genome, as observed when nuclear transfer is performed on readily activated eggs [1,2,4]. We additionally question here the origin of the development defects in the reconstructed embryos.

Methods:

Nuclear transfer experiments were performed on goldfish as described in [3] using cryopreserved caudal fin cells as donor and non-enucleated non-activated oocytes as recipient. Egg activation was delayed for 30 min after nucleus injection. After activation, the reconstructed embryos developed in tap water at 20°C in plastic Petri dishes. Relationship between the first mitosis pattern and the survival rates over embryo development was analyzed under macroscopic magnification. The tubulin organization at the mid-blastula stage (6 hpf) was analyzed using conventional immunofluorescence labeling on whole embryos fixed in methanol. A method was further set up to analyze cell ploidy and microsatellite occurrence on very small samples, so that the ploidy status and the genetic origin of the embryos could be assessed on the same individuals as early as at 24 hpf (15-18 somites stage). More than 360 reconstructed embryos were analyzed in this work.

Results and Discussion:

Of the entire developing embryo observed at the 2 cell stages (70.7 % of the injected oocytes), only 41% displayed 2 neat blastomeres, homogeneous in size. In these embryos, the first cell cycle was not significantly longer than in the control fertilized embryos, and they all reached the mid-blastula stage. Other embryos (40%) did not go through a clear 2 cell stage although they kept developing. At the 4 cell stage, they readily displayed 4 blastomeres more or less regular in size. The remaining embryos (19%) either did not cleave before the 8 to 16 cell stage, or they had asymmetrical cleavages (odd blastomere number). Strikingly, these delayed and abnormal cleavages still produced live embryos at the mid-blastula stage: only 4% of them died before this stage. This indicates that abnormal cleavage did not prevent development, which is consistent with the absence of mitosis checkpoints before mid-blastula stage in fish. Interestingly, chromatin and tubulin organization at the mid-blastula stage was not significantly different between reconstructed embryos and fertilized controls. At 24 hpf, more than 68 % of the embryos normal at the 2 cell stages were still developing. Almost all the embryos which reached the hatching stage belonged to these initially normal embryos. Still, about 20% of the embryos originating from abnormal early cleavages were still developing at 24 hpf, but very few reached the hatching stage.

Although the reconstructed embryos were produced with non-enucleated oocytes, 44 % of them were diploids and only 22 % were triploids. The others were either haploids (13 %), aneuploids (20 %), or tetraploids (1 %). Among the diploids, 54 % were true clones as they displayed only the microsatellite sequences of the donor fish. The other embryos were true hybrids as they

bore a combination of the donor and the recipient microsatellite sequences.

Conclusion:

In this work, 1) we demonstrated for the first time that embryos reaching the mid-blastula stage after somatic cell nuclear transfer could be originating from various early cleavage patterns, and that abnormal early cleavage did not prevent normal development up to mid-blastula stage. This illustrates the great plasticity of fish early development, before the embryonic genome activation and various development checkpoints are set up at the mid-blastula stage. 2) Although nuclear transfer was performed before oocyte activation, in metaphase II cytoplasms still bearing the maternal genome, diploids were produced which were genetically identical to the donor fish. A spontaneous inactivation of the maternal genome operated in these embryos although one could have feared that only hybrids would be produced. This observation raises the question of the underlying mechanisms responsible for such enucleation.

References:

[1]BUBENSHCHIKOVAE.,JU B., PRISTYAZHNYUK I., NIWA K., KAFTANOVSKAYA E., KINOSHITA M., OZATO K., WAKAMATSU Y. 2005. Generation of fertile and diploid fish, medaka (*Oryzias latipes*), from nuclear transplantation of blastula and four-somite-stage embryonic cells into nonenucleated unfertilized eggs. Cloning Stem Cells, 7: 255-264.

[2] GASARYAN K.G., HUNG N.M., NEYFAKH A.A., IVANENKOV V.V., - 1979. Nuclear transplantation in teleost *Misgurnus fossilis* L. Nature, 280: 585-587.

[3] LE BAIL P.Y., DEPINCE A., CHENAIS N., MAHE S., MAISSE G., LABBE C. 2010. Optimization of somatic cell injection in the perspective of nuclear transfer in goldfish. BMC Dev. Biol, 10: 64 p.

[4]LIU T.M., YU X.M., YE Y.Z., ZHOU J.F., WANG Z.W., TONG J.G., WU C.J. 2002. Factors affecting the efficiency of somatic cell nuclear transplantation in the fish embryo. J. Exp. Zool., 293: 719-725.

[5]SIRIPATTARAPRAVAT K., PINMEE B., VENTA P.J., CHANG C.C., CIBELLI J.B. 2009. Somatic cell nuclear transfer in zebrafish. Nat. Methods, 6: 733-735.

[6]WAKAMATSU Y. 2008. Novel method for the nuclear transfer of adult somatic cells in medaka fish (Oryzias latipes): use of diploidized eggs as recipients. Dev. Growth Differ., 427-436.

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

STUDY OF THERMAL REGIME EFFECT ON EUROPEAN EEL (*ANGUILLA ANGUILLA*) MALE REPRODUCTIVE PERFORMANCE DURING INDUCED SEXUAL MATURATION

Gallego V.*, Mazzeo I.*, Carneiro P.C.F.°, Vílchez M.C.*, Baeza R.*, Peñaranda D.S.*, Pérez L.*, AsturianoJ.F.*

*Grupo de Acuicultura y Biodiversidad. Instituto de Ciencia y Tecnología Animal. Universidad Politécnica de Valencia. Camino de Vera s/n, 46022 Valencia, Spain.e-mail: jfastu@dca.upv.es°Embrapa Tabuleiros Costeiros, Av. Beira Mar 3250, 49025-040 Aracaju, Brazil

Introduction:

The European eel, *Anguilla anguilla*, is a teleost fish with a peculiar life cycle. Prepubertal silver eels migrate 4–5000 km crossing the Atlantic ocean from European coastal waters to their supposed spawning grounds in the Sargasso sea, with water temperature around 19 °C. But during this migration eels apparently swim at depths between 200-1000 m and temperatures between 7-12°C, at least during the first part of their oceanic travel [1]. It seems probable that at the sea the gonad development occurs at low temperatures, and the spawning at warmer temperatures. Moreover, the influence of temperature on eel maturation process has been evidenced during the last years by the use of different thermal regimes during hormonal inducing treatments [2, 3].

This study was carried out to verify the influence of different thermal regimes on the reproductive performance of European eel males during the hormonally induced sexual maturation.

Methods:

A total of 317 adult male eels (mean body weight 100±2 g; mean length 40±5cm) were randomly distributed in six 200-L aquaria and submitted to three thermal regimes: T10, 10 °C (first 6 weeks), 15 °C (next 3 weeks) and 20 °C (last 6 weeks); T15, 15 °C (first 6 weeks) and 20 °C (last 9 weeks); and T20, 20 °C during the whole experimental period. Males were hormonally treated for the induction of maturation and spermiation with weekly intraperitoneal injections of hCG (1.5 IU g^{-1} fish). Sperm samples were collected by gentle abdominal pressure. Percentage of spermiating males and sperm parameters as volume, density and motility (using ISAS® system) were also weekly obtained.

Results and discussion:

T20 males began spermating at the 5th week, whereas those from T15 and T10 began later (6th and 10th week, respectively; Fig. 1A). This delay to initiate the spermiation and the lower spermiating percentage showed by T15 and T10 males are a result of a late gonad development caused by the lower temperatures, as occur in European eel females and other fish species under low temperatures [2].

Sperm volume, density and motility are important parameters related to sperm quality. In most species, the success in fertilization is guaranteed with sperm of a reasonable volume and concentration, as well as with a high percentage of motile spermatozoa. In this study all treatments caused, with different timings, a progressive increase of sperm volume that resulted significantly higher in some weeks for T20 males, that produced high volume during a longer period (Fig. 1B). Sperm from T20 fish also showed the highest spermatozoa concentration during almost all the experimental period (Fig. 1C).

Sperm motility is the most used parameter to evaluate sperm quality, and has been correlated with fertilization rates in several fish species [4]. In this sense, sperm samples from T20 fish showed higher motilities than those of T15 and T10 in most of the samplings, with significant differences between the 8th and 11th weeks (Fig. 1D). In conclusion, T20 treatment resulted as the better method to obtain sperm of good quality.

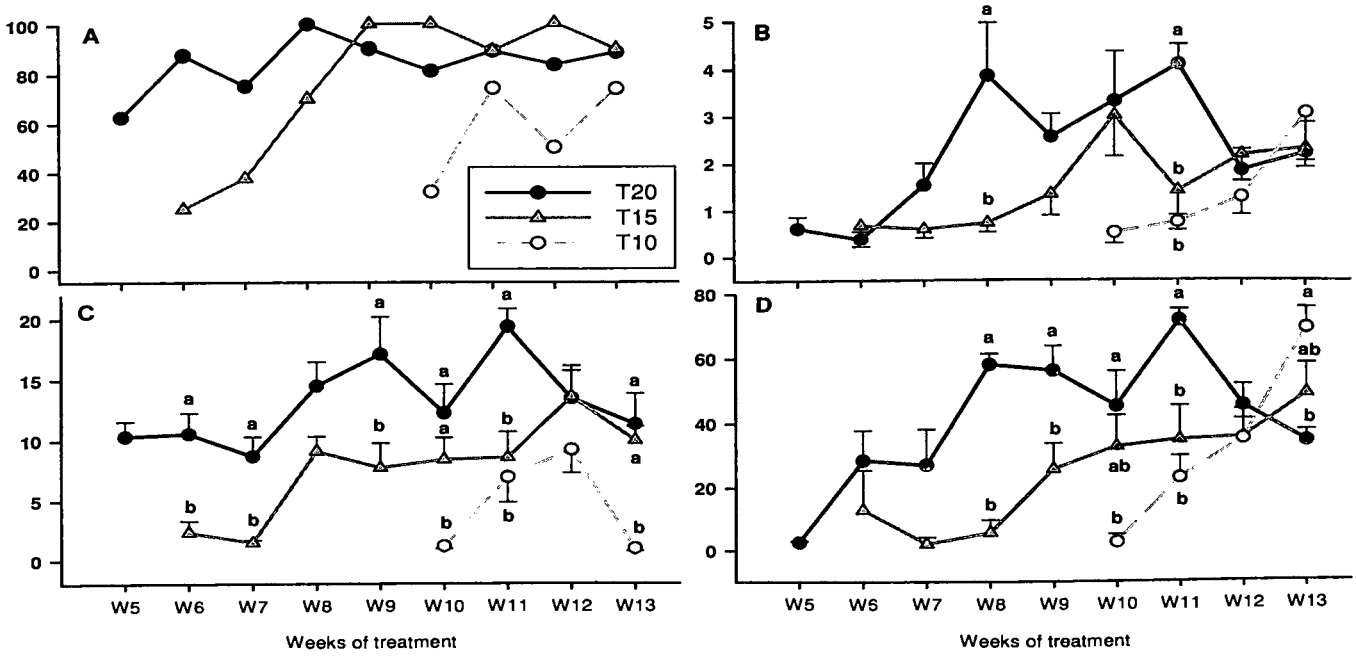
Conclusions:

The results of this work indicate that thermal regime affects the spermiating process, its timing, as well as the sperm quantity and quality of European eel. Best results were obtained when the males were maintained in water at 20 °C during the whole process of hormonal induction, and coincided with results reported in previous studies with European eel males, hCG-treated at this temperature [5].

Acknowledgments:

Funded from the European Community's 7th Framework Programme under the Theme 2 "Food, Agriculture and Fisheries, and Biotechnology", grant agreement n°245257 (Pro-Eel) and Generalitat Valenciana (ACOMP/2011/ 229). D.S.P. and P.C.F.C. have postdoc grants from UPV and PAC-EMBRAPA, respectively. I.M. and V.G. have predoctoral grants from Generalitat Valenciana and Spanish MICINN, respectively.

Figure 1. Semen quality parameters of European eel maintained under different water temperature regimes: A) Percentage of spermiating males (%), B) Sperm volume (ml 100 g^{-1} fish), C) Sperm concentration (10^9 cells ml^{-1}), and D) Sperm motility (% motile cells). Data are expressed as mean ± SEM and different letters indicate significant differences between treatments in every week of sampling.



References:

[1]AARESTRUP, K., ØKLAND, F., HANSEN, M.M., RIGHTON, D., GARGAN, P., CASTONGUAY, M., BERNATCHEZ, L., HOWEY, P., SPARHOLT, H., PEDERSEN, M.I., McKINLEY, R.S. 2009. Oceanic spawning migration of the European Eel (*Anguilla anguilla*). Science, 325 (5948): 1660.

[2]PEÑARANDA, D.S., ASTURIANO, J.F., GALLEGO, V., BALOCHE, S., JOVER, M., DUFOUR, S., PÉREZ, L. 2007. Effect of two temperatures regimes on ovarian development and endogenous gonadotropins in hormonally matured European eel females. Aquaculture Europe 2007. Abstracts book p.448.

[3]SATO, N., KAWAZOE, I., SUZUKI, Y., AIDA, K. 2006. Effects of temperature on vitellogenesis in Japanese eel *Anguilla japonica*. Fish Sci., 72: 961-966.

[4]OTTESEN, O.H., BABIAK, I., DHALE, G. 2009. Sperm competition and fertilization success of Atlantic halibut (*Hippoglossus hippoglossus* L.). Aquaculture, 286: 240-245.

[5]ASTURIANO, J.F., PÉREZ, L., GARZÓN, D.L., PEÑARANDA, D.S., MARCO-JIMÉNEZ, F., MARTÍNEZ-LLORENS, S., TOMÁS, A. AND JOVER, M. 2005. Effect of different methods for the induction of spermiation on semen quality in European eel. Aquac. Res., 36: 1480-1487.

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

EFFECTS OF GONADOTROPIC HORMONE ON THE ACQUISITION OF OVULATORY COMPETENCE IN JAPANESE EEL *ANGUILLA JAPONICA* AND BESTER STURGEON (*HUSO HUSO* × *ACIPENSER RUTHENUS*)

Ishihara M[1], Abe T[1], Kazeto Y[2], Ijiri S[1] and Adachi S[1]

[1]Division of Marine Life Sciences, Graduate school of Fisheries Sciences, Hokkaido University, Hakodate, Hokkaido 041-8611, Japan

[2]National Research Institute of Aquaculture, Tamaki, Mie 519-0423, Japan

Fax: +81-138-40-5546 email: wind-which-quench-fire@hotmail.co.jp

Introduction:

After the completion of oocyte growth, oocyte expresses the ability to undergo final oocyte maturation in response to a maturation-inducing hormone (MIH). The sensitivity to MIH (oocyte maturational competence, OMC) is induced by the stimulation of gonadotropic hormone (GTH), mainly luteinizing hormone (LH) [1]. However, even oocytes acquire OMC, the ovulation is not always induced in response to MIH. The acquisition of ability of ovarian follicles to ovulate under MIS stimulation (ovulatory competence) usually lag behind OMC [1]. It is considered that the ovulatory competence is also induced by LH stimulation, and likely requires independent steps in a transduction pathway from OMC in some part, that are activated by LH. In the eel, 17α, 20β-dihydroxy-4-pregnen-3-one (DHP) has been identified as the MIH. In the sturgeon, 17α-hydroxyprogesterone (17αOHP) has been suggested as a candidate for MIH or the precursor. In the present study, we examined effects of GTH on the acquisition of ovulatory competence in artificially matured Japanese eel, *Anguilla japonica* and spontaneously maturing hybrid bester sturgeon (*Huso huso* × *Acipenser ruthenus*) using previously developed *in vitro* culture system [2].

Methods:

Female Japanese eels were induced sexual maturation by weekly received injections of salmon pituitary extract (SPE). Ovarian fragments during the migratory nucleus stage were removed and pre-incubated in the presence or absence of 250 μg/ml SPE, 1000 ng/ml recombinant Japanese eel follicle stimulating hormone (rFSH) or 1000 ng/ml recombinant Japanese eel LH (rLH) in 1 ml of incubation medium (Cortland solution, pH8.2, 20°C) for 24 hours. These fragments were further incubated with 100 ng/ml DHP for 15 hours and examined germinal vesicle brake down (GVBD) and ovulation rates. In the spontaneously maturing bester sturgeon, ovarian follicles during the migratory nucleus stage were removed in December and April from the same individual. Follicles were incubated in the presence or absence of 100 ng/ml 17αOHP, 1 mg/ml SPE, or co-incubated with 17αOHP and SPE in 1 ml of incubation medium (100% L-15 medium, pH8.2, 15°C) for 48 hours and examined GVBD and ovulation rates.

Fig. 1 GVBD (A) and Ovulation rates (B) after 24 hours pre-incubation and additional 15 hours of incubation with DHP in the Japanese eel ovarian follicles.

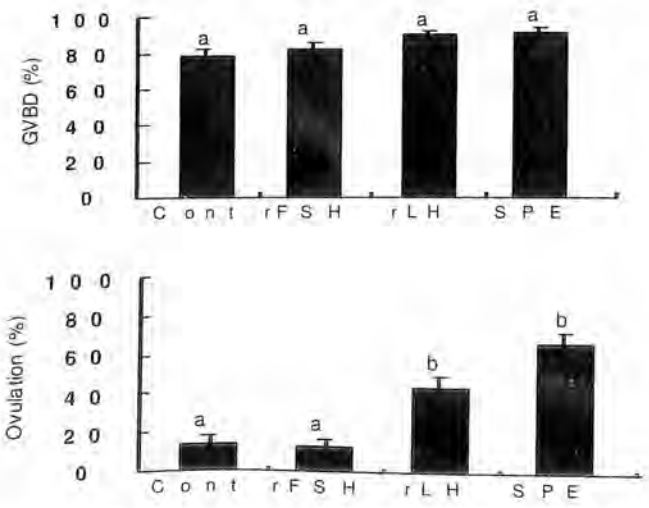
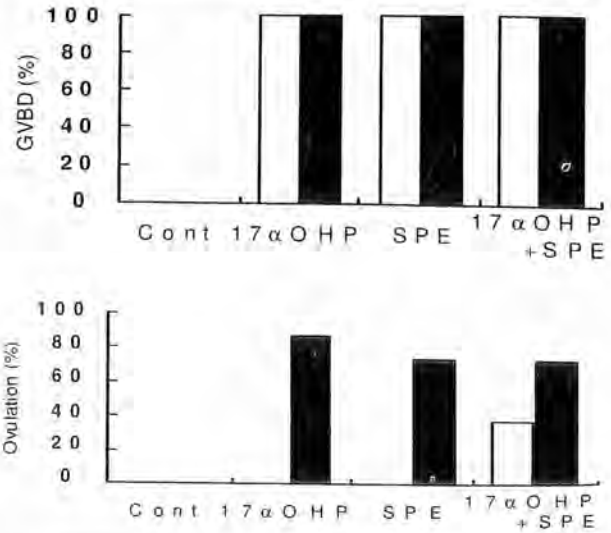


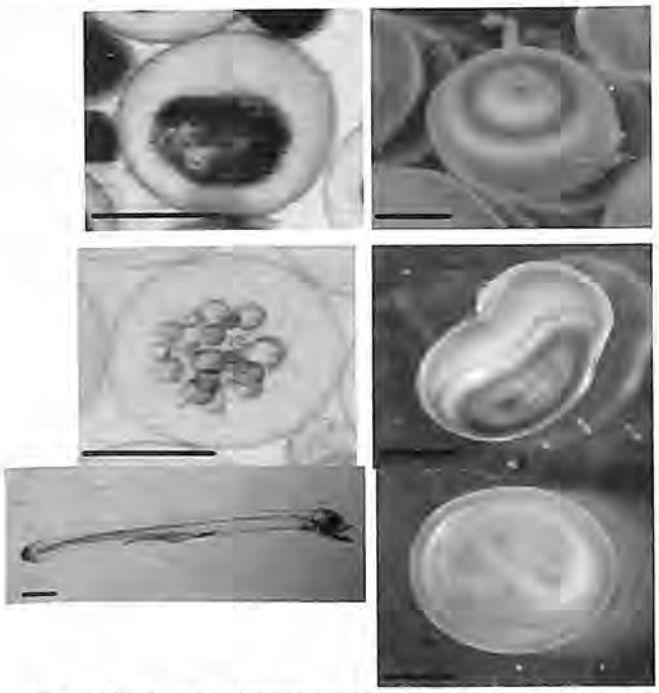
Fig. 2 GVBD (A) and Ovulation rates (B) after 48 hours incubation of bester sturgeon follicles from the same individual at different month.



Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

Fig. 3 Ovarian follicle and larva of the Japanese eel. Each photograph shows that initial follicle (A), ovulated oocyte (B) and the larva (C). Bar indicate 500 mm

Fig. 4 Ovarian follicle and embryo of the bester sturgeon. Each photograph shows that initial follicle (A), ovulating oocyte (B) and the embryo (C). Bar indicate 1 mm



In both eel and bester, ovulated eggs were artificially inseminated to examine the fertility.

Results and discussion:

In the eel, GVBD was not occurred without adding DHP. On the other hand, DHP induced GVBD over 80% of follicles in all pre-incubation groups (Fig. 1A). This indicates that these oocytes had already acquired OMC. In contrast, ovulation rates were clearly segregated among groups of pre-incubation substances. The ovulation rates were low in the control and rFSH, and significantly high in the rLH and SPE pre-incubation groups (Fig. 1B). This suggests that LH but FSH appears to induce ovulatory competence of eel follicles. In the bester sturgeon, 100% of GVBD was induced by 17αOHP and SPE in follicles of both months (Fig. 2A). In the follicles on December, ovulation was induced only in the combination group of 17αOHP and SPE. In April, 17αOHP or SPE alone could also induce ovulation (Fig. 2B). These results indicate that follicles had already acquired OMC since December, however, ovulatory competence had acquired between December and April. Furthermore, ovulatory competence could be induced by SPE. In addition, we demonstrated that eggs obtained from *in vitro* were fertilizable and they could develop normally in the eel and the bester sturgeon (Fig 3, 4).

Conclusion:

This study demonstrated that further processes are required for ovulatory competence after follicles acquired OMC in the Japanese eel and bester sturgeon. Ovarian follicles acquire the ovulatory competence by LH stimulation. FSH had no effect on the acquisition of ovulatory competence in the eel follicles. The sturgeon ovarian follicles could maintain OMC for a long period, at least four months. The ovulatory competence lags months behind OMC. The fact that sturgeon follicles could maintain OMC several months and the ovulatory competence was acquired at a certain point during the long period, indicates this species is a good experimental model for analyzing a mechanism regulating acquisition of ovulatory competence.

References:

[1]PATIÑO, R., THOMAS, P., YOSHIZAKI, G. 2003. Ovarian follicle maturation and ovulation: an integrated perspective. Fish Physiol. Biochem., 28: 305-308.

[2]ABE, T., IJIRI, S., ADACHI, S., YAMAUCHI, K. 2010. Development of an in vitro culture system for producing eel larvae from immature ovarian follicles in Japanese eel Anguilla japonica. Fish. Sci., 76: 257-265.

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

COMPARATIVE STUDY ON INDUCTION OF SPAWNING IN GOLDFISH (*CARASSIUS AURATUS*) BY PROSTAGLANDINS & OTHER INDUCING AGENTS

Jagtap H. S.

Department of Zoology, M.S.P. Mandals, Shri Shivaji College, Parbhani - 431 401 (M.S.) India. E-mail: hjagtap@yahoo.com Fax:02452-221649

Introduction:

In view of the increasing demand both in domestic and export markets especially for freshwater ornamental fishes, if the culture is taken up scientifically and the induced breeding technique is applied in ornamental fishes for repeated (multiple) spawning, to reduce the gap between two spawning periods and enhance production, there is no doubt that it becomes a boon to the national economy. Goldfish, *Carassius auratus* (Linnaeus) one of the best aquarium fish world wide, belongs to the family Cyprinidae and is closely related to Indian major carps. Goldfish was bred by administering human chorionic gonadotropin (HCG) in India first time by Reddy *et al.* [3]. Prostaglandins (PGs) appear to be involved in ovulation, male and female sexual behaviour, gonadotropin (GtH) secretion and thus considerably affect the reproductive physiology of fish (Goetz, [2]; Stacey, [4]; Stacey and Goetz, [5]; Stacey *et al.*, [6]. No study is made by using natural prostaglandin analogues for mass seed production of goldfish due to their high cost, unstable nature, short shelf-life and storage at –70°C. In view of this, in the present attempt was made to evaluate the role of prostaglandins and their analogues (natural as well as synthetic) as induced breeding agents in goldfish, *Carassius auratus*.

Methods:

Mature goldfish brooders were procured from local ornamental fish dealers during winter season. The male and female brooders in the age group of 8-12 months weighing 20-50 gm were properly selected for experiments. Brooders were selected pair-wise. Each selected pair was kept overnight in separate aquarium (36" x 12" x 15") in 50 l of dechlorinated water with temperature 20-22 ^{0}C. Fabricated glass stands woven with nylon mesh were kept at the bottom of aquarium to protect the eggs from being eaten by their parents and pairs were checked for ovulation after 24 h. Non-ovulated females were further subjected to hormone treatment. Prostaglandin- $F_{2\alpha}$ ($PGF_{2\alpha}$); Cloprostenol (Cayman Chemicals, 690 Place Ann Arbor, MI 48108 USA), Iliren (Tiaprost) Hoechst Roussel Vet. GmbHd-65203 Wiesbaden; Prosolvin (Luprostiol) Intervet, International B.V.Boxmeer-Holland, Ovaprim (sGnRHA) Syndel Laboratories, Vancouver, Canada.

Results and Discussion:

Of various prostaglandins ovulatory action of $PGF_{2\alpha}$ was found to be most effective both in vivo and in vitro in variety of teleosts (Stacey and Goetz,[5]. Similar results were obtained in the present study in which natural $PGF_{2\alpha}$ induced ovulation and spawning in goldfish. Tiaprost and Cloprsotenol used in the present study has been characterized as a veterinary luteolytic prostaglandin which is 200 times more luteolytic than natural $PGF_{2\alpha}$ (Dukes et al., [1]).

Conclusion:

Results of the present study concluded that amongst all the prostaglandins (natural & synthetic) used Cloprostetenol and Tiaprost were found more effective as induced breeding agent in goldfish. If the presently preferred technique of induced breeding is employed to increase the production rate it is possible not only to meet the domestic demand but also to enter the international market which will fetch foreign exchange to our country.

References

[1]DUKES, M., RUSSEL W., WALPOLE A. L. 1974. Potent luteolytic agents related to prostaglandin $F_{2\alpha}$.

Table: Spawning response of goldfish, *Carasius auratus* with prostaglandins, their analogues and ovaprim. Data were analyzed by Students t test. Three replicates for each compound (p<0.005)***, (p<0.01)**, (p<0.025)*

Compound	Wt. of fish Mean ± SE (gm)		Dose/gm. body wt.		Eggs/female Mean ± SE	Fertilization % Mean ± SE	Hatching Mean ± SE	Remarks
	Female	Male	Female	Male				
Control	22.16 ±3.99	28.23 ±1.89	--	--	183.33 ± 16.66	55± 2.88	55± 2.88	Natural spawning
Ovaprim	39.77 ±12.90	31.59 ±10.23	0.5µl	0.125 µl	1000 ±115.47	75±2.88	78.33±4.40	Spawning after 10-12hr of injection
$PGF_{2\alpha}$	29.29 ±1.14	28.14 ±3.63	1µg	0.5 µg	200±17.32	20±1.15	Fungal infection	Spawning after 10-12hr of injection
Cloprostenol	24.42 ±3.01	22.09 ±1.29	1µg	0.5 µg	2300 ±104.08***	80±2.51*	91.66 ±1.66**	Spawning after 10-12hr of injection
Tiaprost	40.06 ±1.54	31.66 ±5.48	1µg	0.0µg	3333.33 ±166.67***	81.66 ±1.66*	91.66 ±1.66**	Delayed spawning after 72 hr of injection

Nature, 250 : 330-331.

[2]GOETZ, F. W. 1983. Hormonal control of oocyte final maturation and ovulation in fishes: In: Fish Physiology, IXB (HOAR, W. S., D. J. RANDALL AND E. M. DONALDSON, eds.) pp.117-170, Academic Press, New York.

[3]REDDY, P. S. R., SUBRAMANIAN R., BASKAR I. S., SHENOY A. S., ELAMBARITHY B. 1990. Induced breeding of goldfish, *Carassius auratus* with human chorionic gonadotropin. In: Special Publication Carp Seed Production Technology (P. Keshavanath and K. V. Radhakrishnan, eds.) No. 2 pp. 21-23. College of Fisheries, Mangalore.

[4]STACEY, N. E. 1981. Hormonal regulation of female reproductive behaviour in fish. Amer. Zool., 21 : 305-316

[5]STACEY, N. E., GOETZ F. W. 1982. Role of prostaglandins in fish reproduction. Can. J. Fish. Aquat. Sci., 39 : 92-98.

[6] STACEY, N. E., SORENSEN P. W., DULKA J. G., VAN DER KRAAK G. J., HARA T. J. 1987. Teleost sex pheromones : recent studies on identity and function. In: Proceedings of the Third International Symposium on Reproductive Physiology of Fish (Idler, D. R., L. W. Crim and J. M. Walsh, eds.) pp. 150-153. Memorial University Press, St. John's, Newfoundland.

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

THE LINK BETWEEN BROODSTOCK STRESS AND OFFSPRING DEVELOPMENTAL POTENTIAL IN ATLANTIC COD *GADUS MORHUA* L.

Kleppe, L.[1], Karlsen, Ø.[2], Edvardsen, R.B.[1], Drivenes, Ø.[1], Natário, S.[2], Norberg, B.[2], Taranger, G.L.[1], Thorsen, A.[1], Kjesbu, O.S.[1], Andersson, E.[1], Wargelius, A[1].

[1] Institute of Marine Research, P. O. Box 1870 Nordnes, N-5817 Bergen, Norway. Phone: +47 55 23 85 00, Fax: +47 55 23 85 55, E-mail: lene.kleppe@imr.no.

[2] Institute of Marine Research, Austevoll Research Station, N-5392 Storebø, Norway.

Introduction:

A major challenge in the farming of Atlantic cod (*Gadus morhua* L.) is to ensure a stable supply of high quality eggs and embryos. While broodstock stress can have detrimental effects on egg quality, less is known about the underlying genetic processes that cause embryos to develop abnormally. Maternally contributed RNAs are essential for normal development of the embryo. In this context broodstock stress may affect the incorporation and/or stability of these transcripts and therefore also may lead to abnormal development of the embryo. The aim of this study was to investigate if broodstock stress affects maternal RNA incorporation/composition and stability, in eggs and embryos, and whether this can be linked to embryonic developmental potential in Atlantic cod.

Methods:

Female broodstock cod (n=30) were distributed into 2 tanks prior to spawning, and plasma samples were collected monthly from December 2009 to the end of spawning season (April-May 2010). To mimic prespawning stress in female broodstock, cortisol-treatment was initiated 3 weeks before expected peak spawning. 10 fish were implanted in their body cavity with osmotic pumps containing 15 ng/ml cortisol (pumping rate 2.9 µl/hour for 3 weeks), and 10 fish were implanted with vehicle only (sham controls). 10 fish were kept as untreated controls. At peak spawning all individuals were stripped and eggs were fertilized and incubated until hatching. Samples were collected from unfertilized eggs, 2-cell-stage, blastula and gastrula for further analysis of hormones (testosterone (T), estrogen (E2) and cortisol), gene expression analysis (microarray), and egg/embryo quality parameters.

Results and discussion:

Plasma cortisol levels were low (< 3 ng/ml) in both groups (cortisol-implanted and sham control) prior to implantation of osmotic pumps. In the cortisol-implanted fish a significant increase in plasma cortisol was detected 3 weeks after implantation (mean 59.8+/-27.6 ng/ml) compared to sham controls (< 3 ng/ml). Hence, the osmotic pumps containing cortisol significantly increased cortisol-levels in implanted fish. In agreement with previous reports in cod, plasma levels of E2 increased towards mid-spawning (mean 12.4+/-5.9 ng/ml in March), and thereafter decreased towards the end of the spawning season (mean 1.9+/-1.4 ng/ml at the end of April/start of May). Plasma levels of T had mean values of ~2-3 ng/ml with a marked decrease after mid-spawning (mean 0.6 +/-0.4 ng/ml at end of April/start of May). A slight but significant decrease in both plasma E2 and plasma T was found in cortisol-implanted individuals compared to sham controls. Offspring quality in terms of fertilization rate, hatching rate and cell division symmetry was highly variable between individuals in both the cortisol-implanted group and the sham control group, and no effect of cortisol was found. It is possible that potential effects of cortisol on embryonic development are subtle and may be detected with microarray on the embryos. Another possible explanation is that the window when cortisol-stress affects maternal RNA incorporation is occurring at another stage of oogenesis in cod.

Conclusion:

In this study we successfully elevated plasma cortisol in Atlantic cod by implanting osmotic pumps containing cortisol. No effects were found on the egg/embryo quality parameters measured, however our ongoing microarray-analysis may reveal possible effects in terms of gene expression.

Acknowledgements:

We would like to thank the EU project LIFECYCLE for the funding of this study.

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

INDUCED SPAWNING OF NATIVE THREATENED SPOTTED SNAKEHEAD FISH *CHANNA PUNCTATUS* WITH OVAPRIM

Marimuthu K.[1,2], **Haniffa M. A.** [2]

[1]Department of Biotechnology, Faculty of Applied Sciences, AIMST University, Batu 3[1/2] Bukit Air Nasi, Jalan Bedong Semeling, 08100 Bedong, Kedah Darul Aman, Malaysia
e mail: aquamuthu2k@yahoo.com
[2]Centre for Aquaculture Research and Extension, (CARE), St. Xavier's College, Palayamkottai - 627002, Tamilnadu, India

Introduction:

In aquaculture the most important constraint for large scale cultivation of several fish species is the non-availability of quality seed of uniform size, and free of diseases, parasites and pests at the time of stocking in culture ponds. These strict requirements are seldom fulfilled where the fingerlings are collected from the wild conditions for culture. Further, the wild collected broodstock are reared in captive conditions may not receive appropriate environmental cues for gonad maturation and spawning and these can cause reproductive development to be arrested in late vitellogenesis. For this reason hormonal administration has been attempted for stimulating gamete maturation [1]. Several synthetic ovulating agents in ready made form containing GnRHa and dopamine antagonist such as Ovatide, Ovopel, Dagin and Aquaspawn are becoming very popular nowadays and found to be efficient and successful spawning agents in different fish species [2-6].

The spotted snakehead, *Channa punctatus* (Bloch, 1793), locally known as spotted murrel, is one among the highly priced freshwater food fish species in India. Over the last 10 years, its wild population has undergone a steady decline due to fishing, loss of habitat, introduction of alien species, disease, pollution, siltation, poisoning, dynamite and destructive fishing. These factors not only destroyed the breeding and feeding grounds but also caused havoc to the biodiversity of this important fishery. As a result, according to IUCN status it has been listed among the 66 low-risk near-threatened fish species in India [7]. The fish is well known for its good taste, high protein content and fewer intramuscular spines, high nutritive value, recuperative and medicinal qualities, and is recommended as a diet during convalescence [8].The lack of monsoons also often limits the breeding and seed production. Hence to overcome these problems, induced spawning is thought to be the only alternative method for quality seed production/supply. Therefore, the present study was conducted to investigate the efficacy of a synthetic GnRH, with a dopamine antagonist for the induction of ovulation and the initiation of spawning in *C. punctatus*, and to determine the minimum effective dose of Ovaprim that could be used to spawn and produce seed of the candidate fish species under a controlled captive condition.

Methods:

In total 9 matured female and 18 male fish weight ranging from 63 to 80g, were randomly selected for three hormone dosages. Both male and female fishes in each hormone dosage were administered a dose of 0.2, 0.4, and 0.6 mL of Ovaprim/kg body weight (BW) respectively. Each breeding set consisted of two males and one female. The hormone-administered fishes were then placed in concrete cement tanks for spawning. After spawning, eggs were collected from the breeding tank, and the number of eggs spawned (spawning fecundity) and rate of fertilization were calculated. Two hours post-spawning, a total of 500 fertilized eggs from each breeding set were collected and incubated in glass aquaria to determine the incubation period and hatching rate. The data obtained for mean number of eggs spawned, fertilization rate, latency period, and hatching rate from each hormone dose were analyzed using one-way analysis of variance (ANOVA) to find significant difference among the hormone doses and each treatment mean were analysed by Duncan's multiple range tests ($P = 0.05$) using SPSS package Version.11.

Results and Discussion:

The spawning performance of *C. punctatus* induced at different Ovaprim dosages are presented in Table 1. Partial spawning was observed with a dose of 0.2 ml Ovaprim/kg BW, and complete spawning was noticed in the medium dose (0.4 ml of Ovaprim/kg BW) and high dose (0.6 ml of Ovaprim /kg BW) administered fishes. The latency period of *C. punctatus* ranged from 23.5 to 31.0 hrs at 29 $\pm$ 1.5^0 C in the three doses tested. The latency period was longer than those reported in *H. fossilis* administered with Ovaprim [9] but it was similar to those in *C. striatus* using Ovaprim [10] and in *C. striatus* using Ovatide [11]. The highest number of eggs spawned ($p < 0.05$) was recorded when the females were injected with 0.4 ml of Ovaprim/kg body weight than those injected with other doses. The highest fertilization (97.6%) and hatching rates (96.3%) were also observed

at the medium dose ($P < 0.05$). With regard to hatching rates, no significant difference was noticed between the medium and low doses of Ovaprim administered groups.

Values in each column followed the same superscript are not statistically different ($P > 0.05$).

Conclusion:

From the study, it is evidenced that the synthetic gonadotropin-releasing hormone with a dopamine antagonist at the dose of 0.4 ml /kg BW could be used as an appropriate spawning agent for successful breeding and seed production of *C. punctatus* under captive conditions.

Table 1: Induced spawning of *Channa punctatus* using a synthetic hormone Ovaprim

Hormone dose mL/kg BW	Fish weight (g)	Latency period (hours)	Total spawning fecundity	Fertilization rate s(%)	Hatching rate (%)
0.2	72.41±13.58[a]	29.10 ±1.0[b]	2164±168[a]	83.3 ±2.0[a]	91.3±2.5[b]
0.4	63.36±11.40[a]	24.5±0.5[a]	6538±154[c]	97.6±1.5[b]	96.3±1.5[b]
0.6	76.82±18.50[a]	26.0±2.5[a]	4318±214[b]	78.3±6.6[a]	84.3±5[a]

References:

[1]ZOHAR, Y., MYLONAS, C.C. 2001. Endocrine manipulations of spawning in cultured fish: from hormones to genes. Aquaculture, 197: 99– 136.

[2]PETER, R.E., LIN, H.R., VAN DER KRAAK,G. 1988. Induced spawning in Chinese carps. Proceedings of the Aquaculture International Congress and Exposition, 543-547.

[2]NANDEESHA, M.C., RAO, K.G., JEYANNA, R.N., PARKER, N.C., VARGHESE, T.J., KESHAVANATH, P., SHETTY, H.P.C. 1990. Induced spawning of Indian major carps through single application of ovaprim - C. Page 991 In R. Hirano, and I. Hanyu, eds. The Second Fisheries Forum. Asian Fisheries Society, Manila, Phillippines.

[3]CHEAH, M.S.H., LEE, C.L. 2000. Induced ovulation of the Australian eel tailed catfish *Neosilurus ater* (Perugia) with Ovaprim. Asian Fisheries Science,13:87-96.

[4]DAS, S.K. 2004. Evaluation of a new spawning agent, Ovopel in Induced Breeding of Indian Carps. Asian Fisheries Science, 17: 313-322.

[5]BRZUSKA, E.1998. Ovopel as ovulation stimulator in African cat fish (*Clarias gariepinus* Burchell 1822) female. Scientific papers of Polish Academy of Science, Selected problems No.5: 94-95.

[6]CAMP. 1998. Report of theWorkshop on Conservation Assessment and Management Plan (CAMP) for Freshwater Fishes of India. Zoo Out Reach Organization and NBFGR, Lucknow, India.

[7]HANIFFA, M.A., K. MARIMUTHU, M. NAGARAJAN, A.J. AROKIARAJ AND D. KUMAR. 2004. Breeding behaviour and parental care of the induced bred spotted murrel *Channa punctatus* under captivity. Current Sci., 86(10):1375-1376.

[8]VIJAYAKUMAR, C., S. SRIDHAR AND M.A. HANIFFA. 1998. Low cost breeding and hatching techniques of the catfish (*Heteropneustes fossilis*) for small-scale farmers. NAGA. The ICLARM Quarterly, 21(4): 15-17.

[9]HANIFFA, M.A., J. SHAIK MOHAMMED AND T. MERLIN ROSE. 1996. Induction of ovulation in *Channa striatus* (Bloch) by sGnRHa. Fishing Chimes, 16(5):23-24.

[10]MARIMUTHU, K., D. KUMAR AND M.A. HANIFFA. 2007. Induced spawning and seed production of Striped Snakehead *Channa striatus* using Ovatide. Journal of Applied Aquaculture, 19 (4):95-103.

SPAWNING KINETICS OF INDIVIDUAL FEMALE MEAGRE (*ARGYROSOMUS REGIUS*) AFTER TREATMENT WITH GnRHa IMPLANTS

Mylonas, C.C.*, Mitrizakis, N.*°, Sigelaki, I.* and Papadaki, M.*

* Institute of Aquaculture, Hellenic Center for Marine Research, AQUALABS, P.O.Box 2214, Iraklion, Crete 71003, Greece. Fax +30 2810 337875 email: mylonas@her.hcmr.gr
° University of Crete, Biology Department, Heraklion, Crete 71409, Greece

Introduction:

The meagre (*Argyrosomus regius*) is a new candidate species for European aquaculture [1]. Though broodstocks are maintained by many commercial operations, this species does not spawn spontaneously in captivity, necessitating the development of spawning induction protocols. The present study reports on the use of agonists of gonadotropin releasing hormone (GnRHa) loaded in controlled-release implants for the induction of oocyte maturation and spawning of meagre. Both group spawnings and individual-female spawnings were induced, in order to obtain accurate information on the spawning kinetics and fecundity of this species.

Methods:

Three different spawning induction trials were conducted between May and June 2009. Males and females (n = 10, mean body weight ± SD of 6.9 ±1.9 Kg) were implanted with EVAc GnRHa implants [2] at a dose of approximately 50 μg Kg^{-1} body weight and placed in 9000-l tanks connected to overflow egg collectors. In 2010, six individual females were placed in separate 5000-l tanks with two males each (mean body weight of 8.2 ± 2.2 Kg) during May and June. From every spawning event, an egg sample was collected for the estimation of fecundity and fertilization success. Results were analyzed by ANOVA, followed by Duncan's New Multiple Range test (DNMR), at a minimum significance of $P<0.05$.

Results:

Spawning was initiated 2 or 3 d after GnRHa treatment. In the group spawnings, mean relative fecundity $spawn^{-1}$ ranged from 24,300 to 49,900 eggs Kg^{-1} female body weight and mean fertilization success ranged from 85 to 87%. The number of spawns was between 7 and 17 and mean total annual relative fecundity was 365,000 ± 107,000 eggs Kg^{-1} $year^{-1}$. No statistically significant difference was found in relative fecundity or fertilization success between the three different trials in 2009. In the individualized trials in 2010, the number of spawns per female ranged between 5 and 19, relative fecundity $spawn^{-1}$ ranged between 28,540 and 59,380 eggs Kg^{-1} and fertilization success ranged between 85 to 95% (Table 1).

Relative fecundity was maximal on the second or third spawn and then decreased gradually (Fig. 1). On the contrary, fertilization percentage was high throughout the spawning season. The majority of the eggs (70%) were produced during the first four

Figure 1. Daily relative fecundity ($x10^3$ eggs Kg^{-1} female biomass) and fertilization success (%) of successive spawnings of a single female meagre after GnRHa implantation on June 3, 2010. The arrow refers to the day of the implantation.

