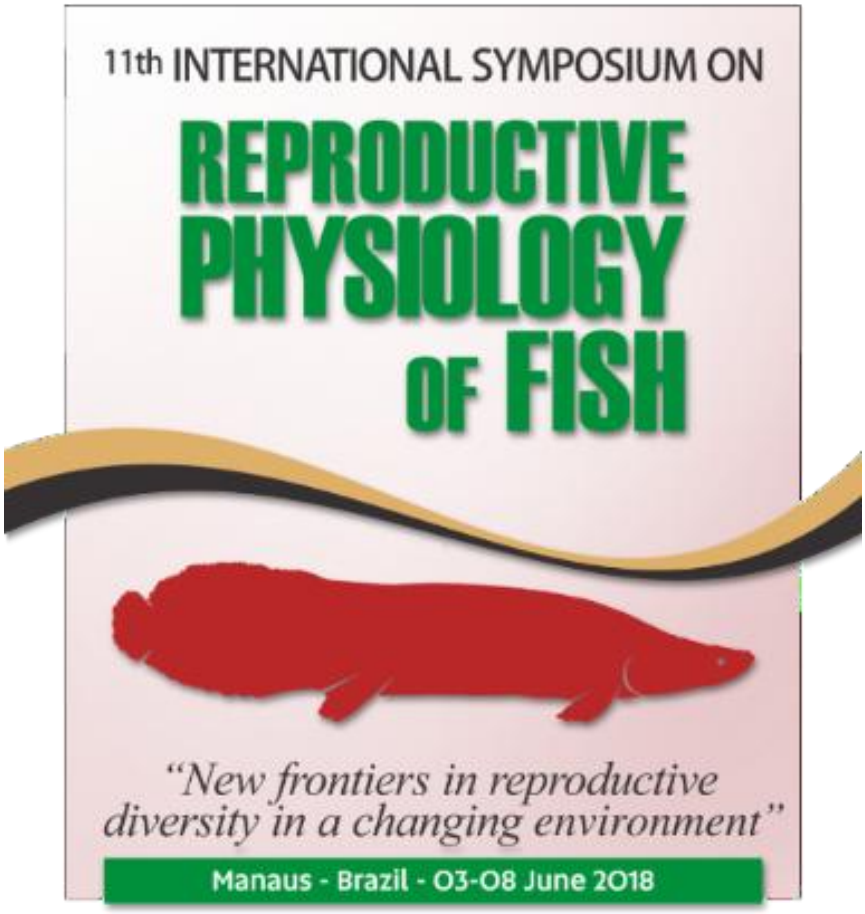
PROGRAM
AND
ABSTRACTS



11 ISRF
2018

Welcome

Dear colleagues, it is our great pleasure to welcome you to the 11th International Symposium on Reproductive Physiology of Fish (ISRPF – 2018), that is taking place in the unique and beautiful city of Manaus and its wonderful Tropical Hotel, located in front of the Negro river.

With more than 2.2 million inhabitants and founded in 1669, Manaus is the capital of the Amazon state and is located at the majestic and emblematic meeting of the Negro and Solimões rivers – also known as the meeting of the waters – that forms the longest and largest river on our planet, the Amazon River.

Its basin holds about 20% of the planet's fresh water and has about 3,000 fish species! Besides being known worldwide for its ecotourism in the heart of the largest tropical forest on the planet, Manaus is also the industrial center and the largest city of the PanAmazon region, an area of almost 8 million square kilometers. Manaus became well known at the beginning of the 20th century during the golden rubber time and its fabulous wealth that also allowed the construction of the legendary Opera House, where the most famous singers of the era held concerts.

Manaus became at that time the second most important Brazilian city after Rio de Janeiro. Thus, do not miss this unique opportunity during the 11th ISRPF to enjoy biodiversity, science and all the emblematic beauty on our planet provided by the tropical forest and particularly by Manaus with all of its natural and cultural treasures. Thanks to innumerous contributions and suggestions we were able to put together and excellent scientific program. We did our best to provide you with a memorable event and wish you and excellent stay with us in early June 2018!

Meeting Symbols

Besides the meeting of the waters the Pirarucu (*Arapaima gigas*) is the fish symbol of the congress.

Arapaima gigas, also known as ***pirarucu***, is a species of arapaima native to the basin of the Amazon River. Once believed to be the sole species in the genus, it is among the largest freshwater fish. The species is an obligate air-breather and needs to come to the surface regularly to gulp air.

This species is among the largest known freshwater fish, commonly measuring 200 cm and exceptionally reaching lengths of up to 450 cm. Adults may weigh up to 200 kg. The *A. gigas* reachs sexual maturity at around 5 years of age, has only one gonad, and has a streamlined body with dorsal and anal fins set well back towards the tail. While the body is mainly gray to gray-green, its Brazilian local name “pirarucu” derives from an indigenous word for “red fish”, thought to refer to either the red flecks on the scales towards the tail, or the reddish-orange colour of the filleted meat.

Presentation/History

The International Symposium on Reproductive Physiology of Fish (ISRPF) is an internationally highly recognized symposium, devoted to discuss and present the most advanced basic and applied knowledge in the field the reproductive physiology of fish and related areas. With its history of four decades, this worldwide scientifically well-established symposium takes place every 4 years, since its creation in 1977 by Prof. Dr. Roland Billard, in France.

The upcoming 11th edition of the ISRPF will be held for the first time in Latin America, a milestone in the development of this conference series with its previous editions in Europe, North America and Asia. To symbolically represent the great diversity of fish species found in South America, and particularly being in the heart of the most abundant and diverse niches of fauna and flora in the world – the Amazon rainforest – the city of Manaus has been chosen to host the 11th ISRPF.

Considering this diversity and the critical and in part even alarming climate and other global environmental changes, the central theme of the 11th ISRPF will be *"New frontiers in reproductive diversity in a changing environment"*. In particular, the term "reproductive diversity" refers to the huge diversity shown by South America species but also alludes to rich diversity of reproductive strategies observed in fish in general.

With respect to the "changing environment", we intend to allude to man-made changes and the consequences of human activities leading to environmental changes, such as the global warming and the direct effects of the temperature but also pH value and CO_2 concentrations on the biology of fish species.

Venue

Tropical Manaus Ecoresort (Tropical Hotel) is the place where researchers and students from all over the world will gather from June 3th to June 8th, 2018 to discuss the most current and exciting topics of fish physiology.

Tropical Manaus Ecoresort is a luxurious eco resort, located at the entrance of the majestic Amazon Forest on the banks of the Rio Negro. The development offers state-of-the-art technology and luxurious accessories (640 apartments), as well as setting the impressive Amazon jungle.

Site: http://www.tropicalmanaus.com.br/

Coordination

Dr. Luiz Renato de França

Director of the National Institute for Amazonian Research (INPA – Manaus/AM – Brazil) and Full Professor at the Federal University of Minas Gerais (UFMG –Belo Horizonte/MG – Brazil).

Brazilian institutions and funding agencies involved:

National Institute for Amazonian Research (INPA – Manaus/AM)

Federal University of Minas Gerais (Belo Horizonte/MG)

São Paulo State University (Botucatu/SP)

Brazilian Agricultural Research Corporation (EMBRAPA) – Western Amazon (Manaus/AM)

Nilton Lins University (Manaus/AM)

Coordination for the Improvement of Higher Education Personnel (CAPES)

National Council for Scientific and Technological Development (CNPq)

Foudation for the State of Amazonas Research (Fapeam)

Committees:

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SCIENTIFIC PROGRAM AT A GLANCE

Monday June 4	Tuesday June 5	Wednesday June 6	Thursday June 7	Friday June 8
PLENARY 1 **GORO YOSHIZAKI** Development of germ cell manipulation technology in fish	**PLENARY 2** **WEI GE** Endocrine and paracrine control of folliculogenesis in the zebrafish – a genetic approach		**PLENARY 3** **ANNA WARGELIUS** Gene editing a way to solve and understand reproductive constraints in salmon farming	**INTEGRATIVE 03** **CHARLES TYLER** The feminization of fish – from individual effects to population level consequences **ORAL PRESENTATIONS (29-32)**
SESSION 01 **MANFRED SCHARTL** Sex determination diversity in fish **ORAL PRESENTATIONS (1-3)**	**SESSION 05** **GRAHAM YOUNG** Regulation of development of previtellogenic ovarian follicles by androgens **ORAL PRESENTATIONS (11-13)**	EXCURSIONS AND OTHER ACTIVITIES	**SESSION 08** **JOSÉ MUÑOZ-CUETO** The gonadotropin-inhibitory hormone system of fish: the case of sea bass (*Dicentrarchus labrax*) **ORAL PRESENTATIONS (22-24)**	**PLENARY 4** **RÜDIGER SCHULZ** Endocrine and paracrine control of spermatogenesis in fish **MEETING HIGHLIGHTS** **PENNY SWANSON**
Coffee Break	Coffee Break		Coffee Break	
SESSION 02 **MINORU TANAKA** How do germ cells determine their own sexual fate? **ORAL PRESENTATIONS (4-5)**	**SESSION 06** **JULIEN BOBE** Molecular portrait of egg quality and novel maternal-effect genes **ORAL PRESENTATIONS (14-17)**		**SESSION 09** **FRANÇOIS CHAUVIGNÉ** New insights into the endocrine regulation of teleost spermiogenesis **ORAL PRESENTATIONS (25-28)**	**CLOSING REMARKS**
Lunch	Lunch		Lunch	Lunch
SESSION 03 **SAMYRA LACERDA** Biotechnological prospects of male germline stem cells in the nile tilapia **ORAL PRESENTATIONS (6-8)**	**SESSION 07** **ADELINO CANÁRIO** Chemical communication in fishes with emphasis on cichlids **ORAL PRESENTATIONS (18-19)**		**INTEGRATIVE 02** **YONATHAN ZOHAR** Who exactly is in control of reproduction in fish? **ORAL PRESENTATIONS (29-31)**	
Coffee Break	Coffee Break		Coffee Break	
SESSION 04 **CONSTANTINOS MYLONAS** Broodstock management and spawning induction of greater amberjack *Seriola dumerili* reared in sea cages in greece **ORAL PRESENTATIONS (9-10)**	**INTEGRATIVE 01** **RONALDO BARTHEM** General pattern of fish migration in the Amazon plan: implications for management and conservation **ORAL PRESENTATIONS (20-21)**		**POSTER SESSION 3** **91-146 (PLUS OP22-36)**	
POSTER SESSION 1 **1-58 (PLUS OP 1-10)**	**POSTER SESSION 2** **59-90 (PLUS OP 11-21)**			

SCIENTIFIC PROGRAM

THURSDAY - JUNE 3
WELCOME RECEPTION (18h00)

MONDAY - JUNE 4	
OPENING CERIMONY (8h30) **PLENARY 1** (8h50) **GORO YOSHIZAKI (JAPAN) (40 MIN)** **Development of germ cell manipulation technology in fish**	
SESSION 1 **SEX DETERMINATION** (9h30-10h45)	**LECTURE 01** **MANFRED SCHARTL (GERMANY) (30MIN)** **Sex determination diversity in fish** **ORAL PRESENTATIONS** Chairs: Mateus Adolfi and Fernanda Loureiro **ORAL 1 (Piferrer, F - Spain)** Methylomic and transcriptomic dynamics during testis and ovarian differentiation in the european sea bass, as assessed by multiplex bisulfite sequencing and weighed correlation network analysis **ORAL 2 (Yamamoto, Y - Japan)** Coexistence of genotypic and temperature-dependent sex determination in cobaltcap silverside **ORAL 3 (Luckenbach, JA - USA)** Sex determination and downstream events associated with gonadal sex differentiation in sablefish
COFFEE BREAK (10h45-11h15)	
SESSION 2 **SEX DIFFERENTIATION** (11h15-12h15)	**LECTURE 02** **MINORU TANAKA (JAPAN) (30MIN)** **How do germ cells determine their own sexual fate?** **ORAL PRESENTATIONS** Chairs: Ricardo Hattori and Minoru Tanaka **ORAL 4 (Paixão, RV - Brazil)** Estrogen receptors are sex-differentially expressed in tambaqui (*Colossoma macropomum*) during sex differentiation

	ORAL 5 (Melo, LH - Brazil) Expression of vasa, nanos2 and sox9 during initial development testicular of nile tilapia (*Oreochromis niloticus*) subjected to sex reversal
LUNCH (12h15-14h00)	
SESSION 3 **STEM CELLS AND SPERMATOGENESIS** (14h00-15h15)	**LECTURE 03** **SAMYRA LACERDA (BRAZIL) (30MIN)** **Biotechnological prospects of male germline stem cells in the nile tilapia** **ORAL PRESENTATIONS** Chairs: Rüdiger Schulz and Rafael Nóbrega **ORAL 6 (Pšenička, M - Czech Republic)** Application of germ cell technologies in sturgeons **ORAL 7 (Iwasaki, Y - Japan)** Production of viable trout offspring derived from germ cells cultured *in vitro* **ORAL 8 (Crespo, D - The Netherlands)** Retinoic acid and 11-ketotestosterone orchestrate germ cell development in zebrafish: characterization of a permissive interaction
SESSION 4 **SPAWNING, FERTILIZATION AND SPERM-EGG INTERACTION** (15h15-16h15)	**LECTURE 04** **CONSTANTINOS MYLONAS (GREECE) (30 MIN)** **Broodstock management and spawning induction of greater amberjack *Seriola dumerili* reared in sea cages in greece** **ORAL PRESENTATIONS** Chairs: Martin Pšenička and Constantinos Mylonas **ORAL 9 (Chakraborty, T - Japan)** Exploring the potentiality of autophagy in fish fertility **ORAL 10 (Xia Hui - China)** Loss of m6a in mettl3 zebrafish mutants disrupts gamete maturation and reduces fertility
COFFEE BREAK (16h15) **POSTER SESSION 1** (16h45-18h15) **FROM POSTER 1 TO 58 (PLUS OP 1-10)**	

TUESDAY – JUNE 5

PLENARY LECTURE 2 (8h30)
WEI GE (CHINA) (40 MIN)
Endocrine and paracrine control of folliculogenesis in the zebrafish – a genetic approach

SESSION 5

OOGENESIS AND VITELLOGENESIS
(9h10-10h25)

LECTURE 05

GRAHAM YOUNG (USA) (30 MIN)
Regulation of development of previtellogenic ovarian follicles by androgens

ORAL PRESENTATIONS
Chairs: Erin Damsteegt and Wei Ge

ORAL 11 (Yilmaz, O - France)
Genome editing reveals developmental dependencies on specific types of vitellogenin in zebrafish (*Danio rerio*)

ORAL 12 (Damsteegt, EL - New Zealand)
The *in vivo* effects of estradiol and 11-ketotestosterone on vitellogenin physiology in the shortfinned eel, *Anguilla australis*

ORAL 13 (Aranyakanont, C - Japan)
17β-hydroxysteroid dehydrogenase type 12 is responsible for maturation-inducing steroid synthesis during oocyte maturation in Nile tilapia

COFFEE BREAK (10h25-10h50)

SESSION 6

OVULATION AND EGG QUALITY
(10h50-12h20)

LECTURE 06

JULIEN BOBE (FRANCE) (30 MIN)
Molecular portrait of egg quality and novel maternal-effect genes

ORAL PRESENTATIONS
Chairs: Julien Bobe and Diego Crespo

ORAL 14 (Klangnurak, W - Japan)
Candidate gene identification of ovulation-inducing genes by *in vivo* induction of oocyte maturation and ovulation in zebrafish

ORAL 15 (Rocha de Almeida, T - France)
A transcriptomic comparison suggests an influence of the domestication process on egg quality in eurasian perch (*Perca fluviatilis*)

ORAL 16 (Gioacchini, G - Italy)
Macromolecular changes of swordfish oocyte at different developmental stage: new insight from fourier transform infrared microspectroscopy (ftirm)

ORAL 17 (Yoshinaga, T - Brazil)
Oocyte quality of nile tilapia (*Oreochromis niloticus*) reared in biofloc system

<table>
<tr><td colspan="2">LUNCH (12h20-14h00)</td></tr>
<tr><td rowspan="2">SESSION 7

PHEROMONES AND BEHAVIOR
(14h00-15h00)</td><td>LECTURE 07

ADELINO CANÁRIO (PORTUGAL) (30MIN)
Chemical communication in fishes with emphasis on cichlids</td></tr>
<tr><td>ORAL PRESENTATIONS
Chairs: Adelino Canário and Matias Pandolfi

ORAL 18 (Sorensen, PW - USA)
Direct evidence that high levels of plasma prostaglandin F2α intimtely associated with ovulaton drive female sexual behavior and pheromone release in a model cyrinid fish, the goldfish

ORAL 19 (Scaia, MF - Argentina)
Intrasexual aggression in cichlids: can estrogens be considered as key elements of the challenge hypothesis?</td></tr>
<tr><td rowspan="2">INTEGRATIVE 01

MIGRATION/REPRODUCTION OF NEOTROPICAL FISH
(15h00-16h00)</td><td>LECTURE 08

RONALDO BARTHEM (BRAZIL) (30 MIN)
General pattern of fish migration in the Amazon plan: implications for management and conservation</td></tr>
<tr><td>ORAL PRESENTATIONS
Chairs: Sergio Batlouni and Gustavo Somoza

ORAL 20 (Burgos-Morán, R - Ecuador)
Advances in the reproduction of Arapaima gigas (cuvier, 1829), in the ecuadorian amazon; a public-private partnership

ORAL 21 (Torati, LS - Brazil)
Cephalic secretion of Arapaima gigas: sex steroids, peptides and proteins suggest roles in chemical communication and parental care</td></tr>
<tr><td colspan="2">COFFEE BREAK (16h00-16h30)

POSTER SESSION 2 (16h30-18h00)
FROM POSTER 59 TO 90 (PLUS OP 11-21)</td></tr>
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<table>
<tr><td>WEDNESDAY – JUNE 6</td></tr>
<tr><td>EXCURSIONS AND OTHER ACTIVITIES</td></tr>
</table>

THURSDAY – JUNE 7

PLENARY LECTURE 3 (8h30)

ANNA WARGELIUS (NORWAY) (40 MIN)
Gene editing a way to solve and understand reproductive constraints in salmon farming

SESSION 8

BRAIN-PITUITARY SYSTEM
(9h10-10h25)

LECTURE 09

JOSÉ ANTONIO MUÑOZ-CUETO (SPAIN) (30 MIN)
The gonadotropin-inhibitory hormone system of fish: the case of sea bass (*Dicentrarchus labrax*)

ORAL PRESENTATIONS
Chairs: José Muñoz-Cueto and Gustavo Somoza

ORAL 22 (Zmora, N - USA)
Does compensation occur in gnrh3 gene knockout zebrafish?

ORAL 23 (Ciani, E - Norway)
Regulation of follicle-stimulating hormone (Fsh) expression during pubertal development in *Salmo salar* parr males

ORAL 24 (Roche, J - France)
Characterization, distribution and role of dopamine receptors in pikeperch during the pre-ovulatory period

COFFEE BREAK (10h25-10h50)

SESSION 9

PITUITARY-GONAD SYSTEM
(10h50-12h20)

LECTURE 10

FRANÇOIS CHAUVIGNÉ (SPAIN) (30 MIN)
New insights into the endocrine regulation of teleost spermiogenesis

ORAL PRESENTATIONS
Chairs: Penny Swanson and Hamid Habibi

ORAL 25 (Andersson, E - Norway)
Loss-of-function of the *fsh* receptor shortens the maturation cycle in male Atlantic salmon

ORAL 26 (Sambroni, E - France)
The mutation of fshr affects reproductive physiology and growth in zebrafish *Danio rerio*

ORAL 27 (Jesus, LWO - Brazil)
Molecular cloning, temporal expression and functional characterization of gonadotropin receptors in the characid fish *Astyanax altiparanae*

ORAL 28 (Kleppe, L - Norway)
Sex steroid production associated with puberty is absent in germ cell-free salmon

LUNCH (12h20-14h00)

INTEGRATIVE 02 **AQUACULTURE AND GENOME-ENVIRONMENT INTERACTION** (14h00-15h30)	**LECTURE 11** **YONATHAN ZOHAR (USA) (30 MIN)** **Who exactly is in control of reproduction in fish?** **ORAL PRESENTATIONS** Chairs: Hanna Rosenfeld and Goro Yoshizaki **ORAL 29 (Yazawa, R - Japan)** Production of functional bluefin tuna sperm using surrogate broodstock technology **ORAL 30 (Gavery, MR - USA)** Investigating the effects of early-rearing environment on sperm dna methylation programming in hatchery reared steelhead (*Oncorhynchus mykiss*) **ORAL 31 (Patil, JG - Australia)** Trojan y genetic manipulation of sex for controling gambusia holbrooki **ORAL 32 (Fernández-Díez, C - Spain)** Reproductive success after genotoxic stress: how fish embryos deal with paternal dna damage
COFFEE BREAK (15h30-16h00) **POSTER SESSION 3** (16h00-17h30) **FROM POSTER 91 TO 146 (PLUS OP 22-36)**	
GALA DINNER (EVENING) 20h00	

FRIDAY – JUNE 8

INTEGRATIVE 03

ENDOCRINE DISRUPTION
(9h00-10h30)

LECTURE 12

CHARLES TYLER (UK) (30 MIN)
The feminization of fish – from individual effects to population level consequences

ORAL PRESENTATIONS
Chairs: Fabiana Lo Nostro and Oliana Carnevali

ORAL 33 (González-Rojo, S - Spain)
Bisphenol A triggers histone hyperacetylation and alters *gper1* expression in zebrafish testicles

ORAL 34 (Forner-Piquer, I - Italy)
Disruption of the gonadal endocannabinoid system in zebrafish exposed to diisononyl phthalate

ORAL 35 (Weber, AA - Brazil)
Reproductive effects of oestrogenic endocrine disrupting chemicals in *Astyanax rivularis* inhabiting headwaters of the velhas river, brazil

ORAL 36 (Batista-Silva, H - Brazil)
Regulation of calcium influx in *Danio rerio* (zebrafish) testis: ionic modification and bis(2-ethylhexyl)phthalate effect

COFFEE BREAK (10h30-11h00)

PLENARY LECTURE 4

RÜDIGER SCHULZ (THE NETHERLANDS) (40 MIN)
Endocrine and paracrine control of spermatogenesis in fish

MEETING HIGHLIGHTS
PENNY SWANSON (USA) (20 MIN)

CLOSING REMARKS

LUNCH
(12h30)

PLENARY ABSTRACTS

PLENARY 1

DEVELOPMENT OF GERM CELL MANIPULATION TECHNOLOGY IN FISH

Yoshizaki, G.

Department of Marine Biosciences, Tokyo University of Marine Science and Technology, Tokyo, Japan.

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We had previously revealed several unique characteristics of germ cell using rainbow trout (*Oncorhynchus mykiss*), including (1) spermatogonia transplanted into the peritoneal cavity of newly hatched embryos can migrate toward recipient gonads, (2) spermatogonia transplanted into male or female recipients to start spermatogenesis or oogenesis and produce functional sperm or eggs, respectively, and (3) diploid germ cells transplanted into triploid recipients can complete gametogenesis. By combining these unique characteristics of fish germ cells, we produced triploid masu salmon (*Oncorhynchus masou*) that yields only rainbow trout gametes. Recently, we have applied this germ cell transplantation technique to several groups of fishes (e.g., *Scianidae*, *Carangidae*, *Adrianichthyidae*, *Cyprinidae*, *Cichlidae*, and *Tetraodontidae*). This germ cell transplantation technology has several promising applications in fish biotechnology. For example, bluefin tunas take 3 to 10 years to mature, and since broodstock individuals often weigh more than 100 kg (market size is much smaller), gamete production for this species is expensive in terms of time, cost, and labor and requires large net cages. However, if bluefin tuna spermatogonia could be transplanted into closely-related smaller fish that sexually mature quickly, it will produce gametes more easily and rapidly in small, land-based fish tanks. This germ cell transplantation technology could also be used to produce eggs and sperm of fish species facing extinction, such as sturgeon, which require at least 10 years of maturing. More importantly, donor germ cells could be cryopreserved in liquid nitrogen without losing their ability to resume gametogenesis in recipients, making it possible to semi-permanently preserve genetic resources of endangered species. Because reliable cryopreservation methods for fish eggs have not yet been established, this method could be a “silver bullet” for preserving valuable genetic resources of endangered fish. This presentation will outline the progress we have made in fish germ cell manipulation over the past 20 years. Furthermore, future perspectives of this technology will be discussed.

PLENARY 2

ENDOCRINE AND PARACRINE CONTROL OF FOLLICULOGENESIS IN THE ZEBRAFISH – A GENETIC APPROACH

Wei GE

Centre of Reproduction, Development and Aging (CRDA), Faculty of Health Sciences, University of Macau, Taipa, Macau, China

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Ovarian folliculogenesis is one of the most dynamic physiological and developmental processes in vertebrates. Despite numerous studies on this issue in mammals, the molecular mechanisms that control follicle development, especially the early phase of folliculogenesis, still remain poorly understood. Gonadotropins are primary endocrine hormones that control ovarian development and function. In addition, various local paracrine and autocrine factors in the ovary also play important roles in this process. Although the endocrine and paracrine controls of folliculogenesis have been studied in fish, there has been a lack of genetic data on their functional importance, mostly due to unavailability of the gene knockout approach in fish models. Using zebrafish as the model and the emerging genome editing technologies, TALEN and CRISPR/Cas9, we have generated a series of zebrafish knockout mutants for both endocrine hormones and local ovarian paracrine factors and analyzed their impacts on folliculogenesis. Using genome editing technologies, we first studied the importance of FSH and LH in follicle development by disrupting fshb, lhb, fshr and lhcgr genes. FSH-deficient zebrafish (fshb-/-) were surprisingly fertile in females; however, the puberty onset or start of vitellogenic growth was significantly delayed. In contrast, LH-deficient zebrafish (lhb-/-) showed normal gonadal growth, but the females failed to spawn and were therefore infertile. In contrast to fshb deficiency, the FSH receptor (fshr)-deficient females showed a complete failure of follicle activation; however, the deletion of lhcgr gene caused no obvious phenotypes. In addition to gonadotropins, we have also investigated potential involvement of local paracrine factors in controlling folliculogenesis. For example, disruption of growth differentiation factor 9 (gdf9) gene led to a complete arrest of follicle development at early stage, suggesting fundamental roles for local factors in orchestrating follicle activation and growth. Our data showed that follicle development in the zebrafish involves both external endocrine hormones and internal paracrine factors in the ovary.

ACKNOWLEDGEMENT This study was supported by grants from the University of Macau (MYRG2014-00062-FHS, MYRG2015-00227-FHS, and CPG2014-00014-FHS) and The Macau Fund for Development of Science and Technology (FDCT114/2013/A3 and FDCT/089/2014/A2).

PLENARY 3

GENE EDITING A WAY TO SOLVE AND UNDERSTAND REPRODUCTIVE CONSTRAINTS IN SALMON FARMING

Anna Wargelius[1*], Lene Kleppe[1], Kai Ove Skaftnesmo[1], Erik Kjærner-Semb[1], Fernando Ayllon[1], Anne Hege Straume[1], Hilal Güralp[1], Birgitta Norberg[1], Tomasz Furmanek[1], Dorothy Dankel[2], Ståle Ellingsen[2], Carl Johan Rubin[3], Tom Hansen[1], Per Gunnar Fjelldal[1], Rüdiger Schulz[1, 4], Eva Andersson[1], Rolf B Edvardsen[1]

[1] Institute of Marine Research, Bergen, Norway, [2] University of Bergen, Bergen, Norway,[3] Uppsala University, Uppsala, Sweden, [4] Utrecht University, Utrecht, Netherlands

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Recent biotechnological innovations offer opportunities to solve major salmon aquaculture sustainability issues. One is the genetic impact of escaped farmed salmon on wild populations, which is considered the most serious long-term negative effect on the environment. Reduced fish welfare associated with precocious maturity represents another obstacle, since robust farmed fish is needed to avoid negative stress phenotypes associated with sexual maturation, such as higher disease susceptibility and osmoregulatory problems. The CRISPR-Cas9 methodology allows editing of specific DNA sequences in any organism, including fish, thus permitting functional studies of genetic traits relevant for aquaculture. We have thus established knock out technology in salmon and are currently exploring knock-in. This in combination with the sequencing of the Atlantic salmon genome allows starting a new era of improved breeding in salmonid aquaculture. Current and future studies are now aiming at elucidating how genetic traits influence for example disease resistance, reproduction, or welfare, and ultimately if and how gene edited salmon can be used in aquaculture to solve major environmental bottlenecks such as escaped fish and diseases. The criticism of GMOs has largely focused on transgenesis, in which genetic material from unrelated species are added to create new or alter traits, such as the growth hormone transgene salmon. Cisgenic editing does not introduce "foreign" DNA, but changes the existing sequence, often into another allele also known from the same species. Thus, clear conceptional differences exist compared to technologies previously used for GMO production. We are therefore also exploring the ethical and legal constraints and possibilities of using gene edited fish for sustainable aquaculture solutions. A first prerequisite for the introduction of gene edited fish to sea cage farming is sterility, to ensure reproductive containment. To induce 100% sterility, we have in this context explored possibilities to produce fish that lack germ cells, thereby avoiding both the risk of genetic introgression with wild populations. Using targeted mutagenesis against the *dnd* gene with the CRISPR-Cas9 methodology, we generated gene-edited fish lacking germ cells and hence fully sterile. However, since these fish lack germ cells, it is challenging to continue breeding this trait. Another path to control the timing of reproduction and reduce genetic introgression is to use fish predetermined to mature late. If escaping, late maturing fish are more likely to die before reaching spawning grounds of wild salmon. We have found a region in the salmon genome largely controlling sea age at sexual maturity. We are currently using gene editing to identify the causative mutation both by knockout of genes in the region and by homologous recombination. The use of gene editing can also help to understand basic reproductive mechanisms in salmon. In this context, we are currently exploring the functions of several proteins involved in reproductive processes, such as the Fsh receptor. In summary, gene editing is not only an important tool for understanding salmon biology, but we also foresee that the potential improvements considering sustainability issues may result in the future, the use of gene edited fish in farming.

PLENARY 4

ENDOCRINE AND PARACRINE CONTROL OF SPERMATOGENESIS IN FISH.

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Pituitary gonadotropins control spermatogenesis in vertebrates and removal of both gonadotropins or their receptors reliably blocks testis maturation and spermatogenesis. However, the capacity of fish testes to compensate for the loss of a single gene/protein function that, in mammals, leads to infertility, is impressive indeed and allows to work with experimental models in teleost species that are not available in mammals so far. This not only applies to gonadotropins or their cognate receptors but also to genes/proteins involved in androgen production or androgen action. These studies improve our understanding of basic biological processes, such as the regulation of germ and somatic stem cell function in the testis. Moreover, results obtained in the course of this work may be relevant to improve sustainability aspects of finfish aquaculture, or for our understanding of how environmental chemicals can disturb endocrine signaling and thereby affect fish reproduction.

In mice, luteinizing hormone (LH) regulates Leydig cell androgen production. Loss of LH, of its cognate receptor (LHCGR), or of the androgen receptor (AR) all result in infertility due to a failure of spermatogenesis during meiosis. Use of a primary testis tissue culture system allowed demonstrating that gonadotropin-stimulated androgen production, or androgens alone, supported full spermatogenesis in eel. Surprisingly, however, experiments in medaka showed that spermatogenesis proceeded normally after removal of an enzyme (Cyp17a1) required for androgen production. Under these circumstances, the production of a progestin (DHP) may have increased, which stimulated spermatogenesis in eel, tilapia and zebrafish. Future work should examine if DHP signaling can compensate for the loss of androgens under these conditions. Also after loss of a functional *ar* gene, zebrafish males still produced some spermatozoa, although testis size and spermatogenesis were compromised. Clearly, the androgen-dependency of spermatogenesis in fish is much less strict than in mammals, and additional ways of regulating spermatogenesis exist that require characterization.

Large-scale gene expression profiling experiments in trout and zebrafish showed that in the absence of androgens, follicle-stimulating hormone (Fsh) had prominent, specific effects on testicular gene expression. An important role for Fsh has been suggested repeatedly in salmonids where most of the pubertal testis growth takes place with measurable Fsh but very low to undetectable Lh plasma levels. We therefore focused our recent work on growth factors regulated by Fsh but not by Lh (or androgens) to investigate Fsh-specific, growth factor-mediated regulation of spermatogenesis, using zebrafish as experimental model. While briefly touching upon published work on Leydig cell-derived insulin-like peptide 3 (Insl3) or Sertoli cell-derived anti-Müllerian hormone (Amh), we will present new work on how Fsh makes use of different components of the evolutionary conserved Wnt signaling system to regulate initial steps of spermatogenesis in a balanced manner; this also involves the insulin-like growth factor 3 (Igf3).

The impression emerging from studies on the regulation of spermatogenesis in fish is that several pathways operate in parallel (e.g. Lh/Fsh – progestin; Lh/Fsh – androgen; Fsh – prostaglandins; Fsh – retinoic acid; Fsh – growth factors [Igf3, Wnt, Amh, Gsdf, Insl3, …?]). All pathways contribute to the regulation of spermatogenesis while no single one of the stimulatory pathways described so far seems to be strictly required. This regulatory redundancy may bring about a certain resilience of the spermatogenic process to the loss of individual pathways.