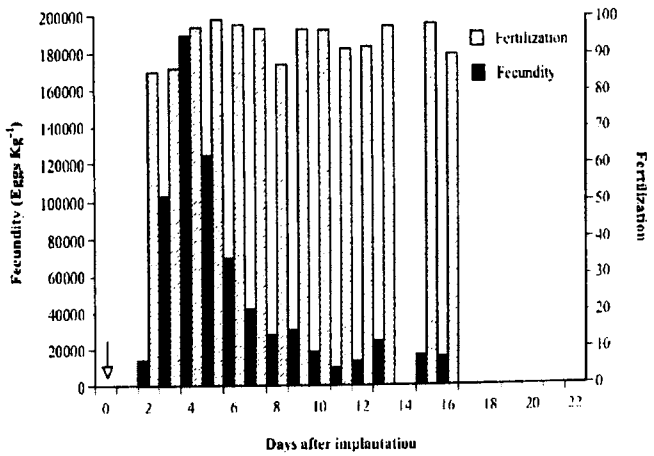


Table 1. Number of spawns, relative fecundity $spawn^{-1}$, total annual relative fecundity and fertilization percentage of the two spawning induction trials conducted in 2010.

No of female	Date of implantation	Spawns	Eggs/ Kg/ Spawn	Eggs/ Kg/ Season	Fertilization (%)
1	05/04/2010	5	33,625	168,127	89
2	05/04/2010	10	28,540	285,401	85
3	05/04/2010	6	59,381	356,286	87
4	06/03/2010	14	49,957	699,394	93
5	06/03/2010	10	52,207	522,066	95
6	06/03/2010	19	30,415	577,886	95

spawning events. No
statistically significant difference was observed in daily fecundity or fertilization success, either between individual females or between different GnRHa implantation dates.

Discussion:

The present study demonstrated that GnRHa implants are very effective in inducing spawning in the meagre, resulting in a large number of consecutive spawns, promoting the asynchronous nature of oocyte maturation of the species. No significant differences in egg production or quality were observed between group and individual spawnings, which is contrary to what is observed in other fish, which often do not reproduce well outside a large group [3]. Meagre can respond well to hormonal induction from May to June with the same effectiveness and egg quality, resulting in a mean annual fecundity of approximately 410,000 eggs Kg^{-1} and a mean fertilization percentage of >75%. The results obtained in the present study will be useful for the broodstock management of meagre in commercial operations, helping hatchery managers plan better and more efficiently their production.

References:

[1]QUÉMÉNER, L., SUQUET, M., MERO, D., GAIGNON, J.-L. 2002. Selection method of new candidates for finfish aquaculture: the case of the French Atlantic, the Channel and the North Sea coasts. Aquat. Living Resour., 15: 293-302

[2]MYLONAS, C. C., ZOHAR, Y. 2001. Use of GnRHa-delivery systems for the control of reproduction in fish. Rev. Fish Biol. Fish., 10: 463-491.

[3]FORNIÉS, M. A., MAÑANOS, E., CARRILLO, M., ROCHA, A., LAUREAU, S., MYLONAS, C. C., ZOHAR, Y. ZANUY, S. 2001. Spawning induction of individualised European seabass females (*Dicentrarchus labrax*) using different GnRHa-delivery systems. Aquaculture, 202: 221-234.

ANDROGENETIC DEVELOPMENT OF BROOK TROUT (*SALVELINUS FONTINALIS* MITCHILL), ARCTIC CHAR (*SALVELINUS ALPINUS* L.) AND THEIR HYBRIDS

Ocalewicz, K*, Dobosz, S.*, Kuzminski, H.*

*Department of Ichthyology, University of Warmia and Mazury in Olsztyn, ul. Oczapowskiego 5, 10-957 Olsztyn, Poland; tel: +48 89 523 39 14, fax: +48 89 523 37 54, e-mail: con@uwm.edu.pl
* Department of Salmonid Research, IFI in Olsztyn, Rutki Poland.

Background:

Interspecies androgenesis is thought to be useful biotechnological method in restoration of extinct or endangered fish species. Unfortunately, generally very high mortality of androgenetic fish during embryogenesis and after hatching is still a limiting factor in application of such approach in species protection programmes. The low survival of androgenetic fish have complex background and seems to be mainly triggered by the quality of eggs, manipulations performed on the oocytes and zygotes and homozygosity for the lethal alleles, among others. Apart from the inappropriate androgenesis parameters, conflict between cytoplasmic factors like maternal mRNAs deposited in the oocytes during oogenesis and male chromosomes may be responsible for the failure of interspecies androgenesis. Therefore, efficient recovery of maternal nuclear DNA in the course of interspecies androgenesis demands the use of fish species which their hybrid progenies are viable and fertile. Moreover, female hybrid individuals when matured might be promising donors of eggs for the recovery of sperm nuclear DNA from one or both parental species. The primary goal of this study was to evaluate suitability of eggs derived from brook trout x Arctic char hybrids for androgenetic development of brook trout and Arctic char individuals.

Methods:

In order to destroy maternal nuclear DNA, part of the brook trout, Arctic char and hybrid oocytes were exposed to 42 000 R of X-ray radiation. Left oocytes were kept for the control variants of the experiments. Batches of irradiated and untreated oocytes were further divided into several batches and inseminated independently with semen collected from brook trout, Arctic char and hybrid males. High pressure shock (7,000 psi for 4 min) was applied 420 minutes post fertilization to the part of the irradiated and inseminated eggs in order to double the haploid chromosomes set in the zygotes during their first mitotic division. Fertilized irradiated as well as untreated eggs which were not subjected to the high pressure shock were left to develop as androgenetic haploid control and normal diploid control variants, respectively. Diploid androgenetic batches consisted of *c.* 1 100 eggs, haploid androgenetic control batches, *c.* 350 eggs and normal diploid batches, *c.* 300 eggs. Survival of embryos was checked at their eyed-stage. Live larvae were counted just after hatching and at the swim-up stage (after five weeks of rearing). Survival of androgenetic progenies after swim-up stage was monitored continually. Randomly chosen androgenetic brook trout and hybrids, and their siblings from the control variant of the experiments were karyologically studied after nine months of rearing to confirm efficiency of the androgenesis conditions.

Results and Discussion:

In all variants of the experiment diploid androgenetic embryos survived up to the eyed stage with the highest rate (39. 25 %) in the case of androgenetic hybrid embryos developing in the enucleated hybrid eggs, and the lowest rate in the interspecies androgenetic variant where enucleated Arctic char eggs were inseminated with brook trout semen (0. 21 %). Apart from the latter experimental variant, androgenetic brook trout, Arctic char and hybrids hatched. Twenty seven (1, 63%) and 107 (6, 15%) live androgenetic brook trout hatched from the brook trout and hybrid eggs were observed at the swim-up stage, respectively. The only androgenetic Arctic chars that survived to the swim-up stage were individuals hatched from the hybrid eggs (5) and brook trout eggs (3). The most successful in terms of survival rates were androgenetic hybrids that hatched from the hybrid eggs (248 individuals - 14, 17%), brook trout eggs (56 specimens - 6, 15%) and Arctic char eggs (6 individuals - 0, 29%). Viable progenies were obtained in all diploid control variants. Unfortunately, during next months of rearing survival of androgenetic progenies decreased dramatically especially among androgenetic hybrid fish. After nine months of rearing, the only survived androgentic fish were brook trout and hybrids: Sf x Sf (10 specimens), H x Sf (10 specimens), H x H (5 specimens), Sf x H (10 specimens), P x H (2 specimes).

Table 2. Survival (% ± SD) of haploid and diploid androgenetic and control brook trout (Sf), Arctic char (Sa) and the hybrid (H) embryos and larvae.

Androgenetic variant	Eyed embryos		Hatched larvae		Swim-up larvae	
	control	2n - andro	control	2n - andro	control	2n - andro
Sf x Sf	92,82 ±1,15	15,98 ±0,85	86,25 ±0,36	2,13 ±0,11	86,25 ±0,36	1,63±0,04
H x Sf	94,80 ±1,27	35,86 ±1,63	91,48 ±2,90	6,77 ±0,31	91,48 ±2,90	6,15±0,51
Sa x Sf	76,71 ±3,17	0,21 ±0,29	68,12 ±4,57	0	68,12 ±4,57	0
Sf x H	94,88 ±3,28	19,05 ±1,67	93,13 ±2,79	4,68 ±0,58	93,13 ±2,79	3,17±0,13
H x H	94,45 ±1,58	39,25 ±1,04	90,66 ±1,24	16,80 ±0,50	90,66 ±1,24	14,17±0,04
Sa x H	57,55 ±4,00	14,86 ±1,10	49,06 ±2,67	1,18 ±0,37	49,06 ±2,67	0,88±0,38
Sf x Sa	75,47 ±4,09	9,96 ±1,39	71,49 ±4,44	0,18 ±0,10	71,49 ±4,44	0,18±0,10
H x Sa	76,55 ±0,53	19,68 ±2,93	72,27 ±0,54	0,64 ±0,13	72,27 ±0,54	0,29±0,10
Sa x Sa	55,57 ±1,56	4,71 ±0,67	43,57 ±1,51	0,13 ±0,18	43,57 ±1,51	0

Results of karyological research exhibited diploid chromosome arm number equaled 84 in the androgenetic brook trout and 82 and 84 in the androgenetic hybrids. Apart from the doubled paternal chromosomes, remnants of maternal nuclear DNA in form of chromosome fragments intraindividually variable in number and size were observed in most of the analyzed fish. The highest numbers of X-ray induced chromosome fragments (0-13) were observed in the androgenetic brook trout and hybrids hatched from the hybrid eggs.

Conclusions:

1. The use of enucleated brook trout x Arctic char hybrid eggs to restore DNA from the brook trout sperm cells is possible.
2. It has not worked out very well in the case of Arctic chars because:
   2.1. quality of gametes matters! Even medium quality gametes should be avoided when induce androgenesis in fish.
   2.2. even slight interspecies differences during fish ontogeny must be taken into consideration and androgenetic conditions adjusted.
3. High survival rates of androgenetic fish during the first weeks after hatching do not assure high survivability during next months of rearing.

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

MOLECULAR MECHANISMS AFFECTED IN *PERCA FLUVIATILIS* OOCYTES IN RELATION TO THEIR REPRODUCTIVE PERFORMANCES REVEALED BY A PROTEOMIC APPROACH

Castets M-D.*, Schaerlinger B.*, Silvestre F.°, Gardeur J-N.*, Corbier C.*, Kestemont P.° and Fontaine P.*

* UR AFPA, Nancy University - INRA, 2 Avenue de la Forêt de Haye, BP 172,
F-54505 Vandœuvre-lès-Nancy, France. Fax: +33 3 83 68 40 01
email: berenice.schaerlinger@scbiol.uhp-nancy.fr
° URBO, University of Namur FUNDP, Rue de Bruxelles, 60, B– 5000 Namur Belgium.

Introduction:

Fish breeding often leads to developmental defects as early mortality or deformities apparition. It is thus of first importance to control fish reproduction. In order to better understand variables influencing and predicting reproductive performances, it is necessary to identify reliable morphological and/or molecular markers that can be used as relevant criteria of "gamete quality", especially oocytes. Indeed, oocytes carry most of the molecular material necessary for the embryos. Lack of incorporation of these compounds influences the correct unroll of embryonic and larval development and thus reproduction success. Up to now, few studies investigated the proteome of oocytes in relation to spawn reproduction performances. In this work, we used the Eurasian perch, *Perca fluviatilis*, as a model to investigate links between morphological criteria characterizing females, spawns or oocytes, embryonic and larval performances and the proteomic signature of oocytes isolated from the corresponding spawns.

Methods:

Perca fluviatilis breeders were reared in experimental facilities in recirculating system before spawning. At the spawning time, 20 egg strands were extensively studied by measuring 14 criteria, in total. Several criteria were measured before the fertilization and characterized females (female weight), spawns (e.g. morphological aspects) and oocytes (e.g. oocyte diameter). After the fertilization, criteria characterizing embryonic and larval performances were studied (e.g. fertilization and hatching rates, larval resistance and deformities). Data were used to perform a Principal Component Analysis (PCA) followed by a Hierarchical Cluster Analysis (HCA) to understand correlations between pre-(illustrative) and post-fertilization (active) variables on one hand and to classify spawns into clusters (called spawn quality clusters) in relation to their embryonic and larval performances on another hand. In the meantime, samples of oocytes from each spawn were collected and snap frozen before the fertilization. Proteins have then been extracted from oocytes of each spawn and analyzed using the 2D-DIGE technology. This study allowed us to compare proteins relative abundances in each spawn and thus to highlight proteins differentially expressed according to their membership to a spawn quality cluster ($p<0.05$). Those proteins were then identified by mass spectrometry (MS/MS) and further analyzed according to their biological function and spawn cluster in order to better understand molecular mechanisms associated to embryonic and larval performances.

Results and Discussion:

From the 20 studied spawns, 14 developed until larval stages while the 6 remaining were not fertilized. The PCA was performed with data obtained with the 14 spawns that led to larvae. It allowed the definition of three independent axes defined based on active variables (embryonic and larval performances). The first axis was strongly correlated to parameters characterizing larval fitness and resistance; the second axis was negatively correlated to the hatching rate; the third axis was correlated to the deformities rate. These data indicate that larval growth and resistance, embryonic performances and deformities apparition are independent and may underlie independent cellular and molecular mechanisms. In a second time, an attempt was done to establish reliable pre-fertilization variables capable of predicting those reproductive performances. Data show that two pre-fertilization predictive characteristics (spawning date and fragmentation rate) were reliable to explain embryonic performances. No such characteristics, among those studied, were relevant enough to assess larval resistance or deformities apparition. These data confirm previous works showing the difficulty to reliably predict reproductive performances using classical morphological criteria measured on breeders, spawns or oocytes. The HCA was performed using the 3 axes of the PCA and the characteristics of the same 14 spawns previously used. It enabled to distinguish two clusters of spawns opposed by their larval performances (7 spawns mainly characterized as strong large larvae (defined as High Quality (HQ) cluster) while the other 7 spawns mainly led to smaller and weaker larvae (Medium Quality (MQ) cluster)). Moreover, the 6 remaining spawns which were not fertilized constituted a third group of spawns identified as the Low Quality (LQ) cluster. We then performed a 2D-DIGE analysis to identify molecular mechanisms underlying performances defects of MQ

and LQ spawns. Among the large number of protein spots highlighted for each spawn, 34 spots showed statistical differences in abundance between spawns representing HQ, MQ and LQ clusters. In total, twenty-five proteins involved in 11 biological functions have been identified. Identification and classification of these proteins according to their biological functions and spawns' quality revealed several molecular mechanisms specifically associated to reproductive performances. Among highlighted molecular mechanisms, two appear of particular interest. Firstly, HSP9 and HSP70 are weakly expressed in LQ spawns compared to MQ and HQ ones. They belong to the Heat Shock Proteins (HSP) family among which one of the roles is to bind to the centrosome, a protein complex regulating the mitotic spindle formation during fertilization. Even if further works are needed, a possible explanation of the fertilization failure in LQ spawns could thus be due to mitotic spindle impairment. Another molecular mechanism highlighted corresponds to the cellular protection against oxidative stress. Indeed, Peroxiredoxins (Prx) are involved in the degradation of the Reactive Oxygen Species (ROS) which are deleterious for cells but are needed for several molecular pathways. As Prx5 and Prx6 are highly expressed in MQ oocytes, the ROS balance may thus be disrupted giving rise to consequences later during the larval development.

Conclusion:

In conclusion, this work shows that, for *Perca fluviatilis*, it is important to assess simultaneously larval resistance, embryonic performances or deformities apparitions to accurately evaluate reproductive performances as they seem to be linked to independent processes. However, few pre-fertilization variables, predictive for reproduction success are associated to these embryonic or larval performances and could constitute relevant predictive criteria. Moreover, the proteomic analysis allowed identification of several molecular mechanisms associated to the reproductive performances in *Perca fluviatilis*. Further works are needed to better characterize those cellular and molecular mechanisms affecting the fish reproduction. In future, proteins associated to these pathways could constitute molecular markers reliable to predict reproduction success and thus to improve fish production.

Reproductive Toxicology & Endocrine Disruptors

ENDOCRINE DISRUPTION IN FISH IN ENGLISH RIVERS: ADDRESSING THE POPULATION LEVEL EFFECTS QUESTION

Tyler, Charles R., Hamilton, Patrick, Paull, Greg, Coe, Toby, Soffka, Marta, Filby, Amy, Lange, Anke.

Biosciences, College of Life and Environemntal Sciences, The Hatherly Laboratories, University of Exeter, Exeter, EX4 4PS, U.K. Email:c.r.tyler@ex.ac.uk

Background:

Feminisation of male fish as a consequence of exposure to endocrine disrupting chemicals (EDCs) has been demonstrated to occur in freshwater ecosystems across the world and in some case the causative chemicals have been identified. One of the most extensive studies on feminisation of wild fish has been on roach (*Rutilus rutilus*) living in English rivers where exposure to wastewater treatment works (WWTW) effluents (and the EDCs they contain) induces a range of feminised phenotypes including intersex condition (the presence of developing eggs in the testis of males). Intersex roach have been found at 86 % of the UK river locations studied and *in vitro* data demonstrate that severely feminised fish have lowered sperm quality. Studies on fish in other river systems across the world have found evidence for feminisation in wild populations and in a range of different species. However, little is known on the impact of feminisation on the subsequent ability of those fish to breed, and essentially nothing is known about the impacts at the level of the population for any species studied. We have been conducting a series of studies on roach and zebrafish (as laboratory models with similar breeding systems to roach) to investigate the effects of the feminised responses seen in wild fish populations on their ability to breed, including responses on breeding behaviours. In this presentation we will provide recent data that provide some significant advancing in addressing the population level effects question of oestrogenic EDCs.

Methods:

We examined the ability of both intersex roach and roach exposed throughout their lives to a WWTW effluent to reproduce when competing with apparently normal males in breeding populations. In the first study roach from effluent-contaminated UK rivers were placed in large tanks, and allowed to breed and after breeding, the level of gonadal disruption, based on the number of oocytes present in the testes, was determined for each male. To assign parentage, both adult fish and fry were genotyped using variable DNA microsatellite loci, thus enabling assessment of the abilities of intersex fish to contribute to the next generation. In the second study, a very similar approach was adopted with fish that had been exposed throughout their lives to an oestrogenic WWTW effluent under controlled conditions. Further experiments have been conducted to investigate the effects of some of the oestrogenic EDCs contained in WwTW effluents on reproductive behaviour in zebrafish and how this impacts on breeding outcome.

Results and Conclusions:

Intersex was found to significantly impair reproductive success. For the most severely feminised fish within each tank, there was a relative decrease of 76%. In the study where fish were exposed to a WWTW effluent (full strength) throughout their lives, females were not impacted in their ability to breed, males however were feminised completely and contained ovaries. Some of these 'sex-reversed' males were able to breed but only with a very low success rate. Exposure to oestrogens was shown to affect behaviours in dominant fish in zebrafish breeding colonies, impacting on aggression and dominance hierarchies and parentage outcome. If such effects occur in wild populations of fish, this could have implications for the genetic structure in populations. In conclusion, our findings suggest that feminisation of male fish and effects of environmental oestrogens on behaviour are likely to be important determinants in reproductive performance and success in wild fish populations living in some freshwater environments.

BISPHENOL A DEPOSITION IN EGGS LEADS TO DEVELOPMENTAL AND GROWTH DEFECTS IN RAINBOW TROUT

Vijayan M. M., Birceanu O.

Department of Biology, University of Waterloo, Waterloo, Ontario, N2L 3G1, Canada

Bisphenol A (BPA), a monomer commonly used in the manufacture of polycarbonate plastics and epoxy resins, is widely distributed in the aquatic environment. We recently detected its presence in local rivers in the vicinity of municipal waste water effluent discharge around the Waterloo region, Ontario, Canada. BPA is a weak xenoestrogen in fish and studies have shown that this chemical interacts with the estrogen receptor in fish. This chemical also accumulates in lipid depots raising the possibility that this xenobiotic may be maternally transferred to embryos. However, the impact of maternal transfer of BPA on development and growth in fish are poorly understood.

We mimicked maternal transfer of BPA by exposing rainbow trout (*Oncorhynchus mykiss*) oocytes to a range of BPA concentrations (0, 0.3, 3, 30 and 300 µg/ml) for three hours. The oocytes were fertilized and water hardened and maintained in clean water thereafter. Growth, development and stress performance of trout, as well as mRNA abundances of several candidate genes involved in growth, stress and reproduction were monitored at specific time points during development. These F1 fish were subsequently bred and the F2 generation embryos were also monitored for growth, development and stress performance to determine generational effects associated with BPA accumulation in eggs.

BPA content in the embryo ranged from <5 ng/embryo at the lower concentrations of BPA exposure to <50 ng/embryo and <400 ng/embryo at the two higher concentrations, respectively. The BPA was quickly cleared from the embryos and they were below detection in all groups by 42 days post fertilization (dpf). Despite the lack of BPA in the embryos, there were clear developmental and growth effects that persisted for over a year post fertilization. These growth effects of BPA corresponded with a disruption of the somatotropic axis function in trout. Also, the BPA deposition in oocytes led to disruption of the acute cortisol stress axis function and this was evident even after one and two years post fertilization. The BPA effect on growth was also evident in the F2 generation.

Taken together, maternal transfer of BPA impairs growth and stress performances in trout that are long lasting, including multigenerational. The talk will highlight some of the molecular responses associated with BPA enrichment of eggs and the possible mode of action leading to the observed generational effects in trout.

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

ZEBRAFISH AS A MODEL FOR INVESTIGATING THE PHYSIOLOGICAL BASIS OF DOMINANCE AND ITS DISRUPTION BY ENVIRONMENTAL CHEMICALS

Filby Amy L, Paull Gregory C, Searle Faye, Bartlett Emily J,Hickmore Tamsin FA, Tyler Charles R.

College of Life and Environmental Sciences, University of Exeter, Hatherly Laboratories, Prince of Wales Road, Exeter, Devon EX4 4PS, United Kingdom.

Introduction:

The zebrafish (*Danio rerio*) is an emerging model for studying social behaviour in fish, as well as an established model in ecotoxicology. In this species, dominance hierarchies are established in both sexes in small groups, and dominant individuals have a greater reproductive success. The physiological basis of dominance, and whether this may act as a target for chemicals discharged into the environment, has however yet to be characterized.

Methods:

We undertook a series of studies to determine the physiological underpinnings of dominance, including its neurological mechanisms. This knowledge was then used to unravel possible mechanisms of effect of the environmental oestrogen 17α-ethinyloestradiol (EE_2) on social hierarchies in this species.

Results:

Comparing the physiology of dominant versus subordinate zebrafish, we found wide-ranging differences, which varied temporally and between the sexes. Dominants were larger in size than subordinates and had both lower plasma cortisol and lower expression of a suite of hypothalamo-pituitary-interrenal (HPI) axis genes. Gene expression profiles in the spleen also suggested differences in immune status between ranks. In females, dominants had a larger gonad and showed different patterns of germ cell development, but this was not the case in males. Dominant males, however, had higher levels of plasma 11keto-testosterone (11-KT) than subordinates. Targeted analyses of 40 genes involved in aggression in the brain identified substantial differences between ranks associated with phenotypic differences in aggressiveness, which occurred mainly in the hypothalamus and telencephalon. These results implied regulatory control of aggression in dominant fish by the hypothalamo-neurohypophysial-system (HNS), serotonin, somatostatin, dopamine, HPI, hypothalamo-pituitary-gonadal (HPG), and histamine pathways. These proposed roles were supported by pharmacological manipulations of various nodes within the axes. Exposure to EE_2 (at 10 ng/L for 10 days) caused 50% of dominant males to loose their dominant rank and show reduced aggression, while the aggression of subordinate males increased, and the consequences of these changes in behaviour for reproductive success were assessed. Differences in sex steroid gene expression, plasma 11-KT, and brain gene expression present between ranks in control fish were no longer present, or were reversed, after exposure, suggesting possible mechanisms of effect of EE_2 on fish behaviour.

Conclusion:

In summary, we characterised wide-ranging differences in physiology that develop in individual zebrafish upon social hierarchy formation. This information is important both for the development of this species as a behavioural model and for understanding behaviour in fish more widely. Oestrogens present in the environment can affect key neurological systems controlling aggression and physiological differences associated with dominance in fish, and this can alter social hierarchies and affect breeding outcomes with implications for fish populations.

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

EFFECTS OF BENZO[A]PYRENE ON THE EXPRESSION OF CYTOCHROME P4501A1, ESTROGEN RECEPTOR β, MEMBRANE PROGESTIN RECEPTOR α, AND SEX STEROID LEVELS IN CHAMELEON GOBY, *TRIDENTIGER TRIGONOCEPHALUS*

In Joon Hwang (1), Young Don Lee (2), Hyung Bae Kim (3), Hea Ja Baek (1)

(1) Department of Marine Biology, Pukyong National University, Busan 608-737, Korea, Fax: +82-51-6295931
email: fire-joon@hanmail.net
(2) Marine and Environmental Research Institute, Jeju, 695-814, Korea
(3) Department of Marine Bio-resources, Gangwon Provincial University, Gangnung 210-804, Korea

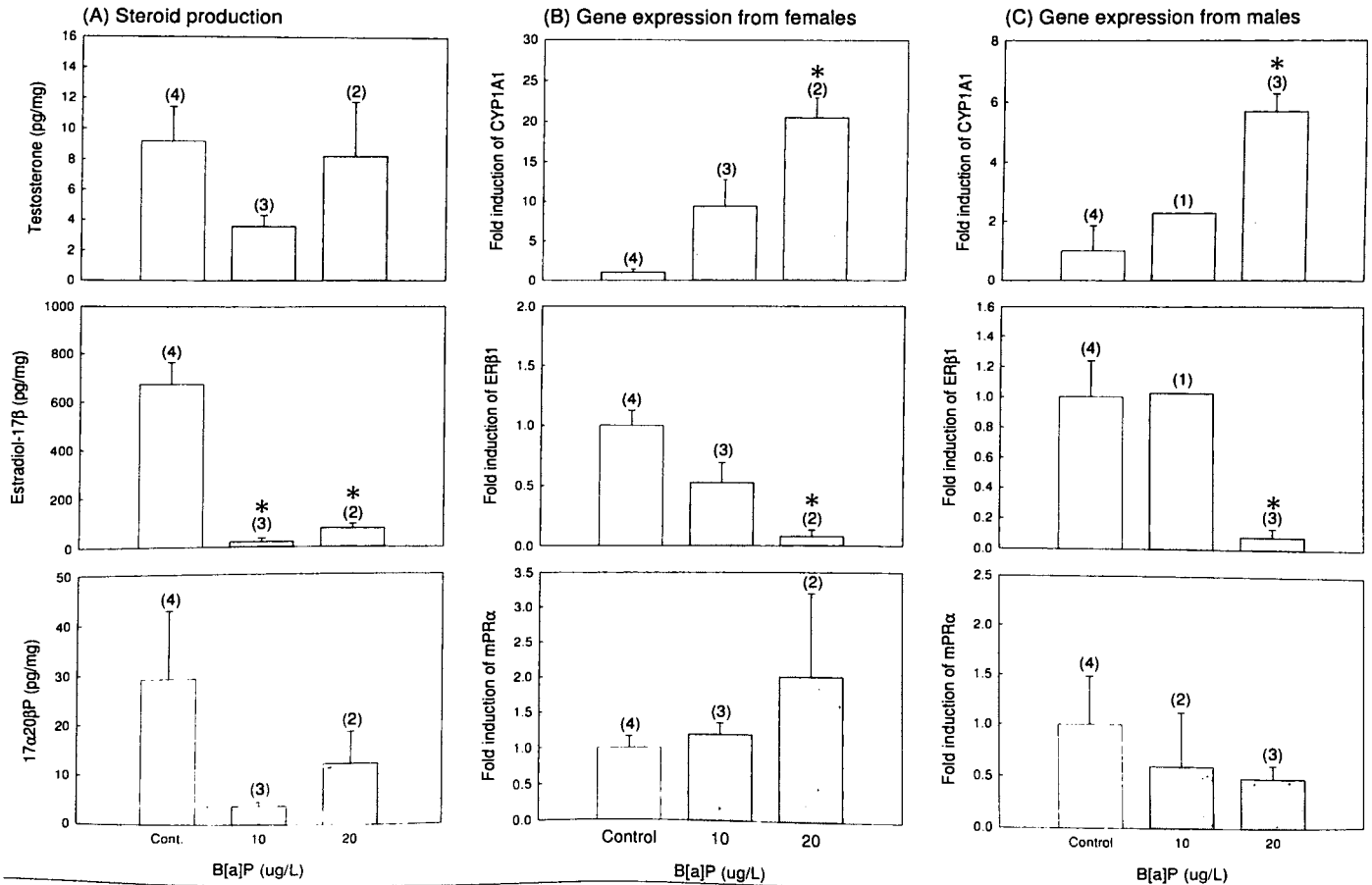
Introduction:

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental contaminants derived from incomplete combustion and crude oil. Benzo[a]pyrene (B[a]P), the representative PAHs, is typically associated with toxicity, B[a]P also has been suggested as endocrine disrupter by negatively impacting reproduction; decrease in egg output, estradiol-17β (E_2) and vitellogenin production. In this study, we investigated the effects of B[a]P on ovarian maturation of chameleon goby, *Tridentiger trigonocephalus*.

Methods:

Matured *T. trigonocephalus* were exposed to waterborne B[a]P (0, 10 and 20 μg/L) for 30 days with renewal rearing condition. After exposure, liver and gonad were fixed, embedded and stained with hematoxylin and eosin for histological observation. Levels of steroids; testosterone (T), E_2 and 17,20β-dihydroxy-4-pregnen-3-one (17α20βP) from ovary were quantified by radioimmunoassay (RIA). As target genes for B[a]P exposure, cytochrome P450 1A1 (CYP1A1) and estrogen receptor β1 (ERβ1) from liver and

Fig. 1. Effects of B[a]P on steroid production from ovary (A), relative expression levels of CYP1A1, ERβ1 from liver and mPRα from ovary (B) and testis (C). Values are mean±SE. Numbers indicated sample size. Data were analyzed using Kruskal-Wallis test and the Bonferroni's adjusment. Asterisks show significant differences from controls ($P < 0.05$).



membrane progestin receptor α (mPRα) from ovary were cloned partially and their expression was quantified by real-time PCR.

Results and discussion:

In histological observations of ovary and testis, there were not any significant differences followed by B[a]P exposure. However, B[a]P caused swelling of lipid droplet from liver in both of females and males. In the production of steroid hormones from ovary, B[a]P significantly decreased E_2 compared to controls. Moreover, B[a]P also decreased the ratio of E_2/T and E_2/17α20βP. Similarly, B[a]P inhibited the aromatase activity in killifish [1] and E_2 production in cuvier [2]. In the transcription of target genes, B[a]P increased transcription of CYP1A1 and decreased transcription of ERβ1 mRNA dose-dependently in liver from females. There were not any significant differences in transcription of mPRα from ovary, although the values were slightly increased with high fluctuation. In males, B[a]P increased the transcription of CYP1A1 and decreased transcription of ERβ1. These results suggested that B[a]P may act as an antiestrogen with inhibition of E2 production and transcription of ERβ1 in chameleon goby. Future study with aromatase activity or vitellogenin production will provide more detailed mechanism of endocrine disruption by exposure to B[a]P.

Conclusion:

The results from this study suggested that ovarian maturation in chameleon goby is affected with increase in transcription of CYP1A1, decrease in E_2 production and transcription of ERβ1 by exposure to B[a]P. Moreover, B[a]P may act as an antiestrogen in the process of maturation of chameleon goby.

Acknowledgement:

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (No. 2010-0017176).

References:

[1]PATEL, M.R., SCHEFFLER, B.E., WANG, L., WILLETT, K.L. 2006. Effects of benzo(*a*)pyrene exposure on killifish (*Fundulus heteroclitus*) aromatase activities and mRNA. Aquat. Toxicol., 77: 267–278.

[2]ZHENG, R., WANG, C., ZHAO, Y., ZUO, Z., CHEN, Y. 2005. Effect of tributyltin, benzo(*a*)pyrene and their mixture exposure on the sex hormone levels in gonads of cuvier (*Sebastiscus marmoratus*). Environ. Toxicol. Pharmacol., 20: 361–367.

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

INTERACTIVE EFFECTS OF INBREEDING AND EXPOSURE TO AN ENDOCRINE DISRUPTING CHEMICAL ON LIFE HISTORY TRAITS IN FISH

Bickley LK[1], Brown AR[1,2], Le Page G[1,2], Hosken DJ[3], Hamilton P[1], Paull,GC[1], Owen SF[2], and Tyler CR[1]

[1]University of Exeter, Hatherly Laboratory, Prince of Wales Road, Exeter, Devon, EX4 4PS, UK. Fax: +44(0)1392 723434.Email: L.K.Bickley@exeter.ac.uk.
[2]AstraZeneca, Brixham Environmental Laboratory, UK.
[3]University of Exeter, Centre for the Environment and Conservation, UK.

Introduction:

The protection of wildlife populations is an implicit goal in environmental risk assessment. It relies on the meaningful extrapolation of data from ecotoxicological studies based on model test organisms to predict population level effects. Such model organisms are often obtained from selectively bred laboratory strains with reduced genetic variation [1]. Whilst this helps to ensure responses are consistent between individuals it may not be reflective of the potential responses of wildlife populations. It has been argued that the use of outbred strains may be more appropriate for ecotoxicology, since they may better represent wild populations [2].

Inbreeding can have negative fitness consequences for natural populations because it causes inbreeding depression, and in some cases this reduced fitness has been shown to be exacerbated under chemical stress [3,4]. Indeed, the few reports available (all of which are on invertebrates) have shown that inbreeding increases the impacts of chemical exposure in laboratory maintained animals.

A significant environmental pressure on wildlife populations is exposure to chemicals discharged as a consequence of anthropogenic activities, and one group of chemicals causing widespread concern are endocrine disrupting chemicals (EDCs). Exposure to EDCs is associated with deleterious impacts on reproduction and other aspects of health in a wide range of wildlife species [5], and may also negatively impact on their population dynamics [6,7,8]. Furthermore, EDCs have the potential to lead to compounding (interactive) effects on inbred populations, since reproductive fitness traits often show most inbreeding depression.

The aim of this research project was to test the hypothesis that inbred zebrafish (*Danio rerio*) differ in their susceptibility to the effects of chemical exposure compared with outbred zebrafish (representing wild populations). This was done via exposing hybrid WIK laboratory strain/Wild Bangladesh zebrafish to clotrimazole, a known EDC and priority hazardous substance [9] and assessing effects on various life history traits, including reproductive competitiveness.

Methods:

Controlled zebrafish matings were conducted to generate inbred (theoretical inbreeding co-efficient F=0.25) and outbred (F=0) family lines. Zebrafish were exposed over a 96 day period (between 37 and 133 days post hatch) to nominal clotrimazole concentrations of 0, 5 and 50 μgL^{-1}. Various end points were recorded to determine effects on somatic growth, sexual differentiation and gonadal development. These included: *in vivo* measurements to determine specific growth rates; sexual differentiation and germ cell development assessed via histology; plasma concentrations of 11-ketotestosterone (11-kt); and the expression of a number of key target genes assessed via quantitative real-time PCR.

Following exposure to 5 μgL^{-1} clotrimazole, competitive breeding trials were conducted and measures of reproductive output (number of eggs spawned and embryo viability) and competitive fertilization success were determined, the latter via paternity assessments of the offspring using microsatellite genotyping.

Results:

Exposure of inbred and outbred zebrafish to 5 μgL^{-1} clotrimazole had no effect on survival, growth or gonadal development. However, exposure of both lines to 50 μgL^{-1} clotrimazole caused a male-biased sex ratio compared with controls (87 % vs. 55 % and 92 % vs. 64 %, for inbred and outbreds, males vs. females, respectively), advanced germ cell development, and reduced plasma 11-kt concentrations. We also found outbred males (but not inbred) exposed to the high level of clotrimazole developed testis that were more than twice the weight of control males. This corresponded with an increase in the relative proportion of Leydig cells, as well as maintenance of the expression of gonadal aromatase (*cyp19a1a*) and insulin-like growth factor (*igf1*), both genes of which were significantly down regulated in inbred males.

In competitive breeding trials, exposure of zebrafish (inbred and outbred) to 5 μgL^{-1} clotrimazole had no effect on fecundity, but significantly reduced embryo viability in inbred (but not outbred) fish. However, this reduction in viability was not observed when inbred and outbred males were directly competing for fertilisation

success within the same tank. Paternity analysis revealed a trend towards a reduction in the proportion of embryos sired by inbred exposed males compared to outbred exposed males. Furthermore, irrespective of clotrimazole exposure, plasma 11-kt concentrations were reduced in inbred compared to outbred males.

Inbreeding depression coefficients indicate the additional stress caused by exposure to clotrimazole amplified the effects of inbreeding on embryo viability (σ = -1.4 and 24.0, for control and exposed fish respectively) and a key male fitness component, siring success (σ = -12.8 and 21.3).

Conclusion:

This is one of the first studies reporting that effects of exposure to an EDC (clotrimazole) on some traits (including sexual development and reproductive success) can differ between inbred and outbred animal strains. We show that inbreeding may be an important consideration in ecotoxicology and that EDCs may potentially affect inbred wild populations differently to outbred wild populations. Our data indicate the importance of better understanding interactions between inbreeding and chemical exposure for environmental risk assessment and protection of wildlife populations.

References:

[1]Coe *et al.*, (2009) Ecotoxicol.
[2]Brown *et al.*, (2009) Phil. Trans. Roy. Soc.
[3]Bijlsma *et al.*, (1999) J. Evol. Biol.
[4]Nowak *et al.*, (2007) Aquat. Toxicol.
[5]Tyler & Goodhead, (2010) 'Silent Summer: The State of Wildlife in Britain and Ireland'
[6]Bryan *et al.*, (1986) J. Mar. Biol. Assoc. U.K.
[7]Kidd *et al.*, (2007) Proc. Natl. Acad. Sci. U.S.A.
[8]Harris *et al.*, (2010) Environ. Health Perspect.
[9]OSPAR, (2005) Background document on clotrimazole. Publication Number: 2005/199.

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

CYCLIC LONG TERM HYPOXIA IMPAIRS ENDOCRINE AND REPRODUCTIVE FUNCTIONS IN GOLDFISH, *CARASSIUS AURATUS*

Bera A., Dasgupta S., Banerjee Sawant P., Chadha N.K., Pal A.K.

Central Institute of Fisheries Education (ICAR), Versova, Mumbai, India, 400061
FAX: +91-2226361573; e-mail: subrata.srt@gmail.com

Introduction:

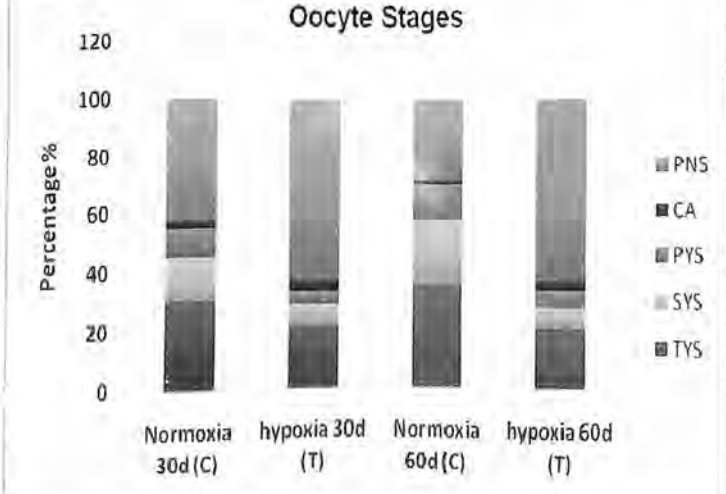
Aquatic hypoxia has been considered as a major environmental concern in the new millennium. Recent studies clearly revealed that hypoxia acts as an endocrine disruptor and reduced reproductive performance of the fish. Wild fish populations generally face hypoxia for variable duration with or without different frequency of daily hypoxic periods. The effect of such cyclic hypoxia on reproduction is not yet elucidated in any fish. Therefore, the present study we have investigated the effect of cyclic hypoxia on gametogenesis and reproductive endocrine function in goldfish, *Carassius auratus,* a well established freshwater model for reproductive endocrinology.

Methods: Adult goldfish were exposed to hypoxia (T, 1.2 mg/l DO) for different durations such as 6h, 9h, 12h, 24h followed by normoxic (C, DO 6.0 mg/l) recovery daily for a total period of 60 days. Gonads were collected for calculation of gonado-somatic index (GSI) and for histological examination. Plasma total cholesterol, HDL cholesterol, LDL cholesterol and triglycerides were estimated by enzymatic colourimetric tests (Merck India Ltd.). Plasma levels of 17 alpha hydroxyprogesterone, testosterone, estradiol, 11 ketotestosterone and vitellogenin were measured by ELISA. Sperm production was estimated by calculating the sperm number and motility score.

Results and Discussion:

The cyclic hypoxia for different duration inhibited gonadal growth by suppressing gametogenesis in both male and female. It significantly reduced GSI values more than 50% which was mainly attributed to reduction of number of vitellogenic oocytes, particularly the tertiary yolk oocytes in the fish exposed to hypoxia (Fig. 1). The hypoxia caused a sharp decline in plasma profile of two key reproductive steroids: estradiol in female and 11 ketotestosterone (11-KT) in male (Fig. 2, 3). The overall inhibition in steroid production pathway played a crucial role in retarding the process of gametogenesis in both the sexes. Plasma vitellogenin (Vg) which is a biomarker of oocyte development was also significantly decreased in female fish resulting hindrance in oocyte growth. On the other hand, significant increase in plasma estradiol may be attributed to higher plasma vitellogenin level in hypoxic male (Fig. 4). The total cholesterol, HDL and LDL cholesterol were significantly reduced in the fish exposed to different duration of cyclic hypoxia (Fig. 5). As cholesterol remains the starting precursor molecule for steroid synthesis, the decrease in its production may be attributed at least to certain extent to the inhibition of steroidogenesis pathway. There was a marked decrease in production of sperm and their motility compared with controls in hypoxic groups, which was associated with decreased 11–KT signaling regulating spermatogenesis. The degree of endocrine disruption and impairment of the reproductive activity

Fig. 1



Parameters		30 Days	60 Days
GSI Female	C	10.54 ± .469	9.21 ± .503
	T	4.63* ± .248	3.99* ± .124
GSI Male	C	3.37 ± .181	3.20 ± .200
	T	1.67* ± .067	1.25* ± .049
Sperm Count (X 10^9)	C	3.38 ± .135	3.15 ± .076
	T	2.03* ± .122	1.88* ± .06
Sperm Motility	C	4.66 ± .210	4.16 ± .307
	T	3.83* ± .307	2.33* ± .210

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

was highest in 24h hypoxia groups followed by 12h and 9h hypoxia groups, whereas 6h hypoxia had significantly lower effect compared to other hypoxia treatments.

adaptive strategy to cope up under cyclic hypoxia by reducing the cholesterol supply for production of sex steroids during active reproductive phase.