LECTURE ABSTRACTS

LECTURE 1

SEX DETERMINATION DIVERSITY IN FISH

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Fish show the greatest plasticity of sex determination (SD) mechanisms amongst vertebrates. In the case of genetic SD this is linked to a similarly high variability of sex chromosome differentiation. While in a handful of species with genetic SD the master SD genes have been identified, their molecular function in directing the development of the bipotential gonad primordium towards testis or ovary is unclear in many cases or incompletely known in the others. To obtain a better understanding of the biological meaning of this diversity we need a deeper understanding of the molecular basis of SD mechanisms and the structure and genetic organization of sex chromosomes across a broad diversity of actinopterygian fish. To identify primary SD genes we used high throughput RAD-tag marker mapping in >40 species as well as transcriptomics, Pool-Seq and genome sequencing to identify sex-specific chromosomal regions and candidate SD genes. This led to the identification of sex-specific markers, allowing to delineate the extent of recombination suppression, which turned out to be highly variable between species. We identified several species with clear cut XX/XY or ZZ/ZW monofactorial systems but also species with more complex sex-determination systems including species with a mix of GSD and ESD and species with potential polygenic systems. In species with available genomic resources, sex-specific markers could be used to assign scaffolds to regions that are supposed to contain the primary SD gene. We identified candidate genes in several species and find that most of them belong to already known factors of the primary SD regulatory network including candidate genes that have not been found so far as being SD genes. We also find that many species harbor very poorly differentiated sex-chromosomes. While the variety of SD genes that can trigger the male or female SD regulatory network and the differentiation of the gonads is now well established, it is only emerging that also the whole system downstream of the primary SD factors is subject to evolutionary change and - like the top SD genes - can vary even between closely related species. Transcriptome profiling of gonads during male and female development and of mature ovaries and testes uncovered an unexpected extent of differential gene expressions in contrast to an obviously striking morphological and physiological conservation of the reproductive organ system.

LECTURE 2

HOW DO GERM CELLS DETERMINE THEIR OWN SEXUAL FATE?

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The studies on germ cells are important not only for basic biology but also for aquaculture. Understanding and manipulation of the mechanism in germ cells lead to a benefit in aquaculture, e.g. a large production of eggs/sperm and/or offspring at the desired time. Most studies on the germ cells, however, have been intensively done using mammals and have provided a general picture of germ cell regulation: germ cells are the special cells destined to develop gametes and are strictly controlled by the surrounded somatic environment. This picture is now a kind of dogma among most researchers. But the recent studies using fish begin to unveil more positive aspects of germ cells. Germ cells can determine how many gametes accommodate in the gonad and which sexual fate to develop, either to eggs or to sperm. Germ cells are even a critical component for feminizing the body which acts downstream of genetic sex determination system. In this talk, we focus on the mechanism of germ cell-autonomous sexual fate decision. The key gene for the sexual fate decision that we had identified was *foxl3*, a gene encoding one of the forkhead transcriptional factors. Germ cells in the female gonad continue to express *foxl3* until they enter meiosis. The disruption of this gene causes sperm production in ovary, indicating that *foxl3* functions as a switch gene repressing the onset of spermatogenesis. In other words, once germ cells commit to spermatogenesis, they go all the way to the end (mature sperm) even in female environment. Therefore it is very interesting to understand the mechanism of the fate decision of germ cells towards sperm in the absence of *foxl3* function. Transcriptome analyses allow us to see the candidate genes. We would like to discuss what is happening when the germ cells are making a decision.

LECTURE 3

BIOTECHNOLOGICAL PROSPECTS OF MALE GERMLINE STEM CELLS IN THE NILE TILAPIA

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In the last decades, studies using spermatogonial stem cell (SSCs) transplantation have contributed significantly to the understanding of the biological characteristics of fish SSCs and their application to developmental biotechnology, as well as to enabling the formation of gene banks, preserving endangered species, and creating opportunities for the genetic improvement of economically important species. Besides that, due to their ability to continuously transmit the genetic information to the subsequent generation, the genetic modification and transplantation of SSCs provide a great opportunity to study the biology of their complex processes, also enabling the generation of transgenic fish strains. In this context, the Nile tilapia emerges as an excellent experimental model to investigate the practicability of new reproductive technologies since this species has been largely responsible for the current expansion of freshwater aquaculture in the world. In addition to the development of a methodology for SSCs transplantation, molecular markers for the appropriate identification and enrichment of Nile tilapia SSCs were previously described. These important studies were still fundamental for the establishment of culture conditions in which Nile tilapia spermatogonia were able to be maintained and amplified in vitro. This system now allows us to investigate important aspects of SSCs, particularly with respect to the genetic manipulation of these cells. Nowadays, nanomaterials are been extensively used to genetically transform cells of diverse origins and this also includes fish cells. For the first time, we have demonstrated that functionalized carbon nanotubes are capable of promoting gene delivery in Nile tilapia SSCs with good efficiency when compared to conventional transfection methods. Also, the ability of lentiviral vectors transducing SSCs in vitro has been investigated. As it results in long-term integration and expression of the transgene, similar to mammals, viral transduction may represent an efficient method to introduce genes into fish male germline. Transduced Nile tilapia SSCs when transplanted into recipient larvae resulted in adult fish that produced sperm carrying the transgene. Stable genetically modified Nile tilapia SSCs could also be obtained using tolls for site-specific genomic integration and the functionality of this approach is now under evaluation. Therefore, this system might represents an important alternative tool for fish transgenesis, avoiding the need of mosaic generation in the transgenesis process.

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LECTURE 4

BROODSTOCK MANAGEMENT AND SPAWNING INDUCTION OF GREATER AMBERJACK *Seriola dumerili* REARED IN SEA CAGES IN GREECE

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The greater amberjack (Seriola dumerili) is a species with a great potential for the Mediterranean aquaculture industry, due to its excellent flesh quality and worldwide consumer acceptability. We describe here broodstock management and spawning induction methods for greater amberjack maintained in sea cages in Greece. Wild captive-reared individuals were maintained under different conditions in various locations around Greece. Broodstocks were fed with raw fish and squid and/or a commercial diet (Skretting, Vitalis CAL). Females eligible for spawning induction (vitellogenic oocytes 650 μm in diameter) were treated with GnRHa EVAc implants. Fish from sea cages were transferred to land based tanks for spawning. Eggs fecundity and fertilization success were estimated every day, and hatching and larval survival to yolk absorption was monitored. Broodstocks held in tanks over the year did not undergo gametogenesis reliably, with <20% of the females being in full vitellogenesis, but with also extensive atresia. On the contrary, in sea cages almost all females were in full vitellogenesis and some were even undergoing maturation and ovulation spontaneously. Egg collection in sea cages was not very successful, and a relatively small amount of eggs was collected over the three years of the experiments. On the contrary, maintaining the broodstocks in cages during the year and then transferring them to land-based tanks for spawning after GnRHa therapy was proven very effective. Males during the three years of the study were not releasing sperm with abdominal pressure, but in most of the cases collection of sperm was possible using a catheter. Concerning sperm quality parameters of all captive-reared greater amberjack, sperm motility was 77±3%, motility duration was 3.7±0.2min, sperm density was 30±2 109 szoa ml-1 and sperm survival was 8±1days, values that are considered appropriate for good fertilization success. Broodstock management and hormonal spawning induction methods have been optimized for greater amberjack maintained in sea cages and transferred to land-based tanks for spawning.

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LECTURE 5

REGULATION OF DEVELOPMENT OF PREVITELLOGENIC OVARIAN FOLLICLES BY ANDROGENS

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Ovarian follicles of teleost fishes undergo substantial increases in size and functionally differentiate during primary and early secondary (previtellogenic) growth. During this time, oocytes are provisioned with numerous maternal factors that support further follicular and/or early embryonic development. Thus, both potential fecundity and important aspects of egg quality are determined prior to the onset of vitellogenic growth. The roles of pituitary gonadotropins in previtellogenic follicle development remain unclear, but work with two teleost models (freshwater anguillid eels, coho salmon) has shown that a non-aromatizable androgen, 11-ketotestosterone (11-KT), promotes the development of previtellogenic ovarian follicles in vitro and in vivo. Recent studies by Lokman and colleagues on New Zealand and Japanese eels have revealed that androgen-induced previtellogenic follicle growth is associated with changes in gonadotropin signaling, steroidogenesis, and lipid uptake and processing. Using coho salmon as a model, we have shown that 11-KT at low concentrations in vitro promotes substantial increases in follicle size, and development to a very early secondary follicle phenotype. Estradiol-17β was without effect on growth at this stage, but both steroids increased the size of the early secondary follicle. To determine the transcriptomic changes accompanying androgen-induced follicle development and to explore the hypothesis that 11-KT functions to sensitize the developing follicle to Fsh and enhance estrogenic signaling, we treated females containing late primary or early secondary follicles with 11-KT. After 3 days of exposure, follicles were subjected to RNA-Seq and pathway analyses. In vivo exposure to 11-KT profoundly altered the transcriptome of primary follicles. The 400+ androgen-sensitive genes identified included those involved in gonadotropin, steroid and growth factor signaling, and cell and ovarian development. Ovarian development, tissue differentiation/remodeling, and lipid metabolism were among the biological functions/pathways potentially altered by 11-KT. The early secondary follicle was even more transcriptionally sensitive to androgen action, with over 3,800 differentially-expressed genes identified. At both stages, 11-KT altered the clathrin-mediated endocytosis signaling pathway, suggesting involvement of androgens in lipid and vitellogenin uptake and processing. Collectively, these studies demonstrate that the actions of 11-KT on previtellogenic follicles include alterations in signaling, metabolism, and morphology that potentially increase the sensitivity of the follicle to FSH, upregulate steroid biosynthesis and signaling, and enhance the molecular machinery for lipid and vitellogenin uptake and processing.

LECTURE 6

MOLECULAR PORTRAIT OF EGG QUALITY AND NOVEL MATERNAL-EFFECT GENES

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Good quality or developmentally competent fish eggs are successfully fertilized and develop normally as viable, non-malformed embryos. However, the detailed mechanisms involved in egg quality remain poorly understood and, at present, no predictive markers of egg quality exist. Early developmental success relies on maternally-inherited molecules deposited in the oocyte throughout oogenesis, and over the last few years, our work has aimed at drawing the molecular picture of poorly competent (i.e. bad quality) fish eggs in order to (1) further uncover the molecular mechanisms associated with low developmental success, (2) reveal new maternal-effect genes that play important roles during early development, and (3) possibly obtain molecular signatures based on maternal mRNA profiles in fertilized eggs that could be used as predictive markers of good quality eggs. A large transcriptomic analysis was performed in zebrafish (Danio rerio) using a high number of egg clutches obtained under normal husbandry conditions that reflect naturally occurring variability in egg quality. The differentially expressed gene profile revealed dysregulation of genes involved in translation and protein synthesis. Interestingly, translation was also one of the main processes dysregulated in a similar study conducted in Sea Bass (Dicentrarchus labrax). Analysis of a smaller number of zebrafish females that consistently produced bad eggs demonstrated a much lower variability in expression profiles that resulted in a higher number of differentially expressed genes identified between good and bad quality eggs. These indicate that while common/general traits of bad quality egg exist, a multiplicity of other factors can dysregulate specific processes that ultimately lead to reduced egg quality. To gain insight into their functions, genome editing using the CRISPR/Cas9 technology was performed to create mutants of two new potential maternal-effect genes, otulina (OTU deubiquitinase with linear linkage specificity a) and slc29a1a (solute carrier family 29, member 1a), that are especially abundant in the ovary. Due to the high efficiency of this technique, mutations could not be transmitted to the next generation and the phenotype was analyzed in F0 females. We observed that both otulina and slc29a1a mutant-derived eggs had very low developmental success. Eggs from mutant females crossed with vasa:eGFP males do not contain GFP, suggesting that they cannot be fertilized. These novel findings showed for the first time that otulina and slc29a1a are essential for the developmental competence of eggs, therefore, are crucial maternal-effect genes. Finally, we applied statistical modeling using Partial Least Square (PLS) regression and genetic algorithm to our data to search for possible molecular signatures. We observed the presence of strong gene signatures, which were statistically robust both in terms of reproducibility and validation by pseudo-data, to link gene expression to the survival rate of eggs in our transcriptomic data. In summary, the molecular mechanisms that control egg quality are complex and probably variable even though some core processes including translation and proteins synthesis appear to play a key role. Yet, some maternal-effect genes have been identified that are important for fertilization. Finally, it appears possible to statistically predict developmental success based on the molecular portrait of the egg.

LECTURE 7

CHEMICAL COMMUNICATION IN FISHES WITH EMPHASIS ON CICHLIDS

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Communication and behaviour are tightly linked and fish use several sensory channels to communicate. Chemical communication is the most ancient form of communication but its role is often ignored because the substances involved are not known. Among the teleost fishes, cichlids are of special interest for the study evolution, for aquaculture and as invasive species in some parts of the world. In the Mozambique tilapia and other cichlids, reproduction and male aggression are mediated through urinary cues tactically released by dominant males. The olfactory potency of male urine depends on the donors' social rank. Females spawn preferentially with dominant males and increase sex steroid production in the presence of male urine. Moreover, dominant males increase urination frequency to signal their dominance status to rivals and reduce male-male aggression. The identity and biological function of the most potent odorants in the urine of dominant Mozambique tilapia males has been elucidated and are important tools to establish the associated neurobiological mechanisms and with potential applications in aquaculture and in population control.

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LECTURE 8

GENERAL PATTERN OF FISH MIGRATION IN THE AMAZON PLAN: IMPLICATIONS FOR MANAGEMENT AND CONSERVATION

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The most important commercial food fishes are the migratory species, which are responsible for at least 87% of the fish landing in the biggest cities of the Amazon basin. Considering the high variability of spatial displacements presented by the fishes in the Amazon basin, the migratory species are considered here as the species that spent part of their life in the channel of river, especially as egg or larvae during the downriver drift migration until they reach the nursery area. The flood pulse synchronize the reproductive period what generally happens in the flood period. Some species spawn at the beginning of the flood period and others at the end. The knowledge of the migration behavior is important for the fisheries management due to the efficiency of the protection of the reproductive and nursery areas to avoid the overfishing. For this reason, the understanding of the general and specific migration pattern of the most important fishery resource is crucial in the maintaining of the relevance of the fishing activity in the Amazon basin. The general pattern of the migration of the most important commercial food fishes in the Amazon plan may be grouped according the length of the migration route. The small species (less than 20 cm) realize generally short distance migration (<400 km). However, the commercial fishery does not exploit this species due to this size, and its migration is generally unknown. The migration of the small catfish Trichomycterus barbouri (Trichomycteridae) (less than 10 cm) is a rare example of the short distance migration studied case. This migration was studied in the Beni River because the local fishermen developed a special technique to catch them while they realize the upriver migration. T. barbouri migrate 370 km between the nursery areas in the Beni wetland, at 180-200 m of altitude, as far as the high Beni River, at 220-400 m of altitude. The long distance migration (>400 and <2.000 km) is very common for medium to large size species of Characiformes and Siluriformes of high commercial values. Studies of Semaprochilodus spp. and Colossoma macropomum migration in the Central Amazon indicating they may migrate upriver 1,300 Km and they may share the spawning area (the meeting of turbid and black or clear water rivers), the nursery area (floodplain lakes), and the feeding area (flooded forest). The last group is the continental-scale migration (>2.000 km), realized by a group of large size species of catfish (Brachyplatystoma). They migrate between the western and eastern Amazon, with the extreme route reaching the Andes piedmont and the estuary. All these species depends of the turbid water rivers and the flood pulse as transport mechanism that carry the egg and larvae from the spawning are to the nursery area.

LECTURE 9

THE GONADOTROPIN-INHIBITORY HORMONE SYSTEM OF FISH: THE CASE OF SEA BASS (*Dicentrarchus labrax*)

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Gonadotropin-inhibitory hormone (GnIH) is a hypothalamic neuropeptide belonging to the RFamide peptide family that was first discovered in quail by Tsutsui and co-workers in the year 2000. Since then, different GnIH orthologs have been identified in all vertebrate groups, from agnathans to mammals. These GnIH genes synthesize peptide precursors that encompass two to four C-terminal LPXRFamide peptides. Functional and behavioral studies carried out in birds and mammals have demonstrated a clear inhibitory role of GnIH on GnRH and gonadotropin synthesis and secretion as well as on aggressive and sexual behavior. However, the effects of GnIH orthologs in reproduction remain controversial in fish with both stimulatory and inhibitory actions being reported. In our laboratory, we have cloned a full-length cDNA encoding two putative Gnihs (sbGnih-1 and sbGnih-2) in the European sea bass, *Dicentrarchus labrax*, and developed molecular tools and specific antibodies to elucidate the tissue and cellular distribution of the Gnih system. The expression of *gnih* was particularly evident in the olfactory bulbs/telencephalon, diencephalon, midbrain tegmentum, rostral rhombencephalon, retina and testis. The immunohistochemical study was highly consistent, revealing Gnih-immunoreactive (ir) perikarya in the same central areas and Gnih-ir fibers that profusely innervated the brain and pituitary of sea bass. Moreover, *in vivo* studies revealed the inhibitory role of centrally- and peripherally-administered Gnih in the reproductive axis of male sea bass, by acting at the brain (on *gnrh* and *kisspeptin* expression), pituitary (on *gnrh* receptors and gonadotropin synthesis and release) and gonadal (on androgen secretion and gametogenesis) levels. Centrally-administered Gnih also affected mRNA levels of neurosteroid-synthesizing enzymes, decreasing 17β-hydroxysteroid dehydrogenase (*hsd17b*) and 3 β-hydroxysteroid dehydrogenase (*hsd3b*) transcript levels and increasing the expression of brain aromatase (*cyp19a1b*). Mirror test analysis has shown that Gnih was able to affect the agonistic behavior of male sea bass. Taken together, our results have revealed the existence of a functional Gnih system in sea bass, and provided evidence of the differential actions of the two Gnih peptides on the reproductive axis of this species, the main inhibitory role in the brain and pituitary being exerted by the sbGnih-2 peptide. Our results have also shown that intracerebroventricular administration of Gnih can inhibit the aggressive behavior of male sea bass by decreasing androgen production and increasing neuroestrogen synthesis in the brain. Recent studies developed in our laboratory also suggest that Gnih might be involved in the transduction of photoperiod and temperature information to the reproductive axis, as well as in the modulation of daily and seasonal rhythmic processes in sea bass.

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LECTURE 10

NEW INSIGHTS INTO THE ENDOCRINE REGULATION OF TELEOST SPERMIOGENESIS

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The molecular and endocrine regulation of spermiogenesis, which controls the developmental transformation and remorphing of post-meiotic haploid germ cells into spermatozoa, has barely been studied in teleosts. An excellent model for studying such regulation in fish is the marine flatfish Senegalese sole (Solea senegalensis), because it exhibits a semi-cystic type of spermatogenesis, where the whole process of spermatid differentiation into spermatozoa occurs in the lumen of the seminiferous tubules. Our investigations to date have uncovered hitherto unknown roles of the pituitary gonadotropins follicle-stimulating (Fsh) and luteinizing (Lh) hormones during spermatogenesis and spermiogenesis in this model organism. By using a set of molecular and cellular approaches, including the production of functional homologous single-chain recombinant Fsh and Lh, we were able to decipher common and distinct molecular pathways induced by each gonadotropin regulating germ cell development and spermiogenesis in the testis. The most striking discovery was that free spermatids express the Lh receptor (Lhcgr) which is activated by Lh to trigger spermatozoon differentiation through the cAMP/PKA signaling cascade and the transcription of genes necessary for the function of spermatozoa, including those necessary for flagellum formation. We subsequently discovered that the expression of Lhcgr in germ cells also occurs in other distantly related orders of teleosts, including Perciformes and Cypriniformes with cystic spermatogenesis. Such findings suggest that this feature may be conserved throughout the teleost crown clade, regardless of the type of germ cell development. The basic knowledge and biotools derived from these experiments have been successfully applied to the development of hormone therapies for captive sole males, in order to enhance sperm production and quality. Here, the results that we have obtained in the last few years will be summarized and discussed in a more global view on the role of gonadotropins and steroid hormones in the regulation of spermiogenesis in teleosts.

LECTURE 11

WHO EXACTLY IS IN CONTROL OF REPRODUCTION IN FISH?

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The conventional wisdom is that reproduction is controlled by the brain-pituitary-gonadal (BPG) axis. A series of brain peptides, including kisspeptins, GnRHs, GnIHs, Neurokinins and others are responsible for the synthesis and release of the pituitary gonadotropins - luteinizing hormone (LH) and follicle stimulating hormone (FSH). LH and FSH, in turn, regulate gonadal steroidogenesis which drives spermatogenesis and oogenesis. However, recent advances in gene editing and functional genomics, using zebrafish as a model, demonstrate that this dogma may be too simplistic, which may have important implications for controlling reproduction in aquaculture. This presentation reviews the use, by our group and others, of knockout technologies for silencing several key hormones along the BPG axis. The expression of the knocked-out peptides/proteins is eliminated for the life of the fish and is hereditary. The mutated fish were raised to maturity and their BPG axis and reproductive performances were evaluated. Using gene editing to knockout in zebrafish the two kisspeptins and their receptors, the two GnRHs, LH and FSH does not lead to loss of reproduction. The mutated fish continue to display varying degrees of reproduction, including full gametogenesis leading to the production of functional gametes (except in LH knockouts, which do not spawn), viable embryos, and phenotypically normal offspring that carry the gene deletions into the next generation and continue to reproduce. These findings are very confusing to fish reproductive biologists and challenge the simple BPG concept. Interestingly, in zebrafish, using laser ablation to entirely remove the cells and neurons of the reproductively most relevant GnRH3 during early development does lead to the arrest of gametogenesis and to reproductively sterile fish. Contrary to the findings in brain and pituitary, genetic silencing of certain reproductive genes in the gonads leads to reproductive abnormalities, total lack of gametogenesis, and sterile fish. Taken together, these findings demonstrate that fish can continue to reproduce despite the absence of key reproductive hormones in the brain and pituitary. This data further suggests that fish have developed back-up strategies and redundancies in which yet-to-be-discovered factors and mechanisms compensate for the lack of the known reproductive regulators, thereby ensuring that reproduction and survival of the species continues despite the lack of the key hormonal players. Studies towards unveiling such compensation strategies will be presented. This new understanding (described above) has important implications for controlling fertility and sterility in aquaculture, which will be discussed.

LECTURE 12

THE FEMINIZATION OF FISH – FROM INDIVIDUAL EFFECTS TO POPULATION LEVEL CONSEQUENCES

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Over 100 000 chemicals are discharged into the environment that derived from human activities some of which are known to alter hormone systems in exposed fish (and other wildlife) affecting developmental processes, including reproduction. More than 1000 chemicals have now been shown to be capable of interacting with hormone receptors or affect enzyme systems involved with hormone biosynthesis or metabolism, illustrating the scale of the issue faced. Around 200 of these so-called endocrine disrupting chemicals (EDCS) have been identified with estrogenic activity and associated with feminised responses in fish, including yolk protein (vitellogenin) induction, reduced sperm production and quality in males, and intersex. Effects of EDCs found also extend to altered sexual behaviours. Some studies have shown reduced reproductive fitness of feminised males. The consequences of these feminised responses for wild fish populations, however, are not well understood. In our research on wild populations of roach (Rutilus rutilus) living in UK Rivers, using an array of molecular tools (including, DNA microsatellites, SNPs and RAD seq), we have not found an impact of exposure to oestrogenic wastewater effluents on the effective population sizes (Ne). We have, however, seen evidence for genetic adaptations to those pollutant discharges. To-date, the focus on environmental oestrogens has predominantly been on the direct effects on gonadal development and reproduction, but oestrogens play much wider roles in fish physiology which in turn can also impact on reproduction. Through the development of oestrogen responsive element transgenic (ERE-TG) biosensor zebrafish (ERE-TG fish) we have shown that a wide range of body tissues including in the liver, heart, muscle and forebrain are responsive to environmental oestrogens with effects on their function. Similarly, applying other transgenic zebrafish fish lines that allow identification of neural activity in the brain, we have shown various environmental oestrogens can directly affect brain activity and we are investigating if these exposures have sensing and/or behavioural outcomes. The final part of this presentation will provide a few illustrative examples of how the combination of EDCs with other environmental stressors may have greater functional consequences for fish and fish populations than for chemical exposures alone, as occurs for chemical risk assessment.

ORAL PRESENTATION ABSTRACTS

SEX DETERMINATION

ORAL 1

METHYLOMIC AND TRANSCRIPTOMIC DYNAMICS DURING TESTIS AND OVARIAN DIFFERENTIATION IN THE EUROPEAN SEA BASS, AS ASSESSED BY MULTIPLEX BISULFITE SEQUENCING AND WEIGHED CORRELATION NETWORK ANALYSIS

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Introduction

In species with polygenic sex determination (PSD), the influence of external factors is evident, with temperature playing a major role. In this context, epigenetic regulatory mechanisms, which integrate genomic and environmental influences are crucial but their actual role remains largely unexplored. In this study, we used the European sea bass (Dicentrachus labrax), a fish with PSD. We investigated both the transcriptomic dynamics during mid-stages of testis and ovarian differentiation and the methylomic lansdcape in key selected genes related to sexual development in juvenile fish previously exposed to elevated temperature as opposed to control temperature.

Methods

For the transcriptomic analysis during sex differentiation, we used a transcriptomic dataset from males and females sampled at 110 days post fertilization (dpf), and at 250 dpf. Weighted Gene Co-expression Network Analysis (WGCNA) and the analysis of transcriptome dynamics were performed. WGCNA allows to determine correlation patterns among canonical genes and signaling pathways previously related to gonad development in fish. Correlation network analysis also applied to a selection of differentially expressed genes (DEG) between sexes at 110 and/or 250 dpf that significantly changed between these two developmental points within each sex. For the methylomic analysis we used a multiplex bisulfite sequencing approach targeting also key genes involved in gonadal development.

Results and Discussion

The WGCNA revealed strong correlation among numerous canonical genes and signaling pathways previously related to gonad development in fish under a stringent statistical threshold. The same analysis applied to DEG between males and females that also change their expression though the development, identified 245 potential candidate genes which could have putative roles in sex differentiation. A comprehensive study of these candidate genes was performed by Gene ontology enrichment analysis, their genomic distribution and heatmap of their expression patterns. The time course expression analysis revealed that active upregulation of pro-male genes is necessary for testis development while ovarian development requires both active upregulation of pro-female genes and concomitant active downregulation of pro-male genes. DNA methylation analysis allowed the identification of the most responsive genes to temperature and used a panel of selected CpGs for Principal Component Analysis. This process allowed the prediction of sex based on epigenetic profiles for the first time with high success. These results reveal a complex epigenetic layer, influenced by both genetic background and early developmental environment, which contributes to the sexual phenotypic outcome.

Conclusion

This study provides a list of candidate genes to further investigate the entangled network of genes that regulate sex differentiation in fish and illustrates the important role of epigenetic regulatory mechanism in mediating environmental influences on gene expression.

ORAL 2

COEXISTENCE OF GENOTYPIC AND TEMPERATURE-DEPENDENT SEX DETERMINATION IN COBALTCAP SILVERSIDE

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Introduction

The coexistence of a male sex-determining gene, the Y chromosome-linked anti-Müllerian hormone (*amhy*) and of temperature-dependent sex determination (TSD) has been described in several Atherinopsids (New World silversides). Such species provide unique models to explore the interplay between genotypic and environmental sex determination and the evolution of these alternative sex determining mechanisms in fish, but little is known in other Atheriniformes. In this study, we extended the search for an *amhy* homologue and the presence of TSD to an Old World atheriniform, the cobaltcap silverside *Hypoatherina tsurugae* (Atherinidae).

Methods

Primer sets were designed based on coding regions of the autosomal *amh* (*amha*) and those that produced more than 1 fragment were selected and amplified in wild females and males to check for sex linkage. The putative *amhy* fragment was sequenced and the transcription level during gonadal sex differentiation of larvae reared at 22ºC (average temperature of spawning season) were measured by q-PCR. To verify the existence of TSD, newly hatched larvae were reared at 18, 22 and 26°C and their *amhy*-based sex genotype and histological sex phenotype were compared. We also screened a wild population in Tokyo Bay for the presence of sex-reversals and estimated the hatching date (by otolith increment analysis; see Miyoshi et al.; this symposium) and the temperature each individual experienced shortly after hatching.

Results and Discussion

An homologue of *amhy* from cobaltcap silverside was successfully cloned. PCR analysis with genomic DNA from wild adults and from laboratory-reared juveniles revealed a high, but not complete association between *amhy* and maleness. *amhy* transcription (in *amhy*-positive larvae) started before and peaked during histological differentiation of the gonads. The high expression of *amhy* early in larval development and the high association with maleness suggest that *amhy* is a male sex determinant in this species. Comparison of the phenotypic and genotypic sex in laboratory-reared individuals revealed that the frequencies of sex-reversed XX-males and XY-females at 18, 22 and 26°C were 0, 11, and 42%, and 67, 10, and 8%, respectively, indicating the occurrence of TSD. Screening of wild juveniles collected in Tokyo Bay in 2014 and 2015 revealed 14-17% XX-males and 8-10% XY-females whereas in 2016 there were 43% XX-males and no XY-females. The birth date/early life water temperature estimation indicated that fish were born later and experienced higher temperatures as larvae in 2016 compared to the other years.

Conclusion

This study shows for the first time the presence of an *amhy* homologue as a genotypic sex determinant and of TSD in an Old World silverside.

ORAL 3

SEX DETERMINATION AND DOWNSTREAM EVENTS ASSOCIATED WITH GONADAL SEX DIFFERENTIATION IN SABLEFISH.

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Introduction

Fish exhibit a variety of sex determination mechanisms and an increasing number of genetic sex determinants, ranging from immune-related to transforming growth factor genes. We have recently studied sex determination and differentiation in a Scorpaeniform species, sablefish (Anoplopoma fimbria). Because sablefish exhibit sexually dimorphic growth and are typically grown in ocean net pens, there has been interest in producing monosex and reproductively sterile stocks for aquaculture. Toward these objectives experiments were conducted to identify their sex determination mechanism and characterize molecular and morphological sex differentiation.

Methods

Sex-reversed female sablefish (neomales) were generated through dietary methyltestosterone treatment, crossed with female broodstock, and progeny testing conducted. An ontogenetic series of samples (whole embryos/larvae, trunks, or gonads) were collected weekly from untreated fish to assess molecular and morphological changes during sex determination and differentiation. Transcripts for the proposed sablefish sex-determining gene, gonadal soma-derived factor (gsdf), were localized by in situ hybridization and expression of X- and Y-copies of gsdf analyzed during early development via qRT-PCR. Other genes associated with sex differentiation were also assessed for comparison of their relative developmental timing of expression.

Results and Discussion

Progeny resulting from control male-female crosses averaged 55% female whereas those from neomale-female crosses were 100% female. This established an XX/XY sex determination system for sablefish. Messenger RNA for gsdf was localized to Sertoli and granulosa cells of testes and ovaries, respectively. Levels of gsdf-X mRNA remained low in embryos and larvae and no sexually dimorphic pattern was observed. Conversely, levels of gsdf-Y increased in males after hatching. Gonadal levels of gsdf increased in both sexes when fish reached ~50 mm fork length (FL) but remained higher in males than females. Increases in gsdf expression coincided with significant elevations in other key genes associated with sex differentiation, including dmrt1 and amh in males, and foxl2 and cyp19a1a in females. Signs of molecular sex differentiation preceded histological signs of sex differentiation, which were observed from ~75-150 mm FL.

Conclusion

Our results indicate that sablefish is an XX/XY species and suggest that male-specific gsdf-Y expression may determine sex during the period of larval development. This is followed by molecular sex differentiation, and ultimately anatomical and cellular changes associated with formation of testes or ovaries.

SEX DIFFERENTIATION

ORAL 4

ESTROGEN RECEPTORS ARE SEX-DIFFERENTIALLY EXPRESSED IN TAMBAQUI (*Colossoma macropomum*) DURING SEX DIFFERENTIATION

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Introduction

Endogenous estrogens play several important roles in the different developmental processes in fish, such as sex differentiation. Their molecular actions are mediated by the estrogen receptors (ERα and ERβ2), which are ligand activated nuclear transactivation factors that control gene expression in all vertebrates. Besides, there is also a subfamily of orphan nuclear receptors closely related to the ERs, namely estrogen related receptors (ERRs) that has been postulated to influence estrogen signaling by either synergizing or competing with ERs in an apparent ligand independent manner. Here we report, for the first time, the identification and initial characterization of ERs and ERRs transcriptional levels in tambaqui (*Colossoma macropomum*) during sexual differentiation.

Methods

The *de novo* transcriptome individual libraries from six headless juvenile tambaqui (3 putative males and 3 putative females) during sex differentiation (from 20 to 33 mm total length) were assembled using the Trinity pipeline. Among other genes differentially expressed between males and females, the estrogen receptors were analyzed and phylogenetically characterized (by full genes identification on the *C. macropomum* genome), and the Coding Sequence (CDS) was used to deduce the protein.