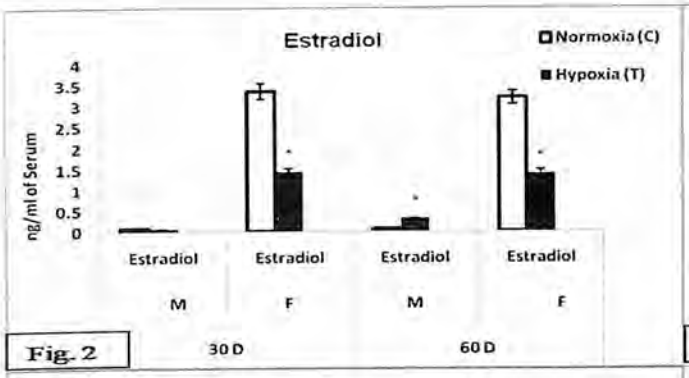


Fig. 2

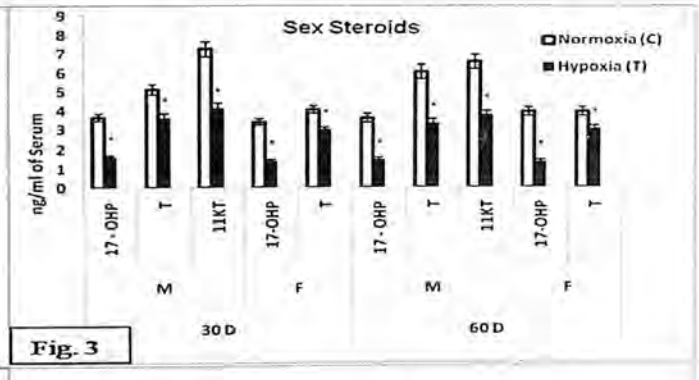


Fig. 3

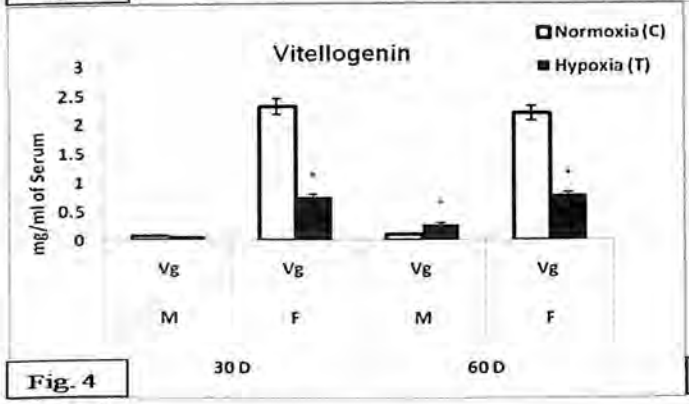


Fig. 4

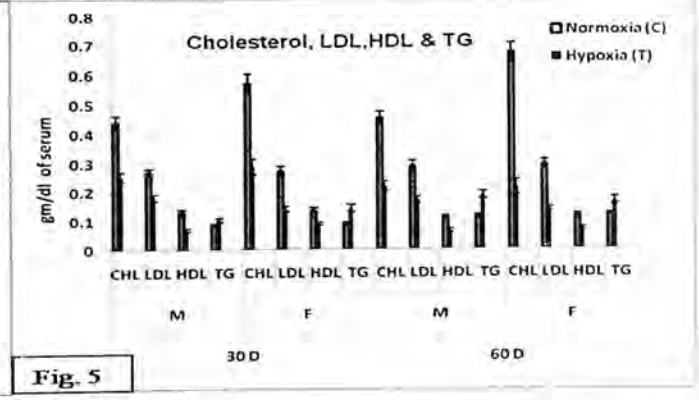


Fig. 5

Conclusion:

The study clearly reveals that cyclic hypoxia caused impairment in reproduction similarly as reported in other fishes exposed to continuous hypoxia (1, 2). However the extent of reproductive suppression under higher hypoxia duration in a cycle was found to be severe similar to continuous hypoxia. Inhibition in steroid synthesis was a prime attributor retarding the gametogenesis process, mainly to reduce the energy expenditure towards reproduction under anaerobic condition. Moreover hypocholesterolemia may be an

References:

[1]WU, R., ZHOU, B., WOO, N., LAM, P., 2003. Aquatic hypoxia is an endocrine disruptor and impairs fish reproduction. Environ. Sci. Technol., 37: 137–1141.

[2]THOMAS, P., RAHMAN, S.M., KHAN, I.A., KUMMER, J.A.2007. Widespread endocrine disruption and reproductive impairment in an estuarine fish population exposed to seasonal hypoxia. Proc. R. Soc. B, 274: 2693-2702.

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

4-NONYLPHENOL IMPAIRS OVARIAN RECRUDESCENCE AND INDUCES ATRESIA IN THE CATFISH *HETEROPNEUSTES FOSSILIS*

Gautam G. J., Chaube R.*, Joy K. P.#

#Department of Zoology, Banaras Hindu University, Varanasi-221005, India. e-mail: kpjoy@bhu.ac.in
*Zoology Department, Mahila Mahavidyalaya, Banaras Hindu University, Varanasi-221005, India. e-mail: chauberadha@rediffmail.com

Introduction:

4-Nonylphenol (NP) is one of the most common pollutants found in aquatic ecosystem arising, due to anthropogenic activities (industrial effluents, discharges from sewage treatment plants, waste water treatment works and agricultural livestock run off) [4]. Recent research has identified NP as the most important degradation product of NPE (Nonylphenol ethoxylates) because of its enhanced resistance to biodegradation, toxicity, ability to bioaccumulate in aquatic organisms [1]. Nonylphenol is estrogenic in various aquatic animals [1, 6, 7]. Investigations in fish have shown that NP causes elevated plasma vitellogenin and zona radiata protein concentrations in both males and females, altered gonadosomatic indices, changes in sex steroids and follicular development and overall adverse effects on gonadal histology [2, 3, 5]. In the present study, effects of long term exposure of NP on ovarian recrudescence and atresia in the catfish *Heteropneustes fossilis* were demonstrated.

Methods:

4-Nonylphenol (99% pure) was purchased from Acros Organics (Geel, Belgium). Other chemicals were of analytical grade and purchased locally. NP was dissolved in acetone and then diluted with water to obtain the required concentrations 64µg/l ($1/25^{th}$ of LC_{50}) and 160µg/l ($1/10^{th}$ of LC_{50}). During resting and preparatory phases, acclimatized mature catfish were divided into three groups of 40-50 fish each. One group was kept as control and the other two were exposed to NP at concentrations 64µg/l and 160µg/l. After 15, 30, 45 and 60 days, the fish were sacrificed; ovaries were sampled and stored at -80°c. Sampled tissues were used for steroid extraction. Estimation of steroid was done with the help of specific ELISA or HPLC. Fish and ovary weights were recorded for the calculation of Gonadosomatic Index (g %). The ovaries of the control and experimental catfish were fixed in Bouin's fixative, embedded in paraffin wax and cut into 7µm sections. Sections were stained with haematoxylin and eosin and observed under microscope. Data were analysed by two way ANOVA, followed by Newman-Keuls test.

Results and Discussion:

Overall significant changes in GSI were noticed during exposure periods in both the concentration groups. In the resting phase, in comparison to the initial control significant change in the GSI was observed. Although there was no significant change in the GSI after 15, 30 and 60 days, it was significantly decreased after 45 days of exposure at 64 and 160µg/l ($p<0.05$). In the preparatory phase, NP exposure produced overall significant effect in a concentration-dependent manner. Low dose (64µg/l) significantly increased the GSI after 15, 30 and 45 days and decreased it after 60 days. Increase in GSI % might be caused by change in the steroid levels. Exposure to NP (64 and 160µg/l) for 60 days had significant effects on the levels of ovarian steroids (estradiol-17β, E_2 and testosterone, T), low dose significantly increased the E_2 level after 15, 30 and 45 days exposure and decreased at 60 days in the resting phase. On the other hand, high concentration significantly decreased the E_2 level in a duration-dependent manner after the exposure. In the preparatory phase, there was a significant decrease in E_2 levels after 15, 30 and 60 days; however a significant increase in E_2 level was observed at 45 day exposure in comparison to the parallel control group. Testosterone level was significantly elevated in both concentration groups after 60 day exposure in the resting phase. In the preparatory phase, there was a significant decrease in testosterone level throughout the exposure duration. Histological examination of the ovary, in all treatments and through duration revealed that NP exposure impaired gonadal development throughout the reproductive season by inducing atresia. In the resting phase there was a significant increase in the atretic oocytes over the exposure period, in a concentration-dependent manner, its potency was low at 64µg/l and high at 160µg/l. Stage I and stage II oocytes were seen in both resting and preparatory phases. However, NP induced gonadal development and stage III, IV and V oocytes were observed after 15, 30 and 45 days of exposure, respectively, suggesting that low dose of NP induces oocyte maturation and ovulation thereby favoring gonadal growth and maturation where as high concentration slowed down gonadal growth and maturation and increased the frequency of atretic oocytes through prolonged exposure period. This study signifies that NP can produce variations in key gonadal steroidogenic and growth regulating pathways that may

lead to imbalance affecting gonadal growth and maturation.

Conclusion:

In conclusion, our investigation suggests that long term exposure of NP impairs gonadal growth and maturation and induces follicular atresia in the catfish in a concentration-dependent manner.

References:

[1]AEL, M., GIGER, W., KOCH, M., 1994. Behaviour of alkylphenol polyethoxylate surfactants in the aquatic environment — I. Occurrence and transformation in sewage treatment. Water Res., 28: 1131–1142.

[2]ARUKWE, A., CELIUS, T., WALTHER, B.T., GOKSOYR, A., 2000. Effects of xenoestrogen treatment on zona radiata protein and vitellogenin expression in Atlantic salmon (*Salmo salar*). Aquat. Toxicol., 49: 159–170.

[3]JOBLING, S., SHEAHAN, D., OSBORNE, J.A., MATTHIESSEN, P., SUMPTER, J.P., 1996. Inhibition of testicular growth in rainbow trout (*Oncorhynchus mykiss*) exposed to estrogenic alkylphenolic chemicals. Environ. Toxicol. Chem., 15: 194– 202

[4]LANGSTON, W., BURT, G., CHESMAN, B., VANE, C., 2005. Partitioning, bioavailability and effects of oestrogens and xeno-oestrogens in the aquatic environment. J. Mar. Biol., 85: 1–31.

[5]LEINO, R.L., JENSEN, K.M., ANKLEY, G.T., 2005. Gonadal histology and characteristic histopathology associated with endocrine disruption in the fathead minnow (Pimephales promelas). Environ. Toxicol. Pharmacol., 19: 85–98.

[6]SERVOS, M.R., 1999. Review of the aquatic toxicity, estrogenic responses and bioaccumulation of alkylphenols and alkylphenol polyethoxylates. Water Qual. Res. J. Can., 31: 123–177.

[7]TALMAGE, S.S., 1994. Environmental and human safety of major surfactants: alcohol ethoxylates and alkylphenol ethoxylates. Lewis Publishers, Boca Raton, FL.

MODULATION OF GENE EXPRESSION IN GONAD AND LIVER OF MALE GOLDFISH EXPOSED TO BISPHENOL A

Hatef A.*, Zare A.§, Alavi S.M.H.*, Habibi H.R.§, Linhart O.*

* Faculty of Fisheries and Protection of Waters, South Bohemia Research Center of Aquaculture and Biodiversity of Hydrocenoses, University of South Bohemia in České Budějovice, Zátiší 728/II, Vodnany 389 25, Czech Republic. Fax +420 387774634, email: ahatef@frov.jcu.cz

§ Department of Biological Sciences, University of Calgary, Calgary, Alberta, Canada.

Introduction:

Bisphenol A (BPA) is widely used for production of polymers such as polycarbonate [1, 2]. Its levels were reported between 0.02–21 ug/L in river water [1]. BPA is known as an estrogen mimicking compound; but there are a few evidence for its anti-androgenic activity depending on dose and period of exposure [3]. Adverse effects of BPA on intersex, vitellogenin (VTG) induction and decrease of androgens and sperm quality have been shown in some studies [1, 2, 3]. The main objectives of the present study was to investigate modulations of gene expression in gonad and liver of male goldfish exposed to environmentally relevant concentrations of BPA.

Methods:

Mature males of goldfish were exposed to BPA at 0.0, 0.2, 2, and 20 ug /L for 90 days during the spawning season. Samples of liver and gonad were collected at days 7, 15, 30, 60, and 90 after exposure. Expressions of ER subtypes, AR, StAR and CYP19Awere analysed in gonad. In liver, analysis of VTG, ER subtypes and AR mRNA expression was performed. Total RNA was extracted from each sample using TriZol Reagent (Invitrogen). cDNA was synthesized from total RNA using an oligo-d(T) anchor and M-MLV reverse transcriptase (Invitrogen) according to the manufacturer's protocol. Quantitative Real-time PCR (iCycler iQ Multicolour Real-time PCR Detection System, Bio-Rad Labratories, Inc) was used for evaluating gene expression level. Relative mRNA expression from the control was used in statistical analysis after testing homogeneity of variance and normality of data.

Results:

Relative ER-beta1 expression in gonad was significantly increased at 20 ug /L after 7 days, while its expression in liver was increased at 2 ug/L after 60 days. Relative ER-beta2 expression either in gonad or in liver was increased at 20 ug/L after 60 days. Relative ER-alpha was increased in liver or gonad at 2 or 20 ug/L after 60 days, but the difference was not significant. Significant increase of relative VTG mRNA expression in liver was observed at 2 ug/L after 60 days exposure. Relative AR mRNA expression in gonad showed significant decrease at 0.2 and 2 ug/L after 7 and 15 days exposure, but increased significantly at 2 ug/L after 90 days exposure. In liver, relative AR mRNA expression did not change within the exposure period. Relative StAR mRNA expression in gonad decreased at 0.2 and 2 ug/L after 90 days exposure. Significant increase of CYP19A was observed aft 20 ug/L after 60 days.

Discussion:

Our previous studies showed that BPA decreased androgen (T and 11-KT) production at low concentrations (0.5–1.5 ug/L) [4]. The observed decrease of androgen production might be corresponding to low cholesterol delivered into steroidogenesis pathway. Because, relative StAR mRNA expression, which deliver cholesterol to the inner mitochondrial membrane to be converted to pregnenolone via P450scc, decreased at low concentrations (0.2 and 2 ug/L) in the present study. The present study also showed that BPA at 0.2 and 2 ug/L decreased the AR mRNA expression that is an evidence for anti-androgenic activity of BPA through AR-antagonist mode of action. However, further studies are required to investigate whether LH involves in regulating anti-androgenic mode of action of BPA. Nelson and Habibi [5] found that ER-beta subtypes are directly regulating VTG production. In addition, ER-beta subtypes can enhance the VTG production through ER-alpha subtype. The present study suggests that BPA at high concentration induce VTG mRNA expression via ER-beta subtypes, because ER-alpha did not show significant increase. Our results also suggested estrogen mimic action of BPA via CYP19A, which convert androgens to estradiol. In the present study, we observed increase of CYP19A when the male goldfish was exposed to 20 ug/L BPA.

Conclusion:

Modes of action of BPA depend on concentration and exposure period. Anti-androgenic mode of action appears at low concentrations through AR-antagonist, but estrogenic activity appears at high concentrations through ER-agonism.

Acknowledgements:

This study was financially supported by GACR 523/09/1793, CENAKVA CZ.1.05/2.1.00/01.0024, 033/2010/Z, 046/2010/Z, and ME10015.

References:

[1]KANG JH, AASI D, KATAYAMA Y. 2007. Bishenol A in the aquatic environment and its endocrine-disruptive effects on aquatic organisms. Critical Rev. Toxicol., 37: 607–625.

[2]VANDENBERG LN, MAFFINI MV, SONNENSCHEIN C, RUBIN BS, SOTO AM. 2009. Bisphenol-A and the great divide: A review of controversies in the field of endocrine disruption. Endocrine Rev., 30: 75–95.

[3]**AKINGBEMI BT,** SOTTAS CM, KOULOVA AI, KLINEFELTER GR, HARDY MP. 2004. Inhibition of testicular steroidogenesis by the xenoestrogen bisphenol A is associated with reduced pituitary luteinizing hormone secretion and decreased steroidogenic enzyme gene expression in rat Leydig cells. Endocrinology, 145: 592–603.

[4]HATEF A, ALAVI SMH, ABDULFATAH A, FONTAINE F, LINHART O. 2011. *In vivo* effects of Bisphenol A on sex steroids and vitellogenin production in male goldfish (*Carassius auratus* L.) at environmentally relevant concentrations .Reprod. Biol. Endocrinol. In revision.

[5]Nelson ER, Habibi HR. 2010. Functional significance of nuclear estrogen receptor subtypes in the liver of goldfish. Endocrinology, 151: 1668–1676.

AGRICULTURAL IMPACT ON REPRODUCTIVE PERFORMANCE OF CEYLON STONE SUCKER (*GARRA CEYLONENSIS* BLEEKER, 1863)

Jayakody A. Sumith[1]., Kelly R., Munkittrick[1]

1Canadian Rivers Institute and Department of Biology, University of New Brunswick, P.O. Box 5050, Saint John, NB, E2L 4L5, Canada. 2Permanent Address: Office of the Registrar of Pesticides, Department of Agriculture, 1056, Getambe, P.O. Box 49, Peradeniya 20400, Sri Lanka. Fax +1 506 648 5811 E-mail: sumith.ja@unb.ca.

Introduction:

Agricultural impact on the associated ecosystem is primarily diffused in nature. There are various potential sources of stressors on fish such as physical disturbance (e.g., suspended solids), organic enrichment (e.g., farm-yard waste), toxic materials (e.g., pesticides) and development activities (e.g., channel modification). Munkittrick and McMaster [1] have developed and applied an effects-based assessment approach that involves measuring the accumulated environmental state of the system to evaluate the impacts of multiple stressors on fish populations. Environmental conditions, which impaired growth and development or reproductive success would become evident in populations and the patterns of indicator responses that depart from the reference state is used to identify the candidate stressors [2]. The intended use of biological criteria is to evaluate the effects of agricultural non-point source pollutior on fish performance integrity and ecological success. We studied the usefulness of Garra ceylonensis (Family: Cyprinidae) -an endemic, algivorous freshwater fish species- as a biological indicator of agricultural pollution in Sri Lanka.

Methods:

Replicate sampling sites in the Uma-oya were selected from 3 catchment elevation levels (viz. upper, middle and lower) representing an elevation gradient design. The approximate land catchment area above each level was 48, 98 and 740 km2, respectively. All 12 sites were considered deteriorated in the Uma-oya, due to intensive exploitation for agriculture. A modified subjective scale [3] on agricultural impacts was defined for five criteria (area above under cultivation, hydrological regime, stream connectivity, morphological conditions, and nutrient organic inputs) and coded as 1= high impact, 2= medium impact and 3= low impact. Four altitudinal categories were defined: 1= sites >1,150 m; 2= >1,050 <1,150 m; 3= >950 <1,050 m; and 4= <950 m above MSL. Two reference sites in the Knuckles streams were 217 and 482 m above MSL. Despite different elevation and reproductive seasonality of Garra spp., the reference sites were considered surrogates for population performance assessments, since acceptable reference locations devoid of human impact within the Uma-oya catchment could not be found. Samples sizes were selected based on a priori power analysis. Only adult specimens of females >8.5 cm and males >7.0 cm of total length were captured. In the laboratory, voucher specimens were measured for length (L) (±0.1 cm) and weight (W) (±0.001 g) and dissected for gonad weight (±0.001 g). Five clumps of post-vitellogenic egg masses (preserved in 70% alcohol) were separated from 3 different regions (i.e., anterior, mid and posterior) along the vertical direction, blotted-dried and measured for weight (±0.001 g) and counted. Diameters (µm) of 10 eggs from each clump were measured under calibrated microscope.

Results and Discussion:

Both contamination and elevation categories showed significant differences ($p<0.001$) in fish size (L and W) and gonad size (GSI) in females. Interestingly, two categories appear to be responsive in opposite directions where significantly heavier and bigger fish were associated with high contamination category ($p<0.001$) and there was a significant size increase (in terms of length and weight of female fish) at elevations below 1,050 m. Similarly, gonad sizes were reduced in the high contamination category and were also reduced at low elevations ($p<0.001$). The larger and heavier females had significantly higher fecundity (number of eggs per female) at high contamination sites. At high contaminations, low GSIs were combined with large body sizes, as compared with bigger and heavier females below 1,050 m that had significantly ($p<0.001$) higher fecundity than higher elevation females. It has been hypothesised that larger body size in females could ensure more accommodative capacity for increased egg production [4]. Although, the regression of GSI and body weight of pre-spawning females of G. ceylonensis was highly significant among elevation categories (ANOVA $F=62.95$, $p<0.001$, $n=612$), there was a significant negative trend of fish weight (W) and GSI (Pearson correlation -0.143, $p<0.001$) implying high elevation females are trying to maximize reproductive investment over lower elevation individuals. Or higher elevation females may have earlier maturity and larger gonadal investment in response to eutrophication and increased food availability. Similarly, the largest and heavier male fish were observed in high contamination category ($p<0.001$) and there was a gradient response in

fish weight in both contamination and elevation gradients. Male fish weight was also significantly negatively correlated for all elevations (r2=76.0% ANOVA F=22.11 DF=8 p=0.002). The fish above 1,150m elevation showed the smallest size male fish ($p<0.001$) compared to the lower elevations. Both contamination scale and elevation gradient were indifferent in gonad size in males ($p>0.05$). The male GSI in all sites except lower-most site in the Uma-oya had larger gonads compared to Knuckles reference population showing strong eutrophication (nutrient enrichment) effect.

Conclusion:

Our results showed strong eutrophication response of reproductive investment in both male and female G. ceylonensis suggesting increased food availability would be a primary determinant of reproduction. However, sub-optimum gonad investment and fecundity of females at sites associated with high agricultural inputs may be due to impaired resource partitioning. Subsequent testing will examine the responses of reproductive steroids and other indicators of reproductive health to agricultural exposures.

Acknowledgements: IAEA 15079/R0 research grant to JAS and NSERC-CRC 950 to KRM

References:

[1]MUNKITTRICK, K.R. AND MCMASTER, M.E. 2000. Effects-driven assessment of multiple stressors using fish populations, pp. 27-66. In: Multiple Stressors in Ecological Risk and Impact Assessment: Approaches to Risk Estimation (FERENC, S.A. and FORAN, J.A., (eds). Pensacola, FL, SETAC-Press.

[2]GIBBONS, W.N. AND MUNKITTRICK, K.R. 1994. A sentinel monitoring framework for identifying fish population responses to industrial discharges. J. Aquatic Ecosyst. Health, 3: 227-237.

[3]MAYON, N., BERTRAND, A., LEROY, D., MALBROUCK, C., MANDIKI, S.N.M., SILVESTRE, F., GOFFART, A., THOMÉ, J-P. AND KESTEMONT, P. 2006. Multi-scale approach of fish responses to different types of environmental contaminations: a case study. Sci. Total Environ., 367:715-731.

[4]ABOU-SEEDO, F. AND DADZIE, S. 2004. Reproductive cycle in the male and female grey mullet, Liza klunzingeri in the Kuwaiti waters of the Arabian Gulf. Cybium, 28(2): 97-104.

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

COMPARATIVE RESPONSIVENESS OF TEST AND SENTINEL FISH SPECIES TO NATURAL AND SYNTHETIC OESTROGENS

Lange Anke (1), Katsu Yoshinao(2) (8), Miyagawa Shinichi (2), Ogino Yukiko (2), Urushitani Hiroshi (2), Kobayashi Tohru (3), Hirai Toshiaki (4), Shears Janice A.(1), Nagae Masaki (5), Ohnishi Yuta (6), Oka Tomohiro (7), Tatarazako Norihisa (7), Iguchi Taisen (2), Tyler Charles R. (1)

(1) Biosciences, University of Exeter, UK (2) Okazaki Institute for Integrative Bioscience, NIBB, NINS, and Department of Basic Biology, School of Life Science, Graduate University for Advanced Studies, Okazaki, Japan (3) Lab Mol Reproductive Biology, Institute for Environmental Sciences, University of Shizuoka, Japan (4) Department of Biosciences, Teikyo University of Science and Technology, Uenohara, Japan (5) Faculty of Environmental Studies, Nagasaki University, Japan (6) Environmental Risk Research Center, Institute of Environmental Ecology, IDEA Consultants, Inc., Shizuoka, Japan (7) Environmental Quality Measurement Section, Research Center for Environmental Risk, National Institute for Environmental Studies, Tsukuba, Japan) (8) Laboratory of Reproductive and Developmental Biology, Graduate School of Science, Hokkaido University, Sapporo, Japan

Introduction:

Oestrogens are necessary for ovarian differentiation in many vertebrates and they promote the growth and differentiation of the adult female reproductive system. The actions of oestrogens are mediated through ligand-activated transcription factors, oestrogen receptors (ERs). Globally, feminization responses in wild male freshwater fish have been shown to be caused by exposure to chemicals discharged into the environment (natural and synthetic oestrogens, and oestrogen mimics). Fish have been reported to show differences in their responses and thus susceptibility to oestrogens, but empirical studies that directly address this hypothesis are lacking.

Methods:

The comparative responsiveness to natural and synthetic oestrogens was assessed using in vitro ERα activation assays in a range of fish widely used as test (including, zebrafish, fathead minnow, medaka) and/or environmental sentinel species (including, roach, stickleback, carp). In vivo exposures to 17α-ethinyloestradiol (EE2; nominal 2 and 10 ng/L) via the water were also conducted for 7 fish species over a 7 day period measuring hepatic vitellogenin (VTG) mRNA induction as an ER-mediated response.

Results:

There were differences in the responses of the different fish ERαs to natural steroid oestrogens; zebrafish ERα was most responsive and carp and stickleback ERαs were the least responsive. The different fish ERαs tested also showed differences in their responsiveness to EE2 with the following sensitivity order: zebrafish>medaka>roach>fathead minnow>carp> stickleback. VTG mRNA induction in vivo occurred at an exposure of 2 ng EE2/L for male rainbow trout and at 10 ng EE2/L for males of all other species tested. The order of responsiveness for VTG mRNA induction (level of induction) by EE2 was rainbow trout>zebrafish>fathead minnow>medaka> roach>stickleback >carp.

Conclusion:

Our data clearly show that there are species differences in the ERα responsiveness (in vitro) to natural and synthetic oestrogens and species differences in the sensitivity and responsiveness to EE2 for VTG mRNA induction. Overall, the in vitro responses via ERα equated well with in vivo responses to EE2 in different species showing in vitro screening of chemicals using fish ERα is indicative of oestrogenic (VTG) responses in vivo.

IN VITRO EFFECTS OF ENDOCRINE DISRUPTING CHEMICALS ON THE GONADAL STEROIDOGENESIS IN AN INDIAN MAJOR CARP, *LABEO ROHITA* (HAM.)

Magadalenal N. N., Prabhu R., Inbaraj R. M*.

Department of Zoology, Madras Christian College, Tambaram, Chennai, 600 059, India
e-mail: inbarajmoses2004@yahoo.com

Introduction:

Environmental chemicals of natural and synthetic origin can interact with the endocrine system and alter development and reproduction in wildlife [1] and humans [2]. A wide range of chemicals were shown to have an affinity for estrogen receptors from various species. These contaminants also appear to influence the endocrine system by altering enzymes that enable hormone synthesis and hormone metabolism. The effects of these endocrine disrupting chemicals have been extensively reported in fish; they include inhibition of gonad growth, reduction in the number and quality of germ cells, feminisation and masculinisation [3]. In the present investigation, an attempt has been made to study the in vitro effects of bisphenol-A (BPA) and butylhydroxy anisole (BHA) on the gonadal steroidogenesis in an Indian major carp, Labeo rohita.

Methods:

Oocytes and testicular fragments were collected during three stages of the gonadal development. Incubations were performed with 3 ml of culture medium containing steroid precursor progesterone for about 24 h at 18°C. BPA and BHA at a concentration of 5 µM were added to the incubation medium. Steroids synthesised in the incubation medium were extracted with dichloromethane. Steroids were analysed and quantified by High Performance Liquid Chromatography.

Results and Discussion:

BPA and BHA show remarkable inhibitory effect on ovarian and testicular steroidogenesis during all three stages of the gonadal development. This inhibition of steroid production by BPA and BHA is of both physiological and toxicological significance. BPA was found to be more estrogenic when compared to BHA. The decreased E2 synthesis by the oocytes confirms that BPA and BHA have the potential to inhibit ovarian aromatase, the enzyme that converts T to E2. The stimulation of 17,20β-P synthesis by BPA in mature oocytes confirms that BPA enhances the activity of 20β-HSD (Fig. 1). BPA and BHA showed inhibitory effect on androgen production by different stages of testicular fragments. BPA significantly decreased T and 11-KT production. The effect of BPA and BHA was not significant on E2 production. Decreased T synthesis observed in the present investigation confirms that BPA and BHA have remarkable effect on 17β-HSD activity (Fig. 2). Taken together, these results indicate that the decreased steroid hormone biosynthesis observed in Labeo rohita, following exposure to BPA and BHA, may lead to reduction in reproductive capacity of the fish.

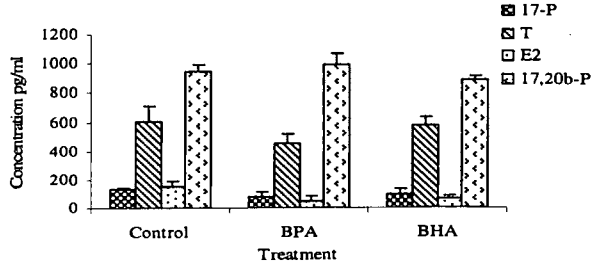


Fig. 1. Effect of BPA and BHA on in vitro steroid production by matured oocytes.

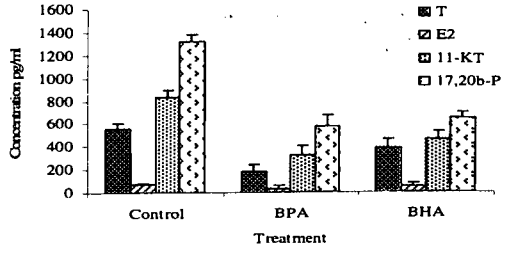


Fig. 2. Effect of BPA and BHA on in vitro steroid production by spawning testicular fragments.

References:

[1]GUILLETTE, L.J., GUNDERSON, M.P. 2001. Alterations in development of reproductive and endocrine systems of wildlife populations exposed to endocrine-disrupting contaminants. Reproduction, 122: 857–864.

[2]OHTAKE, F., TAKEYAMA, K., MATSUMOTO, T., KITAGAWA, H., YAMAMOTO, Y., NOHARA, K., TOHYAMA, C., KRUST, A., MIMURA, J., CHAMBON, P., YANAGISAWA, J., FUJII-KURIYAMA, Y. AND KATO, S. 2003. Modulation of oestrogen receptor signaling by association with the activated dioxin receptor. Nature, 423: 545–550.

[3]ARUKWE, A. 2001. Cellular and molecular responses to endocrine modulators and the impact on fish reproduction. Mar. Pollut. Bull., 42(8): 643–655

EFFECT OF CADMIUM SULPHATE ON PROTIEN AND CARBOHYDRATE LEVEL IN BRAIN AND OVARY OF FRESH WATER FISH *HETEROPNEUSTES FOSSILIS* (BLOCH)

Mishra A.*, Kumar A., Rawat A.

Department of Animal Applied Sciences, Babasaheb Bhimrao Ambedkar University, Lucknow, 226025, India
Email: drabhamishra@gmail.com

Introduction:

Cadmium is a non essential heavy metal and has been listed in the "Black list" of European community. Among the various heavy metal pollutants, cadmium merits special attention due to its potential hazards to aquatic biota as well as to human being. Cadmium alter morphological, physiological and biochemical parameters in fishes [1]. Such effects change digestive enzyme activities [2], decreased immunity state and efficiency of food assimilation [3]. The present report was based on toxicity effects of cadmium sulphate on biochemical property of brain-ovary of freshwater fish *Heteropneustes fossilis.*

Methods:

The live fish *Heteropneustes fossilis,* of similar body weight were acclimatized under normal photoperiod and temperature. Feeding was stopped before 24 hr of experiment. Fish were treated with different concentrations of cadmium sulphate (10, 50, 100, 500 and 1000 mg/l) for 24 hr to see its lethality and sub-lethal concentration (50 mg/l) was used to see the duration effect. A control set was maintained side by side for comparison. The fishes were sacrificed after completion of concentration and duration studies and dissected out the required tissues i.e. brain and ovary. Protein and carbohydrate estimation in both the tissue was done by the Lowery's and Anthrone reagent method respectively.

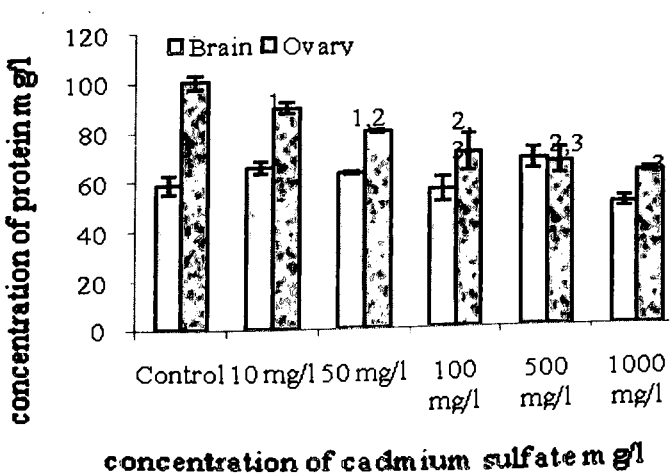
Results and Discussion:

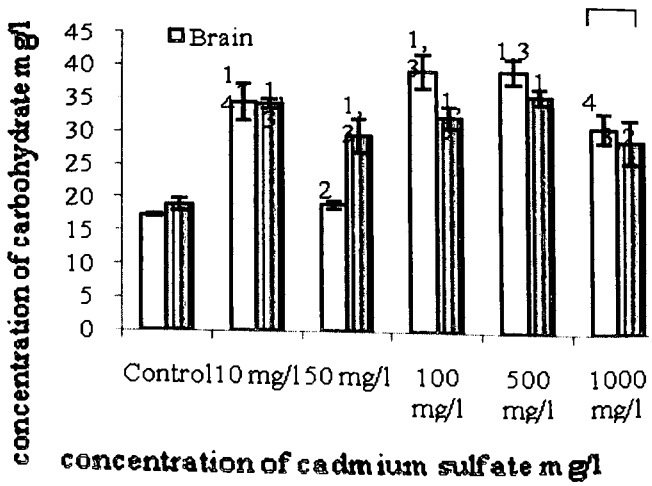
Different concentrations of cadmium sulphate (mg/l) altered the protein and carbohydrate level in brain-ovary in different manner. With the increase of concentration and fish mortality (Table 1), protein concentration in the ovary was decreased while brain showed not much significant variation (Figure 1A). But as for carbohydrate concerned it showed fluctuations but with high values as compared to the control group (Figure 1B) in 24 hr. This may be due to immediate extra energy requirement of fish due to stress features (panting, surface activity, high mucous secretion etc) [4]. Stress caused by cadmium increased the glucose content in blood, because of intensive glycogenolysis and the synthesis of glucose from extrahepatic tissue protein and amino acid in many fishes [5]. When sub-lethal concentration i.e. 50mg/l (Table 1) was used

Table 1. Percent mortality of Heteropneustus fossilis after treatment of various concentration of cadmium sulphate for 24 hr (number of animal per group, n=10).

	Concentration of cadmium sulphate (mg/l)									
	0	10	50	100	500	600	800	900	1000	1200
% Mortality	0	0	0	0	20	20	40	60	100	100

Figure 1: Effect of different concentration cadmium sulfate on brain and ovarian protein and carbohydrate level after 24 hr duration (n=5). Superscripts showed significant difference among different groups (One way ANOVA followed by Newman Keuls' test).

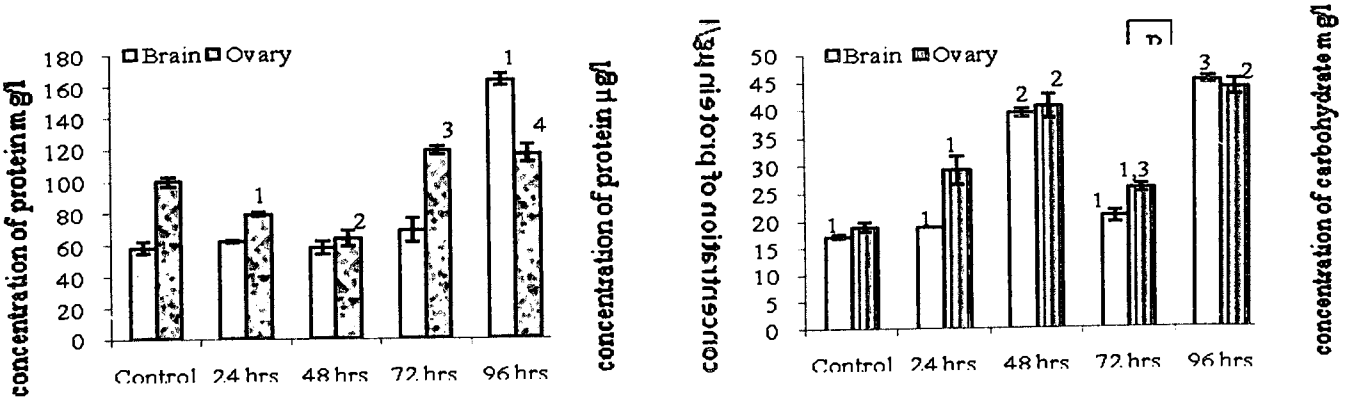




Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

for duration effect study, there was a significant increase in protein-carbohydrate content of brain-ovary with the duration (Figure 2A,B). This might be an indicator of environmental adaptation [4].

Figure 2: Effect of sub-lethal cadmium sulfate (50 mg/l) on brain and ovarian protein and carbohydrate level after 0, 24, 48, 72, 96 hr intervals (n=5). Superscripts showed e significant difference among different groups (One way ANOVA followed by Newman Keuls' test).



Conclusion:

The heavy metal caused deleterious effect on fishes and altered the biochemical characteristics. In sub-lethal, it may not be fatal for an individual organism but it does affect the biochemical parameters resulting in disturbance to whole community and tropic level of food chain, ultimately the ecosystem.

References:

[1]WRIGHT, D. A. AND WELBOURN P. M. 1994. Cadmium in the aquatic environment: a review of ecological, physiological, and toxicological effects on biota. Environ. Rev., 2: 187-214.

[2]VAGLIO, A., LANDRISCINA, C, 1999. Changes in liver enzyme activity in the teleost *Sparus aurata* in response to cadmium intoxication. Ecotoxicol. Environ Saf., 43: 111-6.

[3]MIKRYAKOV, V. R., ZABOTKINA, E. A. 2001. Response of fish immune system upon water pollution by toxicants and water acidification. Moscow, Nauka., pp. 126.

[4]SASTRY, K.V., SUBHADRA, 1982. Effect of cadmium on some aspects of carbohydrate metabolism in a freshwater catfish , *Heteropneustes fossilis*, Toxicol. Lett.,14: 45-55.

[5]LARSSON , A., HAUX, C. 1982. Altered carbohydrate metabolism in fish exposed to sub-lethal of cadmium. *J.* Environ. Biol., 3: 71-81.

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

TISSUE BIOACCUMULATION OF INSECTICIDES AND PLASMA CORTISOL IN THE CATFISHES DURING NON-BREEDING SEASON FROM NORTH INDIA

Nigam, S.K. and Singh, P. B.

Ganpat Sahai Postgraduate College, Sultanpur-228001, India
Fax: 05362240170 email:pratap_b_singh@rediffmail.com

Introduction:

Pesticide residues in wild captured fish have been reported by workers [1,2]. Cortisol in fish has been identified as a metabolic hormone. Since pesticides cause reproductive and metabolic dysfunctions, it is necessary to monitor the tissue bioaccumulation of HCHs and plasma levels of cortisol in some edible catfishes of the un-polluted ponds of Gujartal, Jaunpur (considered as reference site) and the polluted rivers, the Gomti and Ganga during non-breeding season. The objective of this study was to compare the insecticide bioaccumulations in brain, liver and abdominal muscles, and plasma levels of cortisol as stress hormone during non-breeding season. Taking the above facts into account the persistence of chemicals like organochlorine insecticides (OCs) in these rivers, and their eventual incorporation in the food chain finally to humans are necessitated. It is imperative to monitor the presence of residues of toxic chemicals and stress hormone owing to contaminants in order to keep wild fish species in the riverine resources edible for human beings.

Methods:

For the comparative study of HCHs in brain, liver and abdominal muscles and plasma cortisol levels were collected from the captured catfishes of reference site (*Rita rita* and *Bagarius bagarius*) and polluted rivers the Gomti (*Rita rita* and *Bagarius bagarius*) and the Ganga (*Rita rita* and *Clupisoma garua*) during non-breeding season in order to assess the status of pollutants causing stress. In all thirty fish were used in this study from all sites. Blood was taken by caudal vein in separate heparinized culture tubes for pesticide residue and hormone assay. The method of extraction was used with little modification as have been described earlier [3].

Results and Discussion:

The bioaccumulation of ∑HCH was higher in the catfish captured from the river Gomti than the other catfishes captured from the river Ganga indicating that the Gomti is more highly polluted than the Ganga in relation to HCHs pollution. It is suggested that among catfishes there are degrees of pesticide bioaccumulation of HCHs depending upon habit and habitat of the fish. The catfish which were captured from the bottom of the rivers showed high degree of bioaccumulation than the catfish which are not found at bottom of the river. The reason may be due to higher levels of presence of pesticide and water flow is less than the surface water. The findings of the present investigations showing bioaccumulation of HCH in catfishes of the Gomti and the Ganga support the above observations. It is also suggested that these pollutants which caused reproductive dysfunctions through disruption of gonadal steroidogenesis [3], might also have similar effect on the stress response through disruption of interrenal steroidogenesis.

Conclusion:

The catfishes of the Ganga have higher ∑HCH, indicating both the rivers are highly polluted as compared to the reference site. The plasma level of cortisol was suppressed more in the catfishes captured from the river Ganga than the fishes of the river Gomti affecting the growth of the fishes. It is suggested that fish containing pesticide residues beyond the permissible limit must be avoided as food by human beings because it causes health as well as reproductive problems. It is also imperative to monitor insecticide pollution level in both Gomti and Gangetic ecosystem. Such measures would minimize their use in the catchment areas and protect the riverine fishes from the adverse impact of insecticidal pollution on their growth and reproduction.

References:

[1] KANNAN, K., TANABE, S. AND TATSUKAWA, P. 1995. Geographical distribution and accumulation features of organochlorine residues in fish in tropical Asia and Oceania. Environ. Sci. Technol., 29: 2673-2683.

[2] ANTUNES, P., GIL, O., FERREIRA, M., VALEA C. AND REIS-HENRIQUES, M.A. 2007. Depuration of PCBs and DDTs in mullet under captivity clean conditions. Chemosphere, 67(9): 558-564.

[3] LINO, C.M., NORONHA, M.I. AND SILVEIRA, D.A. 1994. Chlorpyrifos, ethion, fenitrothion and methidathion residues in chicken. Bull. Environ. Contam. Toxicol., 52: 425-431.

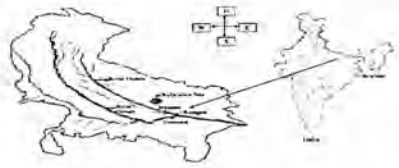


Fig. 1. Map showing sampling sites of unpolluted ponds of Gujartal, Jaunpur (reference site) and polluted rivers (Gomti and Ganga) of north India.
1. Gujartal, Jaunpur : Reference site (Lat. 25.46 N : Long. 82.44 E), captured catfishes [*Rita rita* (Ham.) common name Belgagra and *Bagarius bagarius* (Ham.) common name gonch]
2. River Gomti, Jaunpur : Lat. 25.46 N : Long. 82.44 E, captured catfishes (*Rita rita and Bagarius bagarius*)

Fig. 2. Comparison of isomers of HCH in brain, liver and abdominal muscles of captured catfish between reference site and polluted rivers Gomti and Ganga during non-breeding season. ∑HCH = α+β+γ+δ isomers of HCH. BDL-below detection limit. Results of isomers of HCH of fish tissues from reference site versus fish captured from rivers Gomti and Ganga were compared by Students t-test. The level of significance (P)- *P< 0.001; **P< 0.005; ***P< 0.02; NS. NS- not significant. ANOVA (TW): tissue F-4424.67 P< 0.001; isomers of HCH F-456.52 P< 0.001; tissues x isomers of HCH F-175.61 P< 0.001.

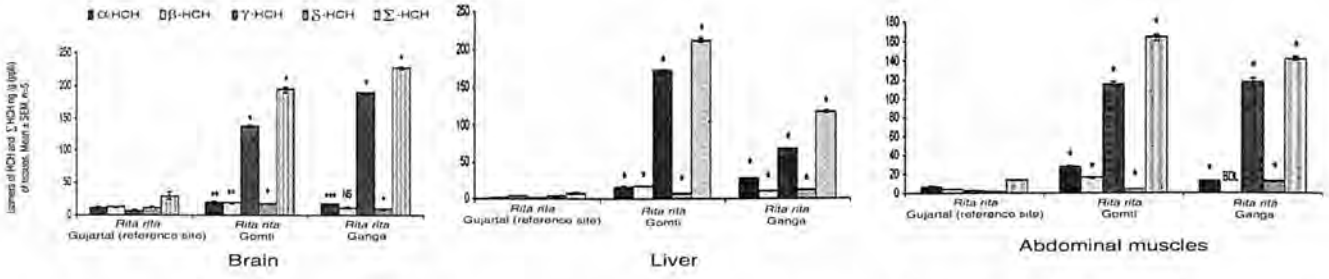


Fig.3. Comparison of isomers of HCH in brain, liver and abdominal muscles of captured catfish between reference site and polluted rivers Gomti and Ganga during non-breeding season. ∑HCH = α+β+γ+δ isomers of HCH. BDL-below detection limit. Results of isomers of HCH of fish tissues from reference site versus fish captured from rivers Gomti and Ganga were compared by Students t-test. The level of significance (P)- *P< 0.001; **P< 0.005; ***P< 0.05; NS. NS- not significant. ANOVA (TW): tissue F-630.74 P< 0.001; isomers of HCH F-248.81 P< 0.001; tissues x isomers of HCH F-39.86 P< 0.001.