Results and Discussion

In sex differentiating *C. macropomum*, both ERβ isoforms (ERβ1 and ERβ2), as well as ERRβ were observed to be differentially expressed in males and females. ERβ1 was exclusively expressed in males (fold change ≥ 2; FDR ≤ 0.05), whereas the ERβ2 isoform was higher expressed in males than females (fold change ≥ 2; FDR ≤ 0.05). The ERβ type binds to estrogens and activates the expression of responsive genes containing estrogen response elements in an estrogen-dependent manner. Interestingly, females expressed significantly higher levels of ERRβ (fold change ≥ 2; FDR ≤ 0.05), which is close related to the ER family, but it lacks the ability to bind to the estrogen. However, ERRs and ERs share common transcriptional target genes, such as the *cyp19a*, whose product converts C19 (androgens) to C18 (estrogens) steroids such as 17β-estradiol, the most active natural ER ligand.

Conclusion

Altogether, these findings raise new consistent hypotheses about sex differentiation in *C. macropomum* to therefore guide further studies. The different transcriptional levels observed for ERs and ERRβ between males and females suggest that feminization in the species might not be fully (if so) dependent of estradiol, as commonly observed in other teleost species.

ORAL 5

EXPRESSION OF VASA, NANOS2 AND SOX9 DURING INITIAL DEVELOPMENT TESTICULAR OF NILE TILAPIA (*Oreochromis niloticus*) SUBJECTED TO SEX REVERSAL

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Introduction
Sexual differentiation and initial gonadal development are essential reproductive processes in Vertebrates, which have great scientific value. In the present work, the expression of Vasa, Nanos2 and Sox9 proteins was examined during initial development of Nile tilapia *Oreochromis niloticus* subjected to sex reversal.

Methods
For this study, 120 larvae of *O. niloticus* with 5 days post hatch (dph) were kept in hatchery without hormonal addition, while 120 larvae were kept in hatchery, fed with commercial ration containing 17-α-methyltestosterone for induction of sexual reversal for males. The fish sampling (N = 15/time) were 21, 23, 25, 30, 35, 45 and 55 dph.

Results and Discussion
The sexual differentiation of Nile tilapia occurred between 20 and 25 dph and sex reversal results in 94% of males, while the control group presented 53% of males. During testicular development, gonocytes (Gon) predominated as germ cells in differentiation, reducing and disappearing after this time. Undifferentiated spermatogonia (Aund) was identified at the beginning of testicular differentiation while differentiated spermatogonia (Adiff) was visualized at the end of this interval. Both spermatogonia increased after sexual differentiation. Somatic cells increased 58% while an extracellular matrix reduced 45% in 55 dph in reversal group, while the control group reduced the cell matrix by 36% after differentiation. The seminiferous tubules and the efferent duct began to develop after differentiation. Vasa was expressed in Gon and Aund, maintaining a cell pluripotency and inhibiting somatic differentiation during initial development. Vasa had its expression increased in Adiff at 55 dph, indicating a possible update at the beginning of gametogenesis. Nanos2 was expressed mainly in Gon and Adiff, ensuring the male fate of the gonad, and in Aund with 35 dph. Sox9 was expressed in the Sertoli cells, increasing significantly at times 45 and 55 dph during the structuring of the gonad, formation of the efferent duct and seminiferous tubules.

Conclusion
The results of this study indicate that the proteins analyzed are essential during initial testicular development of tilapia: Vasa is expressed in germ cells to maintain the pluripotency of these cells and inhibiting somatic differentiation; Nanos2 is expressed in germ cells so that they do not initiate meiosis, thus guaranteeing the male fate of the gonad; Sox9 expression can promote the structuring of the ducts and tubules in the testis and the development of Sertoli cells. These results contribute to a better understanding of the role of Vasa, Nanos2 and Sox9 proteins in the construction of the testis and its relationships between germ cells and somatic cells during initial testicular development.

STEM CELLS
AND
SPERMATOGENESIS

ORAL 6

APPLICATION OF GERM CELL TECHNOLOGIES IN STURGEONS

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Introduction

Sturgeons are one of the oldest, biggest, most valuable and today also most endangered group of fish species. Germ stem cells (GSC), such us embryonic primordial germ cells (PGCs) or spermatogonial/oogonial stem cells can be a key for an effective conservation and possible restoration of these unique and astonishing fishes. In the present research, labelling, development, isolation and transplantation of GSCs were studied in sturgeons.

Results and Discussion

It was shown that the maternally supplied germ plasm, which determines the PGCs, was localized in vegetal pole of oocyte and therefore the PGC specification pattern is similar to that of anuran amphibians rather than teleostean fishes. This knowledge enabled to develop an original PGC labelling method using common cell tracer dye injection into the vegetal pole of 2-8 cell stage embryo. Next, inhibition of maternally supplied RNA using injection of antisense morpholino oligonucleotide against dead end gene or disruption of germplasm using UV irradiation of vegetal hemisphere of fertilized eggs resulted in PGC mis-migration and general sterilization of sturgeon individuals. These methods enables preparation of recipients for germ cell transplantation. Isolation of spermatogonia and oogonia was developed using enzymatic dissociation and percoll purification. It was shown that one sturgeon juvenile (Siberian sturgeon) can provide approx. 1 million of germ cells suitable for transplantation and further development in 60% of recipients. Moreover, these cells are capable for cryopreservation. After freezing/thawing of sturgeon gonadal tissue followed by enzymatic dissociation, above 90% of viable cells were obtained and used for transplantation. The frozen gonadal tissue can be stored in liquid nitrogen for long term or alternatively in -80 °C for short term (in days).

Conclusion

The technique of surrogate production can be applied for conservation and possibly restoration of critically endangered sturgeon species with a long term of maturation and a big body size (e.g. beluga), whereas a more common species with shorter term of maturation and smaller body size (e.g. sterlet) can be used as a recipient (surrogate parent).

ORAL 7

PRODUCTION OF VIABLE TROUT OFFSPRING DERIVED FROM GERM CELLS CULTURED *IN VITRO*.

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Introduction

We previously established a germ cell transplantation technology that could ensure the conservation of endangered species and an efficient production of commercially beneficial seeds. Use of *in vitro*-amplified germ cells would supply donor cells simpler and easier compared with those prepared from live donor fish. In this study, in order to produce live fish derived from *in vitro*-cultured cells, we first cultured the rainbow trout (*Oncorhynchus mykiss*) germ cells *in vitro* and then transplanted the resulting cells into recipient fish. Additionally, we examined whether transplanted cultured cells are able to resume gametogenesis in recipient gonads and produce functional sperm and eggs derived from *in vitro*-cultured germ cells.

Methods

We obtained the germ cells from *vasa-Gfp* transgenic and dominant albino rainbow trout. The initially obtained germ cells were enriched by differentially plating whole testicular cells. These germ cells (primarily type-A spermatogonia labeled with GFP) were cultured in DMEM/F12 media supplemented with growth factors on mitomycin C-treated Sertoli cells derived from rainbow trout testis as feeder cells. When the germ cell cultures attained 80% confluence, the subcultures were passaged at a dilution rate of 1:2. After being cultured for 28 days, the amplified cells were collected with an enzyme solution and intraperitoneally transplanted into wild-type, triploid rainbow trout hatchlings. When the recipient fish was sexually mature, mating studies were performed to evaluate the functionality of the obtained gametes.

Results and Discussion

After 28 days of culturing, we observed that the cultured cells efficiently proliferated and displayed a clear green fluorescence. Furthermore, we confirmed that the transplanted germ cells were incorporated into the recipient gonads with high efficiency and resumed gametogenesis by observing of recipient fish at 20 and 70 days after the transplantation. One and two years post transplantation, some recipient males and females matured and produced gametes. With mating studies, we confirmed that the offspring carried the donor-derived phenotype (albino and GFP-positive). Furthermore, an early development of the subsequent offspring was normal. These results prove that the *in vitro*-cultured germ cells maintain their undifferentiated status for a minimum of one month without losing their ability to proliferate and are capable of differentiating into functional sperm and eggs.

Conclusion

We successfully developed germ cell cultures from rainbow trout and found that the *in vitro*-cultured germ cells can complete gametogenesis in the recipient gonads. Furthermore, the viable offspring can be produced from the cultured germ cells by inseminating the obtained eggs and sperm.

ORAL 8

RETINOIC ACID AND 11-KETOTESTOSTERONE ORCHESTRATE GERM CELL DEVELOPMENT IN ZEBRAFISH: CHARACTERIZATION OF A PERMISSIVE INTERACTION

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Introduction

Spermatogenesis is a complex cellular development that is to the target of several regulatory processes in the vertebrate testis. In teleost fish, the pituitary gonadotropin Fsh (follicle-stimulating hormone) is an important regulator of early steps of spermatogenesis by changing the release of modulatory factors from somatic cell types, such as growth factors and androgens by Sertoli and Leydig cells. Retinoic acid (RA) signaling is crucial for mammalian spermatogenesis, involving stimulation of the expression of the *Stra8* gene. Emerging evidence indicates that RA may also control fish spermatogenesis, although in several species, including zebrafish, *stra8* genes are missing.

Methods

The transcriptome of busulfan-depleted and then recovering testes was investigated by RNA sequencing. In addition, testicular tissue and cell suspensions of untreated males were submitted to different culture conditions, to examine transcript levels of selected genes by qPCR or to quantify germ cell proliferation activity. Also, incubation medium was collected to quantify testicular 11-ketotestosterone and RA release. To determine the incidence of apoptosis, paraffin embedded testis tissue was subjected to TUNEL analysis. To study the cellular localization of testicular *sall4*/*rec8* expression, we used FACS-sorted cell fractions from transgenic Tg (*vasa*:EGFP) testes. Finally, gene knockdown effects were investigated using antisense oligonucleotides (LNA™ GapmeRs).

Results and Discussion

Combining molecular and morphological approaches, we showed that the recovery of spermatogenesis following a cytotoxic insult involved activation of a transcriptional network that included androgen and retinoid signaling pathways. Using different experimental set-ups and pharmacological inhibitors, we demonstrated that retinoids promote spermatogonial differentiation. Also, both the Fsh-stimulated differentiating proliferation of spermatogonia and androgen production were compromised in the absence of RA. Pharmacological inhibition of RA signaling moreover resulted in increased germ cell apoptosis. Finally, while androgen signaling stimulated entry into meiosis, this androgen effect also was compromised when blocking RA synthesis.

Conclusion : For the first time in a non-mammalian vertebrate, we propose a mechanism involving *sall4*/*rec8* to mediate RA effects on spermatogenesis in a *stra8*-independent manner. Taken together, androgen and RA, both responding to Fsh signaling, interact to promote germ cell development during the mitotic and meiotic phases of zebrafish spermatogenesis. It will be interesting to evaluate if an interaction of RA and androgen signaling may be relevant in other vertebrates as well.

SPAWNING, FERTILIZATION AND SPERM-EGG INTERACTION

ORAL 9

EXPLORING THE POTENTIALITY OF AUTOPHAGY IN FISH FERTILITY.

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Introduction

Environmental stressors, gonadal degenerative diseases and tumour development can significantly alter the oocyte physiology, and species fertility and fitness. Gonadal follicular degeneration has been mostly attributed to the genes pertaining to programmed cell death, especially autophagy. Autophagy, a conserved mechanism from yeast to mammals, is a cellular process that delivers cytoplasmic material to the lysosome for recycling, and holds importance in development, tissue/organ repair. It is stimulated when nutrients are scarce, cells are under stress, or damaged organelles needs to be degraded. In this study, we have tried to expand our molecular understanding about the role of autophagy in oocyte and sperm formation, and its association in oocyte degeneration.

Methods

Japanese anchovy and medaka oocytes were analysed *in vitro* for autophagy and ion homeostasis related studies. The steroids/drugs were employed through feed and fertility related experiments were conducted using mating, etc. Tilting mutant medaka were screened from NBRP library, and backcrossing and sibling mating were performed to obtain the respective mutants.

Results and Discussion

To determine the extent of gonadal autophagy induction upon steroidogenic manipulation, we artificially altered the progesterone or estrogen profiles in adult Japanese anchovy. We found that, both these steroids changed the autophagic genes profile, elevated the total degenerating oocyte population and induced Japanese anchovy hatching enzyme (*AcHE) 1b* hyper-demethylation. Overexpression, knockdown and intracellular zinc ion chelation study confirmed the functional significance of *AcHE1b* in autophagy induction via ion-homeostasis. Additionally, we found that sex steroids and their receptors, especially estrogen and estrogen receptors (ER), regulated the autophagic expression in a sex-biased manner. To prove the role of ERs in sex-biased autophagy, we used ERα and ERβ2 knockout (KO) medaka, and analysed the alterations in the autophagic genes and protein expressions. We also found that, autophagy is not only instrumental in germ cell degeneration but also important for oocyte and sperm formation/development in the ER-KO fish.

Conclusion

Cumulatively our data highlights the sex-biased autophagy and ER association, *AcHE*s importance in stress-influenced apoptosis/autophagy cell fate decision, and the immense significance of autophagy in fish fertility.

ORAL 10

LOSS OF M6A IN METTL3 ZEBRAFISH MUTANTS DISRUPTS GAMETE MATURATION AND REDUCES FERTILITY

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Introduction

N6-methyladenosine (m6A), catalyzed by Mettl3 methyltransferase, is a highly conserved epigenetic modification in eukaryotic mRNA. Previous studies have implicated m6A modification in multiple biological processes, but the in vivo function of m6A has been difficult to study, because mettl3 mutants are embryonic lethal in both mammals and plants.

Methods

We have used transcription activator-like effector nucleases and generated viable zygotic mettl3 mutant, Zmettl3m/m, in zebrafish. We also developed a mettl3 overexpressing transgenic zebrafish lines. Using the following techniques such as qPCR, Western blot, LC-MS/MS, GVBD, TUNEL, ELISA, immunofluorescence and phenotypic rescue, we examined mettl3 expression and m6A levels, identified oogenesis and spermatogenesis, assessed the maturation and fertility, measured sex steroid levels.

Results and Discussion

We find that the oocytes in Zmettl3m/m adult females are stalled in early development and the ratio of full grown stage (FG) follicles is significantly lower than that of wild type. Human chorionic gonadotropin-induced ovarian germinal vesicle breakdown in vitro and the numbers of eggs ovulated in vivo are both decreased as well, while the defects of oocyte maturation can be rescued by sex hormone in vitro and in vivo. In Zmettl3m/m adult males, we find defects in sperm maturation and sperm motility is significantly reduced. Further study shows that 11-ketotestosterone (11-KT) and 17β-estradiol (E2) levels are significantly decreased in Zmettl3m/m, and defective gamete maturation is accompanied by decreased overall m6A modification levels and disrupted expression of genes critical for sex hormone synthesis and gonadotropin signaling in Zmettl3m/m.

Conclusion

Our study provides the first in vivo evidence that loss of Mettl3 leads to failed gamete maturation and significantly reduced fertility in zebrafish. Mettl3 and m6A modifications are essential for optimal reproduction in vertebrates.

OOGENESIS
AND
VITELLOGENESIS

ORAL 11

GENOME EDITING REVEALS DEVELOPMENTAL DEPENDENCIES ON SPECIFIC TYPES OF VITELLOGENIN IN ZEBRAFISH (*Danio rerio*)

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Introduction

While multiplicity of vitellogenins (Vtgs) is the norm in fishes, it is usually unknown whether the different types of Vtg are required or have any unique functions. The objectives of this study were to discover which types of Vtg are essential, to discern when their yolk protein products are required during development, and to begin exploring their special physiological functions in the zebrafish, a model teleost expressing an especially diverse array of different forms of Vtg.

Methods

A novel gene knock-out (KO) technology employing a multiple CRISPR approach was used to incapacitate all genes encoding *type-1 vtgs (vtg1, 4, 5, 6,* and *7)* simultaneously, as well as *vtg3* individually. A chimeric gRNA system containing sgRNAs and nCas9n RNA was injected into one-cell stage embryos, which were subsequently screened for double strand breaks at defined target sites using conventional PCR. Detected mutations were confirmed by sequencing. Pure lines carrying desired mutations were produced by stepwise selection and pairing of mutated individuals at each generation. Loss of function of targeted genes was confirmed by real time quantitative PCR (qPCR), Western blotting (WB), and liquid chromatography tandem mass spectrometry (LC-MS/MS). Effects of treatments were detected by phenotypic observations.

Results and Discussion

Mutations of large deletions (1281-1182 bp) on gDNA for *type-1 vtgs*, and also for *vtg3*, resulted in frame shifts of their polypeptide sequences. qPCR, WB and LC-MS/MS results proved the collective KO of *vtg1*, *4* and *5*, and the possible escape of *vtg6* and *vtg7* from Cas9 editing. The same type of analyses confirmed complete incapacitation of the *vtg3* gene. Both KO of *type 3 vtg* and *type 1 vtgs* severely altered survival and development of the respective 4th generation, pure line, mutant progeny. The KO of *vtgs1, 4 and 5* resulted in a major decrease in embryonic survival at 24h, with 100% larval mortality by day 15; KO of *vtg3* resulted in a sharp decrease in embryonic survival by 8h, with a mean larval survival of only 6.25 % by day 22. Remarkably, in both cases, evidence for a compensatory increase in Vtg7 levels was observed. KO of *type 1 vtgs*, and also of *vtg3*, caused deformities such as cardiac and abdominal edema and spinal cord defects at 100 % and 30 % frequencies by day 11, respectively.

Conclusion

The KO of multiple genes encoding different forms of Vtg was achieved, for the first time in any vertebrate, using the zebrafish model. Protein products of collectively knocked-out *vtg1*, *vtg4*, and *vtg5*, are required for late embryonic and larval development and survival. Vtg3, the least abundant form of Vtg in zebrafish and one thought to be mainly a nutrient for late stage larvae in other species, clearly has essential functions in early embryonic development, during body axis formation. Finally, the up-regulation of Vtg7 at transcript and protein levels in *vtg* KO fish may be part of a previously unknown system for regulation of vitellogenesis, a phenomenon which begs further investigation.

ORAL 12

THE *IN VIVO* EFFECTS OF ESTRADIOL AND 11-KETOTESTOSTERONE ON VITELLOGENIN PHYSIOLOGY IN THE SHORTFINNED EEL, *Anguilla australis*.

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Introduction

The unique migratory lifecycle of freshwater eels has restricted research on natural reproductive development to pre-vitellogenesis and the early stages of vitellogenesis. Further complicating the ability to investigate the later stages of vitellogenesis is a captivity-induced reproductive block. Treatment with exogenous pituitary homogenates is routinely used to override this and stimulate further vitellogenic development. The hormonal mediator, estradiol-17β (E_2), is widely considered the main inducer of vitellogenesis, yet previous studies have largely failed to effectively induce vitellogenic uptake in the ovary exclusively with E_2. 11-ketotestosterone (11KT) is known to be an important mediator of aspects reproductive development, specifically ovarian acquisition of lipid, in eels, although its role in vitellogenesis remains unclear. Here we aim to determine the effects of combined or separate treatments with E_2 and/or 11-ketotestosterone on production and ovarian uptake of vitellogenin, in the New Zealand shortfinned eel, *Anguilla australis*.

Methods

Early pubertal eels (~800 g) received slow-release hormone implants containing varying concentrations of E_2 (0, 0.2, 2, 5 mg) with or without 11KT (1 mg). Vitellogenin levels were then determined in plasma, liver and ovarian tissues by histological visualization, qPCR, immunoblotting or immunoradial diffusion. In addition plasma levels of sex steroids, 11KT and E_2, were analysed by radioimmunassay.

Results and Discussion

Plasma steroid concentrations were elevated as expected following implantation with each individual steroid. An as-of-yet to be explained interaction between the two steroids was evident following co-treatment whereby E_2 concentrations were significantly reduced despite a supra-physiological dose being administered (E_2: 105.2 ± 7.6 vs 11KT + E_2: 3.8 ± 0.8 ng/ml). Independently, E_2 and 11KT appear to have opposing effects on hepatic production and plasma levels of vitellogenin, with E_2 stimulating and 11KT seemingly reducing production. Neither steroid showed the ability to induce uptake of vitellogenin into oocytes (as assessed by immunological slotblot and histological examination), despite 11KT treated eels having significantly larger oocytes than placebo treated or E_2 treated eels (with 11KT: 350.4 ± 8.3 vs without 11KT: 239.9 ± 5.4 μm). Interestingly, co-treatment with 11KT and E_2 effectively induced vitellogenin uptake, suggesting that 11KT might facilitate the incorporation of E_2-induced vitellogenin into the developing oocyte.

Conclusion

Our results highlight the potential of sex-steroid co-treatments to induce vitellogenesis in eels, specifically ovarian uptake of yolk, even in the absence of external gonadotropin treatment.

ORAL 13

17β-HYDROXYSTEROID DEHYDROGENASE TYPE 12 IS RESPONSIBLE FOR MATURATION-INDUCING STEROID SYNTHESIS DURING OOCYTE MATURATION IN NILE TILAPIA

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Introduction

17α, 20β-dihydroxy-4-pregnen-3-one (DHP) is considered to be the maturation-inducing steroid in many teleost fish. Carbonyl reductase-like 20β-hydroxysteroid dehydrogenase (CR/20β-HSD) was coined as a candidate enzyme responsible for DHP production during oocyte maturation in fish, including Nile tilapia. However, recently, a novel type of 17β-hydroxysteroid dehydrogenase, type 12-like (hsd17β12L), was identified as the enzyme responsible for DHP production during oocyte maturation in masu salmon. The existence of 17β-HSD12 (presumably the orthologous enzyme of salmon hsd17β12L) was previously reported in Nile tilapia; however its enzymatic activity remains unknown and conversion of the substrate for DHP, 17α-hydroxyprogesterone (17OHP), has not been examined. This study aimed to identify which enzyme, CR/20β-HSD or 17β-HSD12, is responsible for DHP production during oocyte maturation in the Nile tilapia.

Methods

Mammalian expression vectors containing tilapia *hsd17b12* or *CR/20bhsd* were constructed and transfected into HEK293T cells, then incubated with 17OHP for 20 h. After the incubation, DHP concentrations in the media were measured using TR-FIA. In addition, HEK293T cells transfected with *hsd17b12* were incubated with other potential substrates, such as DHP, adrenosterone, androstenedione, estrone, testosterone, 11-ketotestosterone and estradiol-17β. After the incubation, media were analyzed using LC/MS to examine whether this enzyme possessed oxidoreductase activities or not. Finally, full-grown ovarian follicles were subjected to salmon pituitary extract (SPE, 100 μg/mL) or HCG (100 IU/mL) to induce 20β-HSD activity *in vitro*, and enzyme activity was assessed by co-incubation with 100 ng/mL 17OHP. After 2, 4, 8 and 16 h of incubation, media were collected for DHP measurement, while follicles were processed for determination of *hsd17b12* and *CR/20bhsd* mRNA levels by quantitative PCR.

Results and Discussion

HEK293T cells transfected with *hsd17b12* exhibited a strong ability (73.8% yield) to convert exogenous 17OHP to DHP. The cells transfected with *CR/20bhsd* or control vector converted only 7.4% and 7.5% of 17OHP to DHP, respectively. LC/MS analyses demonstrated that 17β-HSD12 did not convert substrates other than 17OHP. Conversion of 17OHP to DHP by ovarian follicles incubated with SPE and HCG peaked at 8 hrs. This finding coincided with a peak in follicular *hsd17b12* mRNA levels, which were significantly higher than those in control incubations. However, the levels of *CR/20bhsd* mRNA remained low and unchanged in all experimental groups.

Conclusion: The present study strongly suggests that 17β-HSD12, and not CR/20β-HSD, is the 20β-HSD responsible for DHP production by ovarian follicles during oocyte maturation in Nile tilapia.

OVULATION
AND
EGG QUALITY

ORAL 14

CANDIDATE GENE IDENTIFICATION OF OVULATION-INDUCING GENES BY *IN VIVO* INDUCTION OF OOCYTE MATURATION AND OVULATION IN ZEBRAFISH.

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Introduction

Ovulation is a critical biological process that prepares fertilizable eggs prior to spawning. During this step matured oocytes rupturing from the follicle layer require multiple factors, which manipulate the phenomenon. Ovulation is mediated by the nuclear progesterone receptor (nPR) through genomic steroid signaling mechanisms in zebrafish.

Ovulation-associated genes are expressed with maturation-associated genes in the ovary. The examination of only ovulation-associated genes in ovarian tissue was impossible. This obstacle severely limits the study of ovulation. However, we previously established a way to select only ovulation-associated genes by using separate *in vivo* oocyte maturation and ovulation inductions. We proposed three ovulation-related candidate genes using microarray analysis in that study. Highly up-regulated genes at the time of ovulation were seen to be associated with apoptosis, and the results support the notion that apoptosis is a potential mechanism for inducing ovulation.

Recently, we began exploring other technologies to analyze gene expression profiling. RNA sequencing (RNA-seq), has been established to reveal the precise cellular transcriptome using sequencing-based methods. RNA-seq is expected to provide a more reliable candidate screen for ovulation-related genes.

Methods

The mean values of gene expression levels in the samples treated with EtOH, DES, Tes, and 17, 20β-DHP from the microarray and RNA sequencing were analyzed by Subio Platform ver. 1.18.4667 (Subio Inc., Amami, Japan). Statistical and non-statistical selection method were used to select genes up-regulated in 17, 20β-DHP treated samples. That is, any genes that showed higher expression in 17, 20β-DHP when compared with other three groups were identified as ovulation-inducing gene candidates. Expression levels of candidate genes were confirmed by qPCR. Genome editing using the CRISPR/Cas9 system was performed on the candidate genes.

Results and Discussion: Genes that up-regulated more than two times in the 17, 20β-DHP-treated group against EtOH- (1), DES- (2) and Tes- (3) treated groups, were selected. Among these genes, significant up-regulation only occurred in the 17, 20β-DHP-treated group, which were selected by Venn Diagram analysis. 35 and 297 of these genes were reported from microarray analysis and RNA sequencing analysis, respectively. By q-PCR analysis, 11 genes are selected as highly possible candidate genes. To show the distinct role of these genes, we started to establish the gene-knock out zebrafish strains. At present, we validated the *in viv*o function of one candidate gene (*stm*) from our list. Unfertilized eggs and abnormal embryos at the early stage of development were produced from *stm* homozygous mutants. *Stm* appears not to play an important role in ovulation, but does support fertilization.

Conclusion: In the present study, 11 genes showing ovulation-specific increases were identified. Genome editing using the CRISPR/Cas9 system of these 11 genes will reveal the roles of candidate genes on ovulation-induction.

ORAL 15

A TRANSCRIPTOMIC COMPARISON SUGGESTS AN INFLUENCE OF THE DOMESTICATION PROCESS ON EGG QUALITY IN EURASIAN PERCH (*Perca fluviatilis*)

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Introduction

Fish populations, both domesticated and wild, are dependent upon the production of good quality eggs. Beside the morphological aspect, the egg quality is determined by its molecular content. In this context, the analysis of the maternal mRNA is a practical approach once they are essential for numerous cellular processes occurring during embryogenesis. The domestication of the Eurasian perch is currently under process and some reproductive issues have been reported, among which variable egg quality. The present study aims at analyzing a potential impact of the domestication process on the egg transcriptomic content and hence on their quality.

Methods

Two broodstock populations were used. The first one, considered as domesticated (D), is originated from breeders reproduced in captivity for several generations in a fish farm. The second one is F1 generation from broodstock caught in the Geneva Lake. Both populations were reared under similar conditions in our facilities from the juvenile stage (3 months) and their reproduction cycle was induced in the same RAS conditions using a photo-thermal program of 10 months. In total, 31 spawn were sampled (13 D and 19 F1). One portion was artificially fertilized with a pool of sperm from 3 males and monitored (survival rates at various stages) to assess egg quality. Another portion was frozen to study the eggs gene expression pattern using microarray analyses.

Results and Discussion

Higher survival rates at 48, 72 and 120hpf ($p<0.05$) was observed in embryos from the F1 population, showing this population produced eggs of better quality. The analysis of mRNA content of the eggs revealed more than 300 genes differently expressed between both populations ($p<0.05$). Interestingly, an unsupervised average linkage clustering analysis of these genes split apart the two populations showing a clear different pattern of gene expression between them. The main differently expressed genes (log2 fold-change>4) are involved in functions as the anterior-posterior patterning and mRNA degradation potentially contributing for the differences in early lethalities in both populations.

Conclusion

The domestication process may cause genetic and epigenetic changes both leading to transcriptomic modifications. As consequence the fish biology is affected in several ways, including egg quality variation. The identification of molecular pathways affected in a domesticated population opens new research areas to better understand the modifications occurring during the domestication process related to gametes quality.

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ORAL 16

MACROMOLECULAR CHANGES OF SWORDFISH OOCYTE AT DIFFERENT DEVELOPMENTAL STAGE: NEW INSIGHT FROM FOURIER TRANSFORM INFRARED MICROSPECTROSCOPY (FTIRM)

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Introduction:

The swordfish is an important commercial species with an extensive seasonal migration and a circumglobal distribution. It is a gonochoristic species, females are multiple spawners with asynchronous ovary. Limited research has been published on the reproductive biology of swordfish from Mediterranean see. As reported in the literature, five different stages can be observed on the base of morphological features, but macromolecular information about cytoplasmic and ultrastructural changes are still lacking. To date, Fourier Transform Infrared Microspectroscopy (FTIRM) is a powerful technique to analyze the macromolecular chemistry of cells. Using multivariate procedures, it is possible to generate chemical maps that provide at the same time and on the same sample, unique biochemical and ultrastructural information. Since several reports applied spectroscopic studies to evaluate biochemical changes associated with oocyte growth and maturation, in this study we applied for the first time FTIRM, to achieve specific macromolecular fingerprint of swordfish oocyte at different developmental stages.

Methods

Fishes were fished by Italian fleets operating in central Mediterranean Sea. FTIRM measurements were carried out by using a Bruker VERTEX 70 interferometer coupled with a Hyperion 3000 Vis-IR microscope equipped with a bidimensional Focal Plane Array (FPA) detector. FTIRM measurements were acquired on oocytes at six different developmental stages: primary, alveoli cortical, early vitellogenic, late vitellogenic, mature and atretic oocytes.

Results and Discussion:

The topographical distribution of lipids, proteins, phosphates and carbohydrates within oocytes at different developmental stages was assessed. In particular, previtellogenic oocyte were characterized by a protein-rich cytoplasm, with central oil globules containing short-chain, unsaturated lipids exclusively, and cortical alveoli reach in glycoproteins. During vitellogenic phase, the oocytes showed small yolk granules around the peripheral cytoplasm, composed of glycoproteins, and proteins rich in tyrosine, glutamate and aspartate. By exploiting FTIRM analysis, it was possible to focus on macromolecular changes of vesicles containing vitellogenin when they blend with lysosomal ones, or when they crosse Zona Radiata (ZR) and plasma membrane. FTIRM analysis let also to obtain macromolecular and ultrastructural information about ZR that becomes already evident in previtellogenic stages, increasing in dimension and changing in composition in late vitellogenic and mature oocytes (with the inner layers mainly proteic and the outer richer on glycoproteins). In addition, the central oil globules change in shape and lipid composition (in terms of unsaturation levels and length of chain). Finally, analysing atretic oocytes it was possible to point out macromolecular changes, in terms of secondary structures and composition, occurring to proteins of ZR and cytoplasm and the increase of saturation levels of membrane lipids.

Conclusion: FTIR analysis let obtain deeper insight into the macromolecular and ultrastructural characterization of swordfish oocytes.

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ORAL 17

OOCYTE QUALITY OF NILE TILAPIA (*Oreochromis niloticus*) REARED IN BIOFLOC SYSTEM

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Introduction

The use of microbial biomass to cycle waste material and provide a supplemental nutrition source to aquatic reared species is the basic concept of biofloc technology (BFT). The BFT has gained great prominence in tilapia aquaculture due to the multiple positive effects of this system in terms of controlling water quality, nutrition, welfare and growth of fish. Positive effects of BFT on reproduction of aquatic cultured species have been described, mainly for shrimps, however, results in the literature are not conclusive for Nile tilapia.

Methods

The experiments were conducted in a greenhouse at Aquaculture Laboratory-UFMG. We used two lines originated from Chitralada Nile tilapia. Each animal received a microchip and were acclimatized for two weeks. Both sex was kept separated in tanks with 800 L of water. In control system, animals were maintained in clear water system with renewal rate of 50% per day. The water renewal in BFT was provided only for evaporation replacement. The animals were randomly divided in control and BFT groups. The stocking density was 33-35 animals/tank. Females "ready to spawn" were induced to spawning by administration of two intramuscular injections of human chorionic gonadotropin (hCG) (first dose = 10% of total dose; total dose = 3,000 IU/kg, dose interval = 18h). The first oocyte collection was done before the beginning of the experiment (n=8 females) and the following three collections were performed from 6 females per each system. These oocyte samples were fixed, counted and the oocyte diameter was measured. The absolute and relative fecundity were calculated.

Results and Discussion

The absolute fecundity of females reared in BFT and Control systems were not significantly different. Relative fecundity was different between treatments ($p<0.05$), where median was 6.66 for BFT and 2.46 for Control. The means of oocyte diameter were 1.92 and 1.80 mm in BFT and Control, respectively ($p<0.05$). In addition, the oocytes of females reared in BFT were bigger than those from females before the treatments. These results suggested BFT enhanced tilapia reproductive performance because the system likely offered a positive nutritional effect contributing to produce bigger oocytes and to improve relative fecundity. We did not find differences between the Nile tilapia lines.