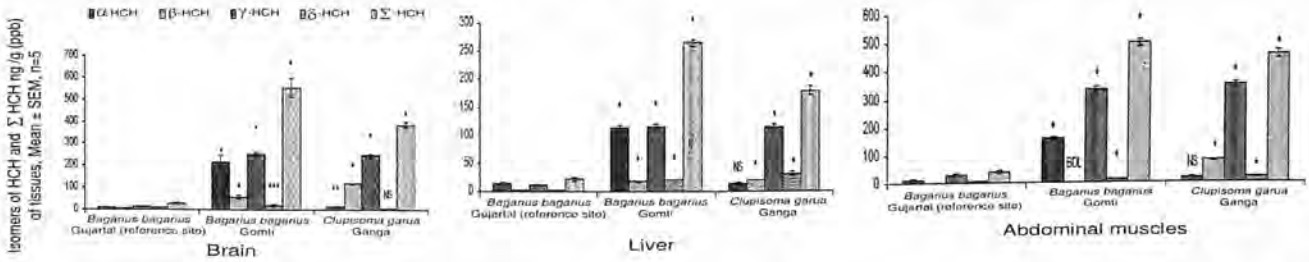
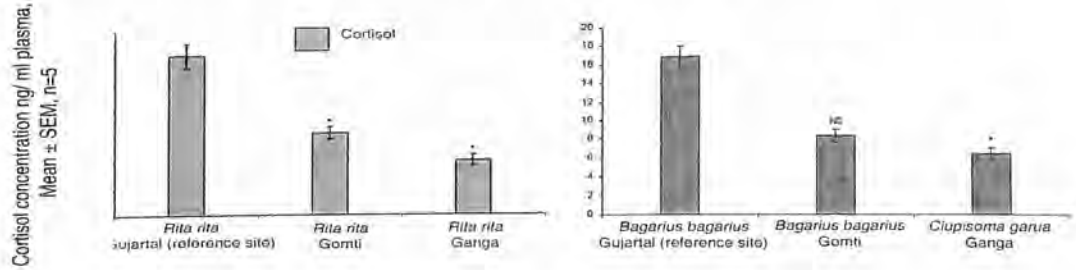


Fig.4. comparison of plasma levels of cortisol during non-breeding season in the catfish captured from the reference site (R. rita and B. bagarius) and polluted rivers Gomti, Jaunpur (R. rita and B. bagarius) and Ganga, Varanasi (R. rita and C. garua).Results were compared from non polluted fish to polluted fish by Students t-test. The level of significance (P)- *P< 0.001. ANOVA (OW): F-655.35 P< 0.001



Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

DEVELOPMENT OF AN ERE-GFP TRANSGENIC ZEBRAFISH FOR ASSESSING HEALTH EFFECTS OF ENVIRONMENTAL OESTROGENS

Okhyun Lee[1], Aya Takesono[1], Masazumi Tada[2], Charles R. Tyler[1]* and Tetsuhiro Kudoh[1]

[1] Biosciences, College of Life and Environmental Sciences, University of Exeter, Exeter, U.K.
[2] Department of Cell and Developmental Biology, University College London, London, UK
* Equal correspondence
Email:c.r.tyler@ex.ac.uk

Background:

Environmental oestrogens are the major group of so-called Endocrine Disrupting Chemicals (EDCs) that can alter hormone signalling in the body and exposure to these chemicals has been shown to impact on reproductive function and alter behaviours in fish. Many thousands of chemicals have now been identified with oestrogenic effects. Understanding the physiological effects of oestrogenic chemicals would be greatly enhanced with in vivo models capable of detecting tissue specific effects of oestrogens with high sensitivity.

Methods:

We have developed a novel Gal4ff-UAS mediated oestrogen responsive transgenic (TG) zebrafish for identifying body system targets of environmental estrogens in real time. The system contains an oestrogen inducible promoter derived from a short stretch of multiple tandem oestrogen responsive elements (EREs) and devoid of any tissue specific enhancer/suppressor elements. To enhance the system's response sensitivity, we used a Gal4ff-UAS system, not previously applied in fish.

Results:

Exposure of the TG fish to oestrogenic EDCs induced specific patterns of GFP expression in a wide variety of tissues including the liver, heart, skeletal muscle, ear, forebrain, lateral line and ganglions, most of which have not been established previously as targets for estrogenic chemicals. Furthermore, we found that different EDCs induced GFP expression with different tissue response patterns and time trajectories suggesting different potential health effects. Importantly, the ERE –TG fish were responsive to oestrogens at environmentally relevant exposure concentrations.

Conclusion:

Our TG zebrafish model provides a new prospect for understanding toxicological effects and health impacts of environmental estrogens in vertebrates.

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

SPERM DENSITY AND SPERM VIABILITY IN WILD MALE FISH ARE INFLUENCED BY DIFFERENT FACTORS

Patrick B. Hamilton[1], Catherine A. Harris[2], Tobias S. Coe[1], Dave Hodgson[1], Tamsin J. Runnalls[2], Susan Jobling[2], John P. Sumpter[2], Charles R. Tyler[1]

[1]. Biosciences, College of Life and Environmental Sciences, Hatherly Laboratories, University of Exeter, Exeter, EX4 4PS, U.K. Email:p.b.hamilton@exeter.ac.uk; [2]. Institute for the Environment, Brunel University, Uxbridge, Middlesex, UB8 3PH, UK

Introduction:

Sperm quality is important for male reproductive success [1], yet little is known of the factors that influence sperm quality in wild fish. Factors known to affect sperm quality in other organisms include age, inbreeding and exposure to pollutants. Feminised male roach (*Rutilus rutilus*) have been reported in freshwater ecosystems receiving wastewater treatment works (WWTW) effluents, resulting from exposure to endocrine disrupting chemicals (EDCs). Wild roach with feminised gonads have reduced sperm quality (sperm motility, sperm density, and fertilization success), compared with less feminised fish from less contaminated sites [2]. However, the influence other factors on sperm quality is not known. For instance, some WWTW effluents are genotoxic [3], so sperm quality might be expected to decrease with age in adult fish living in rivers receiving these effluents, resulting from increased damage to germ cells. In the current study we analysed a large dataset of variables collected from wild fish from effluent-contaminated rivers in order to identify key influences on sperm density and sperm viability.

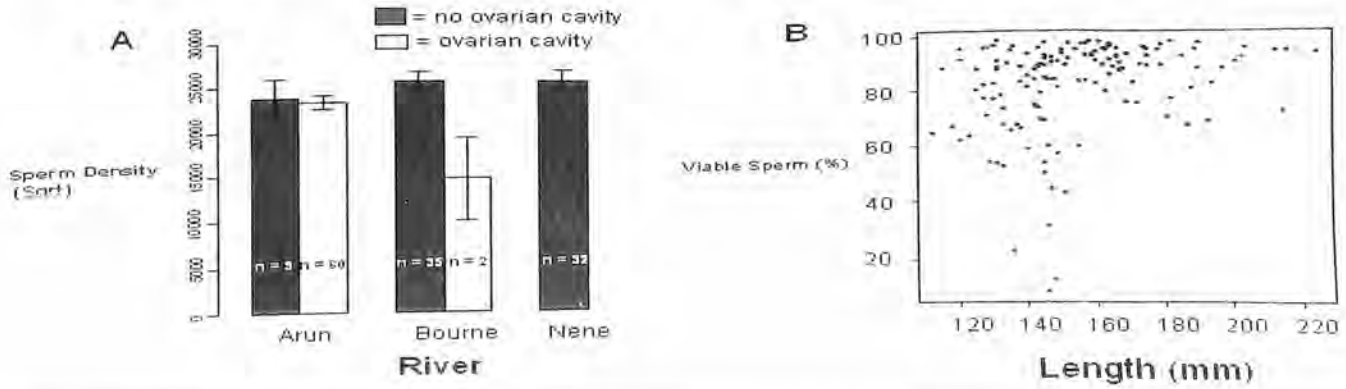
Methods:

Sperm density and sperm viability were examined in 138 adult wild male roach collected from three rivers receiving WWTW effluents. These fish were collected from the River Bourne (2006) the River Nene (2007) and the River Arun (2008) (as per 1). Sperm viability (assessed using trypan blue dye exclusion) and sperm density were recorded for all fish. Gonadal status was assessed using histological techniques and the following feminised phenotypes were recorded separately for each male fish: the presence of developing eggs in the testis of males (the 'intersex condition') and the presence of female-like reproductive ducts (ovarian cavities). The influences of the explanatory variables on both sperm density and viability were assessed using Linear Mixed-Effect (LME) models, using river as a random effect to control for differences between rivers. Variables suspected of influencing sperm quality were included in the maximal models, including body length, skin roughness (due to tubercles, one of the secondary sexual characteristics of male roach), two indices of internal genetic diversity (measures of inbreeding, assessed using 15 DNA microsatellite loci), Intersex Index [based on the number of oocytes present in the testes (4)] and the presence of ovarian cavities.

Results and Discussion:

Based on analyses of the combined dataset from all three rivers, a range of factors were found to influence sperm quality, but the factors that were associated with sperm density and viability differed. There were significant differences in average sperm viability between fish from the three rivers ($F_{(2,137)} = 24.645$, $p < 0.00001$) but there were no significant differences in

Fig. 1. **A**: Relationship between sperm density and the presence or absence of ovarian cavities in fish originating from three rivers. **B**: Relationship between the percentage of viable sperm (untransformed) and fish length. Data are from 138 male fish from three rivers



sperm density ($F_{(2,137)}$ = 1.9365, p = 0.1482). The only factor that was found to influence sperm density was the presence of ovarian cavities, which was negatively associated with sperm density (LME model coefficient = -2354.19, p = 0.0204). However separate analyses of data for each river demonstrated that this relationship was only apparent for the River Bourne (p = 0.0013, given by the hypergeometric distribution with parameters N = 39, n = 2, m = 37) and not the River Arun – Fig 1A. No fish from the River Nene had ovarian cavities. In contrast, sperm viability was significantly correlated with length, with bigger fish having a greater percentage of viable sperm (LME model coefficient = 0.002608, p = 0.0007) – Fig 1B. Sperm viability was also negatively correlated with Intersex Index (LME model coefficient = -0.0326, p = 0.044). We found no statistically significant relationships between roughness and internal genetic diversity with either measure of sperm quality.

Conclusion:

Our results show that the factors that influence sperm density are different to those that influence sperm viability. Sperm viability was affected by the source river, size of the fish and the intersex condition, whereas the presence of ovarian cavities was the only factor linked to sperm density. Our finding that larger roach had a greater percentage of viable sperm is inconsistent with the hypothesis that length of exposure to effluents is a primary factor governing sperm quality, but does not exclude the possibility that effluents harm germ cells. Instead, other factors are more important. For instance, sperm quality may improve with either growth or age of the fish, as has been reported in some mammalian species. These data illustrate the complexities of distilling out the main factors affecting sperm quality in animals (here fish) derived from wild populations.

References:

[1] Harris CA, et al. (2011) The consequences of feminization in breeding groups of wild fish. Environ. Health Persp., 119: 306-311.

[2] Jobling S, et al. (2002) Wild intersex roach (*Rutilus rutilus*) have reduced fertility. Biol. Reprod., 67: 515-524.

[3] Filby AL, et al. (2007) Health impacts of estrogens in the environment, considering complex mixture effects. Environ. Health Persp., 115: 1704-1710.

[4] Jobling S, et al. (2006) Predicted exposures to steroid estrogens in UK rivers correlate with widespread sexual disruption in wild fish populations. Environ. Health Persp., 114: 32-39.

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

EVALUATION OF LIVER HISTOLOGY AND ERYTHROCYTIC MICRONUCLEI AS INDICES OF BISPHENOL A EXPOSURE IN *CHANNA PUNCTATUS*

Pipil S., Rawat V.S. and Sehgal N.

Department of Zoology, University of Delhi, Delhi, India Email id: neetasehgal@mailcity.com

Introduction:

Industrial wastes inflowing into a water body have various detrimental effects on aquatic organisms. Environmental chemicals of natural and synthetic origin can interact with the endocrine system and alter the functioning, development and reproduction in wildlife. Among various other chemicals leaching into water bodies, Bisphenol A, used in the production of polycarbonates, epoxy resins and flame retardants is an estrogenic compound. Various tissues are adversely affected by continuous exposure to estrogenic chemicals. Histological investigation of liver which is the detoxification organ and essential for both the metabolism and the excretion of toxic substances in the body and micronuclei test on fish blood can be used as indicators to determine the health of the species, status of water quality and potential risk they might face. This study was performed in order to assess the effects of Bisphenol A on the liver and red blood cells of the Indian freshwater murrel *Channa punctatus* at sublethal exposure.

Methods:

Adult (body wt. 100-150gms) specimens of the Indian freshwater murrel, *Channa punctatus* were divided into five groups and subjected to four different concentrations of Bisphenol A (1µg/l, 10µg/l, 100µg/l and 1000µg/l). Controls were subjected to the carrier (50% ethanol) alone. Water was renewed daily in the aquaria. Fishes were sacrificed after 15 days and 30 days, and histological studies were made on tissue of fishes. Micronucleated blood cells were also counted in exposed fishes.

Results and discussion:

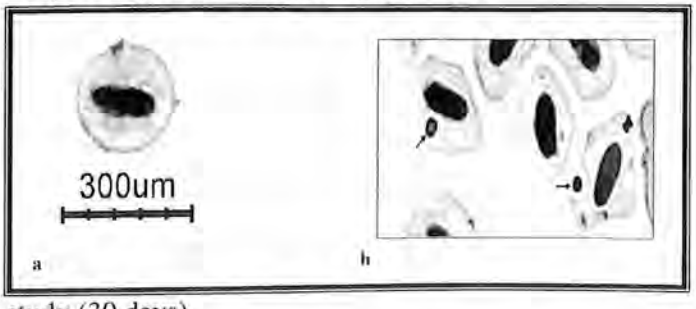
Liver of normal fish is characterized by clearly visible sinusoidal lumen (Fig.1a). *Channa punctatus* exposed to Bisphenol A showed degenerative changes in hepatic cells after 15 days exposure Ffig. 1b). After exposure for 30 days more hypertrophied nuclei were visible alongwith, congestion of blood vessels, hyalinization (hyaline droplet degeneration) and presence of various eosinophilic structures with in the hepatocytes. Sinusoidal spaces were not clearly visible in 30 days treated group (Fig. 1c). The frequency of micronuclei was significantly higher in 100µg/l and 1000µg/l Bisphenol A treated gropus when compared with 1µg/l, 10µg/l bisphenol A treated group and the control group on the 15th day. On the 30th day, micronucleus frequency of 1000µg/l bisphenol A treatment group was significantly different from that of 1µg/l bisphenol a treated and control group. However there was no significant difference in the frequency between 10µg/l, 100µg/l and 1000µg/l bisphenol A treated group.

Conclusion:

The results suggest that Bisphenol A is toxic to liver at all the concentrations in short term (14 days) and long term (30 days) exposure whereas micronucleus can be induced in RBC's on exposure to higher (100µg/l, 1000µg/l) concentrations of Bisphenol A. At lower levels (10µg/l), micronucleus could be induced only in longer duration study (30 days).

Fig. 1 Liver of (a) control fish with clear sinusoidal space (b) fish treated with (1000µg/l) of Bisphenol A for 15 days (c) fish treated with (1000µg/l) Bisphenol A for 30 days.

Fig. 2 (a) Photomicrograph showing erythrocyte from the murrel *Channa punctatus* (1000X), (b) Photomicrograph showing micronucleated erythrocyte from *Channa punctatus* treated with Bisphenol A (1000 X). Arrow indicates micronucleus.



References

[1] IMAM A. MEKKAWY, USAMA M. MAHMOUD , ALAA EL-DIN H. SAYED. 2011. Effects of 4-nonylphenol on blood cells of the African catfish *Clarias gariepinus* (Burchell, 1822).

[2] J.C. VAN DYK, G.M. PIETERSE, J.H.J. VAN VUREN. 2007. Histological changes in the liver of *Oreochromis mossambicus* (Cichlidae) after exposure to cadmium and zinc.

[3] NEVEEN EL-SAID REDA EL-BAKARY, HEKMAT LOTFI EL-GAMMAL. 2010. Comparative histological, histochemical and ultrastructural studies on the liver of flathead grey mullet (*Mugil cephalus*) and Sea bream (*Sparus aurata*).

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

ENDOSULFAN TARGETS GONADAL DIFFERENTIATION IN THE ASIAN CATFISH, *CLARIAS BATRACHUS*

Rajakumar, A., Chakrabarty, S., Singh, R., Senthilkumaran, B.

Department of Animal Sciences, School of Life Sciences-Centre for Advanced Studies, University of Hyderabad, Hyderabad-500 046, Andhra Pradesh, India.

Introduction:

Hypothalamo-hypophyseal-axis is highly conserved among vertebrates which regulates early gonadal development and reproductive cycle in most of the annually spawning teleosts. With growing concern over the use of endosulfan in India and other world nations, in the present study we probed the effect of ensosulfan as an endocrine disruptor, which is known to target gonadal differentiation by acting at the level of gonad and/or brain. To ascertain this, we analyzed the effects of endosulfan and flutamide (as a reference) on catfish gonadotropin-releasing hormone (cfGnRH)-tryptophan hydroxylase (Tph)-gonadal axis. Earlier studies often used adult fish and the impact of these compounds during gonadal differentiation/development was never analyzed in depth at molecular level.

Methods:

Treatment was done by adding endosulfan and flutamide, alone and in combination to the aquarium tanks holding 50 days post hatch catfish juveniles for 50 days. Fishes were then sacrificed to dissect out the gonads and brain for total RNA preparation and histology. Prior to sacrifice, blood was collected, spun and it was used for estimation of estradiol-17β. The quantitative real-time PCR was carried out for the control and treated samples following total RNA isolation and first strand synthesis. All the gene expression patterns were analyzed by relative qRT-PCR

Fig.1A,B. Changes in the expression of various transcripts of gonads/brain after the treatment of endosulfan and flutamide, alone and in combination, in juvenile catfish. *denotes significant difference from control, $P < 0.05$.

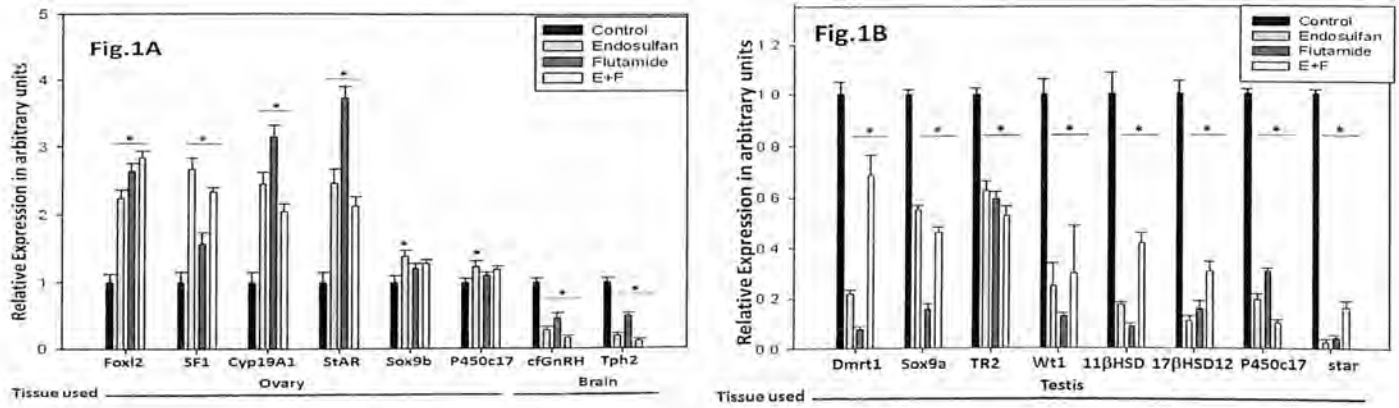
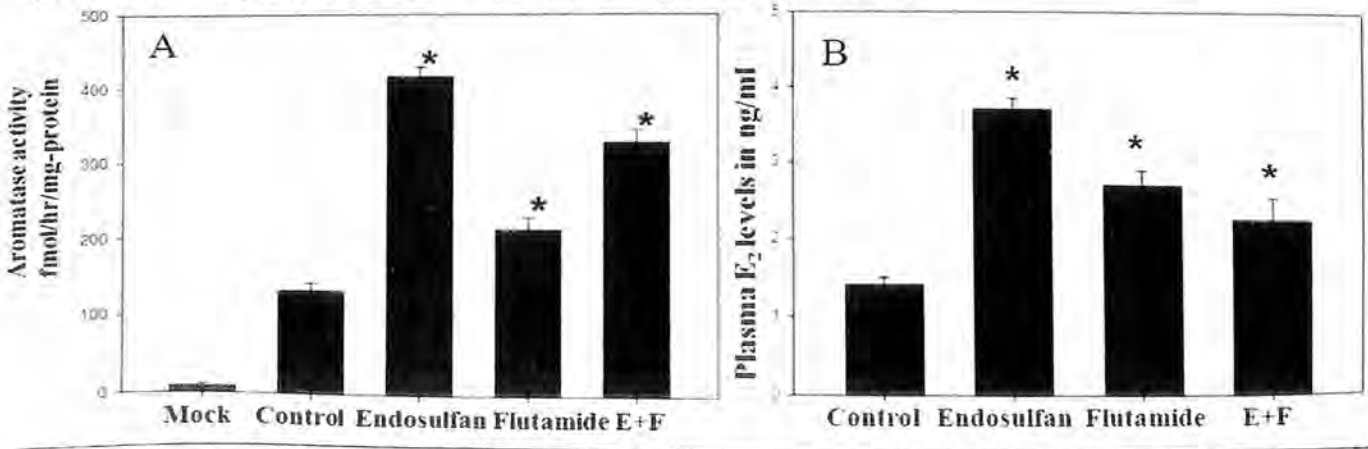


Fig. 2A,B. Aromatase activity and levels of estradiol-17β after the treatment of endosulfan and flutamide, alone and in combination, in juvenile catfish. *denotes significant difference from control, $P < 0.05$. Financial support from DBT (BT/PR11263/AAQ/03/419/2008) grant awarded to BS is gratefully acknowledged.



using SYBR Green detection method except for Tph2 where Taqman probes were used. The real-time PCR specific primers for all the target genes and internal control β–actin were designed such that, at least one of the primers spanned the intron-exon boundary to exclude the amplification from genomic DNA. Radiometric assay was done to estimate aromatase activity in ovary. Immunocytochemistry for tryptophan hydroxylase-2 (Tph) localization was done in catfish brain while hematoxylin and eosin staining was done for gonadal ections.

Results and Discussion:

The results were depicted in figures 1 and 2. Treatment with 2.5ppb endosulfan and 33ppb flutamide, alone and in combination for 50 days enhanced the expression of transcription factors such as *sox9b*, *foxl2* and *Ad4BP/SF-1* in the ovary while down regulated *sox9a*, *dmrt1*,*wt1* and orphan nuclear receptor, *NR2C2* (TR2) in the testis. In the case of females, the expression of *CYP19A1* and *StAR* were increased while *P450c17* expression elevated only in the endosulfan group. Conversely, expression of *P450c17*, *StAR*, *11β-HSD* and *17β-HSD12* was decreased in all the treated males. The expression of Tph and cfGnRH in the brain declined in all the treated females and the impact being maximal in endosulfan-treated fish. Significant reduction of Tph immunoreactivity in the telencephalon-preoptic-hypothalamus region of female brain substantiated our Tph transcript quantification results. The treatment had impact on these correlates in males as well. Histological analysis confirmed modulation of oocyte growth in the treated females and crumpling of lumen in treated males compared to control fishes. Increase in plasma E_2 levels and ovarian aromatase activity in the endosulfan treated females was found to be higher than other groups. These results together demonstrate that the exposure of endosulfan and flutamide modulate the cfGnRH-Tph-gonadal axis, either directly or indirectly.

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

EFFECT OF CHLORDECONE (KEPONE) ON THE BRAIN AROMATASE GENE – A NOVEL *IN VIVO* AND *IN VITRO* APPROACH

Santosh Winkins[1,2], Farzad Pakdel[1], Benjamin Piccini[3], Nathalie Hinfray[3], François Brion[3], Olivier Kah[1], Yann Le Page[1]

[1]UMR CNRS 6026, Université de Rennes 1, Rennes, France
[2] Department of Biotechnology, School of Bioengineering, SRM University, Kattankulathur, Tamilnadu, INDIA
[3]Unité d'Ecotoxicologie, INERIS, Verneuil en Halatte, France
Email : santoshmcc@ktr.srmuniv.ac.in, santoshmcc@yahoo.com

Introduction:

Chlordecone (Kepone) is a polychlorinated hydrocarbon pesticide that was highly used in banana plantations before being banned. However, it is known for being very persistent and is still present in many environmental samples (mainly soils and sediments). Using different *in vivo* and *in vitro* techniques, several studies reported that chlordecone (CD) has a weak agonistic effect on human ERa and a low anti-estrogenic activity on ERb. CD is also known for having neurotoxic effects whose underlying mechanisms are unknown and high risk of prostrate cancer [1]. In this work, we aimed at investigating the estrogenic effect of CD on a well established estrogen-sensitive gene, *cyp19a1b* (aromatase B) whose expression is restricted to radial glia progenitors in fish [2].

Methods:

Three methods were carried out to study the effect of CD. The *in vitro* assay based on human astrocytes transfected with zebrafish ERs (ERa, ERb1, ERb2) and a *cyp19a1b*-luciferase reporter gene [3]. The second method was, zebrafish embryos and larvae exposed for 6 days to similar doses and *cyp19a1b* mRNA expression was studied. Finally, exposure for 5 days of tg(*cyp19a1b*-GFP) transgenic to 10^{-9} to 10^{-6}M CD were studied for GFP expression.

Results:

The results showed that none of the zebrafish ER is activated by CD at 10^{-9}M to 10^{-7}M doses. At the opposite, a mixture of E2 10^{-8}M and CD 10^{-7}M highlight a weak anti-estrogenic activity of CD on both ERs. Similarly, zebrafish embryos and larvae exposed for 6 days to similar doses of CD did not show changes in *cyp19a1b* mRNA expression with CD alone, but a weak anti-estrogenic effect in presence of a mixture of CD and E2 has been detected. Finally, exposure for 5 days of tg(*cyp19a1b*-GFP) transgenic to 10^{-9} to 10^{-6}M CD failed to produce any increase in GFP expression.

Conclusion:

Based on these data, we can conclude that CD within the range of doses used in our study (1nM to 1mM) has a weak anti-estrogenic activity both *in vivo* and *in vitro* on the *cyp19a1b* gene. It must be pointed out that the doses used in the present study are lower than those inducing agonistic Supported by the ANR NEED and the NEMO programs.

References:

[1]Multigner L, Ndong JR, Giusti A, Romana M, Delacroix-Maillard H, Cordier S, Jégou B, Thome JP, Blanchet P. J Clin Oncol. 2010 Jun 21.

[2]Menuet *et al.*, J Comp Neurol. 2005.

[3]Le Page *et al.* Environ Health Perspect. 2006 May;114(5):752-8.effect on Era.

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

EFFECT OF CYPERMETHRIN ON CONJUGATION OF SEX STEROID HORMONES DURING TWO DIFFERENT REPRODUCTIVE PHASES (REPRODUCTIVELY ACTIVE PRESPAWNING AND REPRODUCTIVELY INACTIVE POST-SPAWNING) OF THE ANNUAL REPRODUCTIVE CYCLE IN *HETEROPNEUSTES FOSSILIS* (BLOCH)

Singh[1], V., Srivastava[1], S. S. and Singh[2], P. B.

[1]Department of Zoology, Tilak Dhari Postgraduate College, Jaunpur-222 002 UP
[2]Ganpat Sahai Postgraduate College, Sultanpur-228 001 UP, India
Fax:+91-5362 240170 email: pratap_b_singh@rediffmail.com

Introduction: We have reported that Cypermethrin decreased the plasma levels of unconjugated sex steroids in fish. No information is available on the effect of Cypermethrin insecticide on conjugation of sex steroids in freshwater Indian food fishes at different sexual maturity phases, being the fact that glucuronides and sulfates have very important role in pheromonal behavior and spawning.

Methods:

After acclimation, male sexed fish were divided into 2 batches having 5 fish in each glass aquaria having 20 litres water during prespawning and post-spawning phases. Total 20 fish were used in this study. The sublethal dose (0.02 ppm) was used for 40 days exposure studies as have been reported for this insecticide in *H. fossilis*.

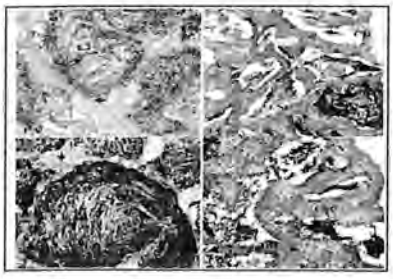
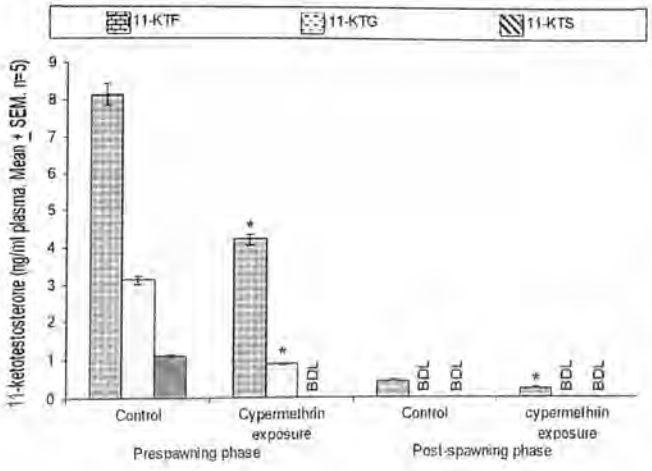


Fig. 1. T. S. of testis showing structural differences of control and 40 day cypermethrin exposed (0.02 ppm) *H. fossilis*.

A. T. S. of control testis showing lumen of testes (LT) filled with mature sperms during prespermiating stage HE x 40
B. Lobules (L) of testes filled with mature sperms (S) and interstitial cells (IC). HE x 400
C. T. S. of testis after 40 days exposure showing cytotoxic damage (CD), condensation of spermatogenic cells (CSC), vacuolization (V) in the tubular epithelium. HE x 40
D. Showing CD, CSC and V and disruption of IC (DIC) in magnification. HE x 400

Fig. 3. Effect of cypermethrin exposure (0.02 ppm) for 40 days on plasma levels of 11KTF, 11-KTG and 11-KTS sex steroid hormones during prespawning and post-spawning phase in *H. fossilis*. Results of cypermethrin exposure on sex hormones during reproductive phases were compared by Student's *t*-test. The levels of siginificance (P) was *P < 0.001; BDL- below detection limit. ANOVA TW : hormones F 1077.17 P < 0.001, phases F 557.04 P < 0.001 hormones x phases F 243.86 P < 0.001



Plasma sex steroids (testosterone-T, 11-ketotestosterone-11KT, 17-hydroxyprogesterone-17P, and 11-deoxycortisol-S) and their conjugates were analysis by ELISA kit (Thermo Electron Corporation, Finland fitted with Ascent Software version 2.6, Multiscan EX). Extraction of unconjugated and conjugated sex steroid hormones was done as per methods described in [1].

Results and Discussion:

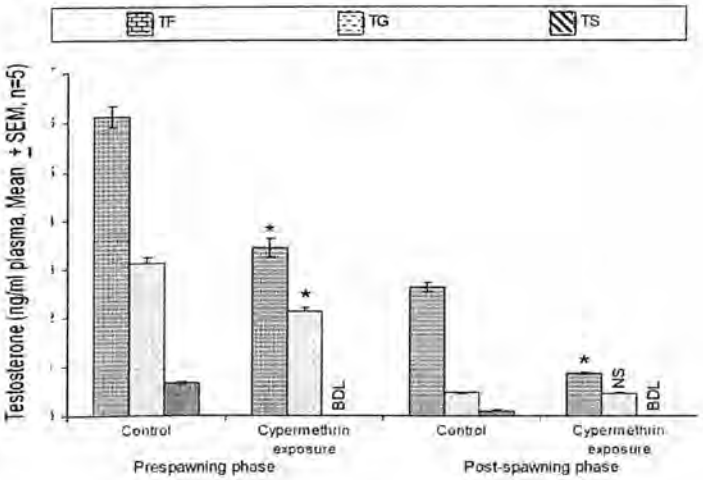
We for the first time reported that the effect of pyrethroid insecticide cypermethrin on conjugation of reproductive sex steroids in male catfish, *H. fossilis* and found that after exposure conjugates have been decreased during prespawning phase of steroidogenesis. The conjugates (glucuronides and sulfates) play very important role in pheromonal behavior and spawning in fish. Our results indicate that when catfish, *H. fossilis* were exposed with cypermethrin there was a decrease in 17P and S production. These results also suggest that the pesticide acts predominantly on the early stage of steroidogenesis, the finding supported by other workers [1,2]. The decrease in unconjugated (free) and conjugated (glucuronides and sulfates) sex steroids after 40 days exposure with cypermethrin indicates that the equilibrium of steroids was perturbed by inhibiting synthesis and release by the testes. The decrease in 11-

KT and T indicates that the synthesis of T is decreased and its conversion to 11-KT is also inhibited which may be due to loss of aromatase activity [1,2].

Conclusion:

The result suggests that cypermethrin causes inhibition of conversion of free to conjugate sex steroids, causing perturbances in the equilibrium/ balance of sex steroid hormones and affecting reproductive physiology.

Fig. 2. Effect of cypermethrin exposure (0.02 ppm) for 40 days on plasma levels of TF, TG and TS during prespawning and post-spawning phase in *H. fossilis*. Results of cypermethrin exposure on sex hormones during reproductive phases were compared by Student's *t*-test. The levels of siginificance (P) was *P < 0.001; BDL- below detection limit. NS- not significant. ANOVA TW : hormones F 553.64 P < 0.001, phases F 1589.86 P < 0.001 hormones x phases F 270.84 P < 0.001



Acknowledgement:

A grant-in-aid (UGC- F. No. 32-485/2006 (SR) dated 28-2-2007 from UGC to PBS

References:

[1]MOORE, A. AND WARING, C.P. 2001. The effects of a synthetic pyrethroid pesticide on some aspects of reproduction in Atlantic salmon (*Salmo salar* L.). Aquat. Toxicol., 52: 1-12.

[2]SINGH, P. B. AND KIME, D.E. 1995. Impact of γ-hexachlorocyclohexane on the *in vitro* production of steroids from the endogenous and exogenous precursors in the spermiating roach, *Rutilus rutilus*. Aquat. Toxicol,. 31: 231-240.

LABORATORY STUDIES ON THE EFFECT OF A PYRETHROID INSECTICIDE ON HISTOPATHOLOGICAL CHANGES IN TESTES OF THE CATFISH, *HETEROPNEUSTES FOSSILIS* (BLOCH) DURING BREEDING SEASON

Vengayil[1] D. T. and Singh[2], P.B.

[1]Department of Science and Technology, Technology Bhavan, New Delhi-110016 [2]Ganpat Sahai Postgraduate College, Sultanpur-228 001
Fax: 05362 240170 email: pratap_b_singh@rediffmail.com

Introduction:

Pyrethroid compounds are considered safe as compared to organochlorine, organophosphate and carbamate compounds owing to its low persistence in nature. The wide use of synthetic pyrethroids is increasing worldwide pollution risks. The synthetic pyrethroids are among the most potent and effective insecticides available, account for more than 30% in the world market [1]. Cypermethrin is a pyrethroid insecticide widely used for pest control programmes in domestic, industrial and agricultural situations because of its low environmental persistence and toxicity. Reports are available which indicate the destruction of interstitial cells of testes in *Glossogobius giuris* after fenthion exposure. Recent review on pesticide-induced reproduction in Indian fish has been reported. It has also been reported that sperm motility is affected by toxicants, osmotic concentration and pH. Sperm biology has been widely studied in domesticated freshwater fish [2, 3]. The quality of sperm is a major contributing factor in the successful production of fish larvae, measurement of its motility could provide a sensitive and accurate bioindicator of pollution [3]. The information available on the effect of cypermethrin induced changes on testes and sperm motility after pesticide exposure are very few.

Methods:

H. fossilis were exposed at sublethal concentration for 45 days during breeding phase (pre-monsoon), of the annual reproductive cycle. After decapitation, testes were dissected, washed in saline (0.6% NaCl) blotted and fixed in Bouin's fluid for histological examination (2). The sperm motility was done just after decapitation (3). The gonadosomatic index (GSI) was calculated as gonad weight x 100/total weight.

Results and Discussion:

Extensive cytotoxic damage and gross condensation of spermatogenic cells by clump formation in testes has been noticed. Testes of *H. fossilis* show significant changes when exposed to cypermethrin. Extensive cytotoxic damage, general inflammatory response and other histological abnormalities are quite prominent. Gross condensation of spermatogenic cells, which are evident by clump formation and appearance of inflammatory lesions are also quite prominent. The tubular epithelial vacuolization increased in cypermethrin treated testes of *H. fossilis*. The interstitial cells were found to be degranulated, accompanied by weak chromophobia and vacuolization in the cytoplasm. The dilution of testicular milt of *H. fossilis* up to 600 times with extender does not initiate motility (forward progression) of the spermatozoa present in it. However, further dilutions, up to 2000 times result in maximum motility of sperm cells in dilution-dependent manner.

Fig.1. T. S. testes showing structural differences of control and 45 d exposed with cypermethrin in *H. fossilis*. T. S. of control testes showing lumen of testes (LT) filled with mature sperms during prespermiating stage HE x 40

E. Lobules (L) of testes filled with mature sperms (S) and interstitial cells (IC). HE x 400

F. T. S. testes after 45 d exposure showing cytotoxic damage (CD), condensation of spermatogenic cells (CSC), vacuolization (V) in the tubular epithelium. HE x 40

G. Showing CD, CSC and V and disruption of IC (DIC) in magnification. HE x 400

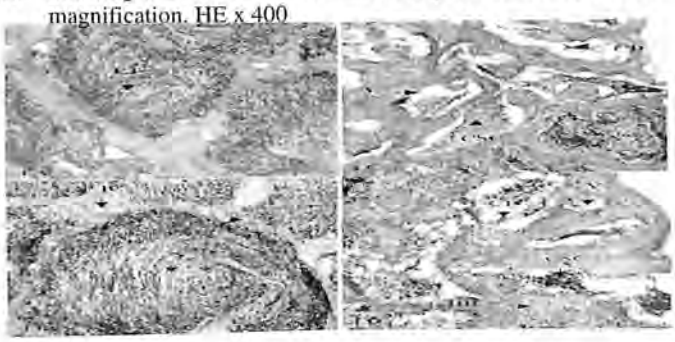
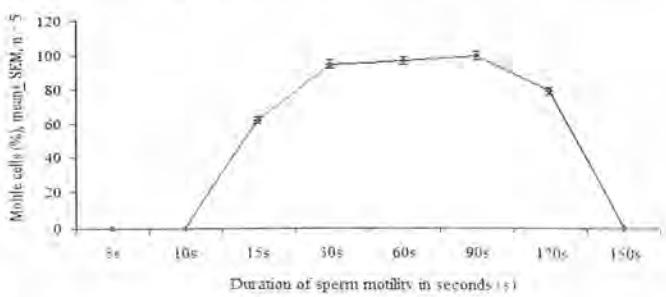


Fig. 2. Percentage of motile spermatozoa and duration of sperm motility after dilution (testis milt : extender, 1 : 2000) in *H. fossilis*.



The motility duration is only for two minutes. The maximum sperm motility has been observed at 90 seconds of duration (Fig 2,3).The decreased motilityof sperm from exposed fish indicated the decreased fertility ultimately decline the fish stock in polluted water or riverine resources.

Fig.3. Percentage of motile spermatozoa at different duration after activation in *H. fossilis* after cypermethrin exposure. Control versus exposed fish were compared by Students t-test. The level of significance (P)- *P < 0.001, **P < 0.01. ANOVA TW : Cypermethrin F = 1376.30, P < 0.001; Motility F = 1150.16, P < 0.001, Cypermethrin x Motility F = 217.83, P < 0.001.

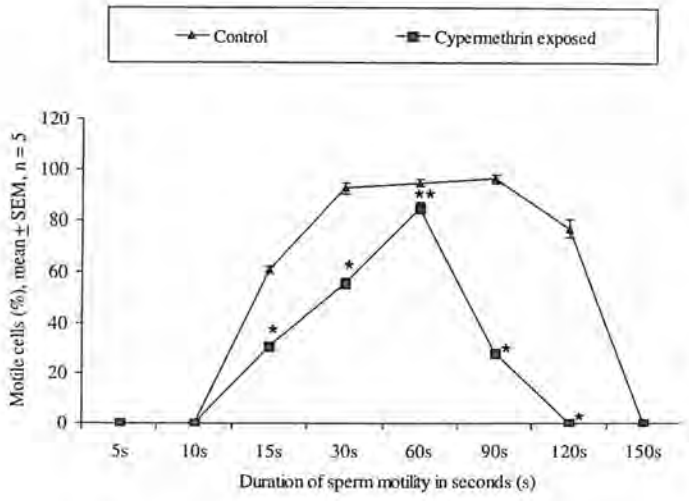


Table 1. Plasma levels of estradiol-17β (E2), and gonadosomatic index (GSI) after 45 days exposure at sublethal concentration (0.02ppm) of technical grade of cypermethrin (94%) during prespawning phase of the annual reproductive cycle in the cat fish, *H.fossilis* (Values are

Batches	Treatments	Female	
		GSI	E2 (ng/ml)
1	Control	6.94 ± 0.42	12.67 ± 0.47
2	Cypermethrin	3.73 ± 0.23*	5.98 ± 0.19*

Control vs cypermethrin treated were compared by students *t*- test. The level of significance (P) - * $P <$ 0.001

Conclusion:

It may be safely concluded that cypermethrin causes the disruption of endocrine system by affecting steroidogenesis via hypothalamo – hypophyseal - gonadal axis. Cypermethrin induced decrease of sperm motility may be owing to inhibition of ATP synthesis in mitochondria. This study will help to monitor the quality of sperm (good or bad) on the basis of scale and duration of motility needed during the production of fish seed or in the field monitoring system as indicator of pollution.

References:

[1] MOORE, A., WARING, C.P., 2001. The effects of a synthetic pyrethroid pesticide on some aspects of reproduction in Atlantic salmon (*Salmo salar* L.). Aquat. Toxicol., 52: 1-12.

[2]SINGH, P.B., KIME, D.E., SINGH, T.P., 1993. Modulatory actions of *Mystus* gonadotropin on γ-BHC-induced histological changes, cholesterol, and sex steroid levels in *Heteropneustes fossilis*. Ecotoxicol. Environ. Saf., 25: 141-153.

[3]SINGH, P.B., SAHU, V., SINGH V., NIGAM, S.K., AND H.K. SINGH., 2008. Sperm motility in the fishes of pesticide exposed and from polluted rivers of Gomti and Ganga of India. Food Chem. Toxicol., 46 (12): 3764-3769.

HISTOMORPHOLOGICAL EVALUATION OF *Pimephales promelas* MALE GONADS AFTER EXPOSURE TO PULP MILL AND DOMESTIC DISCHARGES INTO THE URUGUAY RIVER (FRAY BENTOS-URUGUAY)

Carnikian A.[1,2], **Miguez D.**[2] and **Vizziano-Cantonnet D.**[1]

[1] Laboratorio de Fisiología de la Reproducción y Ecología en peces, Facultad de Ciencias,
[2] Departamento de Aguas y Productos Químicos, Laboratorio Tecnológico del Uruguay.
Laboratorio de Fisiología de la Reproducción y Ecología en peces, Facultad de Ciencias Iguá 4225, Montevideo 11400, Uruguay. Tel +598 2525 8618 7150 email: vizziano@gmail.com

Introduction:

Fish are subject to a broad variety of stressors because their homeostatic mechanisms are highly dependent on prevailing conditions in their immediately surroundings [1]. Moreover, they are suitable models for evaluating endocrine-active compounds in the water column. Fathead minnow (*Pimephales promelas*) Pisces: Cypriniforms has been used as a sentinel organism for ecotoxicity since the sixties for USEPA, Environment Canada and OECD. An increased number of widely used urban, industrial and agricultural chemicals are being found to cause endocrine disruption. In fishes, xenoestrogens can induce female proteins in males, and in some cases, the development of testis-ova [2]. Histomorphological alterations of the testis in Fathead minnow have been previously described for 17β-estradiol [3, 4], 4-nonylphenol and nonylphenol ethoxylate [5]. We studied here the potential estrogenic effect of pulp mill and domestic wastewater discharges located on the Uruguay River on the male gonads of the Fathead minnow, and we compared it to the effect of treatment with 17ß-estradiol (E2).

Methods:

Samples- surface water receiving municipal wastewater was obtained from Fray Bentos stream in March 2010. A sample of final pulp mill effluent was collected by sampling technicians from Laboratorio Tecnológico del Uruguay.

Test Organisms: Fathead Minnow *Pimephales promelas* were cultured at Water & Chemicals department in the Laboratorio Tecnológico del Uruguay according to EPA protocols [6].

Experimental design- Three control and three treated groups, containing two males and four females (7 months old) were exposed in 10L aquaria (n=36) with spawning substrates for 21 days on a semi-static flow with renewal of the testing solution every 48h. Tested solutions were: surface water receiving municipal wastewater, a pulp mill effluent and 150 ng L^{-1} of the estrogen 17β-estradiol (E2). In order to confirm the sensitivity of the experiments the expression of vitellogenin was evaluated in liver of both males and females in control and treated groups. Only E2 induced a significant increase in testis and ovaries vitellogenin expression (Keel, Parodi & Miguez).

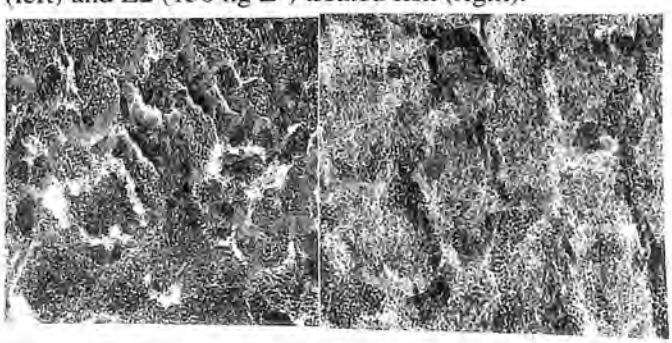
Fish Sampling- On the 21 day fish were weighed and measured and then sacrificed with a cervical incision. Testis were dissected, weighed and fixed in Bouin´s fixative for 24 h and treated by classic histological procedures using Hematoxylin and Eosin (H&E).

Testis Histology- The sections were examined for abnormal findings such as increase in spermatogonias, and signs of testis degeneration as cell apoptosis or vacuolization.

Results and Discussion:

Males were mature and testes contain complete spermatogenic cell stages such as spermatogonia, spermatocytes, spermatids and spermatozoa. For the different experiments, control fish were not all at the same maturation stage. However, none of the treatments delayed or advanced the spermatogenesis when compared with controls. No evidences of histopathological alterations were found for the domestic and pulp mill effluent neither for the E2 concentration applied (Fig. 1). Previous studies on the histopathological effects in gonads of *P. promelas* males exposed to high concentrations (2780 ng L^{-1}) of E2 had shown moderate alterations such as Sertoli cells hyperplasia and hypertrophied with loss of germinal cells, presence of degenerated spermatozoa and occasionally germ cell syncytia [3], vacuolated cells and apoptotic body cells [4]. Vacuolated cells and apoptotic body cells could be attributed to E2 [4], but this alterations were not found in the present study.

Fig.1. Histological sections of testes in the control (left) and E2 (150 ng L^{-1}) treated fish (right).



Conclusion:

We conclude that neither pulp mill nor surface water receiving domestic wastewater altered the testis morphology of Fathead Minnow in the experimental conditions used.

References:

[1]HARPER, C., WOLF J.C. 2009. Morphologic effects of the stress response in fish. ILAR J., 50: (4) 387-396.

[2]GENOVESE, G., DA CUÑA, R., TOWLE, D., MAGGESE, M., LO NOSTRO, F. 2011. Early expression of zona pellucida proteins under octylphenol exposure in *Cichlasoma dimerus* (Perciformes, Cichlidae). Aquat. Toxicol., 101: 175–185.

[3]MILES RICHARDSON, S.R., KRAMER, V., FITZGERALD, S., RENDER, J., YAMINI, B., BARBEE, S., GIESY, J. 1999. Effects of waterborne exposure of 17ß-estradiol on secondary sex characteristics and gonads of fathead minnow (*Pimephales promelas*). Aquat. Toxicol., 47: 129-145.

[4]WOLF, J., DIETRICH, R., FRIEDERICH, U., CAUNTER, J., BROWN, A. 2004. Qualitative and quantitative histomorphologic assessment of fathead minnow, *Pimephales promelas* gonads as an endpoint for evaluating endocrine-active compounds: a pilot methodology study. Toxicol. Pathol., 32: 600-612.

[5]MILES-RICHARDSON, S., PIERENS, S., NICHOLS, K., KRAMER, V., SNYDER, E., SNYDER, S., RENDER, J., FITZGERALD, S., GIESY, J. 1999. Effects of waterborne exposure to 4-nonylphenol and nonylphenol ethoxylate on secondary sex characteristics and gonads of Fathead Minnows (*Pimephales promelas*) Environ. Res., 80: (2) 122-S137.

[6]USEPA. 1988. Guidelines for the culture of Fathead minnows, *Pimephales promelas* for use in toxicity tests. EPA/600/S3-87/001. pp 4.

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

NUTRITIONAL STRESS DURING EARLY SECONDARY OOCYTE GROWTH INDUCES FOLLICULAR ATRESIA AND CHANGES IN OVARIAN GENE EXPRESSION IN COHO SALMON

Yamamoto Y.[(1)(2)(4)], **Luckenbach J.A.**[(2)], **Goetz F.W.**[(3)], **Young G.**[(1)], **Swanson P.**[(2)]

[(1)] School of Aquatic and Fishery Sciences, University of Washington, Seattle, WA 98195, USA
Fax: +1-206-860-3467 email: yojifish@u.washington.edu
[(2)] Northwest Fisheries Science Center, NOAA Fisheries, Seattle, WA 98112, USA
[(3)] School of Freshwater Sciences, University of Wisconsin Milwaukee, Milwaukee, WI 53204, USA
[(4)] Department of Marine Biosciences, Tokyo University of Marine Science and Technology, Tokyo 108-8477, Japan

Background:

Environmental stressors (e.g., reduced food availability, endocrine disrupting chemicals) are known to disrupt endocrine function in fishes, potentially delaying the age of puberty and affecting gamete quality. Recent experiments in our lab have revealed that prolonged fasting of previtellogenic coho salmon (*Oncorhynchus kisutch*) impairs endocrine function and increases follicular atresia [1]. However, little is known about the molecular processes in the ovary that either regulate atresia or are consequences of atresia. We hypothesized that a nutritional stress that perturbs normal ovarian growth and induces follicular atresia would alter the pattern of gene expression in the gonads. Indeed, in a recent fasting experiment we found that expression of some steroidogenesis-related genes (e.g., *steroidogenic acute regulatory protein*) decreased in fasted salmon, while expression of apoptosis-related genes (e.g., *caspase 3, caspase 8 and fas-associated death domain*) were elevated in fasted fish relative to fed fish [1]. The primary aim of this study was to identify additional gene transcripts that are up- or down-regulated in the previtellogenic salmon ovary when ovarian growth is impaired by prolonged fasting. A second goal of the study was to identify candidate genes that might serve as molecular markers of the initiation of atresia.

Methods:

Two year-old coho salmon with ovarian follicles at the late cortical alveolus stage were either fasted or normally fed over a 17-week period starting in March, approximately 10 months before spawning. Fish were subsampled at the initiation of the experiment, and at 3, 9, 14, and 17 weeks after fasting was initiated. The effect of prolonged fasting on previtellogenic follicle growth was monitored by assessing changes in the cellular morphology of ovarian follicles and changes in plasma levels of follicle-stimulating hormone (FSH), estradiol-17beta (E2) and insulin-like growth factor 1 (IGF1), and pituitary FSH content. Reciprocal suppression subtractive hybridization (SSH) cDNA libraries were generated using ovaries from fed and fasted animals collected at week 14. This time point was selected for SSH because it was the earliest time point where fasted salmon showed significantly lower plasma E2 levels and pituitary FSH content, and a higher proportion of follicles either delayed in development or initiating atresia relative to the main cohort of follicles in the ovary. Yet, the stages of the main cohort of follicles were the same in fed and fasted fish at week-14. Therefore, any differences in gene expression at this time point would reflect the effects of disruption of the FSH-ovary axis and atresia onset rather than major differences in development of the main cohort. A total of 480 clones were sequenced per library and annotated by aligning them to the NCBI nr and nt databases. The differential expression of genes identified by SSH was confirmed with quantitative PCR (qPCR) and genes that showed differential expression were analyzed in samples taken over the complete time course of the study (0-17 weeks).

Results and Discussion:

Prolonged fasting reduced body and ovary weight, and increased the proportion of follicles that were either atretic or delayed in stage relative to follicles in normally fed controls. Endocrine analyses showed that fasting reduced plasma IGF1, E2, and pituitary, but not plasma, levels of FSH. The SSH library representing genes up-regulated in the ovary of fasted fish contained genes associated with apoptosis (*programmed cell death protein 4*; *pdcd4*, *lipopolysaccharide-induced TNF factor* and *kruppel-like factor 6*), cortical alveoli components (e.g., *alveolin and serum lectin isoform 2*) and zona pellucida glycoproteins (e.g., *zona pellucida protein X*). The up-regulation of these suites of genes was likely associated with the initiation of atresia or reduced rate of follicle development. On the other hand, the library representing genes up-regulated in the fed ovary contained steroidogenesis-related genes (e.g., *3beta-hydroxysteroid dehydrogenase*; *hsd3b* and *P450 aromatase; cyp19a1a*), TGF-beta superfamily members (*anti-Mullerian hormone*; *amh* and *inhibin alpha; inha*) and cytoskeleton formation-related genes (e.g., *keratin 8*; *krt8*). Thus, genes up-regulated in fed fish were associated with steroid production, cell proliferation and differentiation, and ovarian epithelialization.

Interestingly, ovarian mRNA levels for *hsd3b*, *amh*, *krt8*, and *pdcd4* were significantly different between fed and fasted fish by week 9, before plasma steroid levels and histological differences in ovarian follicle stage were observed. Identification of additional genes from the SSH libraries that are differentially expressed in normally developing versus regressing previtellogenic follicles is ongoing.

Conclusion:

Nutritional stress impaired endocrine function, induced atresia and changed the pattern of genes expression associated with multiple molecular events in coho salmon previtellogenic gonads. This study identified ovarian genes involved in normal previtellogenic oocyte growth as well as those affected by nutritional stress and poor body growth. Genes that were differentially expressed before histological signs of atresia were apparent might be useful markers of early stages of atresia for monitoring impacts of a variety of environmental stressors on ovarian follicle health.

References:

[1]YAMAMOTO, Y., LUCKENBACH, J.A., GOETZ, F.W., YOUNG, G. and SWANSON, P. 2011. Disruption of the salmon reproductive endocrine axis through prolonged nutritional stress: changes in circulating hormone levels and ovarian genes involved in steroidogenesis and apoptosis. Gen Comp Endocrinol.,doi:10.1016/j.ygcen.2011.03.017.

Fish Biotechnology
(Reproductive Manipulations)

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

FISH GONADOTROPIN AGONISTS: APPLICATIONS IN ASSISTED REPRODUCTIVE TECHNOLOGIES

Rosenfeld, H.[1], Zlatnikov, V. [1], and Meiri-Ashkenazi, I.[1]

[1]Israel Oceanographic and Limnological Research (IOLR), National Center for Mariculture (NCM), P.O. Box 1212, Eilat 88112, Israel. Fax: +972-8-6375761 e-mail: hannarosenfeld@gmail.com

Introduction:

Over the years, hormonal approaches employing GnRH, freshly ground pituitaries of reproductively mature fish, and human chorionic gonadotropins, have been used to overcome problems halting final stages of gamete maturation and spawning [1]. Nevertheless, the available therapeutic agents fall short of dealing with induction of early stages of gonadal growth (i.e., vitellogenesis and spermatogenesis). As a result, many commercially important fish, exhibiting a complete failure to undergo gametogenesis, are not at hand for aquaculture uses, and their production is based merely on fishery of wild stocks.

The pituitary gonadotropins (GtHs), FSH and LH are the key regulators of ovarian and testicular function. Therefore , much research efforts have focused so far on developing heterologous eukaryotic systems (i.e., insect cells, mammalian cell lines, and yeast) for the production of fish recombinant GtHs [2]. These have circumvented the need to purify the native hormones from thousands of fish, and provided an important tool for studying the differential functions of LH and FSH. Recently, using yeast (*Pichia pastoris*) fermentation, our group at IOLR-NCM scaled-up the production of biologically active fish recombinant GtHs for therapeutic purposes, providing fine-tuning agents for the regulation of fish sexual maturity and reproduction.

Experimentation with fish LH agonists:

LH, also known as the maturation hormone, is the main GtH responsible for stimulating events leading to final oocyte maturation and ovulation in females and spermiation in males. In an effort to establish fish LH agonist for *in vitro* and *in vivo* experimentation, a recombinant LH chimera, based on a translational fusion of the sea bream (*Sparus aurata*) LH beta and alpha subunits [rsbLH], was produced in the *P. pastoris* expression system. The yeast culture has produced over 100 mg rsbLH per liter of culture supernatant, a quantity that is about 1 to 2 orders of magnitude higher when compared to yields reported for cognate systems [2]. The produced rsbLH stimulated steroidogenesis in gonadal fragments derived from sea bream as well as in those derived from other Perciformes, including the European seabass (*Dicentrarchus labrax*), and the grey mullet (*Mugil cephalus*), pointing to its generic nature. Further *in vitro* studies demonstrated that physiological rsbLH levels (50-100 ng ml^{-1}) increased the frequency of sea bream oocytes undergoing germinal vesicle breakdown, and significantly attenuated the occurrence of atresia among the cultured oocytes. As it has been suggested for the mammalian model, the atretic degeneration could be suppressed directly by LH, or indirectly by paracrine factors, from the somatic cells that mediate the LH signals [3]. *In vivo* trials have demonstrated the hormone's ability to induce spawning in fully mature captive grey mullet, replacing our conventional treatment of GnRH analogue combined with dopamine antagonist [4].

Experimentation with fish FSH agonists:

In most vertebrates FSH has a dominant role in the initiation of gametogenesis and regulation of gonadal growth (i.e., spermatogenesis in males and follicle growth in females). Thus far, therapeutic preparations of FSH are available only for the treatment of human infertility. Using the aforementioned yeast expression system, we produced fish recombinant FSH chimera, based on a translational fusion of the Atlantic bluefin tuna (BFT, *Thunnus thynnus*) FSH beta and alpha subunits [rbftFSH]. Despite the relatively low evolutionary conservation of the FSH molecule among the Perciform species [5], the rbftFSH succeeded to stimulate steroidogenesis in gonadal fragments of heterologous fish species such as sea bream, European seabass, and grey mullet. Exposure of testicular fragments of sexually immature BFT to graded doses of rbftFSH resulted in a dose-dependent increase in the diameter of the seminiferous lobules, and in the proliferation of germ/somatic cells, as compared to untreated controls. These results further confirm previous notion that FSH is an important regulator of spermatogonial proliferation [6]. In addition, our *in vivo* experiments indicate that following injection, elevated rbftFSH levels are sustained over 9 hours in the circulation of the grey mullet. Furthermore, two consecutive rbftFSH injections (150 ng rbftFSH per kg in the grey mullet, spaced two weeks apart) at the onset of the reproductive season (i.e. mid July), significantly improve milt production, in terms of volume and fluidity. This protocol is recently utilized in commercial hatcheries in Israel for a scale-up production of mullet fingerling.

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

Conclusions:

Our work exemplifies the potential of fish GtH agonists, in therapeutic application to alleviate reproductive dysfunctions and expedite the onset of puberty in captive fish. Future studies should focus on optimizing the delivery system for sustained release of GtHs, circumventing the need to use repeated injections, which are labor intensive and stressful to the fish. Thus, industrial production of clinical grade GtHs and development of efficient delivery system(s) for their administration are expected to be the two most relevant improvements to be developed for an intensive aquaculture industry that needs to fill the increasing supply/demand gap for marine fishes.

References:

[1]ZOHAR, Y., MYLONAS, C.C. 2001 Endocrine manipulations of spawning in cultured fish: from hormones to genes. Aquaculture, 197: 99–136

[2]LEVAVI-SIVAN, B., GOLAN, M., AIZEN, J., ELIZUR, A. 2008. Fish recombinant gonadotropins. CYBIUM, Int. J. Ichthyol., 32: 17–21.

[3]ROLAKI, A., DRAKAKIS, P., MILLINGOS, S., LOUTRADIS, D., MAKRIGIANNAKIS, A. 2005. Novel trends in follicular development, atresia and corpus luteum regression: a role for apoptosis. Reprod. BioMed., Online 11: 93-103

[4]AIZEN, J., MEIRI, I., TZCHORI, I., LEVAVI-SIVAN, B., ROSENFELD, H. 2005. Enhancing spawning in the grey mullet (*Mugil cephalus*) by removal of dopaminergic inhibition. Gen. Comp. Endocrinol., 142: 212-221.

[5]YARON, Z., GUR, G., MELAMED, P., ROSENFELD, H., ELIZUR, A., LEVAVI-SIVAN, B. 2003. Regulation of fish gonadotropins. Int. Rev. Cytol. 225: 131–185.

[6]SCHULZ, R.W., DE FRANÇA, L.R., LAREYRE, J.J., LE GAC, F., CHIARINI-GARCIA, H., NOBREGA, R.H., MIURA, T. 2010. Spermatogenesis in fish. Gen. Comp. Endocrinol., 165:390-411.

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

ADVANCEMENT OF THE ONSET OF PUBERTY IN *SERIOLA LALANDI* BY CHRONIC TREATMENT WITH KISS PEPTIDES

Elizur, A., Nocillado, JN, Biran, J, Sivan, B and Zohar, Y.

Faculty of Science, Health and Education, University of the Sunshine Coast, Sippy Downs, Queensland, Australia, 4556 Fax: +61-7-54302889 email: AElizur@usc.edu.au

Introduction:

There is now compelling evidence demonstrating the essential role of the kisspeptin system in vertebrate reproductive function [1]. In mammalian systems, the stimulatory effect of exogenous administration of kisspeptin and its antagonists underpin their essential role in regulating pubertal development. Central and peripheral kisspeptin treatment was effective in inducing precocious puberty in rodents [2,3] and primates [4]. There is growing evidence to the significance of the kisspeptin system in fish. First demonstrated by the molecular cloning of the *Kiss1r* ortholog in *Oreochromis niloticus* [5] Kiss and Kiss receptor genes have now been identified in several perciform, cypriniform, pleuronectiform and salmoniform species. It is becoming apparent that depending on the species, teleosts may have one or two *Kiss1r* genes and either *Kiss1, Kiss2* or both genes [6, 7, 8]. In the present study, we have isolated Kiss1, Kiss 2 and one Kiss receptor genes in *Seriola lalandi*, the yellowtail kingfish (YTK). We investigated the endocrine response of the brain-pituitary-gonadal axis to peripheral administration of slow release implants containing synthetic amidated Kiss1-10 and Kiss2-10 in prepubertal *Seriola lalandi*. The peptides were administered either individually or in combination, to examine for possible synergistic effects.

Methods:

Kiss and Kiss receptor cDNAs were isolated from YTK brains and gonads using PCR. Peptides of the core 10 amino acid Kiss1 and Kiss2 peptide were synthesised by Sigma. Slow release EVAc implants were prepared as described [9]. Prepubertal fish were implanted with EVAc containing Kiss1, Kiss2 or both at a dose of 100ug/kg. Controls were implanted with blank EVAc implants. Implantation was repeated once a fortnight for 4 weeks (during spawning season) or 8 weeks (non spawning season). At the end of the trial fish were weighed, bled and brain, pituitary, gonad and liver were collected for gene expression analysis of key reproductive genes. Gonads were fixed for histological analysis.

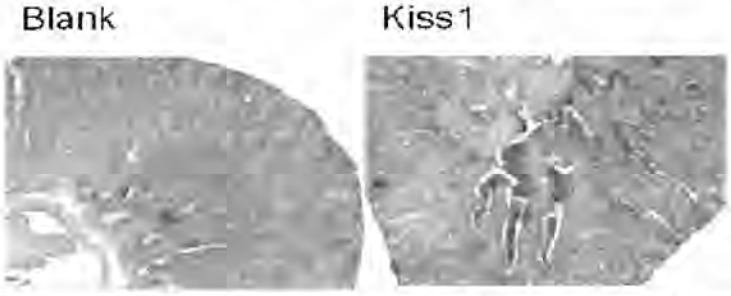
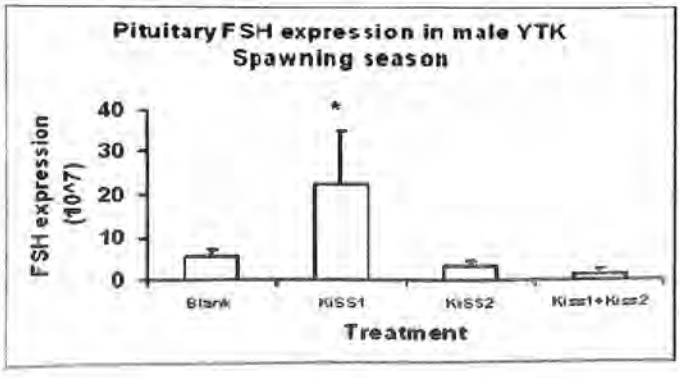


Figure 1



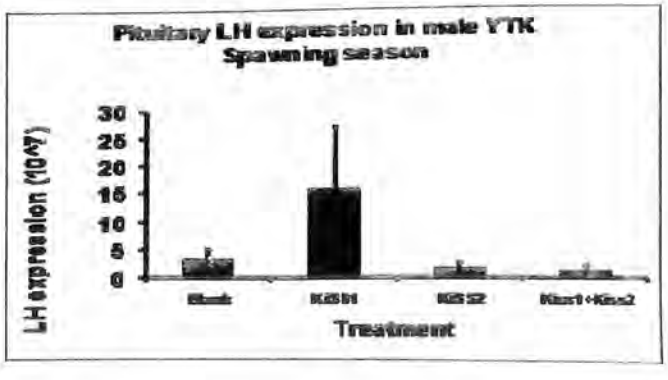
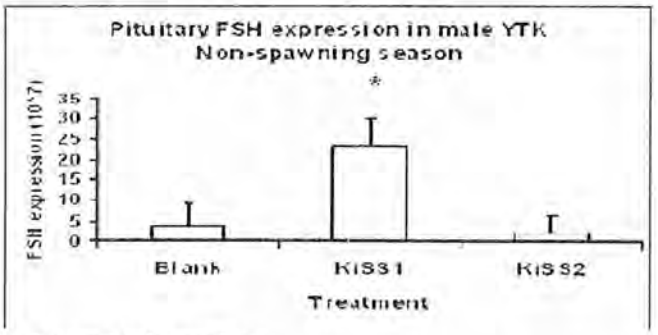
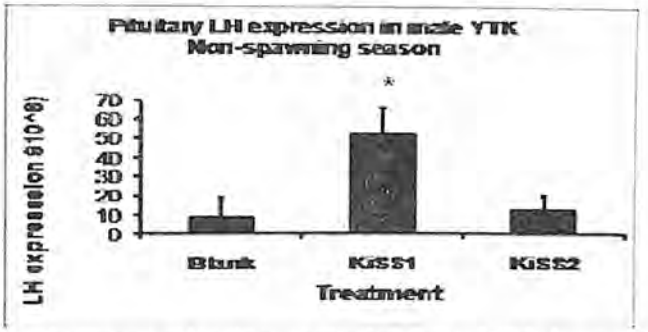


Figure 2





Results and Discussion:

Kiss administration, both during the spawning season and non-spawning season, triggered gonadal development and led to significant up regulation of pituitary gonadotropins. Fig. 1 shows changes in testicular development between Kiss 1 and blank treated males (during the spawning season) and the corresponding up regulation in pituitary FSH. Fig. 2 shows changes in testicular development between Kiss2 and blank treated males during the non spawning season and the corresponding up regulation in pituitary FSH and LH. The results have clearly indicated that Kiss peptides can stimulate gonadal development in prepubertal YTK males. The increase in pituitary gonadotropins gene expression suggests the response to Kiss administration is mediated through the GnRH system.

Acknowledgments:

This work was supported by the Australian Seafood CRC and the University of the Sunshine Coast.

References:

[1]KAUFFMAN AS. 2010. Coming of age in the Kisspeptin Era: Sex differences, development, and puberty. Mol. Cell. Endocrinol., 324: 51–63

[2]NAVARRO VM, et al. 2004. Advanced vaginal opening and precocious activation of the reproductive axis by KiSS-1 peptide, the endogenous ligand of GPR54. J. Physiol., 561.2: 379-386.

[3]NAVARRO VM, et al. 2004. Developmental and hormonally regulated messenger ribonucleic acid expression of KiSS-1 and its putative receptor, GPR54, in rat hypothalamus and potent luteinizing hormone-releasing activity of KiSS-1 peptide. Endocrinology,. 45: 4565–4574.

[4]SHAHAB M, MASTRONARDI C, SEMINARA SB, CROWLEY WF, OJEDA SR, PLANT TM. 2005. Increased hypothalamic GPR54 signaling: A potential mechanism for initiation of puberty in primates. PNAS, 2: 2129–2134.

[5]PARHAR IS, OGAWA S, SAKUMA Y. 2004. Laser-Captured Single Digoxigenin-Labeled Neurons of Gonadotropin-Releasing Hormone Types Reveal a Novel G Protein-Coupled Receptor (Gpr54) during Maturation in Cichlid Fish. Endocrinology, 145: 3613–3618.

[6] OAKLEY AE, CLIFTON DK, STEINER RA. 2009. Kisspeptin signaling in the brain. Endocr. Rev., 30:713–743.

[7]AKAZOME Y, KANDA S, OKUBO K, OKA Y. 2010. Functional and evolutionary insights into vertebrate kisspeptin systems from studies of fish brain. J. Fish Biol., 76: 161–182.

[8]UM HN, HAN JM, HWANG JI,HONG SI, VAUDRY H, SEONG JY. 2010. Molecular coevolution of kisspeptins and their receptors from fish to mammals. Ann. N.Y. Acad. Sci., 1200: 67–74.

[9]MYLONAS CC; et al. 2007. Preparation and administration of gonadotropin-releasing hormone agonist (GnRHa) implants for the artificial control of reproductive maturation in captive-reared Atlantic Bluefin Tuna (*Thunnus thynnus thynnus*). Rev. Fish. Sci., 15:183–210.

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

FRESHWATER AQUACULTURE IN INDIA: TRENDS AND PROSPECTS

J.K. Jena

National Bureau of Fish Genetic Resources, Canal Ring Road, Dilkusha P.O., Lucknoow-226002, India

Aquaculture has become a savior at a stage when production from capture fisheries in the country witnessed almost stagnation. The overwhelming ten-fold growth in just three decades i.e. from 0.37 million tonnes in 1980 to 4.0 million tonnes at present, sharing 50% of the total fish production of 8.0 million tonnes at present, amply justifies the recognition of aquaculture as an industry. While marine farming in the country is almost at its nadir and the land-based coastal aquaculture sector has been contributed by a single group, the shrimp, with production levels hovering just around 0.1 million tonnes, the freshwater aquaculture has been contributing over 95% of the aquaculture production in terms of quantity. The large-scale adoption of scientific carp polyculture together with entrepreneurial initiatives and financial investments has guided the sector to elevate it from a backyard activity vibrant a commercial enterprise.

Although the farming practice has witnessed overwhelming growth in terms of geographical coverage and intensification of farming practice, the three Indian major carps, viz., catla, rohu and mrigal have been continued to form major species group and contribute a lion's share. The introduction of exotic silver carp, grass carp and common carp into the Indian major carp based polyculture system even though added new dimensions to the system owing to their compatibility and high growth rates, they have remained as the second preferred group due to low consumer preference. With the average production under carp farming has been 5-6 tonnes/ha/year in progressive states like Andhra Pradesh, West Bengal, Punjab and Haryana, and several farmers even demonstrating impressive production levels of 8-12 tonnes/ha/year, the sector shows the potential to boost the average national pond productivity from the present level of 2.5 tonnes/ha/yr to a modest level of 4.0 tonnes/ha/yr by 2020, thereby achieving the expected contribution of 7.0 million tonnes from the sector by then.

R&D efforts on species diversification over the years although led to development of protocol for mass-scale breeding and seed production of several indigenous species, their commercial farming remained confined to giant freshwater prawn, *Macrobrachium rosenbergii* and to some extent the catfish, *Clarias batrachus*. Recent years, however, has witnessed drastic reduction in culture area of freshwater prawn with production reduced from about 40,000 tonnes in 2005 to about 10,000 tonnes at present. With availability of technology of seed production and grow-out farming of several medium and minor carps viz., *Labeo fimbriatus, L. gonius, L. calbasu, L. bata, Puntius sarana,* etc.; murrels *Channa striatus* and *C. marulius*; catfishes *Pangasius pangasius, Horabagras brachysoma* and *Ompok bimaculatus* and several other non-conventional candidate species, recent years has observed incorporation of several of these as components in polyculture system. Exotic catfishes, *Clarias gariepinus* and *Pangasianodon hypophthalmus* are some of the recent illegal entrants in freshwater aquaculture, although the latter has been legalized recently for its farming. In spite of ban imposed by Government of India, the farming of *C. gariepinus* has spread all across the due to its high growth rate. Higher growth and production potential of *P. hypophthalmus* have been responsible for large-scale adoption of the species, especially in Krishna-Godavari Delta of Andhra Pradesh. Although the production of the species reached from zero to about 0.6 million tonnes within a span of 4-5 years, unexpectedly it witnessed rather a sharp decline in one year, i.e. 0.2 million tonnes at present, which however was based on market demand.

Availability of required technological back up coupled with critical inputs like seed at the doorsteps has been the major factors for achieving consistent growth rate of 6-7% per year by the sector over last three decades. Further, availability of improved stocks, balanced feed in the form of floating and sinking pellets, formulations against common disease threats, and above all sustained increase in demand for fish as preferred animal protein have been able to take the sector forward. Current research programmes on different aspects of freshwater aquaculture, including domestication and standardization of breeding and culture techniques of non-conventional species, biofertilisation, genetic improvement of the cultivable species, feed development for larvae and grow-out stages, bioremediation, therapeutics and vaccines against dreaded pathogens and above all biotechnological interventions have to play a significant role in sustainability of freshwater aquaculture in years to come to overcome from the challenges of possible water scarcity for aquaculture, water quality deterioration, increase in cost of critical inputs and also competition from other sectors, and above all the climate change. Aquaculture, therefore, need to shift its focus from production to profits and further to production of safe produce on a sustainable basis.

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

GERM CELL TRANSPLANTATION IN MARINE FISH

Yoshizaki G.*, Yazawa R.*, Iwata G.*, Takeuchi Y.°, Morita T+., and Mitsuboshi T.+

*Department of Marine Biosciences, Tokyo University of Marine Science and Technology, Tokyo 108-8477, Japan. E-mail: goro@kaiyodai.ac.jp

°Research Center for Advanced Science and Technology, Tokyo University of Marine Science and Technology, Banda 670, Tateyama-shi, Chiba 294-0308, Japan

+Oita Marine Biological Technology Center, Nippon Suisan Kaisha, Ltd., 508-8 Ariakeura, Saiki, Oita 876-1204, Japan

Introduction:

Germ-cell transplantation has many applications in the fields of biology and animal husbandry, including the creation of broodstock systems, in which a target species can be generated from xenogeneic surrogate parents [1]. We recently developed a germ cell-transplantation system using rainbow trout spermatogonia (SG) [2]. In this system, donor-derived SG were microinjected into the body cavities of newly hatched embryos, which do not have mature immune systems. The intra-peritoneally transplanted SG migrated towards the recipient genital ridges and were subsequently incorporated into them. In male recipients, the transplanted SG underwent spermatogenesis and eventually produced fully functional sperm. In female recipients, the transplanted SG underwent oogenesis and eventually produced fully functional eggs. We further confirmed that the intra-peritoneal transplantation of SG could be successfully performed with recipients of different species [3]. To date, however, the technique has been applied only to freshwater species, which produce relatively large eggs. If it could be applied to marine fish, which produce small pelagic eggs, it could allow for commercially valuable fish species, for which it is difficult to maintain broodstock in captivity (such as bluefin tuna or giant grouper), to be bred using surrogate species that are easier to keep and mature faster (such as mackerel or black-tipped grouper). Therefore, in this study, we aimed to establish a germ cell transplantation system for marine fishes.

Methods:

As materials, we used the nibe croaker (*Nibea mitsulurii*) and yellow tail (*Seriola quinqueradiata*). Donor testes were minced and incubated with 0.5% trypsin in PBS for 2 h at 20°C to dissociate the tissue. The resulting testicular cells were labeled with the red fluorescent membrane dye PKH26 in order to trace them in the recipients. In the study with the nibe croaker, transgenic croaker heterozygously carrying a *Gfp* gene driven by the rainbow trout *Hsc71* promoter was used as a donor. For recipients, 12 days post-fertilization triploid croaker larvae (4 mm in total length) were used. Three weeks after transplantation, the recipients were dissected and the donor cell behavior was observed using PKH26 fluorescence as an indicator. In order to confirm germ-line transmission of donor-derived haplotypes, progeny tests were performed. Genomic DNA was extracted from the resulting offspring and used for PCR against the *Gfp* gene. For yellow tail, the experimental design was similar to the nibe croaker experiments with a few exceptions. First, wild-type non-transgenic yellow tail was used as both the donor and recipient. Second, since a triploidization method for this species has not been established, we used fertile diploid recipients. Germ cell transplantation was performed in 8 days post-fertilization larvae (5 mm in total length). Donor-derived offspring were identified by microsatellite markers.

Results and discussion:

In both the nibe croaker and yellow tail, donor testicular cells showing PKH26 fluorescence were incorporated into the genital ridges of the recipient fish at 20 days post-transplantation. Further, *in situ* hybridization with a *vasa* probe revealed that the PKH26-positive cells incorporated into the recipient genital ridges were germ cells. Although the triploid nibe croaker produced only transparent milt containing extremely small numbers of sperm with abnormal morphology, two out of six recipients receiving transgenic germ cells produced whitish milt containing large numbers of sperm. PCR analyses of the milt using *Gfp* gene primers revealed that the milt of the recipient croaker contained *Gfp*-gene-positive sperm. Furthermore, results of the progeny tests using the milt showed that nearly half of the F1 individuals derived from the triploid recipients possessed the *Gfp* gene. Together with the fact that offspring produced by sperm derived from triploid croaker could not hatch out and eventually died, the resulting hatchlings obtained by the progeny tests were all donor-derived. Their fertilization and hatching rates were comparable to those of the control fish. Furthermore, they grew up normally, and at least their external morphology was normal. These results suggested that by germ cell transplantation, the fertility of triploid recipients was recovered and the recipients produced functional donor-derived mature gametes.

Two and half years after transplantation of yellow tail germ cells into the allogeneic recipients, 9 males and 4 females matured out of 43 recipients (a mixture of males and females). The progeny tests and the following microsatellite marker analyses revealed that the all mature males and females produced donor-derived offspring. The average percentages of donor-derived offspring in the F1 generation was 66.6% (19.6-98.8%) in the case of male recipients, and 63.2% (17.0-97.5%) in the case of female recipients. The resulting offspring grew up normally, and at least their external morphology was normal. The high germ line transmission rates of donor haplotypes using diploid recipients might have been caused by transplanting large numbers of donor germ cells (20,000) into recipients having a relatively small number of endogenous germ cells.

Conclusion:

A germ cell transplantation system was established using fishes belonging to the Scianidae and Carangidae. It is noteworthy that this technique is applicable to fish species producing small pelagic eggs, which is common for most commercially valuable marine species, including tunas and groupers. Currently, we are working on development of a germ cell transplantation system using bluefin tuna.

References:

[1]OKUTSU T, YANO A, NAGASAWA K, SHIKINA S, KOBAYASHI T, TAKEUCHI Y, YOSHIZAKI G. 2006. Manipulation of fish germ cell: visualization, cryopreservation and transplantation. J. Reprod. Dev., 2: 685–693.

[2]OKUTSU T, SUZUKI K, TAKEUCHI Y, TAKEUCHI T, YOSHIZAKI G. 2006. Testicular germ cell can colonize sexually undifferentiated embryonic gonad and produce functional egg in fish. Proc. Natl. Acad. Sci. USA., 103: 2725–2729.

[3]OKUTSU T, SHIKINA S, KANNO M, TAKEUCHI Y, YOSHIZAKI G. 2007. Production of offspring from triploid salmon parents. Science, 317:1517.

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

MECHANISM OF OOCYTE MATURATION AND OVULATION, AND ITS APPLICATION TO SEED PRODUCTION IN THE JAPANESE EEL

Kagawa, H.*, Sakurai , Y., Kazeto Y., Gen, K., Imaizumi, H., Masuda Y.

*Department of Marine Biology and Environmental Sciences, Faculty of Agriculture, University of Miyazaki, Miyazaki 889-2192, Japan. Email: kagawa@cc.miyazaki-u.ac.jp

Introduction:

The Japanese eel, *Anguilla japonica*, is an economically important fish for Japanese food culture and for Japanese freshwater fish aquaculturists and scientists. The grilled eel dish called "kabayaki" is one of the representative dishes of traditional Japanese food culture. However, the decreases in eel resources and the catches of glass eels as seedlings for aquaculture have been a serious concern in recent years in both Europe and East Asia. Thus, technical development to produce eel seeds for artificial cultivation is strongly desired. However, male and female eels are sexually immature under normal cultivated conditions without hormonal treatments. Moreover, silver eels that migrate down the river are also immature. Sexually mature male and females have never been obtained from the wild until recently. Therefore, fundamental information on oocyte maturation and ovulation has not been obtained from naturally matured eels. Development for artificial induction of maturation and seed production in the Japanese eel started almost half a century ago. Finally, *leptocephalus* larvae and glass eels have been produced first in the world from artificially induced mature male and female eels by elaborate and reasonable hormonal treatments [1]. In 2010, "complete culture" system (production of the next generation from broodstocks which are reared from artificially reproduced eggs) has been developed. In the presentation, basic knowledge of mechanism of oocyte maturation and ovulation, and its application to artificial induction of sexual maturation of female eels will be introduced.

Methods:

Full-grown cultivated female eels were obtained by intraperitoneal weekly injections of salmon pituitary extracts (SPE) [2] or by implantation of a single SPE-loaded osmotic pump with a long-term sustained hormone-release system [3]. Ovarian fragments containing immature full-grown oocytes or oocytes at the migratory nucleus stage were taken from a small puncture of abdomen. Matured oocytes and ovulated eggs obtained by the standard methods mentioned previously [2]. For *in vitro* experiments, oocytes were incubated with various hormones and chemicals in 24-well culture plates containing 1ml of L-15 medium.

Results and Discussion:

After a relatively long period of growth (the vitellogenic phase), oocytes undergo maturation, accompanied by several maturational processes in the cytoplasm (such as hydration, lipid coalescence, and clearing of the ooplasm) and in the nucleus (such as germinal vesicle breakdown (GVBD) followed by ovulation. The eel oocytes underwent more than three-fold increase in volume during maturation and ovulation [4]. Wet and dry weight measurements indicated that water accumulation during oocyte maturation is the major factor contributing to the follicular diameter increase. During these processes, the oocytes become buoyant which is essential for their oceanic survival and dispersal as well as for the initiation of early embryogenesis. *In vitro* experiments using inhibitors of aquaporin water permeability ($HgCl_2$), and yolk proteolysis (bafilomycin A1), also indicate that aquaporin facilitates water uptake by acting as a water channel, and yolk proteolysis is essential for water influx into oocytes via osmotic mechanisms. To elucidate the molecular mechanisms underlying hydration during oocyte maturation, we have cloned novel-water selective aquaporin 1 (AQP1b) of the eel. *In situ* hybridization studies with the eel *aqp1b*cRNA probe revealed intense eel *aqp1b* signal in the oocytes at the perinucleolus stage and the signals became faint during the process of oocyte development. Light microscopic immunocytochemical analysis of ovary revealed that the Japanese eel AQP1b was first expressed in the cytoplasm around the yolk globules of oocyte at the primary yolk globule stage and became localized around the large membrane-limited yolk masses which were formed by the fusion of yolk globules during the oocyte maturation phase. These results together indicate that AQP1b, which is synthesized in the oocyte during the process of oocyte growth, is essential for mediating water uptake into eel oocytes during the final oocyte maturation phase. *In vitro* experiments indicate that these hydration processes were first induced in oocytes at the migratory nucleus stage by the gonadotropin, LH, maybe via maturation-inducing steroid, DHP produced in the ovarian follicle. Recombinant eel LH but not FSH, which were produced using a HEK 293 cell,induced a maturational competence (the acquisition of sensitivity of the oocyte

to respond to the MIS of oocytes) in a dose-dependent manner. After acquisition of maturational competence, LH but not FSH induced GVBD and ovulation *in vitro*. SPE (or LH) did not induce *in vitro* GVBD and ovulation in oocytes at the tertiary yolk globule stage (below 700 μm in diameter) which were obtained from female eels just before SPE injection. One day after intraperitoneal SPE injection, oocytes at the migratory nucleus stage approximately 800 μm in diameter underwent GVBD and ovulation *in vitro* in response to SPE. Thereafter, oocyte over 800 μm in diameter became less sensitive to SPE afterwards. These results indicate that oocytes acquire the ability to respond to SPE at the migratory nucleus stage over 800 μm in diameter and furthermore SPE has an essential role for investment and maintenance of the ability of oocytes to respond to SPE. To induce oocyte maturation and ovulation artificially, the following hormonal treatments have been developed from information obtained from *in vitro* and *in vivo* experiments. Eels which have ovaries containing oocytes at the migratory nucleus stage (approximately 700-750 μm in diameter) were injected with SPE to induce oocyte hydration and maturational competence. Eels having competent oocytes (850-900 μm) were injected again (a priming dose) with SPE to induce and maintain maturational competence and ability to respond to gonadotropin. Final treatments of gonadotropin-releasing hormone analog (GnRHa) in combination with SPE given 24 hr after SPE-priming dose to female eels (900-950 μm in diameter) succeeded in induction of spawning in a rearing tank with spermiated male eels injected with human chorionic gonadotropin and OHP. Fertility and hatchability are approximately 80% and 50%, respectively.

Conclusion:

Egg quality obtained from the female eels induced by our hormonal treatment procedure is still not so high and fluctuates among the samples used in this study. Further studies are necessary to elucidate factors associated with egg quality and also to improve our procedures.

References:

[1]TANAKA, H. 2003. Techniques for larval realing. .In: *Eel Biology*. (ed. Aida K, Tsukamoto K, Yamauchi K, 427–434, Springer, Tokyo.

[2]KAGAWA H. 2003. Artificial induction of oocyte maturation and ovulation. In: *Eel Biology*. (ed. Aida K, Tsukamoto K, Yamauchi K, 401–414, Springer, Tokyo.