Conclusion

Our results demonstrated BFT promoted the increase of both relative fecundity and oocyte diameter, which probably enhance the egg quality. These effects of BFT on tilapia reproduction were possibly indirect and were related to better nutritional status of broodstook.

PHEROMONES
AND
BEHAVIOR

ORAL 18

DIRECT EVIDENCE THAT HIGH LEVELS OF PLASMA PROSTAGLANDIN $F_{2\alpha}$ INTIMTELY ASSOCIATED WITH OVULATON DRIVE FEMALE SEXUAL BEHAVIOR AND PHEROMONE RELEASE IN A MODEL CYRINID FISH, THE GOLDFISH

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Introduction

Four decades ago, a series of relatively simple experiments led Stacey and colleagues to propose that when female goldfish (*Carassius auratus*) ovulate, the movement of eggs into their ovisac stimulates synthesis of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), which enters the circulation and acts within the brain to stimulate female spawning behavior. Later, it was proposed that $PGF_{2\alpha}$ metabolites released by ovulated, sexually active fish function as a sex pheromone. However, while $PGF_{2\alpha}$ injection has been shown to elicit female behavior and PGF metabolites have been shown to stimulate male behavior when added to their water, whether $PGF_{2\alpha}$ is actually present in the plasma of female fish and how it might match ovulation and has not been examined. This study established that connection.

Methods

Five experiments were conducted. In one experiment, female goldfish were induced to ovulate and their plasma sampled every 15 min and measured using mass-spectrometry while their sex behavior was monitored. Water was also sampled. In another, fish were stripped of eggs and $PGF_{2\alpha}$ and behavior noted. In a final experiment, the fate of injected $PGF_{2\alpha}$ was noted in fish while their behavior was monitored.

Results and Discussion

Circulating levels of $PGF_{2\alpha}$ increased from 1ng/ml prior to ovulation, to 5ng/ml within 15 min of ovulation (when female behavior was observed) and over 100ng/ml within 6 hours. While non-ovulated fish released less than 1ng/h of various PGF2a metabolites, within 3 h, they were releasing over 1000 ng/hr. Egg-stripped fish had basal levels of $PGF_{2\alpha}$ and did not spawn. $PGF_{2\alpha}$ levels in the plasma of $PGF_{2\alpha}$-injected fish could explain their behavior.

Conclusion

At the time of ovulation, female goldfish start producing large quantities of $PGF_{2\alpha}$ and its metabolites which function as behavioral hormones and are also released as pheromones. Likely, $PGF_{2\alpha}$ plays similar roles in other egg-laying fishes.

ORAL 19

INTRASEXUAL AGGRESSION IN CICHLIDS: CAN ESTROGENS BE CONSIDERED AS KEY ELEMENTS OF THE CHALLENGE HYPOTHESIS?

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Introduction

It has been historically suggested that aggressive behavior in males is mediated by androgens, but recent evidence suggests that the key step regulating this behavior is the aromatization to estrogens. The challenge hypothesis suggests that behavioral interactions lead to an increase in plasma androgen levels in response to social instability, but there is no evidence regarding estradiol levels. Despite the fact that females also display aggressive behavior, this framework has been focused mainly on males and the physiological regulation of female aggression is still understudied. The first aim of this study was to determine whether there is a relationship between sex steroids and intrasexual aggression in males and females of *Cichlasoma dimerus*, a monogamous Neotropical cichlid with bi-parental behavior. Moreover, the second aim of this study was to evaluate if our data supports the challenge hypothesis for both sexes and to determine if estrogens also increase as a consequence of social instability.

Methods

A total of 18 females and 16 males were used for intrasexual dyadic agonistic encounters. Blood samples were obtained before and after each encounter by puncture of the caudal vein to measure steroid hormone levels pre and post conflict. All agonistic interactions in the contest were recorded during one hour and measures of total aggression and submission of each animal were calculated to characterize winners and losers. After trials, fish were euthanized; gonads were dissected, weighted and used to calculate the gonadosomatic index. Plasma testosterone (T), 11-ketotestosterone (11-KT) and estradiol (E2) were determined with commercial ELISA kits.

Results and Discussion

Winner females, but not males, had higher plasma E2 levels than losers before the contest (2.2-fold increase, $p=0.046$), suggesting that initial E2 levels could predict the winner status in females. However, there were no differences in androgen levels in neither sex. During male encounters there was not only a 5.4 and 3.2-fold increase in T and 11-KT, but also 1.5-fold increase in E2 levels ($p=0.016$, $p=0.018$, $p=0.023$, respectively), linking the challenge hypothesis to estrogens. Sex steroids did not increase in response to the dyadic encounter in females and this delta E2 did not correlate with their GSI, suggesting that changes in E2 in females did not depend on their reproductive status.

Conclusion

These results suggest that in this context initial estrogens can predict the outcome of the encounter in females. Moreover, this is the first evidence suggesting that the challenge hypothesis could be referred to estrogens.

MIGRATION/REPRODUCTION OF NEOTROPICAL FISH

ORAL 20

ADVANCES IN THE REPRODUCTION OF *Arapaima gigas* (CUVIER, 1829), IN THE ECUADORIAN AMAZON; A PUBLIC-PRIVATE PARTNERSHIP.

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Introduction

In Ecuador, the development of the cultivation and massive consumption of *Arapaima gigas* (Cuvier, 1829), is still in the beginning phase. Formally, these initiatives have been registered since 2014; although there were reports of related activities since 2008 in the Costa and Amazonian regions of Ecuador. The first Arapaima farming were created with wild fry, intermittently extracted from the natural environment, especially those from lakes located in the lower basin of the Curaray River, in the Kichwa and Waorani territories. In this context, was proposed to aboard this problem, with a research on the adaptation of *Arapaima gigas* in the Ecuadorian Amazon captivity conditions.

Methods

We have carried out monitoring activities of individuals of this species from 2014 to 2017, with age intervals between 4 to 8 years, average weights of 60 kg and standard size of 1.70 m. We choose 4 places: i) the experimental unit of the CIPCA in the Amazon State University, ii) the "Peces Tropicales" company, iii) the ACUATILSA company; and, iv) the Tarqui Association, private and community producers that have undertaken *A. gigas* management initiatives. In each place, breeder candidate animals were selected with age criteria; and, in case that have not found these records, weight and length; whereas in order to meet the criteria, a vitellogenin test can be done, as a test of sexual maturity and identification of females; with the objective to put together reproductive couples.

Results and discussion

Adult animals and levels of sexual maturity were identified throughout the monitoring; some became part of reproductive couples, while others suffered conformation problems due to space restrictions in the premises investigated. Of the individuals identified as adults and sexually mature only one local had constant reproduction since 2014, with eight reproductive pairs formed and a production between 5,000 and 8,000 fingerlings per year. it is expected that there will be feasibility of reproduction in other places and it will continue supporting the reproduction of this species to generate fingerlings for the local productive system with this valuable species

Conclusion

Currently the vitellogenin analysis is the more confident criteria to identify gender whereas are located the populations. However, it is possible to have interferences due the environmental conditions in the reproduction phenomena, that is need identify what critical key factor for inhibition.

ORAL 21

CEPHALIC SECRETION OF *Arapaima gigas*: SEX STEROIDS, PEPTIDES AND PROTEINS SUGGEST ROLES IN CHEMICAL COMMUNICATION AND PARENTAL CARE.

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Introduction

Adults of the Amazon fish *Arapaima gigas* secrete a fluid from the cephalic canals of the lateral line system, whose biological role(s) along the reproductive cycle are unclear. Up-to-date, the biochemical composition of this fluid has been poorly investigated in *A. gigas* as well as other teleosts. Hence, this study aimed to (1) investigate the potential pheromone release through this cephalic fluid, and (2) to characterise its proteome and peptidome by comparing males and females during and outside the parental care phase.

Methods

Sex steroids were investigated in 18 adult couples individually stocked in 330 m^2 earth ponds, which were sampled monthly from Jan to July 2015 in DNOCS research station, Pentecoste-CE, Brazil. Sex steroid concentrations were monitored in blood plasma and cephalic secretion from males (T and 11-KT) and females (T and E_2) using ELISA and RIA methods validated for *A. gigas*. Proteome and peptidome investigations on the cephalic fluid compared parental (n=2) and non-parental (n = 10) males and females using capillary electrophoresis coupled to mass spectrometry (CE-MS) and GeLC-MS/MS analyses.

Results and Discussion

Significant correlations between blood plasma and cephalic secretion levels of 11-KT in males and T in females were observed throughout the study, suggesting a possible novel route of pheromone release in teleosts. Peptidomic analyses revealed 28 peptides were significantly different between males (M) and parental males (PM), 126 between females (F) and parental females (PF), 51 between M and F and 9 between PM and PF. Such results indicate marked physiological changes in the cephalic secretion of parental fish at the peptide level. Finally, 422 proteins were identified with gene ontology analyses revealing 28 secreted extracellular proteins. These included 2 hormones (*prolactin* and *stanniocalcin*) and 12 proteins associated with immunological processes (*serotransferrin, α-1-antitrypsin homolog, apolipoprotein A-I,* and others). As found in parental fish, such proteins could be potentially beneficial to developing offspring during parental care phase.

Conclusion

This study enhances information on the biochemistry of the lateral line system in arapaima, opening research possibilities in fish physiology and chemical communication. Data from this study highlight the complex role that the cephalic secretion of *A. gigas* may play not only on the adults but also on the development of the fingerlings.

BRAIN-PITUITARY SYSTEM

ORAL 22

DOES COMPENSATION OCCUR IN GNRH3 GENE KNOCKOUT ZEBRAFISH?

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Introduction

The recent developments in gene knockout (KO) technologies in fish, especially in the highly studied zebrafish (ZF) model, occasionally contradicted our knowledge established via traditional research methods. Notably, the gene knockout of the hypophysiotropic gonadotropin releasing-hormone (Gnrh3) in ZF is the best example for such a discrepancy. Inheritable *gnrh3* gene KO unexpectedly yielded fertile ZF, even though Gnrh3 neuronal ablation impedes ovulation. These findings have undermined the accepted essential role of Gnrh3 in ZF reproduction, initiating a debate over its dispensability on one hand versus a compensatory mechanism that overcomes Gnrh3 absence on the other. In this study, we investigated the changes occurring in the brains and pituitaries of *gnrh3* KO ZF and propose that a multi-factorial compensation is activated to guarantee proper reproduction.

Methods

Two KO ZF lines, $gnrh3^{-/-}$ and $gnrh2^{-/-};gnrh3^{-/-}$, were generated using TALEN technology, in which both lines were fertile. To reveal differences associated with the lack of Gnrh3, brain and pituitary transcriptomes were compared between wild-type and $gnrh3^{-/-}$ adult males. Identified differentially expressed genes were further examined via *in situ* hybridization. Vasoactive intestinal peptide (Vip) was also tested as a possible replacement for the hypophysiotropic function of Gnrh3 via neuronal immuno-staining in the neurohypophysis, interactions with gonadotropes, and induction of gonadotropin secretion via pituitary incubation *in-vitro* followed by gonadotropin ELISA.

Results and Discussion

Several differentially expressed genes were revealed: In the KO brains, genes encoding peptides such as Gonadotropin-inhibitory hormone (Gnih) and Vip were upregulated. Vip axons populated the neurohypophysis and, like Gnrh3 neurons, directly innervated Lh gonadotropes. Vip peptide induced the secretion of Lh and Fsh from pituitary explants. Moreover, Vip projections intensify in the $gnrh3^{-/-}$ pituitary. In addition, tyrosine hydroxylase, which synthesizes dopamine, is downregulated and monoamine oxidase, which degrades dopamine, is upregulated, possibly lowering dopamine levels in KO fish. Secretogranin 5, a chaperone that enhances protein secretion, is highly upregulated in $gnrh3^{-/-}$ brain and pituitary, indicating that neuropeptide and gonadotropin secretion is augmented in the $gnrh3^{-/-}$ line.

Conclusion

Overall, significant changes occur in the brain and pituitary following the loss of Gnrh3. By removing reproductive inhibitory elements (e.g. dopamine) and supplementing with alternative gonadotropin stimulators, like Vip, these changes probably result in a Gnrh3 independent induction of gonadotropin secretion from the pituitary that in turn enables normal reproduction in the KO line.

ORAL 23

REGULATION OF FOLLICLE-STIMULATING HORMONE (FSH) EXPRESSION DURING PUBERTAL DEVELOPMENT IN *Salmo salar* PARR MALES

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Introduction

Available knowledge suggests that in mammals both Lh and Fsh, produced by the same cell type, are required to trigger the entry into puberty. In fish, where Lh and Fsh are produced by two distinct cell types, evidence suggests a major role of Fsh in the regulation of this developmental process. However, knowledge about the regulation of Fsh synthesis and secretion is still fragmentary. Therefore, we aimed at characterizing receptors specifically expressed in Fsh cells during early puberty.

Methods: In this study, we used Atlantic salmon (*Salmo salar*) as model. During the freshwater phase (parr), some males are able to undergo pubertal maturation and reproduce without migrating to the sea. Those fish are generally called "sneaky spawners" or "precocious males". Using the Norwegian strain Figgjo, we identified and grouped immature and mature parr males using GSI, gonad histology, and steroid plasma levels measured by radioimmunoassay. Afterwards, a 3D mapping of gonadotropin gene expression in the pituitary gland was obtained via fluorescent *in situ* hybridization (FISH) in the maturing fish. Then, qPCR analysis was performed on whole pituitaries for both gonadotropin genes and genes encoding receptors such as those of melatonin, dopamine, gonadotropin releasing hormone (GnRH) and kisspeptin, to look for differentially expressed receptor genes in pubertal fish. Finally, the localization of the receptors was observed by FISH and confocal microscopy.

Results and Discussion: Differently from other investigated strains, which reach maturation after one year, Figgjo fish reach puberty as 0+ in late September only 6 months post hatching. The mapping of gonadotropin distribution confirmed that *lhb* and *fshb* are mostly produced by two different cell types. Although qPCR reveals differential expression of *fshb* between maturing and non-maturing fish, this seems to not be reflected by Fsh cell abundance. qPCR results also revealed the presence of several isoforms of each receptor type in the pituitary. All melatonin, kisspeptin, dopamine, and most of the GnRH receptors were expressed without any variation related to maturational stage nor gonadotropin gene expression levels. However, we observed an increased expression level of GnRH receptor forms 2b1 and 2b2 (*gnrhr2b1, gnrhr2b2*) in maturing parr, coinciding with elevated *fshb* expression, GSI, and steroid plasma levels. Finally, double color FISH confirmed the expression of *gnrhr2b2* in Fsh-producing cells.

Conclusion: In the present study, we report that early maturation in precocious male can occur after only six month after hatching. We identified the presence of *gnrhr2b2* mRNA in Fsh cells of maturing fish suggesting a role of this receptor in the regulation of *fshb* expression during pubertal development.

ORAL 24

CHARACTERIZATION, DISTRIBUTION AND ROLE OF DOPAMINE RECEPTORS IN PIKEPERCH DURING THE PRE-OVULATORY PERIOD.

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Introduction

Dopamine is a biogenic amine exerting a range of pleiotropic actions notably in the reproductive function. In some teleosts, dopamine inhibits luteinizing hormone release at the brain and pituitary levels. Two receptor families mediate the dopamine effects, namely the D_1 and the D_2. To date in teleosts, a maximum of four receptor subtypes have been identified within the family D_1, D_{1A}, D_{1B}, D_{1C} and D_{1E} while the family D_2 encloses five subtypes, D_2, D_{2like} (D_{2l}), D_3, D_4 and $D_{4\text{-related sequence}}$ (D_{4rs}). In pikeperch, a species of interest for aquaculture development, not only the involvement of dopamine in the control of reproduction is equivocal but also the existence, number, location and role of its receptors are totally unknown. To address these questions we gathered the inventory of dopamine receptors (DR) in the brain, investigated a multi-tissue gene expression pattern of these receptors and tested the *in vivo* effects of DR antagonists on sex-steroid production.

Methods

RNA-seq with *de novo* transcriptome reconstruction, functional annotation and phylogenetic analysis were performed to characterize the transcript repertoire of DR in the brain of mature female pikeperch. Then, their gene expression was analysed in a range of tissues. Finally, pre-ovulatory females were injected either with metoclopramide (D_2 family antagonist) or SCH23390 (D_1 family antagonist) and were blood-sampled 24 and 48 hours after injection.

Results and Discussion

From the transcriptome analysis, five cDNA were showed to belong to the D_1 family, two D_{1A}, one D_{1B}, one D_{1C} and one D_{1B} or D_{1C}. Five other cDNA were showed to belong to the D_2 family, two D_2, one D_{2l}, one D_3 and one D_{4rs}. Unlike zebrafish, the subtypes D_{1E} and D_4 have not yet been isolated in pikeperch. As expected, DR D_{1A}, D_{2l}, D_3 and D_{4rs} are mostly expressed in brain parts except for the cerebellum. In many tissues, the gene expression is receptor-dependent, which may indicate specificities in their physiological involvements. Finally, while metoclopramide failed to change the plasma sex-steroid levels, SCH23390 induced an increase of 17β-estradiol and testosterone, thus supporting the implication of D_1 family, but not D_2, in the ovarian steroidogenic mechanisms.

Conclusion

The inter-species and inter-organ differences in the expression of all receptors support the complexity of the dopaminergic actions in teleosts. In pikeperch, their presence in the brain, pituitary and ovary is consistent with their implication in the final stages of reproduction. Nevertheless, further investigations are needed to understand the respective roles of D_1 and D_2 families in pikeperch ovulation.

PITUITARY-GONAD SYSTEM

ORAL 25

LOSS-OF-FUNCTION OF THE *FSH* RECEPTOR SHORTENS THE MATURATION CYCLE IN MALE ATLANTIC SALMON

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Introduction

Timing of puberty is delayed in FSHR knockout (KO) male mice associated with smaller testis and a reduced Sertoli cell number, but the mutant males are still fertile. On the contrary, zebrafish and medaka *fshr* KO males show no clear phenotype with apparently normal puberty and subsequent production of sperm, which was related to the Lh receptor-mediated stimulation of androgen production that in turn promotes spermatogenesis. In salmon, Lh is usually not secreted until close to the spawning period when testis growth has been completed, so that in *fshr* KO salmon, we expected to see a phenotype different from *fshr* KO zebrafish and medaka males.

Methods

We established an *fshr* KO salmon mutant using CRISPR-Cas9 technology. Due to the long generation time of salmon we studied double allelic KO in the F0 generation. To examine maturation phenotypes in males, *fshr* KO and wild-type fish were reared in a common garden under conditions which induce precocious maturation in sea water in one-year old fish (continuous light and 16ºC water temperature for a period of three months). We sampled control and *fshr* KO fish (the gender was identified by *sdy* PCR on fin clips) 1, 2, 5 and 9 months after commencing exposure to the maturation regime. At these samplings GSI, tissue for gene expression (gonad and pituitary) and histology (gonad) and plasma (11-ketotestosterone, 11-KT) was collected.

Results and Discussion

At the first sampling (1 month), all fish displayed low GSI and no effect of the *fshr* KO could be detected on plasma 11-KT levels or stage of spermatogenesis. However, in the samples collected during the last two samplings (5 and 9 months) we observed slightly (5 months) and clearly lower (9 months) GSI and 11-KT in knockout animals compared to control. Further analysis of 11-KT levels, histology and pituitary gene expression (*fshb*, *lhb* and *gnrh4*), suggested that the maturation cycle was shortened in *fshr* KO mutants, since the mutant males with low GSI values showed testes with residual sperm from the previous cycle and were starting already with the next wave of spermatogonial proliferation. We are currently investigating the effects of loss of *fshr* in the testis by quantifying transcripts of genes involved in testis maturation (*fshr*, *amh*, *igf3*, *inha*, *insl3*, *star and cyp17*).

Conclusion

Different from *fshr* KO zebrafish and medaka, *fshr* KO salmon males show a clear phenotype. Mutants produce sperm but display reduced GSI levels and a shortened maturation cycle. This includes an earlier start of testis regression followed by an immediate restart of spermatogonial proliferation.

ORAL 26

THE MUTATION OF FSHR AFFECTS REPRODUCTIVE PHYSIOLOGY AND GROWTH IN ZEBRAFISH *Danio rerio*

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Introduction

Fsh is a pituitary gonadotropin that regulates both gonadal functions, gametogenesis and steroidogenesis, in fish. The biological actions of Fsh are mediated through its binding to a membrane receptor (Fshr) that belongs to the superfamily of the G protein-coupled receptor (GPCR). Previous reports on *in vivo* disruption of the Fshr gene showed that Fsh signaling pathway was dispensable for male fertility but was required for oogenesis. In the present study we generated new mutant zebrafish lines to further investigate the role of *fshr* in reproductive and growth performances.

Methods

We disrupted the *fshr* gene in zebrafish using the CRISPR/Cas9 method. We selected three CRISPR sites in the 10^{th} exon of the gene to cause a large deletion easily detectable by a PCR-based genotyping method. Two mutant lines were generated from independent founders mated with transgenic *vasa*:eGFP females. The mutations were characterized by DNA sequencing in both lines. The deletions led to predicted truncated proteins enable to anchor to the membrane and to transduce Fsh signal.

Results and Discussion

Heterozygous mutant males and females developed an apparently normal gametogenesis and were fertile. However heterozygous zebrafish population ($fshr^{+/-}$) showed a female biased sex-ratio compared to the non mutated siblings. At 27 days post fertilization heterozygous mutants showed higher relative abundance of the gonadal aromatase gene (*cyp19a1a*) and increased levels of transcripts involved in granulosa cell differentiation (*foxl2a2*, *gsdf*, *fshr*, *sox9a*). In addition, transcripts accumulated during late oocyte growth (*vasa*, *sox9b* and *nanos2)* were also detected at higher levels. The relative abundance of s*ycp1* was unchanged indicating that meiosis initiation was normal. In contrast, in homozygous mutant meiosis was altered as revealed by decreased expression levels of *sycp1*. In addition, oocytes did not progress beyond the pre-follicle phase of primary growth. All adult homozygous mutants became males suggesting that impaired oogenesis during early ontogenesis caused sex reversal.

Additional new phenotypes were observed in the homozygous males including lower sperm count and viability. This could result from 11-KT deficiency as revealed by decreased expression levels of *cyp11b2*. Perturbation of steroidogenesis could also explain the increased body weight and length observed in homozygous mutants.

Conclusion

The marked phenotypic perturbations induced by the Fshr mutation confirm that Fsh and Lh have distinct functions. We showed that the loss of one *fshr* gene copy favours the differentiation of granulosa cells and oocyte growth. Fsh signalling pathway is dispensable for Sertoli cell differentiation but remains important for sperm quality and excretion.

ORAL 27

MOLECULAR CLONING, TEMPORAL EXPRESSION AND FUNCTIONAL CHARACTERIZATION OF GONADOTROPIN RECEPTORS IN THE CHARACID FISH *Astyanax altiparanae*

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Introduction

Fish gonadotropins (Fsh/ Lh) and their receptors (Fshr / Lhr) are the protagonists in the regulation of the HPG axis. Knowledge about the endocrine control of reproduction in South American teleosts is still scarce, especially regarding to the structure, patterns of expression and functions of gonadotropins hormones and their receptors. Once we previously characterized the gonadotropin subunits of the South American Characid *Astyanax altiparanae*, herein we report the molecular characterization of the follicle-stimulating, and luteinizing hormone receptors. We investigated their expression patterns in several tissues and in the ovaries. Also, we present some preliminary data about the functional characterization of these receptors.

Methods

Ovaries samples from adults were collected and destined to total RNA extraction, followed by cDNA synthesis and amplification of a partial cDNA for *fsrh* and *lhr*. The full length sequences were obtained by RACE PCR, cloned and sequenced. Several *in silico* and phylogenetic analysis were performed. A tissue screen was performed and the temporal expression of *fsrh* and *lhr* in the ovaries of adult females were analysed in different seasons. Single-chain *A. altiparanae* recFsh and recLh were produced by a commercial company and used in the transactivation assays for Fshr and Lhr transiently expressed in HEK 293T cells.

Results and Discussion

The *A. altiparanae fshr* complete cDNA consists of 2594 nucleotides. Fshr mature protein has 659 amino acids, showing 19 cysteine residues and seven putative N-glycosylation sites. *lhr* complete cDNA consists of 2590 and their mature protein consists of 692 amino acids, containing a total of 27 cysteine residues. Phylogenetic analysis revealed that both receptors are closer to Characiformes than to Siluriformes and Cypriniformes species. Both transcripts were detected only in the gonads. In the ovaries, *fshr* and *lhr* transcripts levels were higher during spring (Nov12) and summer (Jan13), in parallel to high GSI. Then, were down regulated during autumn (May13) and winter (Jul13). In a pilot study, both Fshr and Lhr were activated by hCG and by their cognate ligands. New assays are in progress to evaluate the selectivity of each *A. altiparanae* gonadotropin receptor.

Conclusion

The full length cDNA sequences of *A. altiparanae fshr* and *lhr* were characterized and their mature sequences shown the same organization pattern of glycoprotein hormone receptors family. High *fshr* levels found during the summer (reproductive season) may be associated to the reproductive behavior (asynchronous development with split reproduction) found in this species.

ORAL 28

SEX STEROID PRODUCTION ASSOCIATED WITH PUBERTY IS ABSENT IN GERM CELL-FREE SALMON

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Introduction

The genetic impact of escaped farmed salmon on wild populations is regarded as a major environmental risk. The use of sterile fish would solve this problem. Currently, functionally sterile triploid salmon are tested for commercial use. However, triploid salmon are more sensitive to several environmental factors. Moreover, triploid males still go through puberty, which is associated with elevated plasma sex steroid levels and the entailing welfare hazards related to osmoregulatory problems, reduced growth and increased susceptibility to diseases. Clearly, alternative models should be evaluated. We have recently produced germ cell-free (GCF) salmon by knocking out the *dead end* (*dnd*) gene. However, knowledge on possible effects of the lack of germ cells on processes like puberty and growth, is fragmentary. To address this caveat, we monitored growth and markers for puberty in GCF and wild type (WT) salmon exposed to environmental conditions inducing maturation.

Methods

GCF (*dnd*-knockouts) and WT postsmolts were stimulated to enter puberty in a common garden experiment by exposure to light/temperature, conditions known to induce maturation. Four times during one year we measured length, weight and plasma sex steroid levels, and from the terminal sampling, we measured GSI and the transcript levels of genes related to puberty. The lack of germ cells and mutation of the *dnd* gene was confirmed in all fish.

Results and Discussion

In contrast to what has been shown previously in GCF mouse, zebrafish, loach, medaka and goldfish, none of the GCF salmon entered puberty, whereas 66.7% (males) and 30% (females) WT fish completed or entered puberty, respectively. Expression of genes related to steroidogenesis (*star*, *cyp17a1*, *cyp11β*, *cyp19a1a)*, gonadal somatic cells (*insl*3, *amh*, *igf*3), oocytes (*bmp15)*, gonadotropin receptors (*fshr*, *lhcgr)*, and pituitary gonadotropic cells (*fshb*, *lhb*, *gnrhr4*) showed an immature status. The failure to up-regulate gonadal sex steroid production in male and female GCF fish was also reflected in low or undetectable plasma sex steroids (11-ketotestosterone, estradiol-17β and testosterone). A gender difference (high in females, low in males) was found in the expression of *star* and *cyp17a1* in GCF fish. No clear difference in growth was detected between GCF and immature WT fish, while growth was compromised in maturing WT males.

Conclusion

The Atlantic salmon is the first vertebrate species studied so far, requiring the presence of germ cells for the pubertal activation of gonadal androgen and estrogen production to occur. More work is required to understand the background of the gender difference in the transcript levels of some of the steroidogenesis-related genes (*star* and *cyp17a1*). Finally, while GCF and immature WT fish show no clear difference in growth, growth is compromised in maturing WT salmon.

AQUACULTURE AND GENOME-ENVIRONMENT INTERACTION

ORAL 29

PRODUCTION OF FUNCTIONAL BLUEFIN TUNA SPERM USING SURROGATE BROODSTOCK TECHNOLOGY

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Introduction:

Our ultimate aim is to produce functional bluefin tuna (BFT; *Thunnus orientalis*) gametes from a surrogate broodstock comprising a smaller-bodied species closely related to BFT. If this could be achieved, it would become possible to quickly produce tuna seeds in a land-based tank using surrogate small-bodied parents with shorter generation times. Therefore, in the present study, we attempted BFT spermatogonial transplantation with hybrid little tuna as surrogate hosts.

Methods

Donor cells were prepared from the testes of 3-year-old BFT. Minced testes were enzymatically dissociated by collagenase and dispase. Recipient hybrid little tuna (*Euthynnus affinis* x *E. alletteratus*) were produced by artificial insemination using unfertilized *E. affinis* eggs and cryopreserved *E. alletteratus* sperm. Prepared donor BFT testicular cells were transplanted into the peritoneal cavity of 2,000 hybrid little tuna larvae at 10 days post-fertilization. To evaluate the success of incorporation of donor BFT cells into the recipient gonads, immunohistochemical analysis of the hybrid little tuna was carried out using a BFT-specific antibody. Hybrid little tuna were maintained in a 50 m^3 tank with a semi-closed recirculation system. Spawning was artificially induced in 1-year-old hybrid little tuna using a combination of artificial photothermal controls and administration of a gonadotropin-releasing hormone analog. Presence of donor-derived genome in resulting F1 embryos was investigated by PCR.

Results and Discussion

BFT cells transplanted into peritoneum were detected within gonad in 80% of recipient larvae after two weeks from transplantation, at a rate of 5.0±6.1 (mean±SD) BFT cells per recipient larva. This result indicates that allogenic BFT cells have successfully migrated and were incorporated into hybrid little tuna´s gonads. One year post-transplantation, 68 recipient fish survived, and 15 of these fish were used for artificial spawning induction. We assessed 18,300 embryos (100 pooled sample × 183) using PCR with a BFT-specific primer set and obtained one positive pooled sample. Further, we performed PCR analysis of this sample with another three BFT-specific primer sets; the results provided more evidence that this sample possessed the genome derived from donor BFT. Furthermore, we confirmed that the sample did not contain BFT-derived mitochondrial DNA, which strongly suggests that the positive sample was derived specifically from donor BFT sperm. Together, these data suggested that the F1 hybrid offspring derived from the donor BFT sperm was produced by a surrogate hybrid little tuna.

Conclusion

The present study demonstrates that surrogate hybrid little tuna could produce functional BFT sperm. Although we have still to overcome issues regarding the efficiency of the inter-species germ cell transplantations, this could be a big step forward for BFT gamete production using surrogate broodstock technology.

ORAL 30

INVESTIGATING THE EFFECTS OF EARLY-REARING ENVIRONMENT ON SPERM DNA METHYLATION PROGRAMMING IN HATCHERY REARED STEELHEAD (*Oncorhynchus mykiss*)

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Introduction

Fish conservation hatchery programs intend to produce fish that are genetically and phenotypically indistinguishable from the wild stocks they aim to restore, but this has proven difficult to achieve. There is considerable evidence that steelhead trout reared in hatcheries differ from wild fish in phenotypic traits related to fitness even when wild fish are incorporated as broodstock. Molecular mechanisms underlying these phenotypes and relative contributions of genetic selection and/or environmentally-induced heritable epigenetic changes remain largely unknown. The aim of this work is to examine the effects of early-rearing environment on genetic variation and epigenetic programming in steelhead.

Methods

In an initial study, we described epigenetic variation in hatchery and natural-origin (wild) steelhead from the Methow River. Genetic variation was assessed using Restriction Site Associated DNA Sequencing (RAD-Seq) and variation in DNA methylation was analyzed using reduced representation bisulfite sequencing (RRBS) in both sperm and red blood cells (RBCs). To limit the potential confounding effects of genetic variation, a second study using controlled genetic backgrounds and simulated 'hatchery' and 'natural' environments was performed. Steelhead embryos from 20 families were split across hatchery and natural treatments. After 8 months in the treatment environments fish were tagged and raised to maturity in a common environment. Sperm samples collected from 60 fish were analyzed using RRBS.

Results and Discussion : In the initial study, genetic analysis did not reveal differences between the hatchery and natural-origin fish; however, we found significant differences in DNA methylation in both RBCs and sperm. Using RRBS, we identified both cell-type and origin-specific methylation. We also found a high degree of epigenetic variation among individuals necessitating future studies on how epigenetic and genetic variation interplay to promote such differences, and how much epigenetic variation is inherited. In the second study, hierarchical clustering of genome-wide methylation patterns in sperm show very strong clustering within family regardless of rearing environment. Initial results do not indicate a strong environmental effect; however additional analyses are required to determine statistical power given the strong heritability of methylation patterns. These results highlight a major challenge of epigenetic studies of natural populations, where population structure and kinship among individuals is either not known or controlled for in the experimental design.

Conclusion: Our work is among the first to demonstrate the potential for transgenerational inheritance of epigenetic information in hatchery steelhead by reporting differences in DNA methylation in the male germline. Our findings also emphasize the importance of understanding the effects of kinship among studied individuals in order to properly analyze and interpret DNA methylation data.