[3]KAGAWA, H, KASUGAY, ADACHI J, NISHI A, HASHIMOTO H, IMAIZUMI, H, KAJI S. 2009. Effects of continuous administration of human chorionic gonadotropin, salmon pituitary extract, and gonadotropin-releasing hormone using osmotic pumps on induction of sexual maturation in male Japanese eel, *Anguilla japonica*.Aquaculture, 296: 117–122.

[4]KAGAWA H, HORIUCHI Y, KASUGA Y, KISHI T. 2009. Oocyte hydration in the Japanese eel (*Anguilla japonica*) during meiosis resumption and ovulation. J. Exp. Zool., 311A. :752-762.

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

THE PROTEOMIC AND ANTOXIDANT RESPONSES OF COMMON CARP (*CYPRINUS CARPIO*, L.) SPERM CAUSED BY CRYOPRESERVATION TECHNIGUES

Li P.*°, Hula M.*, Dzyuba B.*#, Rodin M.*, Li Z.H.*°, Boryshpolets S.*, Gela D.*, Linhart O. *

*University of South Bohemia, Faculty of Fisheries and Protection of Water, Research Institute of Fish Culture and Hydrobiology, Zetas 728/II, 38925 Vodnany, Czech Republic. Fax +42-387774634 e-mail: pingli06@yahoo.com
°Key Laboratory of Freshwater Biodiversity Conservation and Utilization, Ministry of Agriculture, Yangtze River Fisheries Research Institute, Chinese Academy of Fishery Science, Wuhan, Hubei, China
#Institute for Problems of Cryobiology and Cryomedicine of the National Academy of Sciences of Ukraine, Pereyaslavskaya str., 23, 61015 Kharkov, Ukraine.

Introduction:

Cryopreservation of fish sperm is an important tool for conservation of biodiversity (gene banks of autochthonous populations), efficient and selective fertilization (maintaining genetic variability of brood stocks for restocking), and for synchronization of artificial reproduction [1]. However, cryopreservation causes irreversible cell damage, resulting in a significant reduction in viable spermatozoa. Multiple nonphysiological processes, such as osmotic and oxidative stress, are crucial factors in spermatozoa cryodamage. Freezing of cells in suspension induces ice nucleation in the extracellular space, creating an osmotic gradient across the plasma membrane between the initially isotonic intracellular solution and the freeze-concentrated extracellular solution. Hence, we designed a series of experiments using sperm of common carp (*Cyprinus carpio,* L.) as a model to understand the effects of the application of different cryopreservation protocols and to examine cryopreservation procedures with respect to osmotic and oxidative stress in common carp spermatozoa. In addition, the spermatozoa motility, membrane integrity, and oxidative stress indicators as well as response of sperm proteome were measured in unfrozen sperm and after freeze/thaw processes.

Results and Discussion:

As a first step towards understanding the effect of cryopreservation procedures on spermatozoa integrity we examined osmotic and oxidative effects on common carp spermatozoa motility; membrane integrity; levels of thiobarbituric-acid-reactive substance (TBARS) and carbonyl groups (CP); and the activity of superoxide dismutase (SOD), glutathione reductase, and glutathione peroxidase (GPx). Prior to experiment, the sperm was diluted in diethyl sulfoxide (DMSO) and ethylene glycol based (EG) extenders, followed by equilibration, freezing, and thawing. Our results showed that equilibration in DMSO extender resulted in a significant reduction of spermatozoa motility, but spermatozoa motility was induced in those spermatozoa following dilution with saline buffer, which usually inhibits undiluted spermatozoa motility. Spermatozoa velocity and membrane integrity significantly decreased with both extenders following freezing and thawing. No significant difference in levels of TBARS or CP, or in SOD activity, was seen in samples equilibrated with either extender. Moreover, the freeze/thaw process induced significantly higher levels of TBARS, CP, and GPx activity, but did not affect the level of SOD. Glutathione reductase activity was inhibited in samples exposed to DMSO extender.

As a second step, we have also used several proteomics methods to characterize the protein components of common carp sperm and to verify whether the cryopreservation procedure, applied to common carp sperm, affected the protein profiles of spermatozoa involved in the control of sperm functions. Additionally, the protein profiles in the associated seminal plasma were also investigated. Finally, the effects of cryopreservation on spermatozoa motility, fertilization, and ova hatching rate were also evaluated. Our results noticed that, following cryopreservation, spermatozoa velocity and motility were significantly lower than in controls. The initial velocity of cryopreserved spermatozoa (15 sec post activation) was similar to that of fresh spermatozoa at 30 sec post-activation, and the initial percent of motile cryopreserved spermatozoa was approximately 40%, less than that of fresh spermatozoa at 45 sec post activation. Both fertilization and ova hatching rates using cryopreserved spermatozoa were lower than the controls. Moreover, investigation of sperm proteome showed that. The number of protein spots detected on the 2DE gels performed on fresh and cryopreserved spermatozoa samples resulted in 187 and 178 spots, respectively. The fourteen protein spots were significantly altered following cryopreservation. Eleven of these were identified: three as specific membrane proteins (N-ethylmaleimide-sensitive fusion protein attachment protein alpha, cofilin 2, and annexing A4) involved in membrane trafficking, organization, and cell movement; six as cytoplasmic enzymes (S-Adenosylhomocysteine hydrolyses, Si:dkey-180p18.9 protein, lactate dehydrogenate B, phosphoglycerate kinase 1,

transaldolase 1, and esterase D/formylglutathione hydrolyses) involved in cell metabolism, oxidoreductase activity, and signal transduction; and two as transferrin variant C and F.

Conclusions:

In conclusion, the results, together with reports of mammal studies, confirmed that the rapid removal of DMSO from spermatozoa resulted in a marked decline in spermatozoa motility, whereas EG was associated with less osmotic damage subsequent to rapid removal. Both CPA effect and the freeze/thaw process can induce oxidative stress in spermatozoa, and DMSO showed more adverse effects than EG with respect to GR and GPx activity. In addition, the antioxidant response of spermatozoa is mainly due to the capacity of GPx to counteract ROS stress of spermatozoa and minimize cryopreservation damages. All data suggest that an alternative CPA, such as EG, should be considered for carp spermatozoa. Furthermore, proteomic analyses of common carp sperm revealed quantitative alterations in protein expression in cryopreserved spermatozoa. The observed downregulation of spermatozoa membrane proteins and cytoplasmic enzymes, together with the accumulation of transferrin in cryopreserved spermatozoa, could be the reason for the observed decrease in spermatozoa velocity, motility, and lower fertilization success as well as in ova hatching rate.

Acknowlegements:

Present study was financially supported by following grants: CZ.1.05/2.1.00/01.0024, MSM 6007665809, LC06073, IAA608030801, ME10015, QH92308, QH82118, QH82119, GAJU 003/2010/Z, 046/2010/Z, and 047/2010/Z, and China grant 200701029.

References:

[1]LI, P., HULAK, M., LINHART, O. 2009. Sperm proteins in teleostean and chondrostean (sturgeon) fishes. Fish Physiol. Biochem., 35: 567–581.

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

TRANSGENERATIONAL KNOCKDOWN OF *DMY* IN MEDAKA, *ORYZIAS LATIPES*

Chakraborty T.[1, 2], **Zhou L.Y.**[2, 3], **Iguchi T.**[1] and **Nagahama Y.**[2, 4]

[1]Laboratory of Molecular Environmental Endocrinology, National Institute for Basic Biology, Okazaki 444-8585, Japan. Fax: +81-564-59-5235 email: tchakraborty83@gmail.com; [2]Laboratory of Reproductive Biology, National Institute for Basic Biology, Okazaki 444-8585, Japan; [3]Key Laboratory of Freshwater Fish Reproduction and Development, Southwest University, Beibei Chongqing, 400715, P.R. China; [4]Institution for Collaborative Relations, Ehime University, Matsuyama 790-8577, Japan.

Introduction:

The Japanese medaka (*Oryzias latipes*) is an excellent and well recognized model for studying developmental processes because of its several advantages over other vertebrate models such as the availability of the Y-chromosome linked sex-determining gene, *dmy* (*Dmrt1bY*) gene, by which male sexual differentiation is initiated in XY embryos. Initiation of *dmy* expression is followed by the arrest of morphological sexual differentiation of male gonads and, in the absence of *dmy*, morphological sexual differentiation occurs in the female gonads. However, no studies have revealed yet how *dmy* acts on somatic and germ cells in XY gonads to induce male sexual differentiation and no targets have been identified for this gene, even though homologues of several male-specific factors such as *gsdf1* and *dmrt1* have been reported to be downstream of DMY. Moreover, several studies have shown that SRY and other testis-determining factors can bring down the ovarian aromatase expression, which further reduces estrogen levels in gonads and directs male development. Developmental biology studies are pillared by functional genomics and reverse genetics, among which knockdown techniques are very popular. Reverse genetics or knockout approaches are well-suited not only to address these issues via the generation of double mutants but also for assigning biological function to uncharacterized genes in a genome. Several such approaches are available for vertebrates, i.e. tilling, morpholino and gripNA etc., but they have several disadvantages. In particular, knockdown at transcriptional/post-transcriptional levels is a costly venture and are mostly intended for mammalian use. Antisense RNA has been traditionally used for knocking down the expression of target mRNAs. Antisense RNA may be introduced into a cell to inhibit translation of a complementary mRNA by base pairing with it and physically obstructing the translation machinery. Generally, antisense RNA still lacks effective design, biological activity, and an efficient route of administration. In the present work, we have developed a DNA vector-based approach to overcome those limitations and used it to investigate the effects of loss-of-function of *dmy*.

Methods:

The AS-RNA expression constructs were designed and evaluated using an online tool. Specific sequences of *GFP/dmy* were amplified and cloned under the control of *cmv* promoter in pcDNA 3.1 vector in an antisense orientation. One- and two-cell embryos of the QurtE strain of medaka were electroporated with antisense constructs. Whole fish samples at 0, 5 and 10 days and gonads of 50 days and adults were used for histological and real-time PCR analysis. Gene inheritance was analyzed using PCR amplification of genomic DNA and Northern blotting.

Results:

To confirm the knockdown effect of the antisense technique *in vivo*, *GFP/ dmy* expression was knocked down in olvas-GFP transgenic/QurtE medaka using a highly specific *GFP/dmy*-antisense construct. No side effect was observed during different stages of development, suggesting the effectiveness of this method. Northern blotting and sequence analyses of small RNAs isolated from F1 generation fish suggest that the antisense RNA-expressing constructs using the siRNA/miRNA pathway were effective to block the expression of respective genes.

Suppression of *dmy* showed the acceleration of both meiosis and mitosis in XY fish, even at 0 days after hatching (dah). At 0 dah, XY fish of *dmy* knockdown group contained 120-140 germ cells. Above that, 40% fish showed 3-5 meiotic cells in their respective gonads. Both mitotic and meiotic proliferations were increased subsequently at 5 (78% fish) and 10 (73% fish) dah. These fish also had comparatively larger gonads containing both individual and clusters of mitotic cells (250-300), similar to XX female medaka at the end of 10 dah. However, normal males only contained individual germ cells at this stage. A complete ovary with an ovarian cavity was formed in both normal females and XY pm-DMYAS females at the end of 50 dah. The *dmy* knockdown and XY sex-reversed fish behaved similar to XX females and produced fertile progeny when mated with XY males.

Our gene expression analyses clearly demonstrated a shift of male-dominated gene expression to a female-responsive gene profile. This shift became permanent, which may be solely because of *dmy* knockdown and give rise to fully fertile sex-reversed XY females. Early set up of meiosis explains the sex reversal in more detail. Overall, this explains that the possible mechanism of *dmy* action is exerted by blocking the *cyp19a1* expression or *RSPO1* expression directly or indirectly by increasing the GSDF1 and SOX9a2 concentrations.

Conclusion:

In this study, stable, cost-effective and gene-specific transgenic RNAi technology was developed and used to investigate the specific role of *dmy* in testicular development. The results clearly showed that *dmy* is the male-determining gene in medaka. Since the RNAi effect is long lasting and inheritable, this will provide a powerful tool for the analysis of gene function of not only embryos, but also phenotypic consequences that develop over longer periods of time. A similar approach can be applied in other nonmammalian vertebrate model organisms, including zebrafish and frogs.

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

INVESTIGATION OF POTENTIAL MOLECULAR MARKERS AND APPROACHES TO CHARACTERIZE AND ISOLATE SPERMATOGONIAL STEM CELLS IN THE NILE TILAPIA (*OREOCHROMIS NILOTICUS*)

Lacerda S.M.S.N., Costa G.M.J., Campos-Jr P.H.A., Rezende-Neto J.V., *Hofmann M-C., França L.R.

Department of Morphology, Federal University of Minas Gerais, Belo Horizonte-MG, 31270-90, Brazil. Fax: +55-31-34992780 email: lrfranca@icb.ufmg.br

*Department of Veterinary Biosciences, University of Illinois at Urbana-Champaign, Illinois, USA.

Introduction:

Undifferentiated spermatogonial population consists of stem and progenitor germ cells, which function to provide the foundation for spermatogenesis. The stem cell component, named spermatogonial stem cells (SSCs), is capable of self-renewal and differentiation. These unique attributes have made them a target for novel technologies to enhance reproductive function in males. First established in mammals, germ cells transplantation is the only functional approach for investigating SSCs biology. In this regard, recently we established successful spermatogonial transplantation in adult Nile tilapia that resulted in donor-derived offspring [1]. In association with in vitro culture, SSCs transplantation have the potential to enhance efficiency of fish production and provide a novel approach to continuously generate transgenic animals, allowing also the preservation of genetic stocks of valuable or endangered fish species. Isolation of SSCs is an essential component for improving the efficiency of these techniques; however, there are no specific molecular markers for SSCs in any vertebrate species, including fish. Therefore, in the current study we aimed to investigate potential molecular markers, particularly those already well established in higher vertebrates [2], which could be properly used to phenotypically characterize and isolate SSCs in the Nile tilapia.

Methods:

Adult tilapia (*O. niloticus*) testes were evaluated by immunohistochemistry and immunofluorescence for the presence of Vasa (germ cell specific marker), GFRα-1 and Notch-1 (undifferentiated spermatogonia surface marker) receptors, and the transcription factor Oct-4 (POU5F1). Reactions were performed according to standard protocols using immunoperoxidase technique and fluorophore-conjugated secondary antibody. For these purposes, the following primary antibodies and the dilutions used were: polyclonal rabbit anti-human DDX/MVH-Vasa (1:200; Abcam), polyclonal rabbit anti-human Notch Homolog 1 (1:100; Lifespan Bioscience), polyclonal goat anti-rat GFRα-1 (1:500; Santa Cruz Biotechnology) and polyclonal rabbit anti-human Oct-4 (1:200; Abcam). Additionally, western blot analyses of protein expression were performed in order to determine the specificity of the antibodies used. The profile and percentage of positive testicular cells were evaluated using flow cytometry (FACS). Using immunomagnetic separation (MACS) we also investigated if some of the surface marker(s) could be used to enrich undifferentiated (SSCs) spermatogonia population.

Results and Discussion:

The flow cytometry analyses showed that the GFRα-1 positive cell fraction comprised approximately 9.5% ± 0.9 (n=3 and three replicates from each fish) of the selected testis cell population evaluated in adult tilapia, whereas Notch-1 positive cells corresponded to 7%±0.8 of the selected testicular cells. Immunohistochemistry and immunofluorescence analysis revealed that GFRα-1 and Notch-1 expression is localized in the seminiferous epithelium, exclusively in single type A spermatogonia (presumptive SSCs). Sertoli cells, differentiating/differentiated germ cells or interstitial cells did not show any evident labeling for these markers. The GFRα-1 and Notch-1 positive spermatogonial cells were found preferentially at the blind ending of the tilapia seminiferous tubules, near to the tunica albuginea, where a high density of type A undifferentiated spermatogonia, characterized by morphological criteria, were previously reported to be located [3]. In addition, these GFRα-1+/Notch-1+ cells showed intense immunoreactivity for anti-Vasa. Using double staining for Notch1 and GFRα-1, we observed that less than one percent of testicular cells were double positive for these markers, suggesting the presence of phenotypically different SSCs populations. The investigation of Oct-4 expression in tilapia testis showed that only type A spermatogonia (presumptively primitive SSCs), also exclusively located at the blind ending of the seminiferous tubules, were positive for this pluripotent marker. Preliminary FACS analyses using double staining for Oct-4 and GFRα-1 showed that the Oct-4 positive cell fraction comprises approximately 6.2% of total selected testicular cells. Interestingly, the entire population of Oct-4 positive cells were also positive for GFRα-1. Considering the promising results related to

GFRα-1 positive staining, we have selected testicular cells for this surfaced marker through immunomagnetic separation. A four-fold relative enrichment of SSCs was obtained in the sorted fraction after MACS separation.

Conclusion:

Taken together, the results found in the present investigation showed that GFRα-1, Notch-1, and Oct-4 are potentially good markers for the SSCs in tilapias. We also demonstrated that an enriched GFRα-1 positive SSCs population from adult tilapia can be obtained using MACS. Therefore, besides providing a better knowledge on SSCs biology in fish, this crucial step represents a very important progress toward the development of new biotechnologies in aquaculture, for instance allowing the generation of transgenic fish using SSCs transfection.

Financial support: FAPEMIG and CNPq

References:

[1]LACERDA, S.M., BATLOUNI, S.R., COSTA, G.M., SEGATELLI, T.M., QUIRINO, B.R., QUEIROZ, B.M., KALAPOTHAKIS, E., FRANÇA, L.R. 2010. A new and fast technique to generate offspring after germ cells transplantation in adult fish: the Nile tilapia (*Oreochromis niloticus*) model. PLoS One, 5:e10740.

[2]PHILLIPS, B., GASSEI, K., ORWIG, K.E. 2010. Spermatogonial stem cell regulation and spermatogenesis. Philos. Trans. R. Soc. Lond. B Biol. Sci., 365:1663-1678.

[3]SCHULZ, R. W., FRANÇA, L.R., LAREYRE, J.J., LE GAC, F., CHIARINI-GARCIA H., NOBREGA, R. H., MIURA, T. Spermatogenesis in fish. Gen. Comp. Endocrinol., 167: 390-411.

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

SEXUAL DIMORPHISM ON THE MORPHOMETRIC CHARACTERISTICS OF PINK SKUNK CLOWNFISH, *AMPHIPRION PERIDERAION*

Abol-Munafi, A. B., Sarmiza, S., Norazmi-Lokman, N. H., and **Abduh, M. Y.**

Department of Fishery Science and Aquaculture, Faculty of Agrotechnology and Food Science, Universiti Malaysia Terengganu, 21030 Kuala Terengganu, Terengganu, Malaysia. email: munafi@umt.edu.my

Introduction:

Pink Skunk Clownfish, *Amphiprion perideraion*, is known as protandric hermaphrodite where it can change its sex from male to female. This species is one of the most popular marine ornamental species that attracts interest of hobbyist due to their unique body coloration, "clowning" behavior, which is actually territorial, and also because of the unusual wiggling swimming motion. *Amphiprion perideraion* is one of the species that have been difficulty to produce in captivity compared to the other Clownfishes due to their aggressive behavior. Until now, there is still lack of report on the production of this species in captivity though the demand is high. In order to breed clownfishes in captivity, they need to be paired [1] which means the ability to identify the fish sex is crucial. This becomes the bottleneck for the production of *A. perideraion* in captivity since breeders have failed to differentiate the sex of the wild fish collected. The only sexing method usually used is by looking at its body size where the females are usually larger than the males. However this method is inaccurate. In order to identify the sex of any organism including fish, the information on its sexual dimorphism is crucial. Therefore, the aim of this study is to differentiate the sexes of *A. perideraion* morphologically by analyzing the morphometric characteristics.

Methods:

Thirty males and females of *A. perideraion* used in this study were collected at Bidong Island, Terengganu. Sixteen morphometric parameters including standard length were taken for each fish (Figure 1). The procedure was then followed with 18 Truss variable measurements (Figure 2). All measurements were taken to the nearest accuracy 0.1 mm. All measurement data were transformed into size-independent shape variables to eliminate the size effect in the data set [3] since all the samples used in this study were different in sizes and the age of the samples is unknown. The measurements were transformed using the following formula: $M_{adj} = M/SL$, where M_{adj} = adjusted measurements; M = original measurements; SL = Standard length [2 and 3]. All the transformed measurements were then analyzed using independent T-test. The statistical analysis was carried out with SPSS 15.0 for Windows.

Figure 1: Sixteen morphometric measurements.

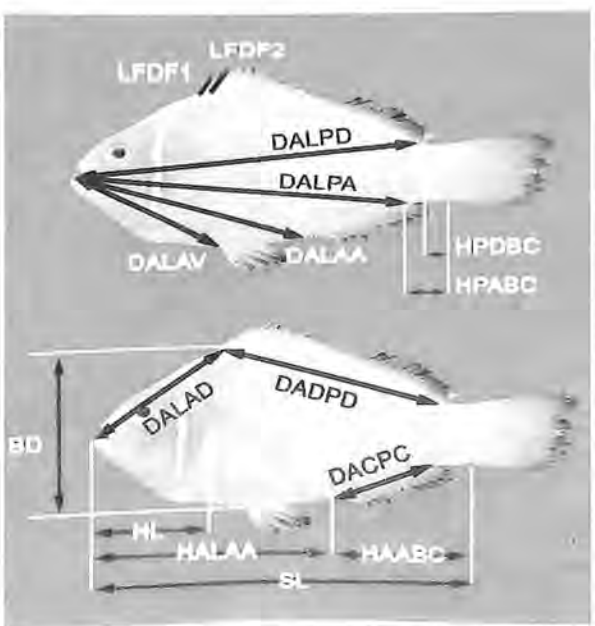
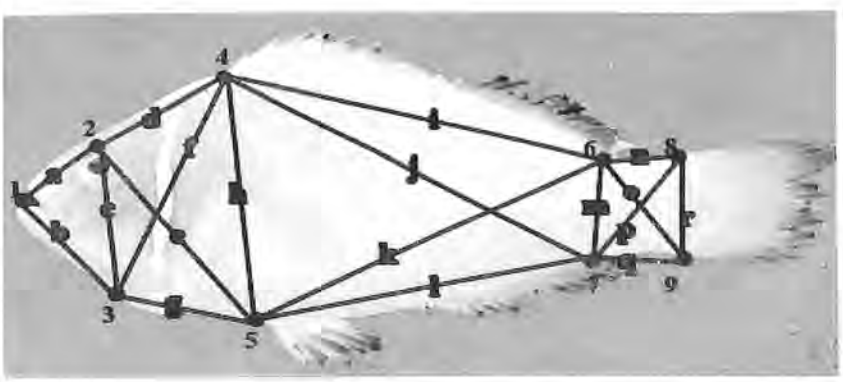


Figure 2: Truss network measurements.



Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

Table 1: Morphometric measurements analysis

Characteristics	Sex	N	P-value	Sig.
HL	F		0.505235	
	M			
BD	F		0.045177	*
	M			
HALAA	F		0.355555	
	M			
HAABC	F		0.355555	
	M			
HPDBC	F		0.719093	
	M			
HPABC	F		0.242554	
	M			
DALAV	F		0.511595	
	M			
DALAA	F		0.455294	
	M			
DACPC	F		0.537125	
	M			
DALPA	F		0.052540	
	M			
DALPD	F		0.000000	***
	M			
DADPD	F		0.537125	
	M			
DALAD	F		0.955514	
	M			
LFDF1	F		0.173292	
	M			
LFDF2	F		0.335199	
	M			

*** Highly significant difference (P<0.001); * Significant difference (P<0.05)

Table 2: Truss network variables analysis

Parameters	Sex	N	P-value	Sig.
a	F		0.500	
	M			
b	F		0.557	
	M			
c	F		0.152	
	M			
d	F		0.017	*
	M			
e	F		0.015	*
	M			
f	F		0.245	
	M			
g	F		0.753	
	M			
h	F		0.157	
	M			
i	F		0.770	
	M			
j	F		0.314	
	M			
k	F		0.519	
	M			
l	F		0.457	
	M			
m	F		0.000	***
	M			
n	F		0.523	
	M			
o	F		0.019	*
	M			
p	F		0.105	
	M			
q	F		0.125	
	M			
r	F		0.000	***
	M			

*** Highly significant difference (P<0.001); * Significant difference (P<0.05)

Results and Discussions:

From the statistical analyses, the male and female of *A. perideraion* were significantly different (P<0.05) at BD (body depth), DALPD (direct distance between the anterior edge of the upper lip and the posterior of the last dorsal fin), d (above eye to origin of dorsal fin), e (above eye to origin of pelvic fin), m (end of dorsal fin to end of anal fin), o (end of dorsal fin to end of caudal fin) and r (upper insertion of caudal fin to end of caudal fin). Table 1 represents the results of the 15 morphometric measurements statistical analysis while Table 2 represents the results of the 18 Truss network variables statistical analysis. The results of both techniques showed that the functional female has greater size at the head and caudal peduncle area. The reason on why the head and caudal peduncle area of *A. perideraion* female are larger than male is still a mystery since the functional role of both part in the sex determination and sex change mechanism of *A. perideraion* are still unknown.

Conclusions:

Amphiprion perideraion is sexually dimorphic at 7 measurements (BD, DALPD, d, e, m, o and r) of its body which occurred at the head and caudal peduncle areas. The morphometric parameter BD was significantly different between sexes ($P < 0.05$) while DALPD was highly significant different between sexes ($P < 0.001$). Based on Truss Network variables, 3 variables d, e and o showed significant different between sexes ($P < 0.05$) while m and r were highly significant different between sexes ($P < 0.001$). Further studies are recommended on the sexual determination mechanism and differentiation process of *A. perideraion.*

References

[1]LIEW, H.J. 2006. Spawning, development and larva rearing of False Clownfish, *Amphiprion ocellaris* under captive conditions. M. Sc. thesis. Institute of Tropical Aquaculture, Universiti Malaysia Terengganu. 135p.

[2]REIST, J. D. 1985. An empirical evaluation of several univariate methods that adjust for size variation in morphometric variation. Can. J. Zool., 63: 1429-1439.

[3]TURAN, C. 1999. A note on the examination of morphometric differentiation among fish populations: The Truss system. Tr. J. Zoology, 23: 259-263.

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

CONTROLLED BREEDING AND SEED PRODUCTION OF SALT WATER FISHES – STATUS, PROSPECTS AND PROBLEMS IN INDIA

Arasu A.R.T., Kailasam M., Sundaray J.K. *, Subburaju R., Thiagarajan G.

Central Institute of Brackishwater Aquaculture, 75, Santhome High Road, R.A.Puram, Chennai 600 028, India

**Kakdwip Research Centre of CIBA, Kakdwip, 24 Paraganas (South), West Bengal-743347, India*

Fisheries play an important role in providing nutritional security for the growing population. The global fish production is in the order of 142 million tones equally contributed by capture and culture fisheries. The Indian fish production is around 7 million tones both from marine and inland sectors more or less equally. The inland fish production is contributed mainly (80%) by the aquaculture. Aquaculture of salt water fishes however, is negligible in the Indian context. One of the major constraints in the development of salt water fish culture is the availability of quality seed in adequate quantity at the appropriate time. Though significant achievements have been made in India in the controlled breeding and seed production of some of the important candidate species suitable for farming like Asian Seabass (Barramundi) (*Lates calcarifer*), Cobia (*Rachycentron canadum*), Grey Mullet (*Mugil cephalus*), Pearlspot (*Etroplus suratensis*), Groupers (*Etroplus tauvina, E. coides*) and Ornamental Fish Spotted scat (*Scatophagus argus*), comprehensive viable technologies are yet to be developed. Technology for the captive broodstock development and acceleration of maturation under controlled conditions providing required parameters has been standardized for some of the candidate species. Protocols and procedures in the induction of spawning through environmental manipulation and exogenous hormonal administration have been successful. Technologies for rearing the larvae and seed production to a limited extent have also been standardized for some species. Species like Asian Seabass has been domesticated and viable technology for the year round seed production is available. The importance of maintaining quality broodstock for meeting the larval requirement and establishment of National Broodstock Facilities and fish hatcheries for the development of salt water fish farming are discussed.

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

SPERM MOTILITY MULTIPLE ACTIVATIONS: PHYSIOLOGICAL BACKGROUND AND PRACTICAL USE IN AQUACULTURE

Boryshpolets S.[a*], **Dzyuba B.**[a,b], **Rodina M.**[a] **and Linhart O.**[a]

a - University of South Bohemia, Faculty of Fisheries and Protection of Waters, CENAKVA, Zatisi 728/II, 389 25 Vodnany, Czech Republic

b - Institute for Problems of Cryobology & Cryomedicin of National Academy of Sciences of the Ukraine, Kharkov, UkraiIntroduction

* - fax: +420387774634, e-mail: boryss00@vurh.jcu.cz

Introduction:

In freshwater fish spawning, sperm motility activation is related to low osmolality of the environment, its duration lasts for short periods and the lost motility is associated with disappearance of fertilizing ability [5]. During artificial reproduction there is the possibility to increase the duration of sperm motility [4] and even restore the motility of spermatozoa being immotile [6]. In this report we summarized our results on studying the physiological backgrounds for sperm motility multiple activation in fish spermatozoa and appearance of spontaneous activations during cryopreservation. Further, we discuss the possible advantage of this phenomenon when necessary to improve the results of artificial reproduction in fish.

Methods:

Fish and sperm sampling- Mature males of Eurasian perch and Common carp were obtained after fish farm pond harvesting and kept in laboratory aquatic systems. Sperm samples were obtained during natural spawning period in case of perch while in carp it was collected after treatment with carp pituitary extract.

Sperm motility parameters- Sperm velocity and motility (percentage of motile spermatozoa) were estimated using an analysis of video records obtained by applying CCD video camera mounted on a dark-field microscope and illuminated with a stroboscopic lamp. Motility was initiated either by sperm dilution in hypotonic media or by freeze-thawing.

Models of sperm multiple activation (1) motility of carp sperm was initiated in hypotonic activating media (100-150 mOsm), after motility stop KCl was added to increase the osmolality up to 300 mOsm and after 20 min the second motility activation by osmolality decrease was initiated; (2) motility of perch spermatozoa was initiated 3 times using stepwise reduction of osmolality of activating solution; (3) motility arisen from freeze-thawing process was observed in carp and perch without transferring them into activating media.

Sperm cryopreservation- Sperm samples were cryopreserved using specific cryoprotective media and freezing methods described previously.

Sperm ATP content measurements- ATP content was evaluated by bioluminescence using a Bioluminescence Assay Kit and multifunctional microplate reader.

Results and discussion:

The different modes of multiple sperm motility activation were investigated. (1) After motility stop following the transferring of carp sperm into isotonic condition the ATP level and cellular volume can be recovered together with ability for the second motility activation in hypotonic condition. During the second activation the gradual increase of motility percentage was observed, while in firstly activated spermatozoa the maximum motility observed right after the start of movement. The rate of motility percentage in secondly activated spermatozoa is associated with individual properties of sperm samples (fig 1.) [1]. These secondly activated spermatozoa have the fertilizing ability [6] and survived cryopreservation demonstrating up to 20% of motility after thawing. The respiration rate during resting period was significantly higher than in immotile sperm and not different from respiration rate during motility or uncoupling condition [1]. That is why we suppose that at least part of ATP produced during reactivation were supported by oxidative phosphorylation.

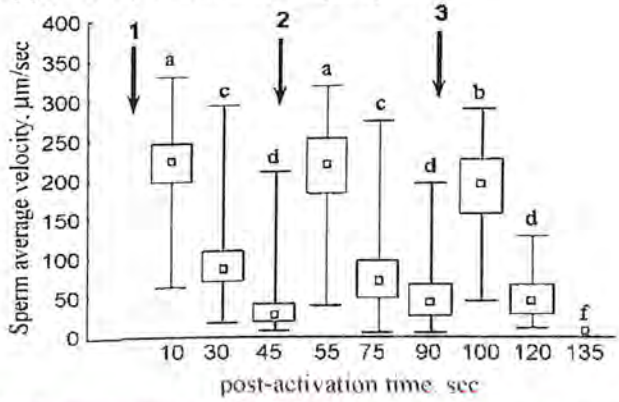


Fig. 3 Perch sperm average velocity during motility activation) in the model of multiple activation

Arrows – first, second and firth motility activation correspondingly

Second mode (2) consists of stepwise decreasing the osmolality of the activating medium. During this kind of multiple activation the total duration of motility is around 3 times longer if compared with activation by sharp osmolality decrease. This phenomenon is based on prolonged and slow ATP consumption during several "waves" of motility arisen due to stepwise environment osmolality decrease (fig.2) [2]. Third mode (3) of multiple activation was observed in carp and perch, when procedure of freeze - thawing itself leads to spontaneous sperm motility activation and endogenous ATP level decrease. Afterwards the sperm could be reinitiated in hypotonic conditions. During this useless activation spermatozoa lost the main part of ATP but still able to be secondly activated and preserve ability for fertilization [3].

Conclusion:

The phenomenon of multiple sperm activation could be the base for the elaboration of the most optimal sperm use because the prolongation of total sperm motility duration potentially and more likely makes the fertilization successful. Spontaneous sperm motility activation during freeze-thawing should be taken into account during cryopreserved sperm use. Finally, conjunction the ability of sperm for multiple activation and its cryopreservation could be at the base of multiple sperm use for fertilization if there is a deficit of valuable individuals' sperm.

Acknowledgements:

Special thanks are expressed to the projects: ME10015, QH82119 and LC06073, CZ.1.05/2.1.00/01.0024, GACR 502/11/0090, GAJU 046/2010/Z.

References:

[1]BORYSHPOLETS S., DZYUBA B., DROKIN S. 2009a. Pre-spawning water temperature affects sperm respiration and reactivation parameters in male carps. Fish Physiol. Biochem. 5:661–668.

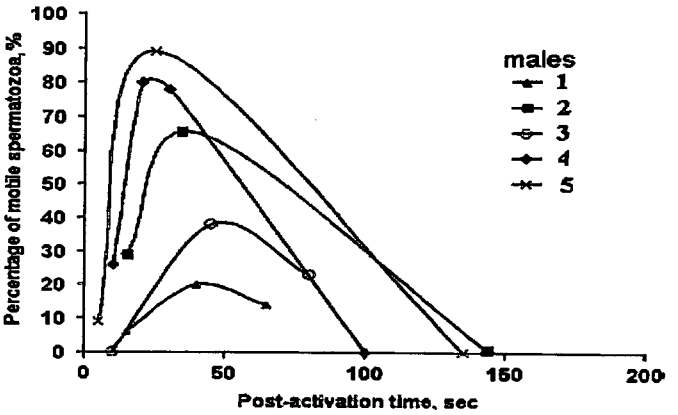


Fig. 1. Graph showing percentage of motile spermatozoa (%) at the second activation obtained for five different males

[2]BORYSHPOLETS S., DZYUBA B., STEJSKAL V., LINHART O. 2009b. Dynamics of ATP and movement in Eurasian perch (*Perca fluviatilis* L.) sperm in conditions of decreasing osmolality. Theriogenology, 72 : 851–859.

[3]BORYSHPOLETS S., DZYUBA B., RODINA M., LI P., HULAK M., GELA D., LINHART O. 2009c. Freeze-thawing as the factor of spontaneous activation of spermatozoa motility in common carp (*Cyprinus carpio* L.) Cryobiology 59: 291–296

[4]COSSON J. 2004. The ionic and osmotic factors controlling motility of fish spermatozoa. Aquacult. Int.,12:69–85.

[5]GINZBURG A.S. 1972. Fertilization in fishes and problem of polyspermia. US Dep. Comm. Nat. Tech. Inf. Ser. Springfield. p366.

[6]LINHART O., ALAVI S.M.H., RODINA M., GELA D., COSSON J. 2008. Comparison of sperm velocity, motility and fertilizing ability between firstly and secondly activated spermatozoa of common carp (*Cyprinus carpio*). J. Appl. Ichthyol.,24:386–92.

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

CLIMATE CHANGE AND FISHERIES IN AFRICA: ISSUES AND CHALLENGES

Fawole, F.J.

Division of Fish Nutrition, Biochemistry and Physiology, Central Institute of Fisheries Education, Versova, Mumbai, 400 061, India. e.mail: femijohn.fnf05@cife.edu.in

Introduction:

It has been ascertained that climatic changes will have a wide range of direct and indirect impacts on aquatic ecosystem, capture fisheries, and aquaculture with a great implications for fisheries-dependent economies if not mitigated. Africa has been considered more vulnerable to the effect of these changes than developed countries due to capacity for adaptability and weak economic power to tackle the problem. As fisheries provide significant feed and seed inputs, the impacts of climate change on them will also affect the productivity and profitability of aquaculture operation. Climatic changes could induce physiological stress on cultured stock and causes metabolic disorder, which would not only affect fish productivity but also increase vulnerability to diseases, impose higher risks and reduce returns to farmers. Fisheries and aquaculture provides an important source of cash income for many poor households, and serve as the main source of food for a billion people worldwide, providing a valuable protein complement to the starchy diet common among the global poor, and essential fatty acid especially n- 3 PUFAs (DHA, EPA). This paper will examine the way in which climatic changes affect fisheries and aquaculture production, and recommend some measures.

Possible effect of climatic changes on fisheries and aquaculture

Fisheries productivity: Global climatic changes are suggested to potentially affect freshwater fisheries by lowering productivity in wild fish populations and in intensive aquaculture systems worldwide [4]. As fishes are poikilotherms, drastic change in their surrounding water temperature will influence their metabolic processes, behavior, migration, growth, reproduction, and survival [9]. Reproduction of fish is often highly sensitive to fluctuations in temperature [5] and so warming can have either a positive or negative effect on egg production, depending on whether the target fish species is close to its thermal optimum. The area and structural complexity of the coral reefs, sea grasses and mangroves that provide shelter and food for many coastal fish species are likely to be altered by rising water temperature, acidification of the ocean, changes in sedimentation from new patterns of rainfall and rising sea levels [8]. Recent declines in fish abundance in the East African Rift Valley lakes have been linked with climatic impacts on lake ecosystems [7]. Lake Tanganyika, for example, has historically supported one of the world's most productive pelagic fisheries. A 30 to 50% decline in clupeid catch since the late 1970s has been attributed partially to environmental factors, because the lake had sustained high yields under similar fishing pressure for the previous fifteen to twenty years, although contrasting views have been expressed [10]. The decline in catch was accompanied by breakdown of the previously strong seasonal patterns in catch, suggesting decoupling from ecosystem processes driven by the weakening of hydrodynamic patterns. These changes in the pelagic fishery are consistent with a lakewide shift in ecosystem functioning [7].

Aquaculture production:

Changes in climate have been reported to induce both direct (e.g. through physical and physiological processes) and indirect (e.g. through variations in fishmeal supplies and trade issues) impacts on aquaculture [2]. It was noted that the physical changes related to climatic changes, i.e. in temperature, solar radiation, current and wave actions, sea level rise, stress due to oxygen deficit, and the frequency of extreme events; will impact physiological, ecological and operational (e.g. species and site selection, containment technologies, etc.) processes. In a work conducted [1] on the effects thermal tolerance and metabolic activity of yellowtail catfish, *Pangasius pangasius,* it was reported that fish show different behavioural responses to temperature variation ranging from, restlessness, escape attempts, unorganized swimming with an attempt to jump out of the aquarium at temperature of between 39-42 °C. However, rapid or dramatic increases in temperature above normal maximum temperatures are expected to have significant negative effects on overall viability of some fish populations [6].

Conclusion:

In general, responses to direct impacts of extreme events on fisheries and its communities are likely to be more effective if they are anticipatory, as part of long-term integrated management planning. However, preparation should commensurate with risk, as excessive protective measures could themselves have negative social and economic impacts [3]. Considering the gravities of this issue, it is high time to discuss on common platform with technocrat and bureaucrats for framing a need-based policy, and strategy to mitigate the stem causes due to climatic changes. There is need for

collaboration among African scientist to cross fertilize ideas and develop a workable strategy on how to mitigate the effects on the continent. Also government at all level should involve both privates sector and civil society for effective implementation of any adopted strategies, and educate the populace, rural and fishing communities on the likely consequences of their action on the planet earth.

References:

[1]DEBNATH. D, A.K. PAL, N.P. SAHU, K. BARUAH, S. YENGKOKPAM, T. DAS, S.M. MANUSH (2006): Thermal tolerance and metabolic activity of yellowtail catfish *Pangasius pangasius* (Hamilton) advanced fingerlings with emphasis on their culture potential. Aquaculture, 258:606-610.

[2]DE SILVA, S.S. & SOTO, D. (2009): Climate change and aquaculture: potential impacts, adaptation and mitigation. In K. Cochrane, C. De Young, D. Soto and T. Bahri (eds). Climate change implications for fisheries and aquaculture. Overview of current scientific knowledge. FAO Fisheries and Aquaculture Technical Paper. No. 530. Rome. FAO. pp. 151–212.

[3]FAO (2008): Climate change for Fisheries and Aquaculture. Technical background document from the Expert Consultation held on 7 to 9 April, 2008, FAO, Rome. Pp 18

[4]FICKE, A.D., MYRICK, C.A. & HANSEN, L.J. (2007): Potential impacts of global change on freshwater fisheries. Rev. Fish Biol. Fisheries, 17: 581–613.

[5]MUNDAY, P.L., G.P. JONES, M.S. PRATCHETT, AND A. WILLIAMS (2008a): Climate change and the future for coral reef fishes. Fish and Fisheries, 9: 261-285.

[6]MUNDAY,P.L.,M.J.KINGSFORD,M.O'CALLAGHAN AND J.M. DONELSON(2008b): Elevated temperature restricts growth potential of the coral reef fish *Acanthochromis polyacanthus*.Coral Reefs, 27: 927-931

[7]O'REILLY, C.M., ALIN, S.R., PILSNIER, P.D., COHEN, A.S. & MCKEE, B.A.(2003): Climate change decreases aquatic ecosystem productivity of Lake Tanganyika, Africa. Nature ,424:766–68.

[8]POLOCZANSKA, E.S., BABCOCK, R.C., BUTLER, A., HOBDAY, A.J., HOEGH-GULDBERG, O., KUNZ, T.J., MATEAR, R., MILTON, D., OKEY, T.A. & RICHARDSON, A.J. (2007): Climate change and Australian marine life. Oceangr. Mar. Biol. Ann. Rev., 45: 409–480.

[9]PÖRTNER, H.O., BERDAL, B., BLUST, R., BRIX, O., COLOSIMO, A., DE WACHTER, B., GIULIANI, A., JOHANSEN, T., FISCHER, T., KNUST, R., NAEVDAL, G., NEDENES, A., NYHAMMER, G., SARTORIS, F.J., SERENDERO, I., SIRABELLA, P., THORKILDSEN, S. & ZAKHARTSEV, M. (2001): Climate induced temperature effects on growth performance, fecundity and recruitment in marine fish: developing a hypothesis for cause and effect relationships in Atlantic cod (*Ghadus morhua*) and common eelpout (*Zoarces viviparous*). Cont. Shelf Res., 21: 1975–1997.

[10]SARVALA, J., LANGENBERG, V.T., SALONEN, K., CHITAMWEBWA, D., COULTER, G.W., HUTTULA, T., KANYARU, R., KOTILAINEN, P., MAKASA, L., MULIMBWA, N. & MÖLSÄ, H. (2006): Fish catches from Lake Tanganyika mainly reflect fishing practices, not climate. Int.Ver. Limnol., 29: 1182–1188.

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

OUT-OF-SEASON SPAWNING OF THREATENED WEATHERFISH MISGURNUS FOSSILIS (L. 1758) USING COMMERCIAL PREPARATIONS CONTAINING GnRH ANALOGUES

Hliwa P.*,Krejszeff S.†, Król J.*, Kozłowski K.‡,Gomułka P.*

*Department of Ichthyology, University of Warmia and Mazury in Olsztyn, OczapowskiegoSt. 5, 10-719 Olsztyn, Poland, Fax: +48 89 5233754, e-mail: phliwa@uwm.edu.pl
†Department of Lake and River Fisheries, University of Warmia and Mazury in Olsztyn, Poland
‡Department of Fish Biology and Pisciculture, University of Warmia and Mazury in Olsztyn, Poland

Introduction:

Weatherfish is the stenotopic fish species dwelling rivers in the Central Europe. Although, weatherfish has no economic value, it is a significant element of the local water ecosystems. In Poland, weatherfish is listed as a threatened species due to the antropogenic degradation of its natural habitat and unfavourable environmental changes. Suggested active protection includes development of efficient techniques of controlled reproduction and fry rearing for the restocking. The study on the efficacy of GnRH analogues in out-of-season weatherfish reproduction is precisely embedded in the range of actions adressed to maintaining of faunistic biodiversity of water ecosystems in Poland.