ORAL 31

TROJAN Y GENETIC MANIPULATION OF SEX FOR CONTROLING GAMBUSIA HOLBROOKI.

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Introduction

The history of trying to deal with destructive pest-fish species on large spatial scales has to date been ineffective with the problem likely to grow more severe around the world in the future. As in aquaculture, sex manipulation approaches could revolutionize the management of pests but are subject to a range of technical, behavioural and ecological limitations and may face challenges of public acceptability. Our work on *Gambusia holbrooki* a pest fish of concern to Australia, takes a systematic approach of evaluating feasibility, assessing public acceptance and making technical advances on Trojan chromosome as a suitable genetic control option.

Methods

To determine Trojan Y dynamics we used a generic model incorporating both genetic and population dynamic determinants for the control of gonochoristic, bisexual vertebrate pests. This was complimented by hormonal sex reversal and application of molecular-genetic tools for generating Trojan carriers and identification of a sex marker respectively. Social acceptance was evaluated using a custom designed survey instrument.

Results and Discussion

We show that the Trojan Y is not only the most effective—about 10 and 20 times more effective compared to a closest gender distorting recombinant approach in terms of time to eradication and cost for total eradication respectively—but also one that remains environmentally benign and socially more acceptable. Both androgen and estrogen treatments effected functional sex reversal in the species and the sex reversed individuals mate and reproduce, with no significant differences in clutch sizes. Hormonal sex reversal and selective breeding suggests that the species is female heterogametic, which has a significant bearing on the control strategy. The study has also generated male and female specific genetic markers, that assisted in the detection of sex reversed individuals and will be critical for monitoring the progress of this or any other sex manipulation strategy for controlling the species.

Conclusion

The study demonstrates that Trojan chromosome is a viable option for controlling *G holbrooki* and the species is female heterogametic.

ORAL 32

REPRODUCTIVE SUCCESS AFTER GENOTOXIC STRESS: HOW FISH EMBRYOS DEAL WITH PATERNAL DNA DAMAGE

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Introduction

Spermatozoa carry DNA damage as a consequence of the spermatogenesis process. Different genotoxicants, as well as some broodstock management practices, particularly short and long-term sperm storage, may increase the chromatin lesions. Different strategies could be adopted upon fertilization to deal to the paternal genotoxic damage. Mammalians activate a zygotic DNA damage response (DDR) that includes cell cycle arrest, DNA repair and alternative apoptosis. This DDR hinders the survival of embryos with unrepaired DNA, protecting the genomic conformity of the reduced progeny. However, fishes, with different reproductive strategies, seem to proceed distinctively. Our previous studies showed a downregulation of apoptotic activity in trout embryos with a defective DNA repairing ability, suggesting that tolerance mechanisms to damaged DNA (DDT) could be activated in order to maintain cell survival and progression with development. In this work we are aimed to analyze the activation of DDR/DDT during zebrafish embryo development.

Methods.

Zebrafish embryos were obtained from control or UV irradiated sperm, carrying more than 10% of fragmented DNA but still preserving its fertilization ability. Embryos were analyzed throughout development. DNA repair activity was assessed by whole mount immunocytostaining (γH2AX and 53BP1foci), apoptotic activity by FITC-Anexin V binding, expression of genes related to DDR by qPCR, postranscriptional activation of p53 by immunodetection of the phosphorylated protein and malformation rates by microscopy inspection and *in toto* cartilage staining.

Results and discussion.

Spawns from damaged sperm displayed a very high rate of multimalformed larvae. Repairing activity was highly enhanced at the mid blastula transition stage in the progeny from damaged sperm, returning to the basal level at later stages. The study of tp53, upstream the DDR, revealed an intense transcriptional and post-translational activation in those progenies. However, the downstream pro-apoptotic factor *noxa* showed a significant downregulation, whereas the anti-apoptotic gene *bcl2* was upregulated, triggering a repressive apoptotic scenario in spite of a clear genomic instability. This repression can be explained by the observed upregulation of *p53* isoform Δ*113p53*, which is known to inhibit *bcl2* transcription.

Conclusion

Our results suggest that tp53, and specifically the expression of the isoform Δ113p53, is involved in DDT pathways, allowing the embryo survival regardless of the paternal DNA damage. DDT could be an evolutionary mechanism in fishes: tolerance to unrepaired sperm DNA could introduce new mutations, some of them potentially advantageous to face a changing environment. Nevertheless, negative consequences in Aquaculture field, devoted to produce genetically consistent cohorts, should be considered.

ENDOCRINE DISRUPTION

ORAL 33

BISPHENOL A TRIGGERS HISTONE HYPERACETYLATION AND ALTERS *GPER1* EXPRESSION IN ZEBRAFISH TESTICLES

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Introduction

Bisphenol A (BPA) is a well-known environmental endocrine disruptor, which mimics estrogens actions binding to estrogen receptors. In males, BPA has been described impairing fertility, interfering with meiotic processes as well as altering the epigenetic pattern, modifying not only the DNA methylation profile but also histone acetylation in gonads. Endocrine disruption and epigenetic profile modification in germ cells during spermatogenesis could modify epigenetic and transcriptomic information contained in mature sperm. Such events could affect the forthcoming progeny upon fertilization, as our group reported when BPA treatment of males promoted heart defects on the non-exposed progeny. The present work is aimed to explore the mechanism of such transmission analyzing the effects of BPA on the epigenetic pattern during spermatogenesis as on the expression of specific transcripts in zebrafish testicle.

Methods

Adult males were exposed to 100 and 2000 ppb of BPA during 21 days. Testis mRNA expression level of different estrogen receptors and genes related to spermatogenesis, apoptosis, cell cycle and epigenetic-remodeling enzymes, was assessed by qPCR. The involvement of the receptor Gper-1 via MAP/ERK pathway was confirmed by Western blot. Apoptosis was determined by TUNEL assay. DNA methylation and histone acetylation levels were evaluated by immunodetection and flow cytometry. Moreover, global acetylation and HAT activity were assessed using commercial kits.

Results and Discussion

Our data showed an upregulation of *gper-1*, an estrogen receptor related to the maintenance of the meiotic arrest in fish oocytes and with the initiation of apoptosis. An increase in the percentage of apoptotic testicular cells similar to that reported in mice or rats was observed and a downregulation of *sycp3* and *ccnb1* genes, suggestive of a reduced meiotic rate, was also noticed. *hdac4* and *kdm6b* genes were also upregulated. We identified changes in the epigenetic profile of testicular cells, particularly in haploid ones, showing an increase in H3K9ac, H3K14ac, H4K12ac and 5mC marks after BPA treatment. However, a reduction in H3K27(me3) was observed after BPA exposure. Evaluation of global histone acetylation also demonstrated hyperacetylation after the two BPA tested doses and an important increase of HAT activity after the highest dose of BPA.

Conclusion: The present study suggests that paternal BPA exposure interferes with Gper1 affecting the expression of key transcripts of spermatogenesis and of epigenetic enzymes. Moreover, we demonstrate that BPA promotes histone hyperacetylation, DNA methylation and transcripts alteration in zebrafish testicular cells, which could impair the correct embryo development of the next generation.

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ORAL 34

DISRUPTION OF THE GONADAL ENDOCANNABINOID SYSTEM IN ZEBRAFISH EXPOSED TO DIISONONYL PHTHALATE

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Introduction

Di-isononyl phthalate (DiNP) is a man-made chemical basically used for the manufacture of Polyvinyl Chloride (PVC). DiNP, as other plastic additives, can be continually leached from the products due to DiNP is not tightly bound with the polymer matrix. In addition, the production of DiNP is increasing since its wide use as Bis(2-ethylhexyl) phthalate (DEHP) replacement. Recently, the endocannabinoid system (ECS) has been pointed out as a new target for the exposure to endocrine disruptor chemicals (EDC's). The ECS is a lipid-based system formed by the cannabinoid receptors, which are activated by the endocannabinoids. Regulating the endocannabinoid levels, a complex enzymatic machinery has been described throughout the body from invertebrates to mammals. The ECS is involved in a plethora of physiological functions, including the reproductive process and fertility. In addition, a cross-talk among the ECS and sex hormones (i.e. estradiol) has been described. Thus, the main objective of the present study was to elucidate whether the chronic exposure to DiNP, at environmental relevant concentrations, affects the gonadal ECS and the reproductive performance in *Danio rerio*.

Methods: Adult zebrafish were chronically exposed to three different concentrations of DiNP: 0.42 μg/L (10-9M); 4.2 μg/L (10-8M); 42 μg/L(10-7M) via water. The Gonadosomatic Index (GSI) was calculated. Gene expression for the ECS pathway was performed, the levels of the endocannabinoids, enzymatic activity and histology.

Results: Our results showed a decrease of the fertilization rate calculated as the number of fertilized eggs per day and per female. Regarding the females, the GSI was decreased, while the levels of endocannabinoids were unaltered. Anyhow, the expression of the genes coding for the ECS receptors and the enzymes regulating the levels of endocannabinoids were generally upregulated. Concerning the males, the GSI was decreased as well as the levels of endocannabinoids deregulated in the testes. At the transcriptomic level, the expression of genes involved in the ECS pathway were significantly affected. In addition, also the expressions of the genes coding for the estrogen and androgen receptors were downregulated. However, both testis and ovary, exhibited any pathological condition after the chronic treatment.

Conclusion: Summarizing, we observed an alteration of the ECS after DiNP treatment in a gender-specific manner. Thus, the deregulation of the ECS may be one of the reasons of the low fertilization rate, since a correct tone of the ECS is essential for a successful reproductive performance. Then, the results herein reported evidenced that DiNP cannot be considered a safer substitute of DEHP.

ORAL 35

REPRODUCTIVE EFFECTS OF OESTROGENIC ENDOCRINE DISRUPTING CHEMICALS IN *Astyanax rivularis* INHABITING HEADWATERS OF THE VELHAS RIVER, BRAZIL

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Introduction

Untreated domestic sewage discharge into rivers and streams contains substances which have the ability to interact with the endocrine system of animals called endocrine disrupting chemicals (EDCs). The Velhas River is the most polluted river in the state of Minas Gerais, south-eastern Brazil. Due to its historical and environmental relevance, the aim of this study was to evaluate the effects of oestrogenic endocrine disruptors on the reproduction of the lambari *Astyanax rivularis*, a small-sized species found in headwaters of the São Francisco River basin.

Methods

Quarterly field samplings were carried out during a reproductive cycle in three streams of the upper Velhas River: S1 (reference site) and S2 and S3 (sites contaminated by untreated sewage). At each sampling site, the fish were caught using 100 m gillnets. Total length (TL), body weight (BW), gonad weight (GW), and liver weight (LW) were measured and the following biological indices were calculated for each fish: gonadosomatic index (GSI = 100 GW/BW), liver somatic index (LSI=100 LW/BW), and Fulton condition factor (K= 100 BW/TL3). The main oestrogenic compounds were evaluated in water using HPLC/MS and molecular, histological and reproductive biomarkers were assessed in liver and gonad.

Results and Discussion

The results showed higher average concentrations of oestradiol (> 200 ng/l) in S2 and S3, oestrone (> 250 ng/l) in S2 as well as oestriol (> 200 ng/l), bisphenol A (> 190 ng/l), and nonylphenol (> 600 ng/l) in S3 compared to S1 (< 70 ng/l for all compounds). In S2 and S3, there was an increase in the proportion of females, higher ELISA levels of vitellogenin (Vtg) and proteins of the zona radiata (Zrp) in liver males. Insulin-like growth factor (IGF-I) levels were lower in S2 males, which also had a smaller body size, a smaller seminiferous tubule diameter, a higher proportion of spermatogonia, and lower proportion of spermatozoa in relation to S1. Histopathological analyses detected an increase in yolk deficient oocytes and over-ripening in the contaminated sites, and these alterations were associated to a reduction of hepatic Vtg levels and a delay in spawning, respectively. Intersex specimens with perinucleolar follicles in a multifocal distribution in the testis were detected in S2 and S3.

Conclusion : These results indicate that chronic exposure to oestrogenic compounds induced endocrine disruption that may affect wild populations of *A. rivularis* in the Velhas River.
Financial support: FAPEMIG

ORAL 36

REGULATION OF CALCIUM INFLUX IN *DANIO RERIO* (ZEBRAFISH) TESTIS: IONIC MODIFICATION AND BIS(2-ETHYLHEXYL)PHTHALATE EFFECT

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Introduction

Calcium (Ca^{2+}) is considered the most important intracellular ion, highly versatile that regulates the performance of cellular processes, including the male reproductive system. In recent years, zebrafish has become an emerging animal model of regulatory mechanism of ion homeostasis and endocrine disrupting compounds impacts on male fertility. Nowadays, the environment is exposed to numerous chemicals that are considered endocrine disruptors, among them is bis(2-ethylhexyl)phthalate (BEHP).

Objectives

To study the influence of different concentrations of Ca^{2+} exposed in aquatic environment in the in vitro Ca^{2+} influx in 30 and 60 minutes, as well as the in vitro effect of BEHP in zebrafish testis.

Material and Methods

The fish *Danio rerio* (zebrafish) were kept in the aquatic environment for 12 hours with low Ca^{2+} concentration (0,02 mM) and high Ca^{2+} concentration (2 mM), the control group was considered the aquarium water. Subsequently, euthanasia was performed and the testis was dissected to study the measurements of Ca^{2+} influx: The testis was incubated in vitro at 30 and 60 minutes with radioactive Ca^{2+} ($^{45}Ca^{2+}$) at 0,1 μCi/mL. After, it was treated in vitro with BEHP at 0,01 and 1 μM for 30 minutes and the Ca^{2+} channel blocker nifedipine was used (CEUA nº PP00968).

Results and Discussion

It was observed that in 30 and 60 minutes, the groups with low and high Ca^{2+} concentrations led to the modification in vitro Ca^{2+} influx in relation to the control group. Furthermore, it was observed that only the BEHP treatment at 1 μM stimulated the Ca^{2+} influx in zebrafish testis when compared to the control group, however, this effect was completely blocked by nifedipine.

Conclusion

Ca^{2+} influx in the testis is highly regulated since low and high Ca^{2+} concentrations in the fish environmental (aquarium) alter the in vitro Ca^{2+} influx. The acute effect of BEHP on Ca^{2+} influx is mediated by voltage-dependent Ca^{2+} channels in the testis. Taking it together, these data suggest that the acute effect of BEHP at low concentration disturb the Ca^{2+} homeostasis that is critical for the testis functions.

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POSTER PRESENTATION ABSTRACTS

SEX DETERMINATION

POSTER 1

RETINOIC ACID INDUCES MEIOSIS AND REGULATES EXPRESSION OF GENES RELATED TO SEX DETERMINATION IN MEDAKA

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Introduction : Sex determination (SD) is a complex and diverse developmental process that leads to the decision whether the bipotential gonad anlage will become a testis or an ovary. The timing of meiosis entry is one of the first recognizable differences between male and female in vertebrates. The germ cells go into meiosis first in females and later in males. In mammals, this event has been shown to be regulated by retinoic acid (RA). This polar small molecule induces in the germ cells the expression of the pre-meiotic marker *Stra8* (stimulated by retinoic acid gene 8), which is necessary for meiosis initiation. Interestingly, genome analyzes have shown that the majority of fish (including medaka) lack *stra8*, adding a question mark to the role of RA in meiosis induction in this group. We investigated in medaka (*Oryzias latipes*) a possible signaling function of RA during the SD period in embryos and in reproductively active gonads of adults.

Methods : We generated transgenic medaka reporter lines of the main genes involved in RA metabolism (*aldh1a2*, *cyp26a1* and *cyp26b1*), and a transgenic RA-responsive reporter line containing 12 copies of the RARE together with eGFP (12XRARE). In addition, the *cyp26a1* gene was disrupted using the TALEN method. We performed several RA treatments in embryos, cells and testis organ cultures, and checked for gene expression by qRT-PCR.

Results and Discussion : RA mediated transcriptional activation in germ cells of both sexes much earlier than the SD stage, however, no such activity was noted during the critical stages of SD. In adults, expression of the RA metabolizing enzymes indicates sexually dimorphic RA levels. In testis, RA acts directly in Sertoli, Leydig and pre-meiotic germ cells. In ovaries, RA transcription regulating activity is highest in meiotic oocytes. Expression analyzes of embryos treated with exogenous RA showed induction of *dmrt1a* at the gonad levels and an increase of *amh* levels. Both genes are known to be involved in the regulation of germ cell proliferation and differentiation. Disruption of the *cyp26a1* gene leads to an early entry of meiosis and oocyte formation in XY medaka larvae, as well as upregulation of *dmrt1a* in adult testis and downregulation of *foxl2* in adult ovary.

Conclusion : RA is important in meiosis induction and gametogenesis in adult medaka. Moreover, contrary to common expectation, RA induces sex related genes that are involved indirectly in meiosis inhibition. We showed for the first time that RA can be involved in induction of meiosis entry, and regulates genes related sex determination and differentiation depending on the sex and the developmental stage in a *stra8*-independent model organism.

POSTER 2

TRANSCRIPTIONAL REWIRING OF THE MEDAKA SEX DETERMINING DMRT1 GENE DUPLICATE AFTER CO-OPTION OF NESTED TRANSPOSABLE ELEMENTS

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Introduction

Sex-determination relies on the proper control of a hierarchically structured network of genes. Our analysis shows that the medaka master male determiner *dmrt1bY* was subjected to a profound rearrangement of its regulatory landscape concomitantly to the acquisition of a dominant position within the sex-determining network.

Results and Discussion

Requiring a complete rewiring of the regulatory network, this evolutionary innovation was brought about by the exaptation of a transposable element (TE) called *Izanagi*. This element acts as a silencer to turn off the *dmrt1bY* gene after it has fulfilled its function. Above this simple feedback regulation, we found that another TE, *Rex1*, has jumped into *Izanagi*. *Rex1* brought in a preformed regulatory element for the transcription factor Sox5. Sox5 has not been implicated in sexual development in any metazoan. We demonstrate its critically implication in gonadal development of medaka and possibly of mice. Sox5 medaka mutants have complete female-to-male sex-reversal.

Conclusion

Our work reveals a dual role for *sox5* during sex determination: first being an evolutionary conserved important regulator of germ cell number in medaka, and second, *de novo* regulating *dmrt1* transcriptional activity during primary sex determination after it has been recruited following transcriptional rewiring of *dmrt1* promoter due to exaptation of a transposable element.

POSTER 3

THE BIZARRE MASTER SEX DETERMINANT OF SALMONIDS TRIGGERS ITS ACTION BY DIRECTLY HIJACKING THE CONSERVED GONADAL DIFFERENTIATION PATHWAY.

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Introduction

Evolutionary novelties require rewiring of transcriptional networks and/or the evolution of new gene functions. Sex determination (SD), as one of the most plastic evolutionary processes, requires such novelties. But studies on the evolution of vertebrate SD revealed that new master SD genes are always recruited from genes involved in the downstream SD regulatory genetic network. Only one exception to this rule is currently known in vertebrates with the intriguing case of the salmonid master SD gene (s*dY)*, which arose from the duplication of an immune-related gene. This leaves the open question on how such a gene from outside the classical sex-differentiation cascade can still function as a SD gene.

Methods

Yeast two-hybrid screening was performed on a late differentiating rainbow trout testicular library using as bait the coding sequence for SdY. Cell transfections (co-localization and luciferase assays) with *sdY*, *sf1* and *foxl2* plasmid constructs were performed by incubating cells with Polyethylenimine in HEK 293T cells or by electroporation in RTG2 cells. Co-immunoprecipitations were carried out after transfection of flagged SdY and Foxl2 proteins in HEK293T cells. Expressions of *sdY*, *foxl2a*, *foxl2b* and *cyp19a1a* were measured by qPCR, and *in-situ* hybridization was performed using digoxygenin labeled PCR-amplified RNA probes.

Results and Discussion

Here we show that SdY directly bridges with the classical vertebrate sex-differentiation cascade by specifically interacting with the forkhead box domain of the female determining transcription factor, Foxl2. In cooperation with Sf1, the SdY:Foxl2 complex prevents the activation of the aromatase (*cyp19a1a*) promoter. By blocking a positive loop of regulation needed for the synthesis of estrogens in the early differentiating gonad, SdY impedes a preset female differentiation pathway, allowing testicular differentiation to proceed.

Conclusion

These results suggest that innovation at the top of vertebrate sex-determination pathways is rather constrained in order to cope with the regulation of the classical sex-differentiation cascade.

POSTER 4

TEMPERATURE EFFECT ON SEX DETERMINATION IN PEJERREY (*Odontesthes bonariensis*) IN THE WILD

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Introduction

It is known that pejerrey (*Odontesthes bonariensis*) has a strong temperature sex determination (TSD). Experimentally, it was demonstrated that the proportion of females gradually changes from 100% at 15–19C to 0% at 29C when larvae are reared at different temperatures between 1 and 5 weeks after hatching (TSD window). The objective of this study was to analyze the effect of water temperature on sex ratio in pejerrey larvae in the wild.

Methods

Newly hatched pejerrey larvae were sown in floating cages of 1 m^3 in September, October, November and December 2016 in *La Salada de Monasterio* lake (35° 44'S, 57° 53'O), *Buenos Aires province, Argentina*. Water temperature (WT) per hour was recorded through data loggers placed in the cages. After three months 50 juveniles per cage were captured and taken to the laboratory. They were sacrificed on ice and the gonads were observed using a microscope to establish the phenotypic sex. Sex proportions were calculated and analyzed in relation to the water temperature recorded during TSD window.

Results and Discussion

In the juveniles of September cages, the proportion of males, was 53% (mean WT during TSD window: 13.9 ± 1.3° C), of October: 43% (mean WT: 18.6 ± 3.0° C), of November: 55% (22.1.7 ± 1.5° C) and of December: 65% (25.7 ± 1.9° C). These results showed that the proportion of males at low water temperatures (September and October cages) mismatch with the experimental proportions where all females were observed. On the other hand, the proportion of males at higher water temperatures (November and December cages) was similar to the found in previous laboratory experiments.

Conclusion

These findings lead us to conclude that in the wild and especially at low water temperatures, another factor (food availability, thermal fluctuation, etc) besides temperature could be related to sexual determination in pejerrey.

POSTER 5

EVOLUTION OF THE SEX DETERMINATION PATHWAY IN THE GENUS *Oryzias:* A COMPARATIVE TRANSCRIPTOME ANALYSIS

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Introduction : In vertebrates, fish are the group with the highest diversity of sex determining genes. This variability is observed not only in species from distant branches of the phylogeny, but also within the same genus. The genus *Oryzias* is a well studied paradigm for this phenomenon. To understand how the sex determination genetic network evolved in closely related species, we analysed the genome and the transcriptome of adult gonads of four species of *Oryzias*: (1) *O. latipes* (XX/XY system where the sex determining gene (SD) on the Y is *dmrt1bY*, (2) *O. curvinotus* (XX/XY system with the same SD gene *dmrt1bY* on a homologous Y*), O. luzonensis* (XX/XY system but with a different SD gene, *gsdfY,* on a different linkage group) and *O. javanicus* (SD gene still unknown, ZZ/ZW sex determination system).

Methods : Three gonad samples of each sex were analyzed by RNA-seq in all four species. All sequences were mapped to the *O. latipes* reference genome using the RNA-seq aligner STAR and differentially expressed genes between testis and ovary were detected using DESeq2 (Bioconductor/R) for each species. Functionally enriched pathways and GO categories were found using the functional annotation tool DAVID (https://david.ncifcrf.gov/).

Results and Discussion : Histology of the adult gonads in four species showed similar structures. A global transcript analysis revealed enriched categories for genes involved in metabolic pathways also across all four species. Histogram analysis for an overview of the transcriptome data (considering Log Fold Change (LFC)>(3), Base Mean>100) showed that *O. javanicus* ovaries and testis are more similar to each other and were separated from the other species. Genes involved in gonadal development showed unexpected divergence in expression pattern between species. Several genes that are preferentially expressed in males of one species are expressed in females in the other species. *O. javanicus* has more genes exclusive differentially expressed in females (e.g. *activinRII*) and males (e.g. *cyp46a1, ptger3*) than the other species. The *fsta (follistatin a)* gene, a well-characterized "female" gene is highly expressed in ovary of *O. javanicus*, while in *O. latipes* this gene is higher expressed in males. Even though *O. latipes* and *O. curvinotus* share the same SD gene, the expression pattern of several genes critically involved in gonadal development are different. Analysis between the genome of *O. latipes*, *O. curvinotus* and *O. luzonensis* showed a conserved synteny for *dmrt1*. *dmrt1* in *O. luzonensis* is higher expressed in females, while in *O. latipes* and *O. curvinotus* the expression pattern is similar but it is higher expressed in males. Interestingly, the transcriptome showed a LFC significantly higher for *dmrt1* in *O. javanicus* males compared with the other species.

Conclusion: The final outcome showed a similar morphology of the gonad, despite of having different sex determining genes and regulatory pathways. The WZ species *O. javanicus* exhibited considerable differences in gonad gene expression compared to the XY species *O. latipes, O. curvinotus* and *O. luzonensis*.

POSTER 6

THE TAMBAQUI (*Colossoma macropomum*) TRANSCRIPTOME AT SEX DIFFERENTIATION STAGE

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Introduction

As females of tambaqui (*Colossoma macropomum*) are heavier than males at harvest, farming all-female populations would be more profitable. Unraveling the genetic and physiological mechanisms involved in sex determination and sex differentiation is then a fundamental objective in order to achieve sex control in this species. Therefore, we produced and assembled individual transcriptome libraries of juveniles sampled just prior to the gonadal sex differentiation in order to identify genes putatively related to testicular or ovarian differentiation in tambaqui.

Methods

Ten juveniles (20 to 33 mm - total length) were decapitated and the remaining trunk was used to extract total RNA (Trizol). Genomic DNA was removed (RQ1 RNase-free kit; Promega). Total RNA was sequenced (Illumina – HiSeq 2000) and a *de novo* transcriptome was assembled using the Trinity pipeline. Based on the transcription of classical genes involved in sex differentiation as well as genes exhibiting similar transcription profiles, the fish were grouped into two different clusters (putative males, n = 3; and putative females, n = 7). For differential expression analysis between the groups (by Trinity pipeline), data of only three females were used. Finally, the sex differentially expressed genes were manually checked out in the four remaining females' data.

Results and Discussion: The juveniles were grouped into male-like group (MLG) and female-like group (FLG), according to the expression of classical (sex differentiation) genes. The main functional categories of genes (according to Gene Ontology) significantly enriched in the MLG were cellular process, metabolic process and catalytic activity. More specific terms within these categories are the metabolic process of fatty acid, steroids and biosynthesis of steroids. Among these genes, their encoded proteins include the enzymes HSD3β e HSD17β3, which are important enzymes involved in testosterone synthesis. The main functional categories significantly enriched in the FLG were developmental process, metabolic process and biological regulation. Some terms involve organ development, biosynthetic process and transcriptional factors, such as the *fox* family (*foxl2* exclusively expressed in the FLG and *foxo3* upregulated in the FLG) and the WNT/βcatenin/FST signaling pathway. The data indicates that androgen synthesis is involved in testicular differentiation while *fox* family and *wnt* seem to be the main path for ovarian development.

Conclusion: Based on gene transcription, the testicular differentiation in tambaqui seems to be androgen dependent. On the other hand, the formation of ovary is trigged by transcription factors classically involved in structural development of organs, as already identified in female-developing of other teleost's species. Moreover, the transcriptome libraries assemblies will now serve as basis for further investigation to unravel the mechanisms that drive these processes in tambaqui, our main native species.

POSTER 7

LABELLING GERM CELLS IN MEDAKA RELATED SPECIES FOR CELL PROLIFERATION AND SEX DETERMINATION STUDIES

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Introduction

The genus Oryzias includes 32 species with XX/XY and ZW/ZZ sex determination systems, different sex determining genes and environments (fresh and sea water). Among them, *O. latipes* has been intensively studied at molecular and genetic levels. Our lab has recently revealed that germ cells, regulated downstream from *Dmrt1bY*/*DMY* (a switch gene for male sex determination), are a critical component for feminizing the gonad. The XY embryos with more germ cells develop into female while less number of germ cells during embryogenesis causes masculinization even in the absence of Y chromosome. In other related-species, however, the importance of germ cells for the feminization remains unclear.

Methods

Four related medaka species (*O. pectoralis, O. dancena*, *O. javanicus* and *O. luzonensis*) which have different sex determination systems, sex determining genes and environments (fresh and sea water) were selected for this study. Sex typing protocols were standardized based on the sex determining gene and sex-linked markers information. Microinjection conditions for each species were standardized based on that already established for *O. latipes* and, one-cell stage embryos were microinjected with a mRNA-encoding EGFP conjugated with *nanos3*-3'UTR from *O. latipes* which drive the EGFP expression in germ cells. Germ cell migration and proliferation were analyzed by fluorescence microscope. In addition, co-injection and germ cell expression of EGFP and Cre recombinase-mCherry (mRNAs) allowed us to generate germ cell-less medakas.

Results and Discussion

The four medaka (Oryzias) related species were successfully sex-typed by conventional PCR followed by electrophoresis. However, a less-time consuming typing method is desirable. Therefore, a sex-typing protocol by qPCR is being currently standardized. In addition, germ cells in the related-species were successfully labelled with both/either EGFP and/or mCherry fluorescence, facilitating the analysis of germ cell migration and proliferation during embryonic development as well as the reduction of germ cell number in the Oryzias species.

Conclusion

Fluorescence-labelled germ cells by injecting a *nanos3*-3'UTR-conjugated reporter, is proposed as an easy way for studying germ cell migration, proliferation and depletion *in vivo*. The germ cell depletion protocol established in our study, will further provide us insights about whether the gonad-feminizing ability of germ cells in *O. latipes* (recently reported by our lab) is also present and a well-conserved mechanism across the Oryzias species, independently of their genetic sex. Likewise, whether this mechanism is conserved even across vertebrate species.

POSTER 8

EFFECTS OF SEX REVERSAL ON ARGININ-VASOTOCIN NEURONES IN THE BRAIN OF NILE TILAPIA NEOMALES (*Oreochromis niloticus*)

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Introduction

Nile tilapia (*Oreochromis niloticus*) is a gonochoristic fish in which sex determination results from both genetic and environmental factors. During the critical period of sex differentiation, female juveniles (genetically XX) can be masculinized by high water temperature treatment (36°C). It is thus possible to obtain genetically functional XX males. Such individuals have already been found in the wild, but the consequences of this sex-reversal on the brain are still unclear. Arginin-vasotocin (AVT) is a neuropeptide involved in the control of social behaviours and reproduction. It is well known that the number and/or size of these AVT expressing neurones vary between sexes in sex-changing fish, or between different male fish morphs. The aim of this study is thus to determine the effects of temperature-induced sex reversal on AVT expressing neurons and on sex steroid concentrations in XX neomales.

Methods

Four families were obtained by artificial crossings; XX female oocytes were separated into two batches; one being fertilized by a XY male, the other by a XX neomale. XX progenies were separated into two groups; one of them being reared at high temperature (36°C) from 10 to 30 dpf in order to induce sex reversal. The other XX batch and XY progenies were reared at ideal temperature (28°C). Progenies from three experimental groups (XY males, XX males, XX females) were then reared in the same conditions (density, food ratio) throughout development. At 6 months of age, individuals from the four families were weighted and sexed. Among these animals, females, males and neomales were randomly selected; their blood was collected before they undergone intracardiac perfusion with 2% paraformaldehyde. Testes were weighted and stored in formalin 10%. Some of these individuals were selected within a body weight ranging from 100-150g, to avoid possible effects of fish size on AVT neurones. Selected brains were cryosectioned at a 20µm thickness on two alternate slide series. Brain slices were immunostained and are being analysed for the number and size of AVT expressing neurons. Serum was assayed for testosterone, 17β-oestradiol and 11-ketotestosterone with radio-immunoassay. Testes were cut at 5µm thickness, stained with hematoxylin-eosin and are being analysed for developmental stage.

Results and Discussion

The statistical analysis revealed a significant group difference with XY males showing mean body weight between lighter females and heavier neomales. However, groups did not differ for gonadosomatic index (GSI). Brains, gonads and serum samples are currently being analysed.

Conclusion

In the present study we confirm previous data which suggest that temperature-induced sex reversal could affect the growth of XX neomales, even though GSI does not seem to be affected.

POSTER 9

PRELIMINARY DATA ON THE SEX RATIO IN FULL-SIB FAMILIES OF TAMBAQUI ***Colossoma macropomum*****.**

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Introduction

Techniques to control sex ratio in fish farming are essential in many instances such as to develop breeding programs or to produce monosex populations when differential growth or timing of puberty exists between the sexes. Despite of recent data showing that females of Tambaqui *Colossoma macropomum* have higher growth rates, the sex determination system and sex ratio in the species are still unknown. This preliminary study aimed to investigate the sex ratio in full sib families of *C. macropomum* to gain insight into the sex determination system and differential growth related to sex.

Methods

Four families of *C. macropomum* were obtained through artificial fertilization and reared in closed indoor systems while fed *ad libitum* until reaching gonadal differentiation to allow gender confirmation. These families were produced in Tocantins (F1, n = 160; F2, n = 80) and Amazonas (F3, n = 53; F4, n = 51). At the approximate bodyweight of 260.6 ± 74.8 g, fish were anesthetized and sacrificed, a piece of gonad collected and fixed in 10% formaldehyde for routine histological analysis aiming at sex identification.