Methods:

In total, 31 specimens (17 females and 14 males) of 19.5 ± 2.1 length and 30.2 ± 8.6 g weight were caught in November 2006 by means of electrofishing or trap net from Sątopy-Samulewo artificial water body (former polder) (NE Poland), transported to the laboratory and placed in the tanks supplied with the tap water. Water temperature in the tanks was 8°C and did not vary from the water temperature in Sątopy-Samulewo. During the next 44 days, fish were stimulated by the gradual change of photoperiod (from 10L:14D to 15L:9D) and increase of temperature up to 19°C. Then, weatherfish were selected by sex and divided into three experimental groups: fish stimulated by the intraperitoneal injection of Ovopel (mGnRH)(6 females and 5 males), Ovaprim (sGnRH) (6 females and 5 males) and left without any treatment - control group (5 females and 4 males). Collected groups of eggs were fertilized with the mixed sperm obtained from males. Sperm motility was assessed subjectively under light microscope (magnification 500×). Fertilized eggs were incubated on Petri dishes in water of temperature 20.0±0.1°C, pH 7.7 and oxygen saturation over 80%. Survival rates were evaluated 24 hr after fertilization and soon after hatching. Moreover, the morphological development of the hatched fish was examined. Results were analyzed with the test of significance of structure coefficients, non-parametric Mann-Whitneyand chi2 tests using Statistica 9.1 software.

Results:

The eggs from 83% of females and the sperm from all the males were obtained 12 hours after Ovopel injection. The relative weight of the eggs ranged from 3.2 to 7.8% of the female body weight and was significantly higher than eggs provided in the course of Ovaprim stimulation ($p<0.05$).The time of latency strongly varied in the case of Ovaprim stimulated fish. After Ovaprim treatment ovulation was induced in all the females, however the ovulation time ranged from 36 to 42 hours. The sperm was obtained from 80% of males 24 to 36 hours after Ovaprim injection .The mean motility of the sperm from mGnRH stimulated males was 68% (±12 SD) and from those stimulated with sGnRH 77% (±8 SD). A small amount of the sperm was obtained from the only one male from the control group. Sperm motility was estimated as about 65% and was not significantly different from stimulated groups . However, the mean percentage of alive and normally developed larvae in the control group (4.5%) was significantly lower ($p<0.001$) from Ovopel (72%) and Ovaprim (14.2%) stimulated groups.

Conclusion:

Ovopel was the most efficient GnRH analogue applied for the stimulation of weatherfish reproduction due to the best synchronization of the ovulation, the highest percentage of spermiation, the biggest relative weight of the obtained eggs and the highest larvae survival. Our study proved that the procedure of out-of-season reproduction stimulated by GnRH analogues is efficient and may increase the chance of success of weatherfish reintroduction in Polish waters.

CONSTRUCTION OF EST DATABASE FROM OVARIES OF WILD MATURING EELS

Ijiri S.[1], Tsukamoto K.[2], Chow S.[3], Kurogi H.[3], Gen K.[4], Tanaka H.[4] and Adachi S.[1]

[1]Division of Marine Life Sciences, Graduate school of Fisheries Sciences, Hokkaido University, Hakodate, Hokkaido 041-8611, Japan

[2]Department of Marine Bioscience, Atmosphere and Ocean Research Institute, The University of Tokyo, 5-1-5 Kashiwanoha, Kashiwa, Chiba 277-8564, Japan.

[3]Coastal Fisheries and Aquaculture Division, National Research Institute of Fisheries Science, Fisheries Research Agency, Yokosuka, Kanagawa 238-0316, Japan.

[4]Aquaculture Biology Division, National Research Institute of Aquaculture, Fisheries Research Agency, Minami-Ise, Mie 516-0193, Japan.

Fax: +81-138-40-5545 email: ijiri@fish.hokudai.ac.jp

Introduction:

Since a research project for artificial production of the Japanese eel, *Anguilla japonica*, was commenced in the late 1960s, a continuing process of trial and error has finally achieved the production of second-generation larvae in 2010. However, quality of eggs obtained through controlled maturation is still highly variable, and the early survival rates of the larvae are usually extremely low. The low egg quality may be caused by abnormal oocyte development induced artificially by salmon pituitary injections. To address this issue, we began seeking wild maturing eels in their spawning area.

Methods:

On receiving a success in capturing post-spawning Japanese eels with their gonads appeared to be degenerated in 2008 [1, 2], a large-scale trawling survey was conducted by four research cruises in the following spring to summer, at the southern part of the West Mariana Ridge. During a 2009 trawling survey, four males and four females of the Japanese eel in spawning condition and two mature males and one female of the giant mottled eel (*Anguilla marmorata*) were caught [3]. Trawling surveys were also carried out in 2010, and one female of *A. marmorata* in maturing condition was captured. Total RNA was extracted from the ovaries of *A. japonica* and *A. marmorata*. Oligo-dT primed cDNA libraries were used for sequencing. Sequencing was performed using Genetic Analyzer II (GAII, Illumina) and 454 FLX Titanium (454FLX, Roche). Sequence reads obtained by GAII were assembled by Velvet assembler, then the GSII contigs and sequence reads obtained by 454FLX were assembled together into contigs by using TGICL clustering software.

Results and discussion:

Two of three female *A. japonica*,t captured in the new moon night, were in a condition just after spawning. The ovaries possessed clear post-ovulated follicles and oocytes in early to mid-vitellogenic stage. This ovary in the early to mid-vitellogenic stage was used for sequencing. The third female had still ovulated oocytes (eggs before spawning). The eggs seemed to be over-ripened judging from the over-conflated oil droplet. The electrophoresis profiles of the RNA extracted from the eggs seemed to occur its fragmentation. *A. marmorata* captured in 2010 had ovary in late-vitellogenic stage with oocytes that occur transparent in their peripheral region. The late-vitellogenic ovary was not obtained in the wild *A. japonica*, therefore, *A. marmorata* ovary was used for sequencing as a reference. From *A. japonica* ovary in the early to mid-vitellogenic stage, GAII single sequencing run produced 14,372,520 reads, with average length of 43 nucleotides. These reads were assembled into 63,622 contigs, with average 214 bases. A half-plate run of 454FLX sequencing produced 320,429 reads, with average length of 293 nucleotides. These reads were assembled into 98,310 contigs including 85,673 singlets, with average length of 447 bases. The contigs constructed from GAII sequences and sequence reads from 454FLX were assembled together by TGICL clustering software. This assemble produced 128,840 contigs, with average length of 341 nucleotides (Table 1). From *A. marmorata* ovary in the late-vitellogenic stage, GAII single sequencing run produced 34,612,320 reads, with average length of 76 nucleotides. A half-plate run of 454FLX sequencing produced 615,163 reads, with average length of 414 nucleotides. These reads were assembled into 91,691 contigs including 65,455 singlets (Table 2). An attempt for cross mapping between *A. japonica* and *A. marmorata* short sequence reads produced by GAII

Table 1 Sequence reads and contigs from *A. japonica* ovary

	Number of reads	Average read length	Number of contigs	Average contig length
GAII	14,372,520	43	63,622	214
454FLX	320,429	293	98,310	447
GAII + 454FLX	-	-	128,840	341

Table 2 Sequence reads and contigs from *A. marmorata* ovary

	Number of reads	Average read length	Number of contigs	Average contig length
GAII	34,612,329	76	not yet	not yet
454FLX	615,163	414	91,691	not yet

resulted in 71 % and 62 % of GAII reads of *A japonica* and *A. marmorata* could be mapped onto contigs of *A. japonica*, respectively.

Conclusion:

EST database was constructed from wild matured *A. japonica* and maturing *A. marmorata* ovaries by the aid of two types of next generation sequencing. GAII produced higher number of short sequence, in contrast, 454FLX produced longer sequences but in lower number. Clustering of these sequence reads together appeared to accelerate formation of contigs. The result from that almost same percentage of GAII sequence reads from *A. japonica* and *A. marmorata* could mapped onto *A. japonica* contigs may indicates that *A. marmorata* sequence reads can be used for transcriptome analyses in *A. japonica* ovaries.

References:

[1]CHOW, S., KUROGI, H., MOCHIOKA, N., KAJI, S., OKAZAKI, M., TSUKAMOTO K. 2009. Discovery of mature freshwater eels in the open ocean. Fish. Sci., 75: 257-259.

[2]KUROGI, H., OKAZAKI, M., MOCHIOKA, N., JINBO, T., HASHIMOTO, H., TAKAHASHI, M., TAWA, A., AOYAMA, J., SHINODA, A., TSUKAMOTO, K., TANAKA, H., GEN, K., KAZETO, Y., CHOW, S. 2011. First capture of post-spawning female of the Japanese eel *Anguilla japonica* at the southern West Mariana Ridge. Fish. Sci., 77: 199-205.

[3]TSUKAMOTO, K., CHOW, S., OTAKE, T., KUROGI, H., MOCHIOKA, N., MILLER, J.M., AOYAMA, J., KIMURA, S., WATANABE, S., YOSHINAGA, T., SHINODA, A., KUROKI, M., OYA, M., WATANABE ,T., HATA, K., IJIRI, S., KAZETO, Y., NOMURA, K.,TANAKA, H. 2011. Oceanic spawning ecology of freshwater eels in the western North Pacific. Nature Communications 2: Article 179.

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

POOR REPRODUCTIVE SUCCESS IN A SWEDISH ARCTIC CHARR BROODSTOCK - BIOLOGICAL OR ENVIRONMENTAL EFFECT?

Jeuthe H., Nilsson J. and Brännäs E.

Department of Wildlife, Fish and Environmental Studies, Swedish University of Agricultural Sciences, Skogsmarksgränd, Umeå, Sweden. Fax +46-907868162 e-mail: henrik.jeuthe@slu.se

Introduction:

The Swedish Arctic charr (*Salvelinus alpinus*) breeding programme started in 1985, described in [1, 2]. A native strain of charr from Lake Hornavan in northern Sweden (66° 14' N, 17° 300 E) was selected to form the basis of this breeding programme. Since the start of the programme no individuals of other origin have been included and the strain has been kept at the Fisheries Board Research station in Kälarne, central Sweden. The result of 25 years of selective breeding is a fast-growing, late-maturing fish with high pigment retention in the fillet. However, the reproductive success is far from satisfactory. Gamete quality is generally low with high frequency of opaque eggs and watery sperm. The spawning period is often prolonged and poorly synchronised within and between sexes, leading to increased handling and hence stress of the fish as well as a more difficult stripping procedure. Fertilisation and hatching rates are erratic and on average very low compared to other salmonids in Scandinavian aquaculture, typically between 30 and 70 percent. The question is whether this problem derives from biological features of the strain itself or from suboptimal holding conditions. This was tested by rearing a split broodstock at two separate facilities and comparing reproductive success.

Methods:

Part of the 2005 generation Arctic charr from the Swedish breeding programme was divided into two replicates of equal family distribution. One group was kept at the Kälarne station, the other was sent to a commercial fish farm in Slussfors, 300 km further north. The two groups were reared until sexual maturation at age 3.5 and stripped at two locations. The Kälarne broodstock was stripped on the site while the Slussfors broodstock was transported to a hatchery 130 km further south three weeks before the first fish was stripped. After fertilisation the eggs from Slussfors were transported back to Kälarne and incubated under equal conditions as their resident relatives. Environmental conditions at the two facilities differ mainly in two aspects: temperature and light. Both are supplied with lake water of ambient temperature, but the location of the Slussfors facility enables favourably lower summer temperatures not exceeding 16°C while temperatures may reach over 20°C in Kälarne. The fish are kept in indoor tanks with artificial lighting at natural photoperiod in Kälarne while Slussfors is an outdoor net pen facility with natural light.

Results:

Differences were found between the groups in synchronisation of spawning period and hatching rates. The Kälarne group exhibited a prolonged stripping procedure, 10 workdays extending just over a month at temperatures between 6-10°C, while the Slussfors stripping was completed in two sessions one week apart at 3-5°C. Spawning also occurred earlier in Slussfors than in Kälarne, October 9-15 and October 17 to November 19 respectively. Survival of the eggs differed significantly between the two groups, with hatching rates of 0.62 ± 0.22 and 0.38 ± 0.20 for Slussfors and Kälarne, respectively.

Conclusion:

There is a strong environmental factor to the low reproductive success of the studied Arctic charr broodstock. The high summer temperature in Kälarne together with the poor synchronization of the ovulation resulted in low egg quality. The hatching rates increased nearly two fold with more favourable temperature and light conditions in Slussfors, but were still below 70 percent which is considerably lower than other salmonids. Salmon and rainbow trout typically have a hatching rate of more than 90 percent under similar conditions. There are other factors such as the effect of handling stress and broodstock diets that need in-depth investigation regarding the critical environmental factors and holding conditions of the Arctic charr broodstock.

References:

[1]ERIKSSON, L.-O., ALANÄRÄ, A., NILSSON, J. AND BRÄNNÄS, E. 2010. The Arctic charr story: development of subarctic freshwater fish farming in Sweden. Hydrobiologia , 650(1): 265-274.

[2]NILSSON, J., BRÄNNÄS, E. AND ERIKSSON L.O. 2010 The Swedish Arctic charr breeding programme. Hydrobiologia, 650(1): 275-282.

HOW PHYSIOLOGICAL STATUS AND IMMUNE DEFENSE ARE AFFECTED BY PHOTO-THERMAL REGIMES AND DOMESTICATION PROCESS IN CAPTIVE EURASIAN PERCH?

Mandiki S.N.M.[1], Henrotte E.[1], Milla S.[1], Douxfils J., Wang N., Rougeot C.[2], Vandecan M.[2], Mélard C.[2], Kestemont P.[1]

1. Department of Biology, URBO, University of Namur, Rue de Bruxelles 61, B-5000 Namur, Belgium. +32-81724362 robert.mandiki@fundp.ac.be
2. CEFRA, University of Liège, Chemin de la Justice 10, B-4500 Tihange, Belgium.

Introduction:

While high reproductive performances are actually obtained in perch *Perca fluviatilis* using different rearing systems [1], high disease outbreaks during or after the spawning season is still one of the major constraints for sustainable development of perch farming in the European countries. Such increase in disease incidence has been observed in some fish species and was related to depression in immune system during winter or after migration for reproduction [2], but the relationship with reproductive events and domestication is poorly described.

Methods:

Experiment 1 was carried out to study to what extent culture conditions inducing gonad maturation affect seasonal changes in physiological status and immune system. In order to inhibit or stimulate gonad maturation, two groups of captive breeders (250-300g) were maintained in constant (CC) or natural (NC) temperature and photoperiod regimes from January to March. Experiment 2 investigated whether domestication level influence stress and immune status at the end of oogenesis and along the spawning season comparing two successive generations (F1 and F4) of captive breeders in March, April and May. For both experiments, each population was tested in duplicate 100L-tanks of 10 breeders each in RAS conditions. Physiological stress status was evaluated by plasma cortisol and glucose, while immune status was investigated by plasma lysozyme activity, spleen superoxide production, and plasma total immunoglobulin. In addition, body condition parameters (K1), spleen and gonad indices were checked.

Results and discussion:

No body condition parameter was affected by the photo-thermal regimes or domestication level. Stress status was relatively lower in NC breeders than in CC ones. Indeed, plasma glucose increased significantly from January to March for both groups but values were higher ($P<0.05$) in CC breeders than in NC ones, especially in February (364±102 vs 182±34 µg/ml). Cortisol level did not indicate high stressful status in both populations but values were higher ($P<0.05$) in CC breeders than in NC ones in February (Figure 1A). Previous studies reported higher levels (120-180 ng/ml) of plasma cortisol in perch subjected to acute stress [3-4]. Regarding changes in non-specific immune defence, plasma lysozyme activity was lower ($P<0.05$) in NC fish than in CC ones but values increased in March (Figure 1B). In contrast, superoxide production by spleen was higher in NC breeders than in the CC group, especially in February (Figure 1C). In experiment 2, stress status differed between the two breeder generations with an increase in plasma cortisol observed in May only for F4 ($P<0.05$,

Figure 1: Changes in plasma cortisol (A), plasma lysozyme activity (B) and spleen superoxide production (C) levels in Eurasian perch breeders maintained in constant (CC) or natural (NC) temperature and photoperiod regimes from January to March. Values with different letters are significantly different between groups and over time ($p<0.05$).

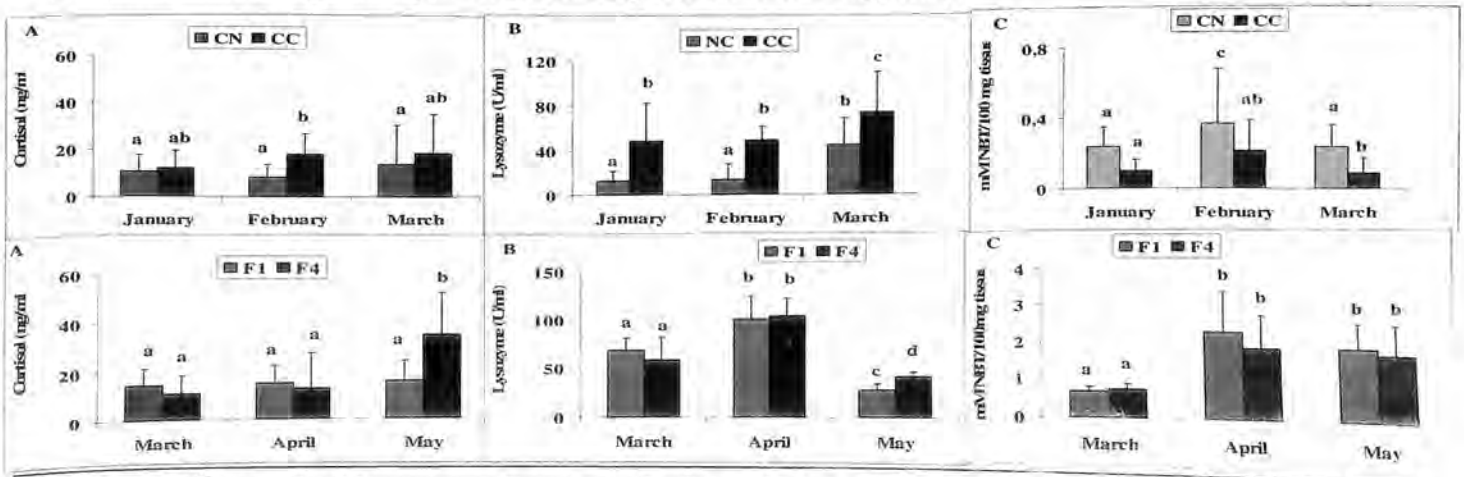


Figure 2A). Moreover, plasma glucose level was higher (P<0.05) in F4 (290±67 µg/ml) than in F1 (163±27 µg/ml) due to a decrease over the time in the latter. Lysozyme activity increased during the spawning time and decreased afterwards (P<0.05, Figure 2B). The same profile was observed for plasma total Ig in both populations (data not shown). Spleen superoxide production was lower in March and increased (P<0.0.5) afterwards in both populations (Figure 2C). Differences in stress status and immune compounds between the two generations of breeders corroborate a recent report on captive juvenile perch [4].

Conclusion:

Photo-thermal culture conditions (inducing gonad maturation) or domestication level influence both stress and immune indicators in Eurasian perch. Specifically, some immune compounds were lowered during gonad maturation or spawning processes, while other remained unaffected. It seems likely that these changes are not only related to variations in photo-thermal conditions but also to an intrinsic trade- off between reproductive processes and immune system. More investigations are still needed to describe the relationship between the reported changes in immune system and the increase in disease incidence during the spawning season.

Figure 2: Changes in plasma cortisol (A), lysozyme activity (B) and spleen superoxide production (C) in Eurasian perch breeders at the end of gonad maturation, during and after the spawning season. Values with different letters are significantly different between groups and over time (p<0.05).

Acknowledgements: This research was supported by the Belgian National Funds for Scientific Research (FNRS)

References:

[1]FONTAINE P., PEREIRA C., WANG N., MARIE M., 2006. Influence of pre-inductive photoperiod variations on Eurasian perch *Perca fluviatilis* broodstock response to an inductive photothermal program. Aquaculture ,255: 410-416.

[2]KORTET R., TASKINEN J., SINISALO T., JOKINEN I., 2003. Breeding-related seasonal changes in immunocompetence, health state and condition of the cyprinid fish, *Rutilus rutilus*, L. Biological Journal of the Linnean Society, 78: 117-127.

[3]ACERETE L., BALASCH J.C., ESPINOSA E., JOSA A., TORT L., 2004. Physiological responses in Eurasian perch (*Perca fluviatilis*, L.) subjected to stress by transport and handling. Aquaculture, 237: 167-178.

[4]OUXFILS J., MANDIKI S.N.M., MAROTTE G., WANG N., SILVESTRE F., MILLA S., HENROTTE E., VANDECAN M., ROUGEOT C., MELARD C., KESTEMONT P., 2011. Does domestication process affect stress response in juvenile Eurasian perch *Perca fluviatilis*? Comp. Biochem. Physiol., Part A, 159: 92–9.

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

LONG TERM EFFECTS OF MASCULINIZING TREATMENTS ON THE REPRODUCTIVE CHARACTERISTICS OF GREY MULLET (*MUGIL CEPHALUS*)

Meiri-Ashkenazi, I.[1], Solomonovich, R.[1], and Rosenfeld, H.[1]

Israel Oceanographic and Limnological Research (IOLR), National Center for Mariculture (NCM), P.O. Box 1212, Eilat 88112, Israel. Fax: +972-8-6375761 e-mail: irismeiri@gmail.com

Introduction:

The grey mullet, *Mugil cephalus,* are fished and farmed world-wide. To avoid the continuous pressure on the wild populations, we developed at IOLR-NCM successful breeding [1] and larval rearing protocols for captive mullets, along with developing formulated growout feeds for their rearing to market size. Following a successful completion of this research, improved economics of production is expected by culturing all-female mullet populations, largely due to their highly prized roe used for preparing a seafood delicacy called "Karasumi". To achieve this goal, the current study has adopted the indirect feminization strategy [2], involving the masculinization of genotypic females, and crosses of the produced neomales with normal females, to produce a female monosex mullet population. Previous studies have demonstrated successful sex reversals in mullet using both an androgen to masculinize [3] and an oestrogen to feminize [4] the fish. However, there was no information whether the masculinized fish turned to be functional phenotypic males at maturation. Therefore, our specific objectives were to follow the sex-reversed fish to sexual maturity and to evaluate their phenotypic sexual stability and fertility.

Methods:

Experimental Fish - Hatchery produced mullet fry at our facility (IOLR-NCM) were maintained in 1- 5 m^3 tanks, supplied with ambient (Gulf of Eilat, Red Sea) seawater at 40 ppt salinity and subjected to natural fluctuations of photoperiod, light and temperature. Fish were fed daily at the rate of 1.5% of their body weight using a 35% crude protein and 7.2% lipid diet, according to our (IOLR-NCM) feed formulation and feeding regime.

Induced masculinization - A masculinized phenotype was obtained by exposing for 4 months, mullet fry, at 3 age categories (3, 6 and 9 month old), to food supplemented with either: (i) methyltestosterone (MT; 15 or 10 mg MT/Kg of food) or (ii) Fadrozole (100 mg/Kg food). In order to study the phenotypic sexual stability, the 6 month old group that obtained the primary treatment of 15 mg MT/Kg food was divided randomly into two groups: one received repeated MT exposure via a slow release vehicle (administered via ethylene-vinyl acetate copolymer [EVAc] implants; 5 mg MT/pellet) at 2, 3 and 4 years after the primary treatment. The second group was used as untreated controls, which obtained only the primary treatment (see above). Sex was determined by vitellogenin dot blot analysis and/or gonadal biopsies [1]. Presence of milt in male mullets was checked by applying gentle abdominal pressure and a sample of milt was collected to evaluate sperm motility, morphology and spermatocrit.

Results and discussion:

As in wild stocks, the sex ratio among control mullet groups did not differ significantly ($P > 0.05$) from the expected 1:1 male:female ratio. These results rule out the occurrence of skewed sex ratios due to culture conditions, and are consistent with those of previous studies carried out in Taiwan [3, 5] and North Carolina [6].The results of the masculinizing studies further define, in mullets, the period of 6 to 9 month of age, as a labile phase when the differentiating gonads are most susceptible to androgens.

The most potent treatment [MT-6] gave rise to 100 % males upon the completion of sexual differentiation. Nevertheless, at sexual maturity (3 years after treatment) markedly lower male percentages (70%) were observed in this group, suggesting that the grey mullet can spontaneously sex reverse. Interestingly, higher male percentages (90%) were detected among retreated fish boosted with MT containing EVAc implants. The milt produced by the latter fish revealed characteristics (i.e. sperm count, motility and morphology) resembling those found for untreated males.

Conclusion:

The grey mullet appears to have high sexual plasticity also away from the sex-differentiation period, which is atypical to most other gonochoric fish that exhibit fixed sexuality once sex differentiation is being completed. Similar sexual plasticity was recently documented also in trout [7], which attests to a spontaneous sex reversal and stresses the need to preserve functional phenotypic sex by repeated treatments. Besides their aquaculture applicability, our results propose the grey mullet as an important model for further studying germ cells plasticity in fish.

References:

[1]AIZEN, J., MEIRI, I., TZCHORI, I., LEVAVI-SIVAN, B., ROSENFELD, H. 2005. Enhancing spawning in the grey mullet (*Mugil cephalus*) by

removal of dopaminergic inhibition. Gen. Comp. Endocrinol., 142: 212-221.

[2]PIFERRER, F. 2001. Endocrine sex control strategies for the feminization of teleost fish. Aquaculture, 197:229-281.

[3]CHANG, C.F., HUNG, C.Y., CHIANG, M.C., LAN, S.C. 1999. The concentrations of plasma sex steroids and gonadal aromatase during controlled sex differentiation in grey mullet, *Mugil cephalus*. Aquaculture, 177:37-45

[4]CHANG, C.F., LAN, S.C., PAN, B.S. 1995. Feed administration of estradiol-17 beta stimulates female differentiation in juvenile grey mullet *Mugil cephalus*. Zool. Studies, 34:257-264.

[5]CHANG, C.F., LAN, S.C., CHOU, H.Y.1995. Gonadal histology and plasma sex steroids during sex differentiation in grey mullet, *Mugil cephalus*. J. Exp. Zool., 272:395-406

[6]BICHY, J.B. 2004. A life history assessment on the reproduction and growth of striped mullet, *Mugil cephalus*, in North Carolina, MA thesis, North Carolina State University.

[7]YOSHIZAKI, G., ICHIKAWA, M., HAYASHI, M., IWASAKI, Y., MIWA, M., SHIKINA, S., OKUTSU, T. 2010. Sexual plasticity of ovarian germ cells in rainbow trout. Development, 137(8):1227-30.

DIETARY LEVELS AND METABOLIC PATHWAYS OF ARACHIDONIC ACID POSSIBLY ASSOCIATED WITH REPRODUCTIVE DYSFUNCTIONS IN SENEGALESE SOLE (*SOLEA SENEGALENSIS*)

Norambuena F.[1] **Morais S.**[3] **Callol A.**[2] **Mackenzie S.**[2] **Bell J.G.**[3] **Estévez A.**[1] **Tocher D.R.**[3] and **Duncan N.**[1*]

[1]IRTA-Sant Carles de la Rápita, Tarragona, Spain
[2]Universitat Autónoma de Barcelona, Barcelona, Spain
[3]University of Stirling, Stirling, UK
*Corresponding author: IRTA-Sant Carles de la Rápita, Ctra. Poble Nou Km 6, 43540-Sant Carles de la Ràpita, Tarragona, Spain Fax +34-977744138 email: neil.duncan@irta.cat

Introduction:

The spawning performance of cultured sole broodstock (G1 generation) is poor, compared to wild counterparts. Studies in IRTA indicated that the problem was behavioural and particularly male G1 broodstock did not complete courtship with the liberation and consequent fertilisation of eggs. This situation may be explained by inadequate broodstock nutrition and studies were performed to define nutritional differences between wild and G1 broodstock and examine optimal levels of arachidonic acid (ARA) affecting metabolic pathways. ARA is an essential fatty acid precursor of 2-series prostaglandins (PGF2α and PGE2) which are produced by cyclooxygenase enzyme activity (COX2) [1,2]. Prostaglandins (PGF2α) are released by females as pheromones to trigger courtship behaviour and to increase milt production in males [3,4].

Methods:

The essential fatty acid profile (EFA), cyclooxygenase (COX2) activity and prostaglandin levels (PGF2α, PGE2, PGF3α and PGE3) were analyzed in muscle, liver, gills, testis, ovary and blood from wild (n=22) and cultured (n=25) broodstock. Further studies were focused on assessing optimal dietary levels of ARA in an experiment in which cultured fish were fed three experimental diets with different ARA level (0.7%, 2.5%

Figure 1. Prostaglandins PGF3α (mean ± SEM) in fish tissues of wild and cultured fish female (A) and male (B).* denote significant differences ($p<0.05$)

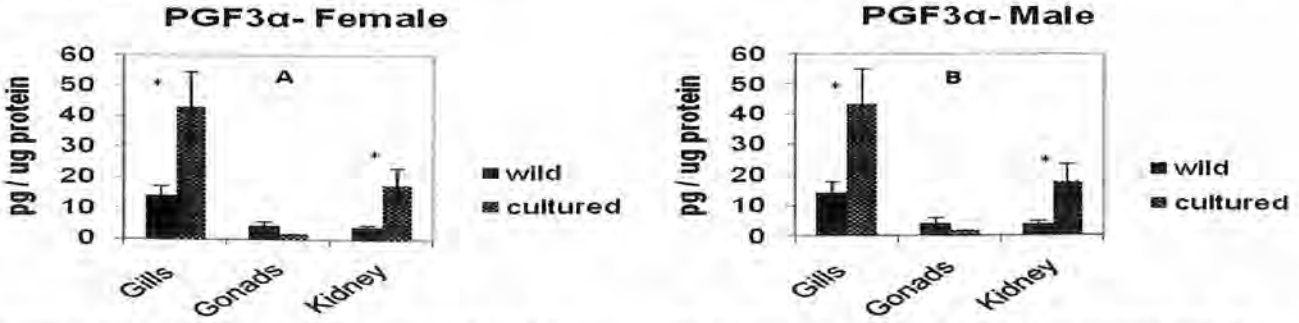
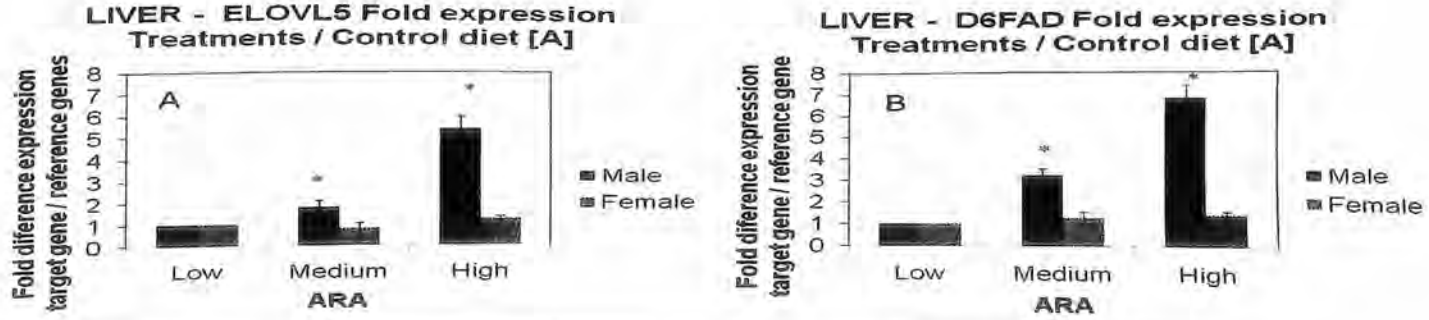


Figure 2. Relative expression of elongase (A) and desaturase (B) in liver of female and male of Senegalese sole in response to changes in dietary arachidonic composition (Diet A, B and C), normalised by the expression of UBQ and RPS4 (reference genes). * denote significant differences, calculated by REST ($p<0.05$)



and 5.2% ARA of total fatty acid in diet, TFA, diet low, medium and high respectively). Samples of mid intestine and liver were collected for studies of fatty acyl desaturase (D6FAD), elongase (ELOVL5), and cyclooxygenase (COX2) gene expression.

Results:

Wild fish showed a significantly higher ARA content than cultured fish. The testis in wild males had 7.8% ARA, a 5-fold higher content than cultured fish, and a similar trend was observed in liver. Concurrently, significant differences in EPA/ARA ratio were observed with higher ratios in cultured fish. The RT-PCR tissue distribution showed that COX2 was expressed mainly in gills, testis, and oviduct. COX2 (Q-PCR) in the liver of cultured fish had 13-fold lower expression than wild fish. Levels of prostaglandins (PGF3α) in cultured fish were high, especially in gills and kidney (Fig. 1). High levels of ARA occurred together with relatively high amounts of 22:4n-6 and 22:5n-6. The dietary experiment showed that the expression of D6FAD and ELOVL5 in the liver showed differential nutritional regulation. Levels of D6FAD and ELOVL5 transcripts were significantly higher in the liver of fish fed high levels of ARA compared with the low group (control) (6.8-fold in D6FAD), but differences in gene expression were only observed in males (Fig 2). The same trend was observed in the levels of COX2 transcript in response to dietary ARA content (2.8-fold higher with high ARA).

Conclusion:

Wild Senegalese sole accumulated more ARA in tissues and metabolised ARA to 22:4n-6 and possibly 22:5n-6 to a certain degree. An increase in dietary ARA levels supplied to cultured sole produced a similar accumulation and an increase in gene expression of D6FAD and ELOVL5, although this regulation was only observed in males. An increase in ARA dietary levels also produced similar changes in COX2 expression although in general wild fish show higher expression of COX2 compared to cultured fish. Differences in prostaglandin production were also observed and PGF3α levels in blood were significantly higher than other PGs. The differences in levels of ARA and its potential effects on eicosanoid metabolic pathways will be discussed in relation to differences in reproductive success between wild and cultured sole.

References:

[1]SMITH, W. L. (1989) Biochem. J., **259**, 315-324
[2]TOCHER, D. R. (2003) Rev. Fish. Sci., **11**, 107-184
[3]WADE, M. G., KRAAK, G. V. D. (1993) Gen.Comp. Endocrinol., **90**, 109-118
[4]SORENSEN, P. W., AND STACEY, N. E. (2004) New Zeal. J. Mar. .,Fresh **38**, 399-417

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

MORPHOMETRIC CHARACTERISTICS ANALYSIS BETWEEN THE MALE AND FEMALE OF PROTANDROUS FALSE CLOWNFISH, *Amphiprion ocellaris*

Norazmi-Lokman, N. H., Abol-Munafi, A. B. and **Asma, N. A.**

Department of Fishery Science and Aquaculture, Faculty of Agrotechnology and Food Science, Universiti Malaysia Terengganu, 21030 Kuala Terengganu, Terengganu, Malaysia. email: lokhakim@umt.edu.my

Introduction:

False clownfish, *Amphiprion ocellaris*, are known to be protandric hermaphrodite where it could change its sex from male to female. This clownfish species is one of the most popular marine ornamental species and has a very high market demand. As new and advance technology in aquaculture is developed, the ability to breed *A. ocellaris* in captivity has become possible. Unfortunately, the lack of information especially on its sexual dimorphism has affected the broodstock production of *A. ocellaris* in captivity since the sex of hatchery produced fish cannot be identified. Breeders still depends on broodstocks collected from wild where the sex is distinguished by its body size since usually the females are larger than males. To date there is still no scientific report on the sexual dimorphism of *A. ocellaris* available. The ability to identify and differentiate the sexes of *A. ocellaris* is crucial especially for broodstock development program since *A. ocellaris* needs to be paired for breeding [2]. Study on the morphometric characteristics differences between the sex of *A. ocellaris* is very important so that the process of sexing *A. ocellaris* in captivity will be efficient and not wasteful in terms of money and energy. Therefore this study was conducted to analyze the morphometric characteristics between the male and female of *A. ocellaris*.

Methods:

Twenty five males and females of *A. ocellaris* was collected at Bidong Island, Terengganu. Sixteen morphometric parameters including standard length were measured to the nearest 0.1mm. This is followed by the measurements of 18 Truss variables. The morphometric parameters are shown in Fig. 1 while the Truss network variables are shown in Fig. 2. All measurements were transformed into size-independent shape variables to eliminate the size effect in the data set since all the samples used in this study comes in different sizes with unknown age [4]. The measurements were transformed using the formula: $M_{adj} = M/SL$ (M_{adj} = adjusted measurements; M = original measurements; SL = standard length) [3 and 4]. All the transformed measurements were then analyzed by using independent T-test. The statistical analysis was carried out with SPSS 15.0 for Windows.

Results and Discussions:

Among the 15 morphometric measurements, 2 parameters were significantly different ($P<0.05$) between the female and male of *A. ocellaris*: DALAV (direct distance between the anterior edge of the upper lip and the anterior insertion of the first pelvic fin) and DADPD (direct distance between the anterior insertion of the first dorsal fin and the posterior insertion of the last dorsal fin). For the Truss network variables, 7 variables showed a significant different ($P<0.05$) between the sexes. The 7 Truss network variables were particularly b (snout to end of mouth), e (above eye to origin of pelvic fin), f (end of mouth to origin of dorsal fin), h (origin of dorsal fin to origin of pelvic fin), l (origin of pelvic fin to end of anal fin), o (end of dorsal fin to end of the caudal fin) and p (end of anal fin to upper insertion of caudal fin). Table 1 shows the results of the 15 morphometric measurements statistical analysis while Table 2 shows the results of the 18 Truss network variables statistical analysis. This shows that the process of changing sex from male to female in *A. ocellaris* is accompanied by rapid growth at this nine body parts. Clownfish modify their sizes to ensure harmony in social groups. They will only change sex followed by their body size if the social hierarchy is disturbed such as in the case where the female dies or being removed from the group [1]. The detail explanation on why this phenomenon occur are not possible at present since the functional role of all the

Figure 1: Sixteen morphometric measurements.

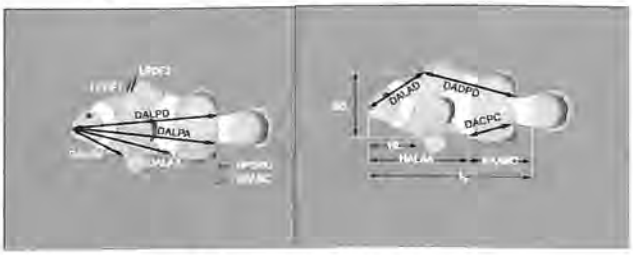
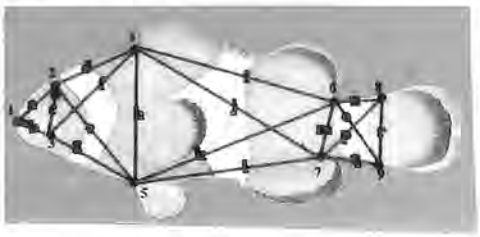


Figure 2: Truss network measurements.



mentioned body parts in the sex determination and sex change mechanism of *A. ocellaris* are still unknown.

Table 1: Morphometric measurements analysis

Parameters	Sex	N	Sig.
HALAA	F	25	0.079290
	M	25	
HAABC	F	25	0.188388
	M	25	
DALAV	F	25	0.004240*
	M	25	
HPABC	F	25	0.552210
	M	25	
HPDBC	F	25	0.363683
	M	25	
BD	F	25	0.067977
	M	25	
DALAA	F	25	0.816583
	M	25	
DALPA	F	25	0.580507
	M	25	
DALPD	F	25	0.654392
	M	25	
DALAD	F	25	0.681018
	M	25	
DADPD	F	25	0.036701*
	M	25	
DACPC	F	25	0.435465
	M	25	
LFDF1	F	25	0.242164
	M	25	
LFDF2	F	25	0.148389
	M	25	
HL	F	25	0.267168
	M	25	

Note: Data are based on transformed measurements
*P<0.05

Table 2: Truss network variables analysis

Parameters	Sex	N	Sig.
a	F	25	0.400
	M	25	
b	F	25	0.002*
	M	25	
c	F	25	0.837
	M	25	
d	F	25	0.469
	M	25	
e	F	25	0.003*
	M	25	
f	F	25	0.002*
	M	25	
g	F	25	0.248
	M	25	
h	F	25	0.002*
	M	25	
i	F	25	0.217
	M	25	
j	F	25	0.440
	M	25	
k	F	25	0.059
	M	25	
l	F	25	0.013*
	M	25	
m	F	25	0.126
	M	25	
n	F	25	0.853
	M	25	
o	F	25	0.031*
	M	25	
p	F	25	0.011*
	M	25	
q	F	25	0.679
	M	25	
r	F	25	0.101
	M	25	

Note: Data are based on transformed measurement
*P<0.05

Conclusions:

A. ocellaris shows a clear sexual dimorphism where 2 morphometric parameters, DALAV and DADPD were significantly different (P<0.05) between the female and male. For the Truss network variables, 7 variables, b, e, f, h, l, o and p showed a significant different (P<0.05) between the sexes. Further studies are recommended on the sexual determination mechanism and differentiation process of *A. ocellaris*.

References:

[1]BUSTON, P. 2003. Size and growth modification in clownfish, sex change is not the only way these fish achieve dominance - they grow into the role. Brief communication- Nature,424:10 July 2003.

[2]LIEW, H. J. 2006. Spawning, development and larva rearing of False Clownfish, *Amphiprion ocellaris* under captive conditions. M. Sc. thesis. Institute of Tropical Aquaculture, Universiti Malaysia Terengganu. 135p.

[3]REIST, J. D. 1985. An empirical evaluation of several univariate methods that adjust for size variation in morphometric variation. Can. J. Zool., 63: 1429-1439.

[4]TURAN, C. 1999. A note on the examination of morphometric differentiation among fish populations: The Truss system. Tr. J. Zoology, 23: 259-263.

ISOLATION OF STURGEON PRIMORDIAL GONOCYTES AND SPERMATOGONIA AS MATERIAL FOR BIOTECHNOLOGY

Psenicka M.*, Saito, T. °, Fujimoto T. °, Arai K. °, Yamaha E #

*University of South Bohemia in Ceske Budejovice, Faculty of Fisheries and Protection of Waters, South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Zatisi 728/II, 389 25 Vodnany, Czech Republic. Fax +42-387774634 e-mail: psenicka@frov.jcu.cz

°Laboratory of Aquaculture, Genetics and Genomics, Faculty of Fisheries Sciences, Hokkaido University, Hokkaido 041-8611, Japan

#Nanae Fresh-Water Laboratory, Field Science Center for Northern Biosphere, Hokkaido University, Hokkaido 041-1105, Nanae, Japan

Introduction:

Most of sturgeons belong to endangered or critical endangered species (The IUCN Red List of Threatened Species) mostly due to caviar and complications with their reproduction characteristics such as late maturation. Sturgeon gametes are unique in term of their morphology and physiology. Sturgeon eggs covered with very thick layers are perforated by tens of micropyles at animal pole, while their spermatozoa are equipped with acrosomes, which fish spermatozoa usually lack. It was suggested that the sturgeon acrosome serves like an anchor and a spear [1]. Genetic and cytogenetic manipulations with sturgeons are very difficult. Therefore it would be very suitable to develop a more efficient system of sturgeon reproduction by means of biotechnology and micromanipulations with germ line cells and embryos. Primordial germ cells (PGCs) or subsequently spermatogonia are the only cells in developing embryos with potential to transmit genetic information to the next generation. Therefore they have a potential to be of value for gene banking and cryopreservation, particularly via the production of donor gametes with germ–line chimeras [2]. Nevertheless the first step for successful transplantation of these germ line cells is their visualization and isolation.

Methods:

Visualization of PGCs is usually done *in vivo* according to Saito et al. [3] using injection of GFP-zebrafish nos1 3'UTR mRNA to the area of egg with PGC precursor localization.

Results:

In our study electron microscopic observation revealed that only the vegetal pole of sturgeon egg contains a germ plasm-like structure, nuage (Fig. 1), which are suggested to be the precursors of germinal granules, therefore an injection of the fluorescent probe for PGCs visualization was applied into the vegetal pole in 1-4 cell stage embryo, while the embryos were incubated at 14°C. The PGCs were then localized using fluorescence stereomicroscope between gut and

Fig. 1 shows sturgeon egg envelope (E), mitochondria (M), cortical granule (C), yolk (Y) and nuage (arrow).

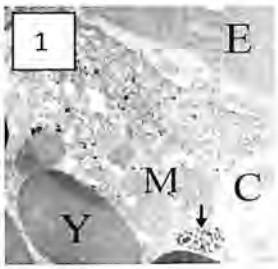


Fig. 2 shows PGCs (arrow) in sturgeon embryo after hatching.

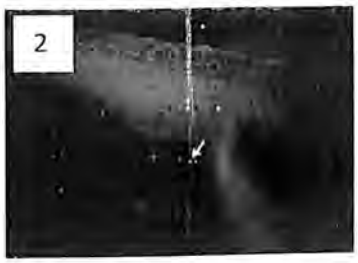


Fig. 3 shows isolated spermatogonia from layers of 10-30 % percoll, stained with antispermatogonia antigen 1, a) under light and b) under fluorescence.

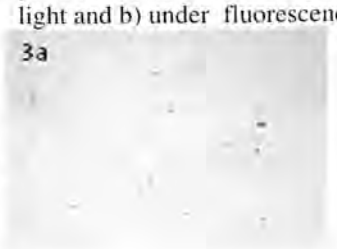
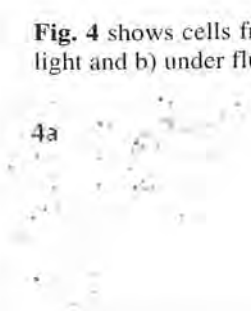


Fig. 4 shows cells from layers of 30-50 % percoll, a) under light and b) under fluorescence.



pronephric duct in the base of yolk extension region at 5th day after fertilization. The cluster of PGCs then migrated to the posterior region of yolk extension. The PGCs were later spread along the all yolk extension from 6th to 13th day (Fig. 2).

Spermatogonia could be an alternative of PGCs for biotechnology. They were isolated from immature testes of 2-4 years old sturgeon males, which did not produce any spermatozoa. The testes was cut into small pieces and incubated in 0.2% collagenase for 2 hours. The obtained homogeneous suspension was filtered through 50 µm filter. The cell suspension was then sorted by percoll gradient (10, 15, 20, 25, 30, 35, 40, 50 %) and stained with antispermatogonia-specific antigen 1 originally made for Japanese eel by Kobayashi et al. [4] combined with Anti-Rabbit IgG–FITC antibody. The cells obtained from layer 10-30% of percoll solution showed the specific fluorescent signal and shape of spermatogonia. The layers below contained cells without almost any signal with spermatocyte-like size (Fig. 3).

The isolated germ-line cells can be then cryopreserved according to Okutsu et al. [5]. To make germ-line chimeras, the PGCs or spermatogonia are isolated from donor and transplanted into the host embryos. The hosts become germ-line chimeras if the transplanted cells successively migrate to genital primordium and differentiate into functional gametes. Donor genotypes can be then restored in the next generation. Currently, the methodology is quite well elaborated for teleost species, but almost nothing was done on sturgeon. We suggest that the long generation interval of sturgeon might be extremely shortened if species that mature earlier are used as the surrogate host and produce donor gametes.

Acknowledgements:

Present study was financially supported by GACR no. P502/10/P426.

References:

[1]PSENICKA, M., RODINA, M., LINHART O. 2010. Ultrastructural study on the fertilisation process in sturgeon (Acipenser) function of acrosome and prevention of polyspermy. Anim. Reprod. Sci. 117: 147–154.

[2]YAMAHA E, MURAKAMI M, HADA K, OTANI S, FUJIMOTO T, TANAKA M, SAKAO S, KOMÁRA S, SATO S, ARAI K. 2003. Recovery of fertility in male hybrids of a cross between goldfish and common carp by transplantation of PGC (primordial germ cell)-containing graft. Genetica, 119: 121-131.

[3]SAITO, T., FUJIMOTO, T., MAEGAWA, S., INOUE, K., TANAKA, M., ARAI, K., YAMAHA, E. 2006. Visualization of primordial germ cells in vivo using GFP–nos1 3'UTR mRNA. Int. J. Dev. Biol., 50: 691–699.

[4]KOBAYASHI T, KAJIURA-KOBAYASHI H, NAGAHAMA Y. 1998. A novel stage-specific antigen is expressed only in early stages of spermatogonia in Japanese eel, Anguilla japonica testis. Mol. Reprod. Dev., 51: 355–361.

[5]OKUTSU, T., SHIKINA, S., KANNO, M., TAKEUCHI, Y., YOSHIZAKI, G. 2007. Production of trout offspring from triploid salmon parents. Science, 317, 1517.

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

CRYOPRESERVATION OF MALE GONAD OF CARPS, *LABEO CALBASU* AND *LABEO ROHITA* FOR LONG TERM STORAGE AND ITS UTILIZATION IN AQUACULTURE

Sahu A. D.*, Bhol C.*, Routray P.* and **Sundaray J. K.†**

*Aquaculture Production and Environment Division, Central Institute of Freshwater Aquaculture, (I.C.A.R), Kausalyaganga, Bhubaneswar-751002, India.

† Kakdwip Research Centre, Central Institute of Brackishwater Aquaculture, West Bengal, India.

e-mail: routray30@yahoo.co.in

Introduction:

Benefits to fisheries' conservation programs include the ability to maintain gene banks of species [1, 3, 4]. Cryopreservation of germ cells provides a relatively inexpensive means for long-term storage of specific selected genotypes that have special attributes for aquaculture. Cryopreservation of sperm is considered a valuable technique for developing the artificial reproduction, for maintaining genetic variability of fish brood stock and biological conservation programs [7]. Cryopreservation of sperm for many species including teleosts and other finfish have been developed and are relatively simple and highly successful. Here, we have attempted to cryopreserve the whole excised testis of two carps, *Labeo calbasu* and *Labeo rohita* that would help in the artificial propagation and conservation of fish species. This may open up animal breeding programs with an additional capability of establishing semen cryobanks.

Methods:

Matured male fishes of *Labeo calbasu* and *Labeo rohita* were humanely killed by anaesthsia (1-2 phenoxy ethanol), matured testis were excised, and the weight of the gonad and fish were recorded. Prior to this the male fish were injected with GnRH hormone (Ovaprim) at a rate of 0.4 ml / Kg body weight. The whole intact testes were removed surgically in aseptic conditions for cryopreservation and sperm evaluation after thawing. The GSI was calculated by the following formula, (GSI = gonad weight/body weight X 100). Each testis was clamped with a cord clamp and individually equilibrated in cryoprotectant i.e., 10 % dimethyl sulfoxide (DMSO), placed in centrifuge tubes and cooled at a rate of -15^{0}C/ min until it attained -60 °C [5] before plunging in to liquid nitrogen. After 1 week, thawing was done in a water bath at 38±2°C for 3-4 min following a modified method [2]. The semen from thawed testis was collected by squeezing out the anterior lobes of each testis into a sterile tube. The motility and duration of spermatozoa after freezing was recorded. Estimation of spermatozoa motility was started immediately after dilution and the movement was observed till 4 min. The motility was recorded in a computer using computer aided motility software (Biovis motility software, M/S Expert Vision Pvt. Ltd, India). For fertilization of 10,000 eggs, 0.5 ml of fresh or 1.5 ml cryopreserved sperm was used each time. Fertilization rate was scored 6 hr after activation of eggs with semen from different treatments and control and normally developing embryos at morula stage were selected as fertilized ones.

Results and Discussion:

Fish are the most diverse and numerous groups of vertebrates. Here it was observed that the whole and intact gonad cryopreservation is possible in carps and the spermatozoa can be utilized for *in vitro* fertilization of oocytes under artificial propagation methods. The details of the testicular status and the semen parameters before and after thawing are depicted in Table 1.

The testes of both the carps were bi-lobed having sufficient testicular lobule to hold spermatozoa. Here, after the cryopreservation, fertilization was successfully done with more than 75 % in both the species. In case of emergency or unforeseen situations where the mortality of brood fish is uncontrollable, this method of

Table 1. Testicular status, semen parameters and fertilization ability of spermatozoa of *Labeo calbasu* and *Labeo rohita* before (control) and after thawing.

	Labeo calbasu		*Labeo rohita*	
	Control	**Cryopreserved**	**Control**	**Cryopreserved**
Parameters				
Testis somatic index (TSI)	1.8 ± 0.28	1.72 ± 0.28	1.7 ± 0.34	1.6 ± 0.42
Milt output (ml/ Kg body weight)	7.0 ± 2.0	6.6 ± 1.25	6.2 ± 2.0	6.0 ± 1.25
Motility %	90 ± 5.8	85 ± 6.8	90 ± 5.8	83 ± 6.8
Spermatocrit Value	85 ± 6.0	82 ± 4.0	80 ± 6.0	79 ± 4.5
Osmolality of seminal plasma (mOsm/Kg)	263 ± 6.0	259.25±5.5	263 ± 6.0	266 ± 4.5
Motility duration (sec)	92 ± 4.0	86 ± 4.0	94± 4.3	83 ± 5.2
Fertilization %	90 ± 5.2	76 ± 5.2	90 ± 2.8	75 ± 4.6

cryopreserving the whole gonad of the male fish can be used effectively. This is an essential tool for germplasm conservation also. It is known that the GSI increases with the approach of spawning season and recedes after spawning or towards the end of the spawning season since such changes have been reported in freshwater teleosts [6]. Sometimes, asynchronous spawning also damages the breeding programs and also during table fish production male fishes are sacrificed for food purposes where testis can be recovered and utilized for artificial insemination purposes.

References:

[1]CAROLSFELD, J., GODINHO, H. P., ZANIBONI FILHOS, E., HARVEY, B. J. 2003. Cryopreservation of sperm in Brasilian migratory fish conservation. J. Fish Biol., 63, 472–489.

[2]GUPTA, S. D., RATH, S. C. 1993. Cryogenic preservation of carp milt and its utilization in seed production. In: Proceedings of the Third Indian Fisheries Forum, 11–14 October 1993, Panthnagar, 77–99.

[3]OHTA, H., KAWAMURA, K., UNUMA, T., TAKEGOSHI, Y. 2001. Cryopreservation of the sperm of the Japanese bitterling. J. Fish Biol., 58: 670–681.

[4]RICHARDSON, G. F., WILSON, C. E., CRIM, L. W., YAO, Z. 1999. Cryopreservation of yellowtail flounder (*Pleuronectes platessa*) semen in large straws. Aquaculture, 174: 89–94.

[5]ROUTRAY, P. 2003. Evaluation of cryopreserved milt of a selected carp, *Labeo calbasu* for fertilization and seed production. Ph.D. Thesis, Sambalpur University, Sambalpur, Orissa.

[6]VERMA, D. K., ROUTRAY, P., DASH. C., DASGUPTA, S., JENA, J. K. 2009. Physical and biochemical characteristics of semen and ultrastructure of spermatozoa in six carp species. Turk. J. Fisher. Aqu. Sci., 9: 67-76.

[7]WILDT, D. E. 1997. Genome resource banking: Impact on biotic conservation and society. In: Reproductive Tissue Banking, Karow A., Critser J. (Eds.), New York: Academic Press, pp 399-439.

GROWTH RESPONSE OF FRINGED LIPPED CARP, *LABEO FIMBRIATUS* (DAY) TO A SOYA PRODUCT, NUTRIPRO AQUA

Sherly D [a], **Sambhu C**[b] and **Jayaprakas V**[c]

a. Department of Zoology, All Saints College, Thiruvananthapuram, Kerala, India, email:davidsherly424@gmail.com
b. Faculty of Marine Sciences, King Abdulaziz University, Jeddah, Saudi Arabia
c. Department of Aquatic Biology and Fisheries, University of Kerala, Karyavattom, Thiruvananthapuram, Kerala, India

Introduction:

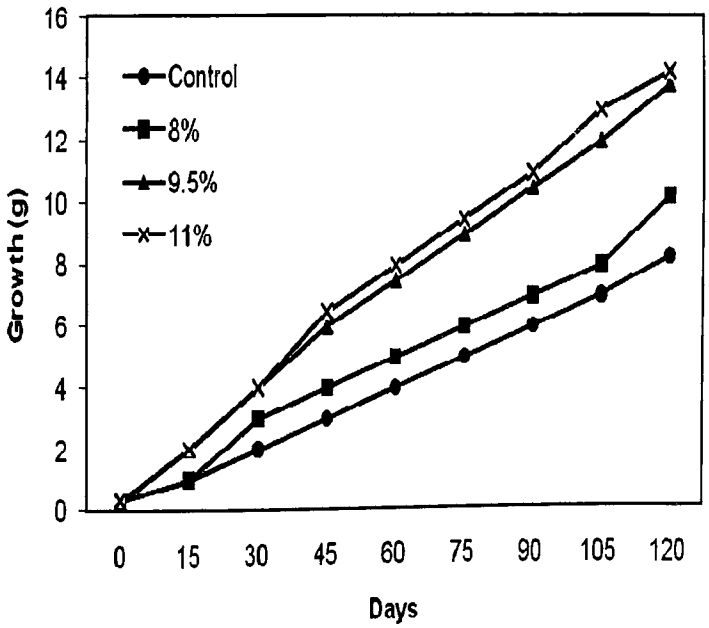
Carp culture is an ancient aquaculture practice and also is an important fisheries subsector in India. The main goal of carp farming is for low input, low cost production of protein food for domestic consumption and cash income [1]. Sustainable and successful freshwater carp culture on scientific basis principally depends upon the use of adequate, economically viable and environment friendly artificial feeds. Since the feed costs vary between 40 to 60% of the total managerial expenditure in carp culture system, provision of artificial feed increases the fish growth and production and results in higher growth rates and yields. With a view to enhance growth, a study was carried out to assess the impact of a soya based feed additive, Nutripro Aqua on growth performance and feed utilisation of fringed lipped carp, *Labeo fimbriatus* an ideal candidate species for composite fish culture.

Methods:

The culture experiment was conducted for a period of 120 days in cement cisterns (size 5x4x1m) without soil bottom. Fishes of uniform size were stocked at the rate of 4/m^2 in each cisterns. There were control and treatment for the experiment and it was replicated thrice. Cisterns provided with a fishmeal based pelleted feed having 40% protein was considered as control and the cisterns offered with a supplementary feed (40% protein) containing Nutripro aqua was designated as treatments. Nutripro aqua was incorporated into the supplementary feed at three different concentrations viz, 8%, 9.5% and 11%. Fishes were administered the experimental feed at a rate of 5% of their body weight twice a day. The quantity of feed to be given was readjusted after every fort nightly sampling. Fishes were sampled once in every 15 days. Feed conversion efficiency (FCE), protein efficiency ratio (PER) and nutrient digestibility were calculated as follows: Feed conversion efficiency (%) = Wet weight gain (g) / Feed consumed (g) x 100; Protein efficiency ratio (%) = Wet weight gain (g) / Protein intake (g) x 100; Apparent nutrient digestibility = Nutrient in feed - Nutrient in excreta / Nutrient in feed x 100. The influence of Nutripro aqua on digestive enzyme activities (amylase, protease and lipase) in the foregut, midgut and hindgut of fishes were estimated. Total activity = Enzyme unit / g tissue;

Specific activity = Enzyme unit / mg protein. DNA and RNA contents in the muscle, liver and brain of the experimental fishes were also estimated. One way analysis of variance (ANOVA) was employed to find out the statistical difference between control and treatment means.

Fig 1: Growth in weight of *L.fimbriatus*



The amylase and lipase activity were highest in the foregut region, while protease activity was higher in midgut region. The highest carcass protein level was recorded in 11% Nutripro-Aqua fed fish while the lowest in control. Nutripro Aqua may induce better growth resulting in protein accretion in the body tissues which in turn is an indication of enhanced protein synthesis [4].

Results and discussion:

The fish fed with a diet containing 11% Nutripro-Aqua exhibited significantly ($p<0.01$) superior growth compared to other treatments and control (Fig 1 and Table 1). Feed additives are reported to exhibit a significant effect on fish growth and production [2]. Similar enhanced growth rate was also observed in catla fingerlings when Nutripro aqua incorporated diet was administered [3]. Feed conversion efficiency and nutrient digestibility were high in the fishes fed with Nutripro-Aqua at 11% and was low in control. The increased nutrient digestibility observed under Nutripro aqua diet may be a reason of the enhanced growth exhibited by the fish in this treatment. RNA/DNA ratio of fishes fed with Nutripro-Aqua showed significant variation from control.

Conclusion:

The growth promoting effect of Nutripro aqua is expressed through stimulation of appetite of fringed lipped carp leading to enhance feed intake, high feed conversion efficiency and improved nutrient digestibility. The high RNA/DNA ratio observed in the rapidly growing fishes suggests this hypothesis. It can be recommended that Nutripro aqua can be used as a viable growth promoter in carp culture.

References:

[1]SAVITA, K., PUSPITA, D., PARTHA, D. AND ANIL, C., 2010. Growth performance of fingerlings of the Indian major carp, *Catla catla* (Ham.) fed with feeds supplemented with different seaweeds, *Pertanika J. Sci. & Technol.* 18 (2): 255-262.

[2]JAYAPRAKAS, V. AND ABRAHAM, K.M., 2002. Influence of Nutripro-Aqua incorporated feed on water quality, primary pond productivity and growth in the culture of pearlspot. *Fish. Technol.*, 39(1): 49-53.

[3]MISHRA, K.. AND SAMANTARAY, K.., 2004. Interacting effects of dietary lipid level and temperature on growth, body composition and fatty acid profile of rohu, *Labeo rohita* (Hamilton). *Aqua. Nutri., 10* (6), 359-269.

[4]KESHAVNATH, P., MANJAPPA, K. AND GANGADHAR, B., 2002. Evaluation of carbohydrates rich diets through common carp culture in manured tanks. *Aquaculture, 8* (3), 169-174.

Table 1: Growth, feed utilization, digestive enzyme activity and RNA/DNA ratio of *L.fimbriatus* fed with different diets

Parameters	Control	8%	9.5%	11%
	M ± SD	M ± SD	M ± SD	M ± SD
Initial length (cm)	2.40±0.22	2.40±0.22	2.40±0.22	2.40±0.22
Initial weight (g)	0.32±0.02	0.32±0.02	0.32±0.02	0.32±0.02
Final length (cm)**	8.21^{a}±0.32	10.20^{ab}±1.29	13.80^{b}±0.34	14.20^{c}±0.51
Final weight (g)**	11.13^{a}±0.51	13.31^{a}±1.73	19.87^{b}±1.02	23.10^{c}±0.71
S.G.R. (%)	3.09±0.56	3.85±0.44	5.04±0.31	5.85±0.01
Survival (%)	80.00±4.00	80.00±4.00	82.00±6.00	80.00±4.00
Feed consumption (g)	21.11^{a}±1.03	22.21^{ab}±2.88	24.01^{c}±1.89	25.79^{d}±1.07
Feed con.efficiency**	18.19^{a} ±2.73	20.23^{b} ±3.07	22.11^{b} ±2.16	23.11^{c} ±2.75
Protein digestibility (%)	64.32±0.88	65.42±0.92	67.11±1.02	69.21±0.86
App.lipid.diges. (%)	76.12±0.99	76.21±1.02	79.61±1.12	80.31±0.29
Dry matter (%)**	63.11^{a}±2.03	64.21^{b}±2.88	64.01^{b}±1.89	66.79^{c}±1.07
Protein in carcass (%)**	67.19^{a}±2.73	68.23^{b}±3.07	70.11^{c}±2.16	71.11^{c}±2.75
Lipid in carcass (%)^{NS}	3.21±0.71	3.33±0.63	3.01±0.59	3.08±0.39

** P< 0.01; a,b,c,d. - Means with the same superscript do not differ from each other (Duncan's test)

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

LHRHa-INDUCED SPAWNING OF THE EASTERN LITTLE TUNA *EUTHYNNUS AFFINIS* IN A 70-M^3 LAND-BASED TANK

Takeuchi Y.*, Sato K.°, Yazawa R.°, Yoshikawa H.*, Iwata G.°, Kabeya N°., Shimizu S. ° and Yoshizaki G.°

*Research Center for Advanced Science and Technology, Tokyo University of Marine Science and Technology, 670 Banda, Tateyama, Chiba 294-0308, Japan.
FAX: +81 470 20 9021 e-mail: yutakat@kaiyodai.ac.jp
°Department of Marine Biosciences,3 Tokyo University of Marine Science and Technology, Tokyo, Japan.

Introduction:

If small surrogates could be used to produce gametes of Pacific bluefin tuna *Thunnus orientalis* (PBT), then large quantities of PBT seeds could be produced in small facilities over a short period of time. With its small-body size, short generation time, and physiological similarities to PBT, the eastern little tuna (ELT) is considered to be well suited as a candidate for transplanting donor PBT germ cells. However, the technology for inducing spawning in ELT maintained in captivity is not yet well developed. We therefore attempted to control the reproduction of ELT in a 70-m^3 land-based tank (7 m in diameter, 1.8 m deep) by hormonal administration over an extended period.

Methods:

To investigate gonadal development in 1- to 2-year old ELT, the gonads of fish that died during the rearing period were processed for histological analysis. In addition, in the summer of 2010, cholesterol pellets containing LHRHa (L4513, Sigma, St. Louis) at a dose of 100 µg/kg (body weight, BW) were implanted into the dorsal muscles of 2-year old ELT. A non-invasive sex steroid assay using clipped fins was used to determine the sex of the broodstock and whether LHRHa administration affected the amount of 11-keto testosterone (11-KT) and 17β-estradiol (E2). For each spawning event, the time of spawning, the number of eggs collected, and the fertilization and hatching rates were determined. To identify the number of broodstock that participated in spawning events and the spawning frequency of each individual, DNA extracted from the clipped fin samples of the broodstock and hatched larvae were analyzed using three microsatellite loci.

Results:

ELT juveniles (BW: approx. 300 g; total length (TL) 20 cm) caught in the wild on August 2008 at Kushimoto in Wakayama Prefecture, Japan, were reared in a pen until June 2009. Thirty-two 1-year-old ELT were then transferred to a 70-m^3 land-based tank at Tateyama Station in Chiba Prefecture and reared for 15 months until September 2010 when there were 14 fish (BW: approx. 2 kg; TL: 50 cm). In August 2010, ovaries and testes obtained from dead fish contained vitellogenic oocytes with a diameter of 400 µm and sperm, respectively. No spawning behavior was observed in the ELT reared in the tank without the hormonal treatment. In September 2010, LHRHa-pellets were implanted into nine fish (five females and four males) on the same day. Three days post-implantation, fertilized eggs were collected by an egg collector. Spawning events occurred between 15:30 and 19:30 and continued for 9 days and the number of eggs collected in a day ranged from 50,000 to 170,000. The water temperature during this period ranged from 23.7 to 26.6°C. The mean fertilization and hatching rates were 86.8% and 34.4%, respectively. The larvae started to feed on rotifers at 3 days post-fertilization and grew up normally. At least 30 larvae were analyzed by microsatellite DNA markers at each spawning event and the parents of 20.2% (76 out of 376) of the larvae were identified. Parentage analysis revealed that three of the five implanted females produced viable offspring, and one of these females participated in up to nine consecutive spawning events. The sires consisted of two implanted males and one non-implanted male, suggesting that the LHRHa administration was not always necessary for inducing spawning behavior in ELT males. In most cases, the offspring of one female were sired by two males. Video recording of spawning events showed the horizontal spawning dash of a female accompanied by one or two males followed by the release of gametes. Using an enzyme-linked immunosorbent assay, LHRHa treatment was shown to elevate 11-KT and E2 levels in the parental broodstock for up to two weeks after implantation.

Conclusion:

This study showed that LHRHa administration was effective for inducing spawning in ELT maintained in captivity and for producing high quality eggs. Multiple spawning events by both males and females were observed. We therefore propose that ELT may be well suited for use surrogate broodstock for the production BLT seeds via xenogenic germ cell transplantation. We are currently focusing on rearing xenotransplanted ELT juveniles harboring donor-derived BTL germ cells in their gonads.

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

AFTER SIX YEARS OF STOREFISH: ASSESSMENT AND PERSPECTIVES

Teletchea, F.* ,Fontaine, P.°

°Nancy-Université INRA, UR AFPA, Domestication in Inland Aquaculture, 2 Avenue de la Forêt de Haye, BP 172, 54505 Vandœuvre-lès-Nancy, France.
FAX : +33383685598, fabrice.teletchea@lsa-man.uhp-nancy.fr.

Introduction:

In 2005, a research program was launched to evaluate the feasibility of developing a generic approach of the domestication of finfish species that would be helpful to promote the diversification of the production in aquaculture. This program was focused on temperate freshwater fish inhabiting chiefly Western Europe because European inland aquaculture presents several opportunities for diversification (e.g., Eurasian perch, pikeperch, tench, burbot...) and enough reliable information was expected to be available for numerous species. The choice was made to focus on reproduction because its control is a prerequisite for domesticating new finfish species [1].

Methods:

An extensive literature search was performed based chiefly on the Aquatic Sciences and Fisheries Abstracts bibliographic database (ASFA). All reliable information was entered into a new database entitled STOREFISH, acronym for STrategies Of Reproduction in FISH [2]. Today, *ca.* 80% of the 4000 cells of the database have been fulfilled based on the analysis of more than 2000 references.

Results and Discussion:

Based on this database, four different studies were performed. First, the analysis of 29 reproductive traits for 65 species resulted in an original typology that differs significantly from all others proposed earlier [3]. From this typology, a rather regular continuum of ten reproductive clusters emerged with two obvious endpoints. Between two extremes, species could be ordered chiefly according to temperature requirement, spawning season and parental care. Because most clusters are sufficiently homogeneous, it is reasonable to think that extrapolations of biological and zootechnical knowledge acquired on one species would be possible between species belonging to the same cluster, particularly concerning the environmental control of reproductive cycles, and regulation of egg and larvae quality.

Then, two complementary studies were performed in order to test some generalities often stated in the scientific literature. The first study focused on the possible relationships between oocyte diameter and incubation temperature to incubation time [4]. A strong relationship was found between incubation time (t, days) and incubation temperature (T, °C): $t = 186.23e^{-0.197T}$ ($r^2 = 0.87$). A strong dependence of incubation time on oocyte diameter ($\varnothing$, mm) and incubation temperature was also found: $\log_{10}t = 3.002 + 0.599 \log_{10}\varnothing - 1.91 \log_{10}(T + 2)$ ($r^2 = 0.87$). However, $\varnothing$ and T are not related implying that it is impossible to predict the required T, based only on $\varnothing$. Noteworthy, the equations calculated for the freshwater species fitted badly previous datasets of marine species, particularly due to the lack of large eggs comparable to the eggs of some salmonids(e.g. *Salmo*, *Oncorhynchus*, *Salvelinus*) in the marine fish datasets. The second study aimed at comparing the early-life stage strategies between 65 freshwater fish species and evaluating how trade-offs are directed towards first feeding of larvae in spring and summer [5]. t and degree-days (°D) required to reach hatching and mixed feeding were weakly related to $\varnothing$ and strongly to T. These results are chiefly because oocyte diameter and yolk reserves are weakly related and temperature strongly increases tissue differentiation rate, activity of hatching glands and embryo motility. Besides, no link was found between larval size at hatching and °D from hatching to mixed feeding and between °D for incubation and °D from hatching to mixed feeding. These last two results are chiefly because the developmental stages at hatching and at the onset of exogenous feeding are not fixed in ontogeny and are not directly related to either larval size or degree-days for incubation, but more probably are species-specific. Whatever the spawning season, which can take place virtually all year long, the different trade-offs at the early life stages ensure that most larvae are first feeding during spring, when food size and abundance are the most appropriate.

Based on these two studies, it appeared clear that freshwater and marine species display some differences, particularly concerning the early life stages. A fourth study was thus realized to both summarize the main differences (*e.g.*, freshwater fish species have generally bigger eggs that are adhesive and larger larvae at hatching than marine species) and highlight the possible implications for aquaculture practices (*e.g.*, procedures for removing the stickiness of eggs, larval diets, onset of cannibalism) [6].

Conclusion:

Based on the past six years, it became obvious that comparative biology is quite scarcely used in fish biology in general and in aquaculture in particular. Yet, such approaches provide original information, particularly concerning different trade-offs for reproductive traits, and highlight some differences between marine and freshwater species. From a basic point of view, further analysis are required including phylogenetic analysis to better understand when different trade-offs occur within the phylogenetic tree of teleosts. From a more applied view, further experimental studies are required to evaluate more deeply the interest and the potential of these comparative analyses, particularly [3], for domesticating new finfish species.

A larger research program is expected to start next year to take into account two other biological functions (growth and nutrition) in order to be able to study the entire life cycle of fish. Also, new species inhabiting warm waters (*e.g.*, Mediterranean Sea and African freshwaters) will be targeted to study a larger diversity of life cycles and thus help to promote the diversification of finfish production worldwide.

References:

[1] BILIO, M. 2007-2008. Controlled reproduction and domestication in aquaculture. The current state of the art. Part I-IV. Aquaculture Europe, 32-33.

[2] TELETCHEA, F., FOSTIER, A., LE BAIL, P.Y., JALABERT, B., GARDEUR, J.N. AND FONTAINE, P. 2007. STOREFISH: a new database dedicated to the reproduction of temperate freshwater teleost fishes. Cybium, 31: 227-235.

[3] TELETCHEA, F., FOSTIER, A., KAMLER, E., GARDEUR, J.N., LE BAIL, P.Y., JALABERT, B. AND FONTAINE, P. 2009. Comparative analysis of reproductive traits in 65 freshwater fish species: application to the domestication of new fish species. Rev. Fish Biol.., Fish 19: 403-430.

[4] TELETCHEA, F., GARDEUR, J.N., KAMLER, E. AND FONTAINE, P. 2009. The relationship of oocyte diameter and incubation temperature to incubation time in temperate freshwater fish species. J. Fish Biol., 74: 652-668.

[5] TELETCHEA, F. AND FONTAINE, P. 2010. Comparison of early-life stage strategies in 65 European freshwater fish species: trade-offs are directed towards first-feeding of larvae in spring and early-summer. J. Fish Biol., 77: 257-278.

[6] TELETCHEA, F. AND FONTAINE, P. 2011. Particularities of early life stages in temperate freshwater fish species: comparisons with marine species and implications for aquaculture practices. Aquaculture Int., (in press).

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

OPTIMIZATION OF GROWTH CONDITIONS FOR *IN VITRO* CULTURE OF FISH EMBRYONIC STEM CELLS

Tripathy S.*, Sahu A. D.*, Dash C*., Routray P.*

*Aquaculture Production and Environment Division, Central Institute of Freshwater Aquaculture, (I.C.A.R), Kausalyaganga, Bhubaneswar-751002, India.
E-mail: routray30@yahoo.co.in

Introduction:

Different methodologies have been applied to establish *in vitro* culture of embryonic stem (ES) cells from vertebrates. Application of murine *in vitro* conditionS to non-murine mammals has achieved with only partial success. Embryonic stem cells (IMC-ES) derived from early blastula of Indian major carps (IMCs) (*Labeo rohita, Catla catla, Cirrhinus mrigala*) need specific physiological conditions for their long-term propagation while maintaining their undifferentiated status. The pluripotency of ES cells is largely affected by the conditions provided viz., physical, chemical and biological parameters during *in vitro* culture. In the present investigation we standardized various components (physical, biological and chemical) that are responsible for derivation, proliferation, propagation and maintenance of ES cells from the IMCs. Different parameters *viz*., temperature, pH of media, osmolality, amount of oxygen and carbon dioxide, combination of media, concentration of fetal bovine serum, glucose, growth factors and initial seeding density were analyzed. The properties of the ES-cells were further investigated by alkaline phosphatase (AP) activity, immunological evidence and their differentiation.

Methods:

The blastomeres collected from early blastule embryos of *L. rohita, C. catla, C. mrigala* were cultured in condition media composed of Leibovitz-15, Dulbecco's modified Eagle's medium with 4·5 g l^{-1} glucose and Ham's F12 (LDF) in 50:35:15 ratio and 5% FBS. Additional components such as 15 mM HEPES [4-(2-hydroxyethyl)-1-piperazine-ethane-sulphonic acid], 8 nM sodium selenite, 100µM ß-mercaptoetanol, 1mM sodium pyruvate, 1mM non-essential amino acid (NEAA), 100 IU ml^{-1} penicillin, 0.1mg ml^{-1} streptomycin, (Sigma, USA), 1 % FS and 1 % FEE. Cultured ES cells were subjected to several physical (temperature, pH of medium, osmolality, % of oxygen and % carbon dioxide), chemical (different combination of medium, concentration of fetal bovine serum, addition of growth factors) and biological (initial seeding density) parameters that affect proliferation, propagation and maintenance of IMC-ES like cell. Cryopreservation of IMC-ES cells was done using 10 % DMSO (dimethyl sulfoxide).

Results and discussion:

Embryonic stem cells derived from early embryos hold strong potential application in several field of biotechnology [5, 6]. Long-term propagation of stem cells was achieved with partial success in a species-specific condition to maintain the cellular niche [2, 3, 4]. Growth and propagation of primate and murine ES cells need specific *in vitro* condition for their growth [1].

The present results revealed that creating an *in vivo* environment in *in vitro* condition can cause suitable growth and maintenance of cultured ES cells. Among the investigated factors; temperature of 28 °C, pH 7.4; osmolality 270 mOsm / Kg; 40 % oxygen tension, 5 % carbon dioxide, 50:35:15 LDF combination, 10% FBS, 25 mM glucose, 10 ng/ ml of hLIF, 10 ng/ ml of bFGF, $1x10^5$ cells/ml of initial seeding density facilitated the IMC-ES cell growth and propagation up to 40 passages without showing significant differentiation. These ES cells showed extensive alkaline phosphatase activity, positive SSEA-1 expression and differentiated into three cell types of embryonic germ layers. The viability of cryopreseved ES cells was estimated by trypan blue assay and found to be more than 70 % viable with colony forming ability.

The ES-like cells formed maximum colonies at a seeding density of 1 $X10^5$ $cells^{-ml}$ as shown in Fig.1. Similarly, proliferation of IMC-ES cells was noticed maximum at 28 °C.

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

Fig. 1. Formation of IMC-ES colonies at different initial cell density.

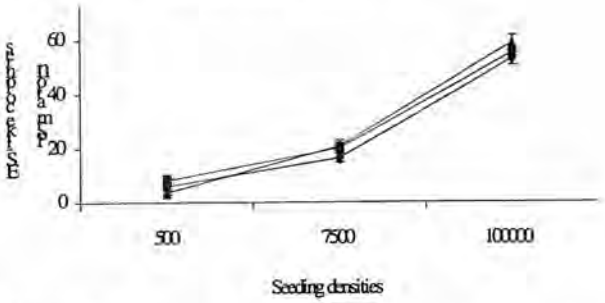
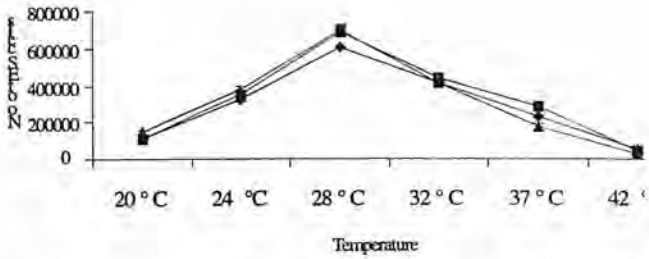


Fig. 2. Proliferation of IMC-ES like cells at different temperatures.



Conclusion:

The embryonic stem cell of fish has got extensive prospects in the transgenesis research in aquaculture. This optimization study will help in derivation and maintenance of ES- like cells in other fishes.

References:

[1]BEATTIE, G. M., BUCAY N., HUMPHREY, R., LOPEZ, A. D., HAYEK, A. 2005. Growth conditions for primate *vs.* murine embryonic stem cells. Stem Cells in Endocrinology. Contemporary Endocrinology, 2005, Part I, 93-111.

[2]CAMPBELL, K. H. S., MCWHIR, J., RITCHIE, W. A.,WILMUT, I. 1996. Sheep cloned by nuclear transfer from a cultured cell line. Nature, 380: 64–66.

[3]DISCHER, D. E., MOONEY, D. J., ZANDSTRA, P. W. 2009. Growth factors, matrices and forces combine and control stem cells. Science, 324: 1673–1677.

[4]DASH, C., ROUTRAY, P., TRIPATHY, S., VERMA, D.K., GURU, B. C., MEHER, P.K., NANDI, S. AND EKNATH, A. E. 2010. Derivation and characterization of embryonic stem-like cells of Indian major carp *Catla catla*. J. Fish Biol., 77: 1096–1113.

[5]GEPSTEIN, L. 2002. Derivation and potential applications of human embryonic stem cells. Circulation Res., 91: 866–876.

[6]ROUTRAY, P., DASH, C., DASH, S. N., TRIPATHY, S., VERMA, D. K., SWAIN, S. K., SWAIN, P., GURU, B. C. 2009. Cryopreservation of isolated blastomeres and embryonic stem-like cells of Leopard danio, *Brachydanio frankei*. Aquacul. Res., 41: 579–589.

Vol. 4 No. S 8 (August 2011) ISSN: 0974- 6846

RECENT PROGRESS IN THE STUDY OF FISH REPRODUCTIVE PHYSIOLOGY
CONCLUDING REMARKS

Yaron Z.

Department of Zoology, Tel-Aviv University, Tel Aviv 69978, Israel
Fax: +972-3-6405606 email: yaronz@post.tau.ac.il

As the following account is written ahead of the symposium it is only based on the general program and the presentation titles.

The organizers have wisely selected the session topics that have been used traditionally in previous ISRPF meetings, although in a different order. Poster sessions, always presenting new and exciting data, have been allotted ample time for meetings and discussions with students and with the new generation of young scientists. The list of titles and addresses indicates a growing number of participants from Korea, Malaysia, New Zealand and especially from South American countries that join the symposium in spite of the discouraging distance.

In the reproductive neuroendocrinology session, kisspeptins continue to play the leading role although new neurohormones are emerging and their site and function along the reproductive axis may open a new front in neuroendocrine research. Steroids and steroidal pheromones affect both circulating vasotocin, brain catecholamines and fish behavior. Endocrine research is no more restricted to small model fish like the goldfish, medaka or zebrafish as hormones of large fish namely Thunnus thynnus and Arapaima gigas are now on the list.

A number of contemporary tools such as transcriptome, next generation sequencing, microRNA and AFLP have been mobilized in attempts to reveal the mechanisms of sex determination, gonadal differentiation or sex change, that are regulated by genetic or external factors such as ambient temperature.

Fish as exothermal vertebrates experience seasonal variations of reproductive parameters that ensure spawning at the optimal season for the survival of their breeding products. The session dealing with these temporal changes presents more examples of this phenomenon together with attempts to reveal the mechanism underlying these changes.

Testicular physiology is represented in the symposium by a review on chondrostean spermatozoa and contributions on the possible effect of peptides belonging to the relaxin/insulin- like family on spermatogenesis, and that of leptin on male early maturation. In addition, new information is presented on fish androgens, spermatogonial stem cells, and the use of somatic gene transfer for studying the gonadotropic regulation of spermatogenesis.

The session on ovarian physiology provides novel information on lipid metabolism and oocyte development, on ovarian lipoprotein receptors, vitellogenesis, yolk proteolysis, and on molecular regulation of aquaporins as related to oocyte hydration.

In the current symposium, the session on spawning has expanded to include effects on gamete quality and early embryogenesis, In addition, the session spans reports on various spawning-inducing agents, gonadotropins and the acquisition of ovulatory competence, and a proteomic approach to study the molecular mechanisms operating in fish oocytes.

The contribution of the symposium to the issue of environmental pollution is presented in the session on reproductive toxicology and endocrine disruptors. The main emphasis of the presentations is on estrogenic pollution, including bisphenol A which interferes with the normal function of both the testis and ovary. Attempts are made to discover fast and reliable criteria for the biological detection of estrogenic pollutants. Pyrethroid insecticides commonly used in agriculture and household disrupt too fish reproduction interfering with steroid metabolism. Other environmental factors such as long-term cyclic hypoxia or nutritional stress can disrupt the normal reproductive process in fish.

Germ cells transplantation and spermatogonial stem cells in teleosts or primordial gonocytes and spermatogonia continue to lead the germ-cell studies in the biotechnology session. Constructing ESR database from the ovaries of eels maturing in the wild may prove essential for future studies of eel puberty. The practical aspect of biotechnology includes presentations on classical spawning induction, sperm cryopreservation, the effect of photo-thermal regime or dietary arachidonic acid on domestication or gonadal dysfunction. In addition, the session includes report on masculinization in mullet and other endocrine manipulations related to aquaculture.

This cursory overview of the program is only intended to raise the reader's curiosity and to encourage the active participation in the lectures and poster sessions.

Author Index

"REGISTERED"
No. TN/CC(S) Dn./398/10-12
Posted at Patrika Channel, Egmore, Chennai-600 008
Registered with Registrar of Newspapers in India,
No.TNENG/2010/33487, ISSN:0974-6846

If undelivered please return to..
The Editor, Indian Journal of Science and Technology
23 (new) - Neelkamal aptt., Flat-14, 3rd Main road, Gandhinagar,
Adyar, Chennai - 600020, India. Ph: +91-9940444695; indjst@gmail.com

Indian Journal of Science and Technology
Access full Proceedings of 9th ISRPF at
http:/www.indjst.org

Highlights of ISRPF

The 9th International Symposium on Reproductive Physiology of Fish (ISRPF) has been scheduled to be in India. The first meeting was the result of a pioneer initiative by the Fish Physiology Laboratory of Dr. Roland Billard in September 1977 at Paimpont, France. In about once in four years the meeting was held in different countries, e.g. Netherlands, Canada, United Kingdom, USA, Norway, Japan, France, and gathered many scientists working in the field of Reproduction of Fish. The last meeting (Saint Malo, France, June 3-8, 2007) attracted the active participation and presentation of more than 350 scientists with their findings.

The ISRPFs are unique in that there is no formal society or professional body that organizes the meeting. The international advisory committee (IAC) of scientists drawn from different countries in collaboration with national and local organizing committees organizes the meeting. The technical sessions of the meetings are discussed and decided by these committees to give a true international flavor and state of art deliberations. These deliberations indeed define and direct future research thrusts and focuses.

The IAC of the 8th ISRPF, St. Malo, France has chosen India from among the strong contenders, to organize the 9th ISRPF at Kochi. It is indeed recognition of the research contributions of Indian workers in reproductive physiology of fish. The Madras Christian College has been chosen for the Institutional support and to organize the International Symposium. India has emerged as the world's second largest producer of farmed fish and has a vast network of government and private establishments engaged in both culture and capture fisheries. Teaching and research in fish biology and fisheries are a part of the academic curricula in Universities and colleges. It is befitting that India has been chosen as the venue of the 9th symposium by the International Advisory Committee, the second meeting of its kind in the Asian continent. The ISRPF attracts a large number of scientists, upcoming young scientists and students underscoring the importance of the meeting. The previous meetings have been all held in different parts of the globe except India. The holding of the 9th ISRPF at Kochi, a premier hub of fisheries and aquaculture operations in the country, brings India in the map of the ISRPFs. This significant scientific gathering will benefit young scientists to find new avenues in their future research and it will greatly benefit the up gradation of reproductive technologies of fish around the globe.

- Organizers

Published by Dr. N. Gajendran on behalf of Indian Society for Education and Environment (ISEE) from 23 (New)-Neelkamal Aptt., Flat-14, 3rd Main road, Gandhi Nagar, Adyar, Chennai - 600 020, India and printed by Mr. G. Ahamed Rafi at TAMCOS, 34/1, Poornamprakasam Road, Balaji Nagar, Royapettah, Chennai - 600 014, India.

Journal Editor: **N. Gajendran**

Special Issue Editors: **K. P. Joy, R. M. Inbaraj, R. Kirubagaran and R. Chaube**

Organized by

MADRAS CHRISTIAN COLLEGE

Tambaram, Chennai - 600059, India, www.9isrpf.org Email: os9isrpf@9isrpf.org

web: www.mcc.edu.in