Results and Discussion

Three studied families had balanced sex ratio (F1-F3; χ-square; $P > 0.05$) while one from an Amazonas hatchery had a significant higher number of males (F4; χ-square; $P < 0.05$). Though an apparent pattern of chromosomal sex determination system appears, the deviation in sex ratio found in one family indicates that the regulation of sex in Tambaqui could involve other factors (such as temperature) like in other teleosts. Males and females had similar growth performance in all families when considering body weight (F test; $P > 0.05$) and total length (F test; $P > 0.05$) during the early period considered in this study, also observed in recent studies on Tambaqui.

Conclusion

This preliminary study indicate *C. macropomum* has a balanced sex ratio with possible influence of environmental factors to be investigated. Future work should use these families to better investigate sex determination system in Tambaqui using next-generation sequencing (*i.e.* RADseq for QTL mapping) and epigenetic approaches.

POSTER 10

A PILOT STUDY ON THE USE OF OTOLITH ANALYSIS TO EXAMINE THE OCCURRENCE OF TSD IN WILD COBALTCAP SILVERSIDE

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Introduction

Verification of the occurrence of temperature-dependent sex determination (TSD) in wild fish is difficult because their past thermal history is unknown. Here we evaluated the usefulness of otolith increment analysis to determine the birth dates and gain insight on the thermal history of wild cobaltcap silversides, in which we previously demonstrated both the presence of a chromosomal sex marker/putative genetic testis determining factor and of TSD in laboratory-reared animals. Otoliths are biominerals located in the inner ears that are responsible for the sense of balance and that grow by consecutive, daily accretion of layers to their external surface. Birth dates were used to estimate the thermal history of juveniles from three yearclasses (2014-2016) caught in Tokyo Bay and this information was compared with the rates of sex reversal in each yearclass.

Methods

Validation of the daily deposition of otolith increments was conducted using specimens from laboratory populations with known birth dates that were reared from hatching at constant (but environmentally relevant temperatures) of 22 and 26℃. Larvae and juveniles were sampled at 2, 4 and 6 weeks after hatching and their otoliths were extracted, mounted, sectioned, and examined under a microscope to count the number of increments. Birth dates of wild juveniles were calculated as the collection date minus the number of otolith increments and the average temperature for the estimated hatching period of each yearclass was inferred from local water temperature charts.

Results and Discussion

The number of otolith increments agreed with the number of rearing days regardless of water temperature. This confirms their daily formation in cobaltcap silverside young and validates their use for daily age determination in juveniles. Sex-reversed XX-males (14-17%) and XY-females (8-10%) were found in 2014 and 2015 whereas the 2016 yearclass had an unusually high proportion of XX-males (43%) and absence of XY-females (see presentation by Yamamoto et al.). The average temperature during the estimated hatching period in 2014, 2015 and 2016 was 23.1±1.5ºC (Jun.8~Aug.8), 25.4±1.7ºC (Jul.7~Sep.8) and 26.2±0.9ºC (Aug.7~Sep.24), respectively. These data indicate that fish were born later and experienced higher temperatures during the first weeks of life in 2016 than in other years and suggest that exposure to relatively low and high water temperatures was behind the formation of sex reversed XY-females and XX-males, respectively. These results are consistent with laboratory results and suggested the occurrence of TSD in wild cobaltcap silverside.

Conclusion

The results of this study suggest that otolith analysis might be a valuable aid to study the occurrence of TSD in wild fish.

POSTER 11

EFFECTS OF ENVIRONMENTAL FACTORS OTHER THAN TEMPERATURE ON THE SEX DETERMINATION OF PEJERREY *Odontesthes bonariensis*, A SPECIES WITH MARKED TEMPERATURE-DEPENDENT SEX DETERMINATION

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Introduction

The pejerrey *Odontesthes bonariensis* is an Atherinopsid species that presents a combination of genotypic and environmental (temperature-dependent) sex determination systems (GSD and ESD/TSD). Low and high temperatures are associated with female- and male-biased sex ratios and masculinization involves the perception of thermal (heat) stress and the release of the stress hormone cortisol, activation of the enzyme 11β-HSD for conversion of cortisol into cortisone, and the by-production of the potent androgen 11-ketotestoretone from its precursor testosterone that is mediated by the same enzyme. Given the involvement of stress, it is possible that other environmental stressors can also affect sex determination in pejerrey. In this study, we tested whether background color, rearing density, and salinity could cause a stress response and affect the sex ratios of pejerrey.

Methods

The progenies from single crosses of pejerrey with XX and XY genotypes were exposed to different background colors (white, black, light blue, dark blue, green, gray, red; two trials), rearing densities (15, 62 and 250 larvae/L; two trials), and salinities (0, 0.05, 0.1, 0.3, 1 and 3%: single trial) during the critical period of sex determination (1-5 weeks after hatching) at 25°C, a mixed sex promoting temperature. Fish were sampled throughout the rearing period for determination of whole-body cortisol titer by ELISA and after completion of gonadal sex differentiation for determination of sex reversal rates. Sex reversals were inferred from histological analysis of the gonads and detection of *amhy* gene as the genotypic sex marker by PCR.

Results and Discussion

Background color did not affect the cortisol titers and the sex reversal rates in a consistent manner although some groups showed elevated cortisol. High rearing density was associated with increased frequency of masculinization of XX fish and higher cortisol titers compared to the intermediate and low densities. The experiment on salinity revealed the occurrence of masculinization at 1 and 3% salinity and conversely of feminization at 0-0.3%. These preliminary results suggest that the factors examined may be sources of stress for pejerrey and support the notion that high levels of stress and cortisol are associated with increasing rates of masculinization. Further studies should examine individual differences in stress responsiveness and analyze the underlying molecular mechanisms for this response and masculinization.

Conclusion

This study shows that environmental factors other than temperature such as rearing density and salinity concentration can elicit a stress response in pejerrey during the period of gonadal sex determination and affect its sex determination.

SEX DIFFERENTIATION

POSTER 12

THE DIFFERENTIAL EXPRESSION OF TWO PARALOG TRANSCRIPTION FACTOR IIIA GENES IN TELEOSTS: *gtf3ab* AS A MARKER OF OVARIAN DEVELOPMENT

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Introduction

Stockpiling of molecules such as rRNAs in fish oocytes is essential to assist ribosomal assembly and protein synthesis in the newly formed embryo. In this way, the massive 5S rRNA expression during fish oogenesis serves as oocyte differentiation marker in the intersex testis formed after exposure to xenoestrogens. This 5S rRNA production is allowed by the general transcription factor IIIA (*gtf3a*) and as in *Xenopus* one single *gtf3a* gene but two transcripts exist, one of them oocyte specific, we wanted to study the possible existence of paralog *gtf3a*genes in teleosts.

Methods

Teleost genomes in ENSEMBL were analyzed to identify gtf3a orthologs. Two paralogs were identified so a synteny analysis was performed to understand the origin of the gene duplication event in teleosts. Then, transcription levels of gtf3aa and gtf3ab were analyzed by qPCR in tissues of adult zebrafish (D. rerio, ZF) and during the first 30 hours of embryo development. Transcription levels were also quantified in whole larvae (26 & 61 dpf), either masculinized or feminized after methyltestosterone (MT) and *17β-estradiol* (E2) exposures, and compared to cyp19a1a, dmrt1 and amh levels. Finally, the promoter methylation level of both genes was studied in testis and ovary by bisulphite sequencing.

Results and Discussion

Teleost genomes present two *gtf3a* paralog genes. *Gtf3ab* aroused from the teleost specific genome duplication event, with specific expression in ZF oocytes. Instead, *gtf3aa* is ubiquitously expressed in all tissues tested. No gonads were observable at 26 dpf in the ZF studied, exposed or not to E2 or MT, with no *gtf3ab* transcription but with detectable *gtf3aa* levels. 61 dpf E2 feminized ZF showed transcription of *gtf3ab* and *gtf3aa* in whole body analyses, while MT masculinized juveniles only transcribed *gtf3aa*. Female g*tf3ab* transcription coincided with that of ovarian *cyp19a1a* and opposite to that of *amh* and *dmrt1*. Maternal *gtf3ab* transcripts were present in zygote but disappeared after embryo genome activation. Opposite, the transcription of *gtf3aa* began with the activation of the zygotic genome (~8 hpf). Bisulfite sequencing of the promoters of both *gtf3a* genes is currently ongoing.

Conclusion

As 26 days exposure to E2 induced no *gtf3ab* transcription in whole juveniles, but transcripts were detected upon ovarian development at 61 dpf, we can consider that *gtf3ab* transcription is a consequence of oocyte production in fish and not a direct result of E2 exposure. Thus, *gtf3ab* expression constitutes a plausible marker of feminization in ZF. Funded: Basque Gov. (IT810-13), UPV/EHU (UFI 11/37), Spanish MINECO & EU-FEDER/ERDF (AGL2015-63936-R & AGL2015-73864).

POSTER 13

RELATIONSHIP BETWEEN LARVAL ONTOGENESIS AND GONADAL DEVELOPMENT IN *Brycon orbignyanus* (CHARACIFORMES, CHARACIDAE)

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Introduction

Knowledge of larval ontogeny is important for fish farming, as well as clarifying the mechanisms of gonadal development are essential for the reproductive management satisfactory. The piracanjuba, *Brycon orbignyanus* is a native species with potential for aquaculture. The aim of this study is to relate the piracanjuba gonadal development with larval morphological development by providing relevant information for future work on sex reversal.

Methods

Larvae of *B. orbignyanus* were collected at intervals of six and 24 hours after hatching (hpe) until the 10th day of life, the Estação de Piscicultura da CESP (Três Lagoas / MS). The same were anesthetized, fixed in 4% paraformaldehyde and 2% glutaraldehyde in 0.1 M Sorensen phosphate buffer, pH 7.2, measured with calipers, processed according to the protocol for light microscopy and stained with hematoxylin / eosin and toluidine blue.

Results and Discussion

Larvae *B. orbignyanus*, hatch with total length (TL) of 4.26 mm, and yolk sac elliptical and large intestine straight and without celular differentiation. The swin bladder and liver cells are absent. The gonadal primordium is already present in the coelomic cavity, consisting of rare elongated primordial germ cells (CGPS) of and few somatic cells, which often are present only involving germ cells, the gonadal primordium therefore is still discontinuous. The hatching in early stage of ontogenesis is common among teleósteos, however, the primordial gonad is not always found in the coelomic cavity at the time of eclosão[2]. The discontinuity of the gonadal primordium lasts up to 79 hpe (10.76 mm CT), after begins to occur intense proliferation of somatic cells which results in a thicker primordium and set at 103 hpe (12.06 mm CT). Concomitant with this step, practically all larval ontogenesis has occurred: the yolk sac was completely consumed and the exogen feeding period was initiated at 30 HPE (6.50 mm CT). The gut is differentiated in all cephalic portion (anterior, middle and posterior). The liver and swin bladder are formed and there is many adipocytes in the coelomic cavity. At 127 hpe (14.29 mm CT), most CGPS has a rounded shape and cytoplasm few eosinophilic. However, up to 223 hpe (29.32 mm CT), the gonad remains a gonadal primordium, thus considered to not to present blood vessels and mitotic divisions of germ cells. Thus, the formation of undifferentiated gonads and sex differentiation in *B. orbignyanus* do not coincide with the transition from endogenous feeding to exógena but occurs after to larval development.

Conclusion

In *B. orbignyanus*, the gonad remains at the stage of gonadal primordium, throughout larval ontogeny.

POSTER 14

IS AROMATASE NECESSARY FOR OVARIAN DIFFERENTIATION IN TAMBAQUI (*Colossoma macropomum*)?

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Introduction

Cytochrome P450 aromatase, encoded by the *Cyp19* gene, is a critical steroidogenic enzyme for the aromatization of androgens into oestrogen. In most teleosts, there are two isoforms of the gene, *Cyp19a* and *Cyp19b*, which encode two structurally different proteins, P450aromA and P450aromB, respectively, with similar catalytic activities. The *Cyp19a* is predominantly expressed in the gonads, while the *Cyp19b* is mainly expressed in the brain, while lower levels of both isoforms are found in both sites and in some other tissues. Here we report for the first time the identification, characterization and expression of *Cyp19a* and *Cyp19b* in tambaqui (*Colossoma macropomum*) during sex differentiation.

Methods

The *de novo* individual transcriptome libraries generated from six headless juvenile tambaqui (3 putative males and 3 putative females) during sex differentiation (from 20 to 33 mm total length) and ovary and testes of immature juveniles (25 and 28 cm standard length, respectively) were assembled using the Trinity pipeline. The Cytochrome P450 aromatases were analyzed and phylogenetically characterized by full gene identification on the *C. macropomum* genome and the Coding Sequence (CDS) was used to deduce the protein.

Results and Discussion

We detected the expression of *Cyp19a* and *Cyp19b* in ovary and testis of immature fish. However, only the *Cyp19b* was expressed in headless juveniles during sex differentiation, with no difference between the putative males and females. The absence of *Cyp19a* transcripts as well as the non-dimorphic (and low) expression of *Cyp19b* seems to be a physiological feature of this phase in tambaqui. Altogether, these data suggest that oestradiol does not have a relevant function at these early stages of sex differentiation. Therefore, these novel findings indicate that the differentiation of ovaries in tambaqui might have another pathway, different from the well-known role of oestrogen in the female differentiation of lower vertebrates.

Conclusion

Taken together, our results indicate that both aromatase isoforms are expressed in tambaqui, but in a life-cycle-specific pattern. During sex differentiation, the equal (and low) expression of *Cyp19b*, in addition to the complete absence of the *Cy19a* in headless juveniles, suggests a lack of oestradiol synthesis in this phase. Further studies are required to discover what is the main force driving the ovary formation in the tambaqui.

POSTER 15

THE INFLUENCE OF WATER PH ON THE SEX DETERMINATION OF TAMBAQUI (*Colossoma macropomum*)

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Introduction

The Amazonian tambaqui (*Colossoma macropomum*) are the most important native fish in Brazilian aquaculture. The species is responsible for more than 38% of the national production of fish. At harvest, females are almost 20% heavier than males, therefore are the most profitable sex to be cultivated in tambaqui farming. In order to provide knowledge supporting the development of new technologies for the tambaqui industry, we aimed to evaluate the influence of pH on the sex determination of tambaqui.

Methods

The experiment was carried out under controlled laboratory conditions. Two independent tests were developed using larvae from different parental background (even different origin). We tested three pH treatments, 6.5 (acid), 7.5 (control) and pH 8.5 (alkaline) in two replicates (n = 350/ polyethylene tank). Tambaqui larvae (12 days after hatching) were maintained in the pH treatments until they were 4 cm standard length (SL), since at this size the gonads are already differentiated (unpublished data). Next, they were transferred to net tanks for growth, until 20 cm SL for sex identification through histology.

Results and Discussion

There was no significant difference between the treatment of pH 8.5 and control (7.5) in both tests ($x^2 = 0$, p 1 and $x^2 = 5.496$, p 0.01 for tests 1 and 2, respectively). On the other hand, more acidic water (pH 6.5) affected the sex ratio, resulting in a larger number of males in both tests ($x^2 = 12.54$, p 0.0003 and $x^2 = 6.87$, p 0.00 for tests 1 and 2, respectively). However, the pH 6,5 significantly increased the fish mortality. The low survival rate strongly suggests that this condition causes stress in tambaqui larvae. Since cortisol is a stress mediator steroid, in addition to being normally involved in the ionic regulation of fish, we assume that the ionic stress may increase the levels of cortisol. The function of cortisol in male formation is well characterized in other species in which environmental features interfere with sex (by inhibition of aromatase, induction of 11-ketotestosteron synthesis and/or causing germ cell apoptosis). Thus, we suspect that the pH effect on tambaqui masculinization is mediated by this steroid.

Conclusion

The pH imbalances the sex ratio in tambaqui. More acidic water leads to more males in the population, whereas neutral and alkaline pH have no influence on the sexual determination of the species. It remains to be elucidated if i) this effect is mediated by cortisol as in other species and ii) if this pH value is relevant in the natural environment, as differentiating larvae survival was very low in this condition. Our data stands out as the first indicating an environmental influence on the sex of tambaqui.

POSTER 16

THE ROLE OF ANTI-MÜLLERIAN HORMONE SIGNALING IN MEDAKA SEX DIFFERENTIATION

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Introduction

Anti-müllerian hormone **(**Amh) signaling is an important effector in the decision whether the bipotential gonad anlage will become a testis or ovary. Studies on the medaka mutant *hotei*, where the *amh* type II receptor (*amhrII*) is mutated, showed an over-proliferation of germ cells and a male-to-female sex reversal. Our hypothesis is that Amh signaling regulates sex differentiation in medaka.

Methods

We produced a knockout line for the *amh* gene of medaka using CRISPR/Cas9 technology. To increase the probability of successful targeting and full knockout, three guide RNAs were designed, in exons 3, 6 and the 3'UTR . Mutants for *amh* was screened by PCR amplifying the mutated target site. To detect and confirm positive mutants, primers were designed which neighbor the target regions to perform the screening and sequencing to check for mutations. To identify sex reversed fish, genotyping of adult fish was done from fin clip DNA with PCR for the *dmy/dmrt1bY* gene. For determining the phenotypic sex we compared secondary sex characters - like dorsal and anal fins of mutants with the phenotype of wild-type fish. To confirm the heritability of mutation as well as to characterize and isolate the mutation, establish stable mutant lines positive G0 fish were crossed to wild-type medaka.

Results and Discussion

Analysis of injected embryos revealed 96,6% efficiency where all three targets were mutated, thereby causing gene disruption. Screening results showed that 82,8% were *amh*+/- and 13,8% *amh*-/- while 3,4% were wild-type. Interestingly, only *amh*-/- underwent sex reversal into male-to-female. All *amh*-/- mutants became sexually mature at 3 months after hatching and showed fertility like wild-type medaka. Crossing of mutants F0 to wild-type was done. In the F1 fish, we retreived one type of mutation in heterozygous animals, one mutation for targets located in exon 3 and one for the 3' UTR region. Interestingly, this mutant has two remarkable phenotypic abnormalities: (1) male-to female sex reversal and (2) gonadal hypertrophy. A to male-to female sex reversal and gonadal hypertrophy was also seen in the *hotei* mutant where *amhrII* is mutated. However, in *Danio rerio*, the mutation of the *amh* gene lead to gonadal hypertrophy like occurs in medaka.

Conclusion

In conclusion, these data confirm a crucial role of Amh signaling during sex determination and differentiation in medaka.

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POSTER 17

STRUCTURAL ASPECTS OF THE FEMALE SEXUAL DIFFERENTIATION OF THE PIRARUCU (*Arapaima gigas*, SCHINZ, 1822)

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Introduction

The pirarucu (*Arapaima gigas*) are the biggest scale Amazonian fish and their farming potential is enormous. However, there are few studies involving the reproductive physiology of pirarucu. Raising fish in confined environments still encounters several adversities, being necessary basic studies for the development of the productive chain, mainly at the early stages of development. Thus, studies related to the gonadal development are essential for the understanding of the reproductive biology of the species.

Methods

To study gonadal differentiation, gonadal tissues from 75 juveniles (from 15 to 52 cm) of *A. gigas* were fixed in glutaraldehyde, dehydrated and embedded in historesin. Sections (5µm) were stained with Toluidin Blue, Metanil Yellow+Periodic Acid Schiff´s+Hematoxylin and Reticulin Method.

Results and Discussion

In *A. gigas*, the gonadal primordium is an elongated paired structure with isolated primordial germ cells (PGCs) scattered among the somatic cells. With the development they become thicker, giving rise to an undifferentiated gonad. However, during the early gonadal stages, the right gonad degenerates and therefore the ovarian differentiation occurs only in the left gonad. In the differentiating ovary, the PGCs differentiate into oogonia. Each oogonium proliferates by mitosis and after enters into meiosis, becoming prophase oocytes. Oogonia and oocytes are surrounded by the pre-follicle cells, forming clusters of the germ cells (germline cysts). At this stage, the pre-follicle cells begin the synthesis of the basement membrane around each cyst, segregating the germinal and stromal compartment. Oocytes initiates primary growth, originating the previtellogenic oocytes, which are gradually and individually involved by the follicle cells. Consequently, each ovarian follicle is completely surrounded by a basement membrane. During this early folliculogenesis, the ovary of pirarucu maintains a compact structure. Then, the ventral side of the ovary suffers invaginations as the ovigerous lamellae develop. Epithelial cells from the gonad periphery present in the invaginations associate with oogonia to form the germinal epithelium. Hence, the newly established germinal epithelium becomes functional. The ovarian tissue develops progressively, increasing the number of previtellogenic oocytes. This pattern of ovarian differentiation seems to be maintained among more basal animals, as already observed in sturgeons and Ostariophysi.

Conclusion: In the pirarucu, the somatic cells, derived from the coelomic epithelium, appear to be involved in the process of germ cells reorganization during gonadal differentiation. Especially when the germ cells of undifferentiated gonads begin to form continuous cords and establish new relationships during the development of both the germinal epithelium and stroma.

POSTER 18

GREEN LED LIGHT IRRADIATION DURING SEX DIFFERENTIATION INDUCED FUNCTIONAL SEX REVERSAL IN MEDAKA *Oryzias latipes*

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Introduction

Light is one of the important environmental factors in aquatic habitats. It has been reported that the color of light can modulate the physiological states of fish. For example, green light promoted growth performance in barfin flounder. In some fish species, environmental factors such as water temperature, pH and rearing density affect sex differentiation. However, the effects of specific light wavelengths on gonadal sex differentiation have benn little known. This study investigated if the specific wavelength light can affect the sex differentiation.

Methods

Medaka Oryzias latipes is a small model fish and their sex determination system is male heterogametic (XX/XY). A sex determining gene named dmy has been identified on the Y chromosome. HdrR-II1 (strain ID: IB178), an inbred strain of medaka provided by NBRP Medaka, shows a different body color between males (scarlet) and females (white) because their pigment gene is located on a sex chromosome. Therefore, body color represents the genetic sex in this species. Medaka fish were reared in fish tanks (31.5×18.5×24.4 cm) equipped with white and green (518 nm) LEDs. There was no significant difference in the intensity (μmol m-2 S-1) of light between the two colors. The photoperiod of the rearing tank was 14 hours light and 10 hours dark. The water temperate in each rearing tank was maintained at 26 ± 1°C. The genetic sex was analyzed by genomic DNA PCR and fish body color. The phenotypic sex was analyzed by gonadal histology and the shape of the anal and dorsal fins. Artificial insemination was conducted between the spermatozoa of sex-reversed males and unfertilized eggs of normal females.

Results and Discussion

Female to male sex reversal was observed under green LED treatment in 3-month-old fish. The appearance rate of neo males (white body color, dmy (-/-), male-specific fin type, and testis) was 22.8%. No sex reversal was observed under white LED treatment. Motile spermatozoa were obtained from neo males. The dmy gene was not detected in the DNA of neo male sperm. Moreover, progeny tests revealed that F1 offspring were all females.

Conclusion

Green LED light irradiation during the sex differentiation period induced female to male sex reversal in genetic females with the neo males producing functional spermatozoa, suggesting that specific light wavelength irradiation could be a trigger for sex reversal in some fish.

STEM CELLS AND SPERMATOGENESIS

POSTER 19

EFFECTS OF IMMUNOSUPRESSANTS ON THE IMMUNE SYSTEM OF RAINBOW TROUT (*Oncorhynchus mykiss*) AND THEIR APLICATION IN ALLOGRAFT TRANSPLANTATION

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Introduction

Germ cell transplantation and testis graft are useful reproduction tools. However, the host immune system may restrict the application of those techniques in allogeneic transplants. Therefore, in this work we aimed to test immunosuppressants-containing emulsions to verify whether they are capable to prevent immune rejection and, thus, promote testis allograft survival in rainbow trout.

Methods

To evaluate the effects of two immunosuppressants in rainbow trout, leucocytes from peripheral blood were cultured and treated with tacrolimus and cyclosporine for the analysis of proliferation/survival in the presence of these drugs. Then, juveniles were treated with a single application of emulsions containing two doses of tacrolimus and two of cyclosporine each. Samples of head kidney were analyzed for assessing the immunosuppressive effects by quantification of immune-related genes (qRT-PCR). In a third experiment, testis fragments were grafted subcutaneously in each side of dorsal region of the donor itself (autograft) and also in other one-year old trouts (allografts). Two groups of allografted animals were treated weekly with tacrolimus-containing emulsions, whereas the remaining group received only emulsions (control group). Samples of head kidney and grafts of each group were collected for mRNA expression and histological analysis.

Result and Discussion

In *in vitro* experiments, tacrolimus and cyclosporine were able to inhibit leucocyte proliferation even under mitotic stimulation. In *in vivo* experiments, two dosages of tacrolimus and a lower of cyclosporine inhibited significantly the expression of *il2* three days post-injection. A higher dosage of cyclosporine was able to inhibit *il2* expression for up to seven days post-injection. In experiments with testis allograft, histological (H.E. staining) and RT-PCR (*vasa* and *txdnc6*) analysis demonstrated the presence of spermatogonial cells in the first week and indicated the presence of spermatids/spermatocytes in the fifth week, respectively, in animals treated with lowest dosage of tacrolimus. In the group treated with the highest dosage and in the control group (without immunosuppressant), no germ cells or their respective markers were detected.

Conclusion

These results suggest that tacrolimus comprises a promising immunosuppressant, with potential applications to testis allografts or germ cell transplantation. Co-administration combining tacrolimus with other immunosuppressive drugs can be evaluated for inhibiting more pathways of the immune system in order to optimize the immunosuppressive effects in host organisms.

POSTER 20

FSH-STIMULATED IGF3 RELEASE ACTIVATES CANONICAL WNT SIGNALING TO PROMOTE SPERMATOGONIAL DIFFERENTIATION IN ZEBRAFISH TESTIS.

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Introduction

Follicle-stimulating hormone (Fsh) is a major regulator of vertebrate spermatogenesis, which targets somatic cell functions in the testis. Previous studies showed that zebrafish (*Danio rerio*) Fsh promoted the differentiation of type A undifferentiated (A_{und}) spermatogonia by stimulating the production of factors advancing germ cell differentiation, such as androgens, insulin-like peptide 3 (Insl3) and insulin-like growth factor 3 (Igf3). In addition, Fsh modulated the transcript levels of several genes belonging to different signaling pathways, including Wnt signaling. Here, we evaluated if and how Fsh makes use of the canonical/β-catenin Wnt signaling to regulate the developmental fate of spermatogonia.

Methods

We examined Sertoli and germ cell proliferation activity by quantifying BrdU incorporation with immunocytochemistry (ICC). The relative section areas occupied by type A_{und} and type A differentiating (A_{diff}) spermatogonia were quantified. Gfp was detected by ICC in testis tissue of transgenic fish. The transcript levels of selected genes in response to recombinant proteins and pharmacological inhibitors were quantified by RT-qPCR in testis tissue. All analyses were applied to testis samples from adult males after primary tissue culture.

Results and Discussion

From the three downstream mediators of Fsh activity that we tested only Igf3, but not 11-ketotestosterone (11-KT) or Insl3, modulated the transcript levels of *cyclinD1* and *axin2*, two β-catenin target genes. Using transgenic zebrafish, in which Gfp expression is controlled by a promoter sensitive to β-catenin, we found that Igf3 activated canonical Wnt signaling in type A spermatogonia. Interestingly, this activation was independent of the release of Wnt-ligands. Pharmacological inhibition of the canonical Wnt or of the phosphoinositide 3-kinase (PI3K) pathway revealed that Igf3 requires β-catenin signaling to promote A_{und} differentiation in a manner involving the PI3K pathway.

Conclusion

Fsh-stimulated Igf3 release activates canonical Wnt/β-catenin signaling in type A spermatogonia via the PI3K pathway, thereby promoting the differentiating proliferation of spermatogonia. This mechanism represents a so far unique example of a pituitary hormone using an evolutionary conserved, local signaling system to regulate cellular differentiation processes in a target tissue.

POSTER 21

VGLL3 AND THE HIPPO PATHWAY ARE REGULATED IN SERTOLI CELLS UPON ENTRY AND DURING PUBERTY IN ATLANTIC SALMON TESTIS.

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Introduction

Understanding the mechanisms that regulate sexual maturition in Atlantic salmon is important. Precocious maturity is a problem causing reduced size, quality and welfare of farmed salmon. Genome-wide association studies demonstrated a strong correlation between polymorphisms in Vestigial-like family member 3 (*vgll3*) and age at maturity in Atlantic salmon (*Salmo salar*). However, molecular mechanisms and roles of *vgll3* in controlling the timing of puberty have remained uncharacterized, as well as relevant tissue and cell types. *Vgll3* is associated with the Hippo signaling pathway, which acts as a regulator of cell proliferation in many tissues. The purpose of this study was to identify the relevant tissue and cell types where *vgll3* is expressed, as well as to determine the timing of the expression. With his information, we aim at improving our insight into the function of *vgll3* in regulating the onset of puberty.

Methods

Gene expression of *vgll3* and Hippo pathway members were analyzed by RNA-sequencing and qPCR in gonads from different pubertal stages in male and female fish, including immature, during and after puberty. Pubertal stages were determined by analyzing GSI, sex steroids and histological tissue sections; proliferation activity in the gonads was assayed using an immunocytochemical approach. To identify the cell types expressing *vgll3*, an *in situ* hybridization analysis was performed on male and female gonad tissue sections.

Results and Discussion

Gene expression analyses showed that *vgll3* and several members of the Hippo pathway were down-regulated during early puberty and remained repressed during the testis growth phase, showing an inverse relationship with the proliferation activity. However, we did not observe a similar relationship in ovarian tissue. Using *in situ* hybridization, we found that *vgll3* is expressed in Sertoli and granulosa cells in testis and ovary, respectively.

Conclusion : Our results show for the first time that *vgll3* and several members of the Hippo pathway are down-regulated during the onset puberty in Atlantic salmon testis and remain suppressed until completion of the seasonal testicular growth. The Hippo pathway has been linked to negative regulation of organ size through limiting cell proliferation. We therefore hypothesize that *vgll3*, together with the Hippo pathway, negatively regulates Sertoli cell proliferation, and thus may act as an inhibitor of testis maturation.

POSTER 22

GENE EXPRESSION PROFILING DURING RAINBOW TROUT ONTOGENESIS REVEALS SUCCESSIVE CHANGES GATED IN TELEOSTS IN GERM STEM CELL MOLECULAR SIGNATURE AND REGULATORY PATHWAYS.

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Introduction: The spermatogonial stem cells (SSCs) are adult male germ stem cells and they represent a narrowed population of undifferentiated spermatogonia. SSC have the ability to continuously self-renew and/or to differentiate ultimately into spermatozoa throughout adult male lifespan. In mammals, there is evidence that SSCs derived originally from primordial germ cells (PGCs) and both cell types are submitted to different micr o-environments formed by somatic "germinal niches" that regulate their survival, migratory properties, self-renewal and/or differentiation. However, regulatory pathways that may be functional in the successive germinal niches remain poorly investigated in Teleosts. Our study was aimed to investigate potential changes in regulatory pathways that may regulate germ stem cell fate during late rainbow trout ontogenesis. The kitlg/kitr and Glial cell line-derived neurotrophic factor (gdnf)/ glial cell line-derived neurotrophic factor receptor alpha 1(gfra1) systems are two major regulatory pathways involved in mammalian PGCs and SSCs proliferation in embryo and adult male, respectively.

Methods: Phylogenetic reconstructions and analysis of syntenic chromosomal fragments were carried out from the genome of different vertebrate species to investigate the evolutionary conservation of the candidate genes and to establish reliable orthologous relationships.The temporal expression profiles of the candidate genes were determined in trout testes from 55 to 180 days post fertilization.

Results and Discussion: All candidate genes were conserved in cartilaginous and bony fishes but the latter showed taxon-specific gene duplications and losses. For instance, the teleost-specific *gfra1a* gene copy was further duplicated in Rainbow trout and Atlantic salmon whereas *gfra1b* gene copy was not found in both genomes and in mRNA expression databases. A different evolutive history was observed with kitr (duplication of the kitra gene copy whereas a single kitrb gene copy was retained in salmoninae). Temporal expression profiles showed high expression levels of paralogous kitr genes (*kitlgb*, *kitrb* and *kitra2*) at 55 days followed by a sharp decreased expression suggesting a role in germ stem cell fate before spermatogenesis onset. Interestingly, the *gfra1a1*, *gdnfa1* and *gdnfb* genes were barely expressed before spermatogenesis onset. Genes involved in the *gdnf/gfra1* regulatory pathways were highly expressed after 90 -120 days corresponding to spermatogenesis onset and the apparition of first progenitors. Gene expression profiling of additional germ cell specific genes indicate that the molecular signature of undifferentiated spermatogonia change during ontogenesis.

Conclusion Our study demonstrates that the germ stem cell entities and germinal environment change drastically during fish ontogenesis, well before and after spermatogenesis onset.

POSTER 23

ARGININE INCREASE THE NITRIC OXIDE PRODUCTION IN FISH TESTIS

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Introduction

Arginine has been studied in the nutrition of mammal breeders, but this is a pioneering study for fish. The role of arginine as a functional amino acid that acts on the necessary metabolic pathways for the maintenance, growth, reproduction and immunity of vertebrates. Arginine, besides being a constituent of proteins, is also involved in the synthesis of polyamines and proline, as substrate for collagen synthesis and nitric oxide, considered a key component of the seminal plasma and spermatozoa.

Methods

Three *Rhamdia quelen* males in sexual recrudescence were used. They were euthanized and had their testes dissected out. The experiment was conducted in two phases: 18 hours (assessment of NO production and percentage of spermatogenic cysts) and seven days (cell proliferation). The testes were fragmented, weighed and placed on a nitrocellulose membrane on top of an agar block. Testis fragments were then incubated in medium with the addition of arginine (5 and 10 mM) or not, 12 replications of each animal were used for each concentration of arginine and time of culture. In order to evaluate cell proliferation, BrdU was added to the medium. After the period of culture, the gonads were fixed in methacarn, included in historesin, sectioned and submitted to BrdU immunodetection. The 18-hour culture medium was frozen for further measurement of NO concentration. The percentage of spermatogenic cysts was obtained by the total count of cysts. The index of BrdU incorporation was determined by counting the number of positive BrdU cells. The concentration of nitric oxide was determined in the medium, using Griess method. Absorbance was determined at 550 nm using a Biotek microplate reader.

Results and discussion

In vitro studies showed a dose-response effect of arginine on the testicular production and release of nitric oxide after 18 hours of culture. Moreover, when investigating the testicular composition, arginine (5mM, 10mM) did not change the frequency of the germ cell cysts after 18 hours of in vitro exposure. Interestingly, cell proliferation was not affected either after 7 days of culture with different concentrations of arginine.

Conclusion

Our studies showed that arginine stimulated NO production in a dose-response manner. This result is an direct evidence that *R. quelen* is able to produce NO, confirming that this compound might have a role on reproduction, and that arginine present relation with this production.

POSTER 24

SEXUAL SPECTRUM OF GERMLINE STEM CELLS IN FISH.

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Introduction

Sex steroids and their receptors are known to harmoniously maintain the reproductive/gonadal physiology. The gonad is a mix of both germ cells and somatic cells. Studies show that, not only the genetic sex, but the environmental changes at any stages of development can influence the germ-somatic cells interaction, thus affecting the gonadal sexuality. Notably, germ cells are considered to be the important building block for reproduction, and any changes in germ cell number or its sex can significantly impact the overall species existence. Several stages of germ cell exists in vertebrates, i.e. primordial germ cell, germline stem cell (GSC), oocytes, etc. Therefore, in this study, we have focused on critically examining the role and effects of each stages of germ cell on the sexual plasticity or sexual spectrum of fish.

Methods

ERβ2 knockout/knockdown medaka lines were generated using previously published protocols. The GSC isolation, maintenance and transplantation were carried out using unique and user-friendly methodology. The methylation and other global analysis were performed using specific kits. Concentrations of steroids and chemicals were predetermined using pilot experiment.

Results and Discussion

In our study, we focused on two major aspects, i.e., when the sex steroids are scarce (early sex differentiation) and when they are plentiful (maturation), in order to, respectively, clarify the effect of GSC on early and maturing/matured gonadal sexuality. Firstly, we elucidated the significance of Estrogen receptor (ER) β2 in germ cell proliferation and sex differentiation in medaka. *ERβ2* knockdown caused germ cell mis-migration and loss due to *SDF1a*/*CXCR4b* chemotaxis alterations. We also found that, calcium ion signalling was critical for estrogen/ER responsive early germ cell maintenance. However, we failed to see any direct effect of *ERβ2* on adult GSC maintenance. Interestingly, we observed strong correlation between estrogen and Oct4 (undifferentiated stem cell marker) positive early gonia proliferation in both sexes. Further analysis confirmed that, Oct4 expressed in the GSCs of both sexes and early oocytes in females. Additionally, we found that the degree of sexual plasticity is linked to the GSC number and Oct4 nuclear localization. We isolated these GSC clusters from both sexes, and validated their stemness *in vitro* and *in vivo*. *In depth* analysis confirmed the DNMT-Oct4-retinoic acid interconnected gonadal stemness regulation, and the differential role of Oct4 in both GSCs and oocytes.

Conclusion

Our study suggests that the sexual spectrum mostly depends on the GSCs, their rejuvenating potential and methylation pattern. It is a step forward towards understanding the sex related disorders and their remedies.

POSTER 25

EFFECT OF GROWTH FACTORS AND GONADAL SOMATIC CELLS ON GERM CELL PROLIFERATION IN STURGEON

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Introduction

Nowadays over 85% of sturgeon species are classified as at risk of extinction. Germ cell transplantation could be an available and rapid method for endangered fishes with large bodies and long life history. To expand sturgeon germ cell populations and sustain the supply for long periods for transplantation, we tried to expand sturgeon germ cell populations *in vitro*. We investigated the effect of gonadal somatic cell on germ cell growth, and then assessed the influence of glial-cell-derived neurotrophic factor (GDNF) and leukemia inhibitory factor (LIF) on germ cell proliferation.

Methods

Purified germ cells were obtained by density gradient centrifugation using Ficoll-Paque Plus® and cultured with/without feeders respectively. The feeder cells, derived from gonad somatic cells of sturgeon, were treated with mitomycin C. Germ cell proliferation was performed by BrdU and Vasa immunocytochemical assays after 7, 14 and 21 d culture. 25 μg/ml LIF and 25μg/ml GDNF were added into the culture medium. Germ cell mitotic activity was checked after 10 d culture. Besides, we investigated the presence of the *vasa*, *dead end*, *nanos1*, *gfra1a*, *grip2* gene transcripts in cultivated cells by quantitative real-time PCR. Finally, the larvae of Russian sturgeon 2 weeks after hatching with FITC labeled endogenous PGCs were used as recipients. 40d cultured cells from sterlet labeled with PKH26 were injected near the presumptive genital ridge of the host.

Results and Discussion: It revealed significant variation ($P<0.001$) in the proliferation of the germ cells in both cultural day and presence/absence of feeders. The interaction between day and feeder was also significant ($P<0.001$). Highest germ cell proliferation was performed when they were cultured without feeder. Whatever feeder cells present or not, BrdU incorporation showed that germ cell propagation was highest after 14 d culture and decreased thereafter. According to the q-PCR analysis of some germ-line specific genes, there was no significant difference of marker gene expressions before and after 10d culture with growth factors ($P>0.05$), confirmed that cultured cells may remain similar condition as those initial fresh germ cells. Cultivated cells without growth factors showed significant difference compared with germ cells before culture($P<0.05$), indicting the conditions of those cells have been changed. Besides, after 40 days posttransplantation, some cultured germ cells were incorporated and developed in the recipient gonads.

Conclusion: Proliferation of germ cells was significantly promoted and maintained for long periods by elimination of gonad somatic cells. LIF and GDNF may help germ cell prolong their proliferation fate.

POSTER 26

REGENERATION OF ZEBRAFISH LINES BY TESTIS CRYOPRESERVATION AND SPERMATOGONIA TRANSPLANTATION

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Introduction

Several thousands of transgenic zebrafish (*Danio rerio*) lines have recently been created by molecular biology tools, with their number increasing each year. This trend is leading to problems regarding storage and maintenance of these transgenic lines. Cryopreservation offers a valuable potential in overcoming storage problems, however the method is hindered by the inability of successful offspring production from cryopreserved eggs.

Methods

Slow-rate freezing of spermatogonia was optimized by testing the effects of various cryoprotectants, their concentrations as well as sugar and protein supplementation on spermatogonia viability in four consecutive experiments. Vitrification was optimized by testing three different equilibration solutions (ES1 – ES3) and three different vitrification solutions (VS1 – VS3) containing various concentrations of dimethyl sulfoxide (Me_2SO), methanol (MeOH) and propylene glycol (PG). The repeatability of the developed protocols was tested on five different zebrafish lines. Fresh, slow-rate frozen and vitrified spermatogonia of *vasa:gfp* zebrafish were then transplanted into the peritoneal cavity of wild type larvae to test the incorporation and proliferation capacity of the frozen spermatogonia. Lastly, to produce offspring, wild type embryos were sterilized by anti *dnd*-moprohlino microinjection, and fresh, slow-rate frozen or vitrified spermatogonia of *β-actin* zebrafish were transplanted into the sterilized recipients.

Results and Discussion

The viability of testicular germ cells slow-frozen with the addition of 1.3 M Me_2SO in the cryomedium was significantly higher than the viability of those frozen with other tested cryoprotectants and Me_2SO concentrations. Different sugar and protein supplementation did not significantly affect the viability of spermatogonia. During vitrification, only the vitrification solutions had a significant effect on the testicular germ cell viability after warming. The highest viability was obtained when combining the VS3 containing equal concentrations of PG and Me_2SO (3 M of both) and either ES1 or ES3. Both slow-rate freezing and vitrification protocols demonstrated favorable repeatability since they yielded viability rates of nearly (or higher than) 50%. Vitrification demonstrated an advantage over the slow-rate freezing protocol since we observed considerably less spermatozoa in the cell suspensions following warming. Observation of dissected recipients 50 days post-transplantation revealed that GFP-labeled germ cells were present in the recipient gonads and the number of recipients containing incorporated donor-derived spermatogonia did not differ among the groups (25 – 30%). Furthermore, viable offspring displaying fluorescent signal was produced indicating the possibility of surrogate-production by transplantation of cryopreserved spermatogonia.

Conclusion

Both slow-rate freezing and vitrification can be used for storage of zebrafish spermatogonia. Both cryopreservation methods yield viability rates of above 50%. Cryopreserved spermatogonia have normal physiological activity and may be used to regenerate transgenic zebrafish lines.

POSTER 27

TARGETING *dnd1* IN STERLETS (*Acipenser ruthenus*) BY CRISPR/Cas9 GENERATES PHENOTYPIC ABNORMALITIES

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Introduction

Sturgeons are commonly known as living fossils that are famous for the caviar. Recently, these archaic giants are facing certain threats for survival due to overfishing and interference in the natural habitats, which in-turn make them "more critically endangered than any other group of fishes". Sterlet (*Acipenser ruthenus*) is a common sturgeon species with the fastest reproductive cycle among sturgeons; therefore, this species have potential to be used as host for surrogate production for the huge and the IUCN red-listed sturgeon species. Ablation of germ cells or germ cell free host is required as prior condition for surrogate production. In order to achieve this, knockout and/or knockdown of dead-end gene (*dnd1*) should be done to disrupt migration and survival of primordial germ cells (PGCs). Previously we have successfully used antisense morpholino oligonucleotide (MO) in sterlets to knockdown *dnd1*; however, due to cost-intensiveness and toxic to cells, we decided to use CRISPR/Cas9, which presents advantages over other genome editing technologies.

Methods

CRISPR consists of two important components *i.e.*, guide RNA (gRNA) and CRISPR-associated endonuclease (Cas9). *Acipenser ruthenus dnd1* (*Ardnd1)* mRNA sequence was used to select target sites and oligonucleotides were annealed and cloned into px330 plasmid. Purified plasmid was diluted with KCL prior injection into eggs at 1-2 cell stage. PGCs are generated in vegetal pole (VP) of sturgeon eggs; thus, we injected fluorescein isothiocyanate (FITC)-biotin-dextran into VP to label them as marker for *dnd1* knockout; whereas purified-diluted plasmid was injected into animal pole (AP). To validate injection method and PGCs labelling, we injected only FITC in control group. In another attempt, we also injected sgRNA and Cas9 into AP of sterlet eggs.

Results and Discussion

PGCs visualization was done at 21 days post fertilization (dpf) under the fluorescent stereomicroscope, and significant difference was recorded in number of PGCs in px330 plasmid injected embryos and control group ($P<0.05$). Intriguingly, px330 plasmid injected embryos exhibited strange abnormal developmental patterns and ultimate mortality. To authenticate whether this was due to plasmid toxicity, we injected un-ligated plasmid into AP and VP of eggs, and no abnormal developmental pattern was recorded in any injected embryo. To compare CRISPR/Cas9 methods, we also injected sgRNA and Cas9 protein into sterlet eggs from two different females and similar results were obtained.

Conclusion: From our present data in sturgeons, we presume that knockout of *dnd1* could have a strong correlation with other important genes contributing in somatic development of embryos. However, further studies are suggested to determine biological mechanisms behind correlation of *Ardnd1* with other genes.

POSTER 28

ALTERNATIVE SPLICING OF *DEAD END GENE*

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Introduction

The Amazon species *Colossoma macropomum* is one of the highly exploited species in Brazil due the appreciated flavor and rapid growth. However, declining of its natural resources due to overexploitation and the climate change is concerned. Although germ cell cryopreservation and transplantation can be a silver bullet to preserve their genetic resources semi-permanently, their germ cell behavior has not been analyzed to date. Therefore, in this study, we isolated tambaqui *dead end* homolog (*Tdnd*) as a molecular marker for germ cells and undifferentiated mitotic germ cells, in order to obtain basic information essential for germ cell transplantation.

Methods

The total RNA was extracted from the gonads of immature and mature tambaqui. RT-PCR was performed firstly with degenerate primers that were designed using the highly conserved regions of *dnd* gene homologs from various fish species. Amplified cDNA fragments were sequenced and 5' and 3' rapid amplification of cDNA ends (RACE) was performed. The whole gonads of tambaqui were fixed using Bouin's solution and used for in situ hybridization with *dnd* probe. To study the alternative splicing of *Tdnd*, the genomic DNA was extracted, amplified by PCR, sequenced and compared to the sequences obtained by cDNA sequencing. To compare the expression profiles of each isoform at different ages of tambaqui, qPCR was performed by $\Delta\Delta$Ct method with β-actin as an internal control.

Results and Discussion

The cloned cDNA had an open reading frame of 1194 bp and the amino acid sequence was inferred to encode 398 amino acid residues. The sequence alignment with other species indicates that the *dnd* gene is highly conserved through the process of vertebrate evolution and the phylogenetic analysis revealed that *Tdnd* sequence belongs to the *dnd* family. RT-PCR products showed that *Tdnd* was detectable only in testes and ovaries, and three transcripts of *Tdnd* were found as a result of the alternative splicing. In situ hybridization showed strong positive signal in oocytes and weakly detected in some spermatogonia. The qPCR showed that the three transcripts presented distinctive profiles of expression, although the relative quantification has shown a wave behavior. While the dnd1 showed higher expression in mature fishes, the relative expression of the variants dnd2 and dnd3 oscillates depending on the fish age.

Conclusion

We successfully isolated tambaqui homolog of *dnd* and confirmed that it can be used as a potential germ cell marker. Further study to determine the function of different transcripts of *dnd* is currently ongoing.

POSTER 29

FUNCTIONAL ACTIVITY OF RECOMBINANT FORMS OF ANTIMÜLLERIAN HORMONE IN EUROPEAN SEA BASS GONADS

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Introduction

In higher vertebrates, anti-Müllerian hormone (AMH), signaling through the type 2 AMH receptor, is required for involution of the Müllerian ducts during male sexual differentiation and for negatively regulating gonadal development in both sexes. Amh and AmhR2 have been described in fish, but the mechanism of Amh signaling and its involvement in gonad development needs further investigation. In European sea bass, a recent study showed higher expression levels of *amh* and *amhr2* during early and final stages of spermatogenesis, while in ovary ligand and receptor showed opposite profiles. As tool for studying the mechanisms of Amh action we aimed at producing specific recombinant mature forms of this hormone

Methods

Sea bass *amh* cDNA was cloned in frame with the α-factor signal sequence into the pPIC9K vector (Invitrogen). The RATR-motif was changed to an EKR site for cleavage of the pro-protein by *Pichia pastoris* Kex2 protease. A six-His tag was introduced before (His-Amh) or after (Amh-His) the mature protein to facilitate purification.
Functionality of the recombinant Amh forms was tested in COS-7 cells co-transfected with expression vectors containing sea bass *amhr2* and the BRE-luc reporter.
Fragments of sea bass testis and ovary were cultured *in vitro* and treated with recombinant Fsh and/or different doses of Amh. Steroid release was analyzed by specific EIAs. Changes in gene expression were analyzed by RT-qPCR

Results and Discussion

Two different his-tagged recombinant sea bass Amh were produced in the *P. pastoris* system and secreted as mature proteins after endogenous processing by Kex2. Both purified recombinant Amh proteins are able to activate the sea bass Amhr2 receptor as recorded by an increase in BMP-responsive element driven luciferase activity.
In testis, Amh was detected in Sertoli cells surrounding spermatogonia; while in ovary it was found in follicular cells surrounding early and late vitellogenic oocytes, but not in pre-vitellogenic oocytes.
In vitro cultured immature sea bass testis produced testosterone and 11-ketotestosterone when treated with recombinant Amh, and increased the expression of several genes coding for steroidogenic enzymes. In early vitellogenic ovaries, stimulation with sea bass Fsh and increasing doses of Amh resulted in an increase of estradiol release and *cyp19a1a* expression.

Conclusion

New functional recombinant forms of sea bass Amh have been produced that activate the AmhR2 receptor and induce steroid production in immature testis and early vitellogenic ovaries showing a clear involvement of sea bass Amh in gametogenesis.
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POSTER 30

AUTOMATIC DETECTION OF SPERMATOCYTES FROM HISTOLOGY DIGITAL IMAGES OF HYBRID FISH FOR SEXUAL MATURATION QUANTIFICATION

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Introduction

The introduction of new species, e.g. fish, into an environmental system can have fatal consequences for that environment, such as deforestation or mass destruction of native species. In order to avoid this, it has been determined that hybridization is one of the most appropriate methods to keep the system stable and increase aquaculture production. However, this has consequences, the animals procreated by hybridization method tend to lose genetic information (introgression / erosion) which is vital for the reproduction process. Taking into account the above, it is necessary to perform a quantitative histological analysis of the morphological characteristics of spermatocytes at different time points in the fish population in order to determine the degree of sexual maturation for the reproduction process of them.

Methods

The aim was to develop an automatic computer method for spermatocytes detection from histology images at 40x. First, a color histogram normalization is applied to improve the image quality in order to highlight image contrast and the differences of staining. Then, color deconvolution process is applied to split the stain components inside the images of hematoxylin and eosin (H&E). Using the hematoxylin color channel, because it highlights the nuclei, a segmentation process is performed using Otsu's method to obtain the binary mask of nuclei regions. Finally, a labeling process to separate each nuclei was done followed by a morphological feature extraction (area, perimeter, circularity).

Results and Discussion

Our preliminary results show that our automatic method improves the H&E images quality for analysis. The automatic method for spermatocyte detection has a good performance achieving a Dice coefficient of 91% in comparison with the ground truth binary masks of nuclei manually annotated by experts.

Conclusion

The developed automatic computational method is able to detect and segment spermatocytes into histology images. This fundamental step is important to extract morphological features of these cells for sexual maturation estimation.

POSTER 31

EFFECTS OF METHIMAZOLE-INDUCED HYPOTHYROIDISM IN ZEBRAFISH SPERMATOGENESIS

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Introduction : Many important physiological functions of vertebrates are controlled by thyroid hormones, such as metabolism, growth, behavior and reproduction. More recently, it has become evident the crossover of the hypothalamic pituitary-thyroid (HPT) and gonadal axes in vertebrates, especially in fish. In zebrafish, T3 stimulated proliferation of type A undifferentiated spermatogonia (A_{und*}) through Sertoli cell production of Igf3 (Insulin-like growth factor 3). Moreover, T3 potentialized the Fsh-induced androgen release by zebrafish testicular explants. To unravel the cross-talk between the HPT and testis, the current study aimed to investigate the effects of methimazole, an antithyroid drug, in zebrafish spermatogenesis.

Methods : Thirty adult male were chemically exposed to methimazole (1mM) for 21 days, and as a control, ten fish were kept in water without treatment. After the exposure period, testes (n = 10) were collected, fixed, included in historesin, sectioned and stained with toluidine blue. Sections were submitted to morphometric evaluation to quantify the frequency of germ cell cysts per area. Blood samples were collected to measure 11-Ketotestosterone (11-KT) levels in the plasma of chemically-induced and control males. 11-KT release capacity of zebrafish testicular explants were evaluated after 18h *ex vivo* culture for both groups. For this purpose, testes were incubated either with 100ng/mL Fsh or 100ng/mL Fsh+65ng/mL T3, and their contralateral ones with basal or 100ng/mL Fsh, respectively.

Results and Discussion: The results showed a significant reduction of A_{und*} in chemically-induced testes, suggesting that absence of T3 may affect the renewal of A_{und*}. Moreover, the frequency of spermatid cysts was also decreased in methimazole-treated males, which may reflect the low amount of spermatozoa formed at the end of the spermatogenic process in the testicular lumen of these males. Interestingly, the amount of apoptosis in the germ cell cysts, especially among spermatocytes, increased significantly when compared to control. 11-KT plasma levels were reduced in hypothyroidism-induced males, suggesting that methimazole has impaired the hypothalamic-pituitary-gonadal axis affecting androgen production and release. In this context, more studies are needed to evaluate whether the effects were attributed to lower levels of androgens or T3. With respect to the steroidogenic capacity, our results showed that Fsh did not stimulate androgen release by methimazole-induced testes, as observed in control. Interestingly, the lower androgen capacity of chemically-induced testes was not nullified when T3 was added with Fsh.

Conclusion: Our results showed that zebrafish spermatogenesis is impaired in methimazole-induced treatment. The observed effects may be directly or indirectly attributed to the absence of thyroid hormones. More studies are needed to unravel the cross-talk between the HPT and testis using this interesting model. Financial support: FAPESP (2017/15793-7 and 2014/07620-7).

POSTER 32

CORTISOL STIMULATES ZEBRAFISH SPERMATOGENESIS BY MODULATING SERTOLI AND LEYDIG CELL FUNCTIONS IN AN ANDROGEN-INDEPENDENT MANNER

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Introduction

The inhibitory role of cortisol on fish reproduction has been demonstrated systematically through *in vivo* experiments by incorporating the hormone into the diet or by implanting cortisol-releasing pellets in the body cavity, as shown in carp, salmon and rainbow trout. Nevertheless, the direct effects of cortisol in the testis remain poorly investigated. The aim of this work is to study the *in vitro* effects of cortisol in zebrafish testis using a well-established organ culture system described for this species.

Methods

We evaluated the germ cell proliferation activity by quantifying BrdU incorporation among types A undifferentiated (A_{und}), A differentiated (A_{diff}) and B spermatogonia. The transcript levels of selected testicular genes were quantified after 18h and 7 days of incubation in response to different levels of cortisol by qPCR. In addition, the androgen release (11-Ketotestosterone, 11-KT) in the culture medium was evaluated in the 18h incubation using an enzyme-linked immunosorbent assay (ELISA). *In situ* hybridization (ISH) was used to localize glucocorticoid receptors (*grα* and *grβ*) mRNAs in testicular tissue.

Results and Discussion

Cortisol at different concentrations (from 0.1 ng/mL to 1000 ng/mL) was unable to modulate androgen release by zebrafish testicular explants. Lower and higher concentrations of cortisol induced changes on gene expression for androgen and glucocorticoid receptors. Further an intermediate concentration of cortisol (100 ng/mL) increased the mitotic index of A_{und}, A_{diff} and B spermatogonia in a long-term zebrafish testis culture. Gene expression analysis showed elevated transcript levels of *nanog*, a marker of undifferentiated spermatogonia, and also increased mRNA levels for meiotic and post-meiotic genes. Glucocorticoid receptors were found in Sertoli and Leydig cells.

Conclusion

Cortisol has a direct effect in zebrafish spermatogenesis by increasing the number of type A and B of spermatogonia through Sertoli and Leydig cell functions, in an androgen-independent manner.

Financial support: FAPESP (2014/16280-5 and 2014/07620-7).

POSTER 33

COOPERATIVE PROLIFERATION BETWEEN SERTOLI AND GERM CELLS IN ZEBRAFISH TESTIS

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Introduction

Interactions between spermatogonial stem cell (SSC) and the surrounding somatic Sertoli cells are essential for the maintenance of the spermatogenic process. In the cystic spermatogenesis, SSCs are surrounded by a given number of Sertoli cells. When a SSC divides, the derived daughter cells have the potential either to remain stem cells or differentiate into committed germ cells. Likewise, it is suggested that cystic surrounding Sertoli cells may accommodate the newly formed germ cells by creating either new cysts or increasing the pre-existing cyst. This study aimed to evaluate the cooperative proliferation between Sertoli and germ cells mediated by endocrine and paracrine signals in the zebrafish testis.

Methods

Sertoli and germ cell proliferation were assessed by quantifying the BrdU incorporation of these cells in zebrafish testes cultivated in the presence of recombinant zebrafish Fsh (rzf Fsh) and recombinant human GDNF (rh GDNF) for 7 days. In addition, RT-qPCR analysis were carried out to evaluate the expression of selected testicular genes in the above treatments. *In situ* hybridization was used to detect stem cell transcripts in zebrafish testes.

Results and Discussion

First, we characterized stem cell transcripts, such as *pou5f3* and *nanog* in spermatogonia belonging to cysts of 1, 2 or 4 cells. rzf Fsh and rh GDNF increased the mitotic index and cyst proportion of type A undifferentiated spermatogonia (A_{und}). However, only GDNF increased proportion of type A differentiated spermatogonia (A_{diff}). Interestingly, Fsh and GDNF stimulated Sertoli cell proliferation in association with BrdU-positive A_{und}. Increased Sertoli cell proliferation associated with A_{diff} was seen only in GDNF treatment. Gene expression analysis showed elevated transcript levels of *igf3*, *nanog* and *nanos3* while *pou5f3* and *gfrα1a* were down-regulated by Fsh. rh GDNF did not change the expression levels of the selected stem cell transcripts.

Conclusion

We conclude that Fsh stimulates proliferation of type A_{und} and supports the formation of new cysts/niche by increasing Sertoli cell proliferation associated with A_{und} and stem cell transcripts. GDNF stimulates proliferation of both types A_{und} and A_{diff} and supports the growing of pre-existing cysts.

Acknowledgement

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POSTER 34

IMPACT OF *PIWIL2* KNOCKOUT IN PRIMORDIAL GERM CELLS USING CRISPR/CAS9 SYSTEM IN NILE TILAPIA *Oreochromis niloticus*

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Introduction

Producing sterile farmed fish through targeted removal of primordial germ cells (PGCs) can provide environmental benefits by preventing potential cross-breeding and introgression between farmed escapees and wild populations and boost productivity in farms through enhanced growth by suppressing sexual maturation. The rationale for this research was to better understand the roles played by selected genes on PGC maintenance and survival, and spermatogenesis in Nile tilapia then to explore the viability of gene editing for inducing sterility.

Methods

A panel of germ cell-specific genes was studied during Nile tilapia ontogenic development to identify suitable candidate genes that can be targeted to disrupt PGCs. Based upon results, *piwil2* was selected as a target for functional analysis using CRISPR/Cas9 gene editing methodology. Two guideRNA (gRNA) constructs were designed to target the conserved domain of PIWI. Three concentrations of gRNA (100, 150 and 250 ng/µl) and 500 ng/µl of Cas9 were tested in 3 independent tilapia egg batches for each gRNA. The treatment mortality was recorded at 3 days post fertilisation (dpf), and genomic DNA of each larvae was extracted to screen mutants by qPCR melt curve analysis. Based on the result, *piwil2* gRNA2 was selected and injected at 150 ng/µl with 500 ng/µl of Cas9 to create *piwil2* mutant larvae. The physiological impact of *piwil2* knockout on PGCs survival was then investigated in 6 dpf mutant larvae. Treated larvae were screened for mutation rate using melt curve analysis, and the confirmed mutant larvae trunks were analysed by histology to confirm effects on PGCs.

Results and Discussion

While there was no apparent difference in mortality associated with the gRNA concentrations, mutation rates were significantly influenced by the constructs (3.3 ± 5.6 % vs. 94.4 ± 7.9 % for gRNA1 and 2, respectively). This demonstrated the importance of gRNA design on the gene editing efficacy. Histological observations confirmed an apparent lack of PGCs in 15 mutant larvae (corresponding to 29 % of analysed larvae) while 13 others (25 %) showed atrophic and/or locally restricted PGCs and 24 (46 %) were similar to control larvae. Melt curve analysis confirmed a high proportion of complex mosaic mutations that require further characterisation to associate complex genotype with physiological response. Overall, these results suggest *piwil2* plays an important role in survival of PGCs during early larvae development in Nile tilapia.

Conclusion

This is the first demonstration of *piwil2* gene editing using CRISPR/Cas9 system, resulting in putative sterile fish in a commercially important farmed species. Further studies are still required to confirm long-term sterility in mutants and overcome technical and regulatory hurdles to apply the methodology to large-scale commercial operations.

POSTER 35

TESTICULAR EXPRESSION PROFILE OF THE OESTROGEN RECEPTOR (ERα AND ERβ) AND CYP19 IN THE LAMBARI A*stYanax rivularis*

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Introduction

Although oestrogen action is essential for maintenance of male fertility, studies regarding the distribution and expression of aromatase (CYP19) and oestrogen receptors (ERα and ERβ) in testis are restricted to mammals and rarely investigated in fish species. The aim of the present study was to analyse the expression pattern of ERα, ERβ and CYP19 in testes of *Astyanax rivularis* along testicular maturation.

Methods

Quarterly field samplings were carried out during a reproductive cycle in a stream of the upper Velhas River with a good conservation status. For analyses of the spermatogenesis, samples of the middle region of the left testis of each fish were fixed in Bouin's fluid for 8 to 12 h and then kept in 70% ethanol for histological processing. The samples were submitted to immunohistochemical analyses for localization of those three protein along testicular maturation. Samples from each sampling months were subjected to indirect ELISA assay for ERα, ERβ, and CYP19 expression. The following polyclonal primary antibodies (Santa Cruz Biotechnology, Inc. USA): rabbit anti-human ERα (MC-20, sc-542), rabbit anti-human ERβ (H-150, sc-8974) and rabbit anti-human CYP19 (H-300, sc-30086).

Results and Discussion

The results of immunohistochemistry demonstrated labelling of CYP19 in Leydig cells and acidophilic granulocytes, but spermatogonia, Sertoli cells, spermatids and spermatozoa were also labelled. ERα was more widely distributed than ERβ being found in all developmental germ cells phases. On the other hand, ERβ was found only in spermatogonia and spermatocytes. Both ERs were expressed in the Leydig and Sertoli cells. During testicular maturation, ELISA levels for CYP19, ERα and ERβ followed the gonadosomatic index (GSI) with significant higher values in the ripe stage.

Conclusion - Together, our results demonstrate expression patterns of CYP19, ERα and ERβ in the testis of *A. rivularis.*

Financial support: FAPEMIG

POSTER 36

MOLECULAR CHARACTERIZATION OF KIT LIGAND AND BONE MORPHOGENETIC PROTEIN 4 AND THEIR RECEPTORS IN THE EUROPEAN SEABASS.

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Introduction

In mammals, kit ligand and its receptor kit, integrate the Kit system, which plays an important role in the control of gametogenesis such as the proliferation, survival and differentiation of germ cells. Bone morphogenetic protein 4 (BMP4), a member of the TGF-β superfamily, has also been described as an important actor in the regulation of gametogenesis. In fish, two forms of the kit ligand (*kitlga* and *kitlgb*) and two forms of the receptor (*kita* and *kitb*) have been described in the ovary of zebrafish, but little is known about the Kit system in males. Although *bmp4* has been cloned in some fish species, their study has been restricted to its function during embryogenesis, and therefore its role in the control of gametogenesis is not known yet. The aim of the current work was to clone and characterize the European seabass (*Dicentarchus labrax* L.) *kitlg*, its receptor *kit*, as well as *bmp4*, and their type-I (*bmpr1a*) and type-II (*bmpr2*) receptors as a first step to study their involvement in the control of the initial stages of spermatogenesis in fish.

Methods

Blast analysis against the NCBI whole-genome shotgun contigs (wgs) database for *D. labrax* (accession numbers CBXY010000001 to CBXY010037781), was performed to find the sequences of *kitlg, kit, bmp4, bmpr1a and bmpr2*. The deduced mRNA sequences obtained were cloned by PCR using testis and/or ovary cDNA and specific primers designed to exons of the respective genomic sequences. The resulting amplicons were sequenced to confirm their identity. The phylogenetic analysis of the amino acid sequences were performed with MEGA 7 software. The gene expression patterns for tissue specificity were analyzed by non-quantitative RT-PCR.

Results and Discussion: One *kitlg* and two kit receptors (*kita* and *kitb*) sequences with open reading frames (ORF) of 810 bp, 2973 bp and 2871 bp encoding 269, 990 and 956 amino acids, respectively, were found in the European seabass genome. One *bmp4* and one type-I receptor (*bmpr1a*), but two type-II receptor (*bmpr2* and *bmpr2-like*) sequences with ORF of 1209 bp, 1575 bp, 3207 bp and 3630 bp, coding for 403, 525, 1069 and 1210 amino acid residues, respectively, were also found. Tissue specificity analysis revealed expression of *kitlg*, *kita*, *kitb*, *bmp4*, *bmpr1a*, *bmpr2* and *bmpr2-like* in almost all tissues studied, including ovary and testis. However, expression of *kita*, *kitb*, *bmp4* and *bmpr2-like* appeared to be higher in testis than in ovary.

Conclusion : In the present study we have characterized the European seabass Kit system as well as *bmp4* and their type-I and type-II receptors. The preliminary gene expression results suggest that both, the Kit system and Bmp4, can play an important role in the control of gonadal development in this species.

POSTER 37

EXPRESSION OF KIT LIGAND, BONE MORPHOGENETIC PROTEIN 4 AND THEIR RECEPTORS IN THE EUROPEAN SEABASS TESTIS DURING THE REPRODUCTIVE CYCLE AND UNDER UNILATERAL ORCHIECTOMY CONDITIONS.

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Introduction : In mammals, kit ligand, its receptor kit and bone morphogenetic protein 4 (BMP4) play an important role in the control of spermatogenesis. KIT mediates proliferation, survival and differentiation of type A spermatogonia, while *Kit* is specifically expressed in differentiating spermatogonia. BMP4 also plays an important role inducing spermatogonia differentiation and stimulating *Kit* receptor expression. However, little is known about the roles of Kit system and Bmp4 in fish males. The aim of this work was to study the expression of *kitlg*, *kita*, *kitb* and *bpm4* and their type-I (*bmpr1a*) and type-II (*bmpr2* and *bmpr2-like*) receptors in testis of the European seabass (*Dicentarchus labrax* L.) to determine their involvement in the control of the initial stages of spermatogenesis.

Methods: Testis samples from immature to fully matured stages were collected at different times of the reproductive cycle. Also, testis samples from fish submitted to unilateral orchiectomy (ULO) or SHAM operated in mid-September when testes contained only type A and early type B spermatogonia, were collected at 0, 2, 7 and 12 weeks after the removal of one of the testis. Levels of expression of *kitlg*, *kita*, *kitb*, *bpm4*, *bmpr1a*, *bmpr2* and *bmpr2-like* were analyzed in all testis samples by quantitative real time PCR.

Results and Discussion : In mid-September, all studied genes showed high levels of expression, suggesting that Kitlg and Bmp4 can be involved in the regulation of early stages of spermatogenesis. Then, the levels of expression of *kitlg*, *kita*, *bpm4*, *bmpr2* and *bmpr2-like* decreased to the lowest levels in November and December, and increased again in March and April at the end of spermiogenesis. At this time, however, *kitb* and *bmpr1a* showed the lowest levels of expression. Fish submitted to ULO, presented higher levels of expression of *kitlg*, *kita*, *bmp4* and *bmpr2* than SHAM operated fish at 12 weeks after surgery, when testis were occupied by cysts at all developmental stages. However, the presence of free spermatozoa filling the tubular lumens was higher in ULO than in SHAM individuals. It is likely that at this time, Kitlg, Kita and Bmp4 could rapidly induce type A spermatogonia differentiation to provide the remaining testis of ULO males with new spermatogenic cysts and, therefore, fully maintain production of spermatozoa. In contrast, the levels of expression of *kitb* of ULO fish were similar to the SHAM fish having the lowest levels at 12 weeks after surgery, suggesting that Kitb would not participate in this process.

Conclusion: The results of the present study strongly suggest that Kit ligand and Kita together with Bmp4 play an important role in the control of spermatogenesis of the European seabass, likely during the early stages.

POSTER 38

INTRAPAPILLARY XENOGENEIC GERM CELL TRANSPLANTATION FROM GOLDFISH (*Carassius auratus*) INTO ADULT COMMON CARP (*Cyprinus carpio*) TESTES

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Introduction

Germ cell transplantation is a promising technique in reproductive biotechnology and aquaculture. The germ cell transplantation consists of isolating germ cells (including the putative spermatogonial stem cells) from a fertile donor and transplanting them into the testes of a sterile recipient. This technique can be used to produce commercially valuable fish using surrogated parents, conservation of endangered species and for understanding the spermatogonial stem cell biology.

Methods

Herein, we describe the establishment of an intrapapillary xenogeneic transplant of germ cells from sexually mature goldfish (*C. auratus*) males into the testes of common carp (*C. carpio*), which had been cytoablated with a thermo-chemical treatment (two doses of busulfan at 40mg/kg, at 35°C). To follow the development of transplanted germ cells in the recipient testes, donor germ cells were labeled with PKH26, a fluorescent cell membrane dye, prior to transplantation.

Results and Discussion

Our results demonstrated that thermochemical treatment caused an effective suppression of spermatogenesis and a pronounced germ cell loss. Transplanted spermatogonial cells were able to colonize the recipients' testes, resume spermatogenesis and presumably generate spermatozoa within eight weeks after transplantation. These findings suggested that recipient testes provided suitable conditions for the survival, colonization, proliferation and differentiation of the donor spermatogonia from a related species.

Conclusion

In summary, we conclude that thermo-chemical treatment caused an effective suppression of common carp spermatogenesis and a pronounced germ cell loss, which is suitable for establishing common carp as recipient for germ cell transplantation. This study indicated that recipients' testes exhibited a high degree of plasticity to accept and support xenogenic donor germ cells. Goldfish germ cells were able to migrate, colonize, proliferate and presumably differentiate into common carp recipient gonads.

Acknowledgments

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POSTER 39

CHARACTERIZATION OF UNDIFFERENTIATED SPERMATOGONIA IN THE LAMBARI FISH *Astyanax altiparanae*: PREVIOUS RESULTS ON THE ROLE OF AMH IN THE REGULATION OF THE SPERMATOGONIAL NICHE

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Introduction

Undifferentiated type A spermatogonia (A_{und}) are the foundation of fish spermatogenesis. This cell population includes the spermatogonial stem cells that are located in a specific region of the testes, the spermatogonial niche, which regulates the function of these cells. Among the factors that regulate the spermatogenic process are mainly gonadotropins, and some growth factors produced by Sertoli cells, such as the Anti-Müllerian hormone (AMH). AMH has already been found in Sertoli cells involving early germ cells and appears to have an inhibitory effect in the spermatogonial differentiation, keeping the initial germ cells in their undifferentiated state. This study made the characterization of A_{und} spermatogonia, their niche and their S-phase label-retaining cell properties and, in addition, we intend to understand the role of AMH in the regulation of spermatogonial niche and spermatogenesis in *Astyanax altiparanae* testes.

Methods

For the characterization of undifferentiated spermatogonia, light microscopy techniques, transmission electron microscopy and morphometry (n=50) were applied. To determine the spermatogonial niche, the spatial distribution of 500 undifferentiated spermatogonia was evaluated. To evaluate the S-phase label-retaining cell properties, immunostaining with anti-BrdU was performed after 6 pulses of BrdU with a 12-hour interval between each pulse. Molecular analyzes of *amh* were started with the cloning of a fragment from degenerate primers using RT-PCR methods. Analyzes continued in order to obtain the 5' and 3' ends using the RACE technique.

Results and Discussion

Two types of undifferentiated spermatogonia have been described: A_{und*} and A_{und}. A_{und*} spermatogonia have an irregular nuclear envelope, decondensed chromatin, one or two nucleoli, and nuages in the cytoplasm; meanwhile, type A_{und} have a round nucleus. A_{und*} is preferentially distributed neighboring the interstitial compartment, whereas A_{und} is located in the intertubular area. Undifferentiated type A spermatogonia were able to retain BrdU over a long period, suggesting that these cells have a long cell cycle and potential stem cell candidates among them. For molecular characterization of *amh*, the cloning results have so far: 211 bp fragment obtained with RT-PCR technique and ~ 1000 bp fragment obtained with RACE technique; both fragments have more than 90% of similarity with the species used as model, *A. mexicanus*.

Conclusion

Based on these findings, undifferentiated type A spermatogonia may be characterized as putative stem cells in *A. altiparanae* testis and the regulatory effects of *amh* must be further investigated. This work will contribute to studies on the stem cell biology of this promising Neotropical experimental model.

POSTER 40

ISOLATION, *IN VITRO* STUDY AND STEM CELL MARKERS FOR TYPE A SPERMATOGONIA IN A SPECIES OF CHARACIFORMES

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Introduction

Curimbatá, *Prochilodus lineatus*, is a migratory species of South America. The knowledge about the biology of type A spermatogonia is still poorly understood in fish. These cells are still characterized by morphological criteria, due to the lack of specific markers, limited availability, and difficult accessibility. Thus, their physiological properties are still poorly understood. In this context, adequate identification and isolation of type A spermatogonia are essential to better understand the behavior of these cells after culture. The main objective of the present work was to establish a protocol for the characterization, isolation and culture of type A spermatogonia using molecular markers specific for these cells in fish.

Methods

To this end, adult *Prochilodus lineatus* testes were collected and digested enzymatically, and the resulting testicular suspension was separated by a Percoll discontinuous gradient, followed by differential plating. Cell cultures obtained were maintained for 15 days and cultured cells were analyzed by immunofluorescence method with anti-Vasa, anti-GFRα1 and anti-OCT4 antibodies, which are markers for spermatogonial stem cells. Spermatogonia enrichment method was also performed by flow cytometry.

Results

Percoll discontinuous gradient centrifugation followed by differential plating not only promoted the removal of spermatocytes, spermatids, spermatozoa, red blood cells and other somatic cells, but also enriched the pool of type A spermatogonia. An active process of proliferation of the type A spermatogonia was observed in the cell cultures of 15 days, resulting in prominent cell agglomerates that were characterized according to different stem cell markers.

Discussion

The positive characterization of these markers by immunocytochemical protocols and cell separation analyzes, allied to specific cell culture conditions, allowed some aspects of spermatogonial regulation to be clarified, such as survival and proliferation.

Conclusion

In the present study, type A spermatogonia were identified and isolated from *Prochilodus lineatus.* The protocols were effective, since we were able to isolate, maintain in culture and characterize these cells with specific molecular markers for germinal stem cells. The understanding of the regulation of the *in vitro* germ cell maintenance process may contribute to the enhancement of *in vivo* and *in vitro* reproduction techniques of fish species that are important for aquaculture and / or are endangered.

POSTER 41

IMPROVEMENT OF PROTOCOLS THAT INCREASE SPERM PRODUCTION IN THE FLATFISH SOLEA SENEGALENSIS BY USING RECOMBINANT GONADOTROPINS

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Introduction

Sperm production of captive Senegalese sole, Solea senegalensis, remains a major hindrance to commercial production of this highly valued species. New biotechnological approaches involving efficient hormonal therapies that increase sperm production are thus needed to establish manageable in vitro fertilization protocols. Homologous recombinant gonadotropins, follicle stimulating and luteinizing hormones (rFsh and rLh, respectively), produced in mammalian host cells, have recently arisen as promising candidates. Previous trials on juvenile fish using these hormones indicated that treatment with rFsh stimulates spermatogenesis, whereas rLh potentiates spermatozoa differentiation. The aim of the present work was to set up a protocol using rFsh and rLh to enhance sperm production in adult sole males.

Material and methods

Senegalese sole males (~1 kg) were injected with rFsh (9 or 18 μg/kg) each week for 5 weeks, and on the 6th week treated with a single injection of rLh (9 or 18 μg/kg). Twenty-four hours after rLh injection, gonadotropin and androgen levels were determined by enzyme-linked immunosorbent assay (ELISA), and semen quantity and quality were evaluated by computer-assisted sperm analysis (CASA). Trials were carried out in early spring (temperatures from 13ºC to 17ºC), and in autumn under a constant temperature of 12°C.

Results and Discussion

Treatment of males with rFsh and rLh during spring was not effective at inducing sperm production, possibly because of an advanced stage of sexual maturation of the males, as reflected by the high basal plasma levels of Lh (17 ng/ml). However, under constant low temperature in autumn, when circulating Lh is much lower (3 ng/ml), the dose of 9 ug/kg rFsh and rLh generated a four-fold increase in sperm production, which was not observed with the 18 ug/kg dose. It seems likely that, as previously found in juvenile fish, sustained treatment with high doses of rFsh diminished Leydig cell survival. Nevertheless, the motility, progressivity and velocity of spermatozoa were enhanced after rLh treatment during both spring and autumn.

Conclusion

The data indicate that combined treatments with rFsh and rLh increase sperm production and quality in Senegalese sole adult males. However, the effectiveness of these hormonal therapies appears to be highly dependent on the reproductive stage of the males, and the temperature during which rFsh-induced spermatogenesis takes place.

POSTER 42

SEX STEROIDS IN MALES OF *BRYCON ORBIGNYANUS* AT DIFFERENT AGES IN THE REPRODUCTIVE PERIOD

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INTRODUCTION

Numerous endocrine factors linked to growth and metabolism may influence the events leading to puberty and gamete production. Interaction between growth and reproduction occurs in several vertebrates and may be particularly evident at given stages of the life cycle in fish. Thus, the main of this study was to characterize the plasma profile of the major sex steroids, 17β-Estradiol (E2), 17α-hydroxyprogesterone (17α-OHP), Testosterone (T) and 11-Ketotestosterone (11-KT) in the reproductive period of *Brycon orbignyanus* at different ages.

METHODS

The experiment was carried out in a commercial fish farm, where the animals were in tanks separated by age (12, 24 and 48 months). The sex of the 48-month-old animals (n=4) were identified by the sexual dimorphism of the species. For the determination of the sex of the animals with 12 (n = 20) and 24 months (n=20) of age the histology was used. To measure the hormones, blood was collected from each animal by puncturing the caudal region. Blood samples were centrifuged and plasma was used for quantification of the E2, 17α-OHP, T and 11-KT plasma by ELISA using commercial 11-KT telephones kit (Cayman) and human for E2, 17α-OHP and T (Interkit).

RESULTS AND DISCUSSION

Three stages of gonadal development were observed: immature, developing (12 and 24) and animals in regression (48). In immature fish with 12 months, E2 showed the highest level (11.81 ng / ml-1; $P < 0.05$) highlighting its action on the proliferation and renewal of spermatogonia by mitosis, characteristic of this stage. Testosteron showed higher levels ($P < 0.05$) in fish at 24 and 48 months (0.18; 0.21 ng / ml-1). This data shows that testosterone is acting as a negative feedback, inhibiting the release of FSH and positive LH feedback, important during spermatogenesis and spermiogenesis. The results of 11-KT and 17α-OHP suggest that, in the studied ages and stages of development, spermatogenesis and spermiogenesis are occurring through meiotic divisions, since 11-KT controls the initiation of maturation, as well as to 17α-OHP, which has active in the initiation of meiosis and spermiation. 17α-OHP is a precursor of 17α, 20β-dihydroxy-4-pregnen-3-one (DHP), which acts on spermatogenesis by both mitosis and meiosis, and is essential for the final stage of maturation.

CONCLUSION

Therefore, E2 is fundamental for the process of proliferation and renewal of spermatogonia by mitosis. Already T, 11-KT, and 17α-OHP, act with greater expression during spermatogenesis and spermiogenesis, characterized by meiotic divisions.

SPAWNING, FERTILIZATION AND SPERM-EGG INTERACTION

POSTER 43

PROGESTIN INDUCTION OF SPERM HYPERMOTILITY IN SOUTHERN FLOUNDER THROUGH mPRα IS MEDIATED BY ACTIVATION OF MULTIPLE SIGNALING PATHWAYS

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Introduction

Development of commercial aquaculture for many marine flatfish species such as southern flounder is severely limited by poor male reproductive performance, especially reductions in sperm motility and fertility, but the underlying causes are largely unknown. A clearer understanding of the mechanisms regulating sperm motility would enable the development of more reliable and efficient methods to improve sperm quality and spawning of southern flounder and other marine flatfish species. Our previous studies have shown that acute treatment of southern flounder and Atlantic croaker sperm in vitro with a teleost progestin hormone (20β-S) causes rapid induction of sperm hypermotility and increased fertility through activation of progestin membrane receptor alpha (mPRα) coupled to a stimulatory G protein and increases in cAMP levels. In the present study the potential involvement of several intracellular signaling pathways in the sperm hypermotility response to 20β-S was investigated using specific pharmacological tools.

Methods

Aliquots of freshly collected sperm, diluted in physiological saline, were preincubated with inhibitors (I) or activators (A) of Egfr (I:AG1478, A:EGF), Mapk/Erk1/2 (I: U0126), Pi3k/Akt (Pi3k I:Wortmannin, LY294002, Akt I: ML9), ACY/cAMP (I: dd-Ado, A: forskolin), Pde (I: Cilostamide), or calcium (L-type channel I:verapamil) signaling pathways for 30 min. prior to treatment with 20β-S or Org 02-0 (specific mPR agonist) for 1 min. and motility activation with a hyperosmotic medium. Sperm motility was observed under a microscope and recorded for 1 min with a video camera and swimming speed was calculated using motion analysis software. Calcium influx into flounder sperm was measured using Fura-2 with a fluorescence plate reader and phosphorylation of Akt and Erk 1/2 by Western blot analysis.

Results and Discussion

The induction of sperm hypermotility by 20β-S and Org 02-0 was blocked by prior treatments with inhibitors of Egfr, Mapk, Pi3k/Akt, Pde, and Acy. On the other hand treatment with activators of Egfr (EGF) and Acy (forskolin) mimicked the effects of the progestins and induced sperm hypermotility. These results indicate that progestins induce sperm hypermotility through Egfr/Mapk/Erk1/2, Pi3k/Akt/Pde, and Acy/cAMP pathways. The progestins caused rapid calcium influx into sperm, an effect mimicked by EGF and forskolin and blocked by verapamil which also blocked progestin induced sperm hypermotility.

Conclusion

Progestins activate multiple signaling pathways through mPRα in flounder sperm to induce calcium influx and hypermotility and enhance fertility. Practical methods to increase the reproductive performance of flounder broodstock could potentially be developed through pharmacological stimulation of these pathways.

POSTER 44

PROGRESSION OF SPAWNING COMPETENCE AND EGG QUALITY WITH AGE IN CAPTIVE-BRED HĀPUKU (*Polyprion oxygeneios*)

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Introduction

The wreckfish hāpuku (*Polyprion oxygeneios*) is a high-value commercial species found in the waters of the southern hemisphere. A research breeding program was started to develop the species as for aquaculture in New Zealand, that showed the amenableness of wild caught broodstock to reproduce in captivity. The resultant availability of captive-bred (F1) fish provided the first opportunity to investigate the early development of reproductive physiology and performance in this species. Here, we provide a broad description of the development of spawning competence in this little-known species.

Methods

Two cohorts (2008 and 2010 year-classes) of F1 broodstock were kept in 20-40 m^3 photothermal controlled tanks from 4 years of age. The fish were given pelleted broodstock feed, except for the 2010 cohort at 4-5 years. The 2008 cohort was part of a study to describe the physiological control of reproduction (reported in Wylie et al, 2017a, b), and were periodically handled for gonad biopsies, measurements, blood sampling and hormonal induction (GnRHa and hCG). The 2010 cohort acted as a minimal disturbance group for comparison. Spawning occurred between August and December, with sufficiently viable eggs carried through incubation and larvae rearing. Production and quality parameters of eggs and larvae for each batch (e.g., fertilization, hatch and survival rates) were recorded. Eggs from selected batches were also genotyped for parentage analyses.

Results and discussion

We observed clear evidence of age related progression in reproductive competence in hāpuku. In general, vitellogenesis and ovulation are first detected at 4-5 years (> 5 kg body mass) but no spontaneous spawning occurred. By 5-6 years, females produced greater proportions of late vitellogenic oocytes that spontaneously ovulate following exogenous GnRHa induction. Although overall spawning quality was poor, fertilizable eggs resulting in viable juveniles were obtainable through stripping. At 7-8 years GnRHa implant consistently induced spontaneous spawning that resulted in non-trivial amounts of high quality eggs and fingerlings. By 9 years, spontaneous spawning of high quality eggs occurred without exogenous hormone. Between 7-9 years, egg and fingerling production increased accompanied by relative increases in quality parameters. Interestingly, those with earliest attainment of reproductive competence occurred in fish with the highest growth rates, but importantly those subject to minimal handling.

Conclusion

The ongoing research program has now demonstrated that captive-bred (F1) broodstock are a viable and sustainable source for high quality gametes in hāpuku. However, commercial-level natural production does not take place until at least 7 years.

POSTER 45

PHYSIOLOGICAL POLYSPERMY IN STURGEONS.

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Introduction

Fertilization is essential for sexual reproduction for animals and plants, although the mechanisms of factors determining which type of fertilization in different animals occurs are not clear. In most animals only one spermatozoa participates in karyogamy, producing monospermic fertilization, whereas in some eggs numerous spermatozoa can penetrate in the cytoplasm and cause polyspermy. It is considered that it is fatal if more than one spermatozoon fuses with the oocyte: polyspermic embryos develop abnormally and perish before hatching or can develop to abnormal larvae which die.
Sturgeons are representing unique biological characteristics: multiple micropyles, polyploidism, and fertilization between interspecies and intergenera species. Fertilization is monospermic due to size of micropyles, which is slightly bigger than a diameter of a spermatozoon's head and enlarging of a perivitelline space after fusion of egg with spermatozoa, whereas big amount of micropyles and highly concentrated sperm suspensions can lead to polyspermy.

Methods

Several fertilizations were performed to produce polyspermic fishes and to discover the reason of their appearance and survival rate: *Acipenser ruthenus* (2n) x *Acipenser ruthenus* (2n), *A. ruthenus* (2n) x *A. gueldenstaedtii* (4n) and fertilization of gametes from three parents at ones: *A. baerii* (4n) x *A. ruthenus* (2n) x *A. gueldenstaedtii* (4n). Atypically developed embryos (3, 5, 6, 7, 9, 10 cells) were collected separately to a study in a detail. Ploidy of germinated larvae was evaluated by flow cytometry, survival rate of atypically divided embryos was noted.

Results and Discussion

Normally divided embryos (2-, 4-, 8-, etc.) had an inherent for *A. ruthenus* and hybrids of *A. ruthenus* x *A. gueldenstaedtii* ploidy: 2n and 3n respectively. Abnormally developed *A. ruthenus* embryos had mosaic 1n/2n ploidy, *A. ruthenus* x *A. gueldenstaedtii* hybrids were 2n/3n and the majority of hybrids from three species (88%) were mosaic with 2n/3n, 2n/4n, 2n/5n ploidy. Polyspermic organisms had mostly the same shape as a monospermic and survived well (similar to a control).

Conclusion

This study demonstrates that physiological polyspermy is happening in sturgeon and producing viable mosaics. This appreciate phenomenon shows that sturgeon eggs can be easily fertilized by several spermatozoa even from different species at once and produce a viable progeny.

POSTER 46

OVERCOMING REPRODUCTIVE DYSFUNCTIONS IN CAPTIVE GREY MULLET (*Mugil cephalus*): AN EXPANDED TOOL BOX FOR SUCCESSFUL BREEDING

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Introduction

The grey mullet (*Mugil cephalus*), a euryhaline marine teleost, is widely cultured in brackish and fresh water fishponds. Under captive conditions, mullet brooders do not spawn spontaneously, while both genders display reproductive dysfunctions. Spermiating males are rarely observed and in most cases the produced milt is highly viscous and fails to fertilize the eggs. Females are often arrested in the early stages of vitellogenesis, or fail to undergo oocyte maturation and ovulation. Our previous study, underlined dopaminergic inhibition as a major barrier arresting spontaneous spawning in captive mullet broodstock. This inhibition was alleviated by combined treatment with GnRHa and dopamine receptor antagonist (DA). Within the framework of the European project DIVERSIFY, yeast-produced recombinant gonadotropins (r-FSH and r-LH) were employed as therapeutic agents to further enhance gametogenesis and improve spawning success in captive mullet broodstock.

Methods

Biopotent recombinant gonadotropins, r-FSH and r-LH, were produced using Pichia pastoris yeast fermentation. ELISAs were established to measure LH and FSH in grey mullet. In the first experiment, adult captive mullet were injected at the onset of the reproductive season with either (i) saline (control), (ii) r-FSH or (iii) r-LH. Three weeks later, all fish were killed, gonadosomatic index (GSI) was calculated and blood and tissues (pituitary, gonad and liver) were sampled. Treatment effects on gametogenesis were evaluated by gonadal histology, mRNA quantification of target genes and hormone measurements (LH, FSH, sex steroids). In the second experiment, captive mullet brooders were injected with r-FSH combined with DA. Reproductive performance in hormonally treated and saline-injected control fish was compared and tested in triplicate replicates.

Results and discussion

Results of the first experiment indicated a stimulatory effect of the r-FSH treatment on captive mullet gametogenesis. The r-FSH treated fish exhibited significantly higher GSI values, advanced gonadal development and increased circulating sex steroid levels, when compared with control fish. Furthermore, the r-FSH treatment was found to positively affect the synthesis and accumulation of pituitary LH while evoking a negative feedback loop on pituitary FSH content. Similarly, results of the second trial indicated that the combined r-FSH/DA treatment induced synchronized gonadal maturation in the brooders. This gave rise to a relatively high abundance of spermiating males and fully mature females suitable for the spawning induction trials. Following successful spawning, the resultant high quality eggs and larvae gave rise to large numbers of robust juveniles.

Conclusion : FSH agonist appears to play an important therapeutic role accelerating gonadal development and facilitating breeding in captive grey mullet broodstock.

POSTER 47

INSEMINANT DOSES FOR *Steindachneridion parahybae* USING FRESH AND CRYOPRESERVED SPERM

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Introduction

The procedures sperm cryopreservation is applied in some neotropical fishes with high rates of motility after thawing, indicating great quality of sperm, however, the success of cryopreservation in fish should be verified after oocyte fertilization, for so, the protocols need to be standardized, such as the inseminant doses, important parameter to be evaluate in artificial reproduction procedure, principally in endangered species, such as the surubim-do-Paraíba, *Steindachneridion parahybae* (Siluriformes: Pimelodidae) considered endangered on Brazilian red list. Thus, the aim was evaluated different inseminant dosages to oocytes fertilization of *S. parahybae* with fresh and cryopreserved sperm.

Methods

For cryopreservation was used a semen pool of 12 males diluted (1:3) in cryoprotectant solution (8.5% glucose, 0.25% whole milk powder and 10% methanol) and bottled in 4.0mL straws and subjected to freezing (22h in Dry-shipper and 24h in Liquid N). After thawing (30s at 25^{o}C), the computadorized sperm motility was evaluated by free CASA (ImageJ). According to the values of motility and sperm concentration, the inseminating doses of $1.0x10^5$, $7.5x10^5$, $14.0x10^5$, $20.5x10^5$, $27.0x10^5$ of mobile spermatozoa oocyte^{-1} were estimated and applied. As a control, fresh semen (pool) from three other males were used with the same inseminating doses as above. A conical incubator (1.5L) containing 1g (322) of oocytes (pool of three females), fertilized with 10mL of water and with the respective inseminating doses was considered as an experimental unit (in triplicate - n=30). The fertilization rates were estimate 8h after fertilization. The results subjected at ANOVA and t-student test at 5% of significance.

Results and Discussion

For each semen used (fresh and cryopreserved) was not verify differences ($P>0,05$, Anova test) on the inseminant doses applied, for so were observed the values of 56.85±8.66 and 41.64±13.48% of oocytes fertilized for fresh and cryopreserved sperm, respectively. When compared in each sperm doses, was verified differences ($P<0,05$, t-student test) in fertilization rates only to $1.0x10^5$ spermatozoa oocyte^{-1}, with 53.92±6.39% to fresh and 34.36±6.66% of cryopreserved sperm. For others inseminating doses, no differences between fresh or cryopreserved were observed ($P>0.05$). These values present important applications of *S. parahybae*¸ possibility the economy of genetic material, without less of oocyte fertilization, furthermore, it is clear that cryopreservation reduces the fertilization potential of spermatozoa and the application of larger inseminating doses may minimize this.

Conclusion

To artificial fertilization of *S. parahybae* oocytes with cryopreserved sperm it is recommended the use of 750.000 sperm mobile oocyte^{-1}.

POSTER 48

SOMATIC CELL NUCLEAR TRANSFER IN RAINBOW TROUT (*Oncorhynchus mykiss*): WHAT MAKES IT DIFFERENT FROM FERTILIZATION WITH A SPERMATOZOA ?

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Introduction

Somatic cells are a highly convenient diploid support for biodiversity conservation: they carry the genome of both parents, and they can be collected whatever the age or sex of the donor. In fish, cryobanking of somatic cells is highly valuable because eggs and embryos cannot be cryopreserved. With those cells, the regeneration of the donor genotype will however rely on the nuclear transfer technology in which the donor cell nucleus is injected into a recipient oocyte. Somatic cell nuclear transfer (SCNT) has been performed in fish since the 1960s, mainly in Cyprinidae (carp, goldfish and zebrafish), Adrianichthydae (medaka) and Cobitidae (loach). Although survival rates after nuclear transfer are low, SNCT is a promising technology of reconstruction. No study has been reported in Salmonidae, despite their importance in aquaculture.

Methods

We used rainbow trout oocytes, arrested at metaphase II as recipients and rainbow trout somatic cells isolated from caudal fin as nucleus donors. Nuclear transfer was performed on non-enucleated oocytes, in order to reduce the development rate variability induced by enucleation. Oocytes were incubated in Fish Ringer (300 mOsm/kg) in order to keep them inactivated and to maintain their quality during the whole injection process. The donor cell was delivered through the micropyle, which is the route that the spermatozoa use to enter the oocyte, using a 15µm needle. Development was then initiated by transferring oocyte in fresh water.

Results and Discussion

All steps sought to imitate as much as possible the fertilization process. Injecting the egg through the micropyle required its visualization. We observed that the position of the lipid globules is quite independent from the micropyle position. Therefore, the micropyle had to be found based on the slight changes in light diffraction rather than by the egg internal structures underneath. After egg activation with water, some vitellus coagulation was induced, likely because of alteration of egg plasma membrane upon needle puncture during injection. However, no other suitable medium for egg activation could be found. We also demonstrated that early development (up to 8 days post fertilization) could be estimated only once the chorion is removed. In all, although all those necessary steps were mastered, no development could be induced on more than 100 SCNT. Interestingly, control oocytes which were injected with medium only, before normal fertilization were also unable to initiate any development. Those results suggest that the penetration of the needle and/or the medium used are deleterious to the oocytes. This had never been observed in other species.

Conclusion : This first SCNT attempt shows that Salmonidae are quite refractory to this technology, and a better understanding of the alterations induced at the animal pole is required. **Acknowledgements**: Financial support of “Investissements d’Avenir “ANR-11-INBS-0003 (CRB-Anim 2013-2019) and COST AQUAGAMETE FA 1205.

POSTER 49

INACTIVATING MUTATION OF THE THYROID HORMONE ACTIVATING DEIODINASE TYPE 2 IN ZEBRAFISH SEVERELY DISTURBS MALE AND FEMALE REPRODUCTION

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Introduction:

The vertebrate reproductive process is mediated by different factors, amongst which thyroid hormones are of crucial importance. The key player is the biologically active hormone 3,5,3'-triiodothyronine (T_3). Its bioavailability in different tissues is locally controlled by deiodinating enzymes. In fish, the main activating enzyme is deiodinase type 2 (Dio2). To unravel its role in reproduction, we generated a mutant zebrafish line, completely and specifically lacking Dio2 activity due to a 3 amino acid deletion upstream of the active site of the enzyme.

Methods:

The reproductive phenotype of Dio2 knockout (Dio2KO) zebrafish was assessed at the level of offspring production (fecundity/fertilization rate), gametogenesis (histological stage counting), functioning of the hypothalamic-pituitary gonadal axis (quantitative PCR analysis of different genes) and sex steroid secretion (enzyme-linked immunosorbent assay of holding water samples). Rescue experiments were performed by systemic T_3 supplementation.

Results and Discussion:

Homozygous Dio2-deficient zebrafish display a delay in sexual maturity and the duration of the reproductive period is substantially shortened. Females lay remarkably low amounts of eggs and males have problems with fertilization, pointing to reproductive defects in both sexes. While the process of oogenesis appears largely normal, mature oocytes accumulate in mutant ovaries due to inhibition of ovulation. This finding was substantiated by measuring the ovary size, which was significantly enlarged in Dio2KO fish from their sexually mature stage onwards. Spermatogenesis is more strongly affected, showing amongst others significantly lower amount of spermatogonia in Dio2KO fish during the reproductive period. Furthermore we found that regulation of the male hypothalamic-pituitary-gonadal axis is disturbed, including upregulation of kisspeptin 2 (*kiss2*) and insulin-like growth factor 3 (*igf3*) and decreased secretion of (11-keto)testosterone. These low sex steroid levels also perturb courtship behaviour. As Dio2KO zebrafish suffer from an overall hypothyroidism, we tried to evaluate the relative importance of T_3 uptake versus local T_3 production in the gonads by increasing systemic T_3 levels. Treatment started in juveniles resulted in normalization of reproductive activity in both sexes.

Conclusion: We provide the first evidence of a link between loss of Dio2 activity and reproductive dysfunction. While ovulatory problems mainly summarize the female aspect, male reproduction is affected at different levels. These data clearly reveal the critical contribution of TH activation by Dio2 for fish reproduction.

POSTER 50

DESLORELIN IN REPRODUCTION OF *Colossoma macropomum*

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Introduction

Hormonal induction of teleost fish maintained in captive is a technique very useful for fish protein production and germplasm conservation. The hypothalamic/pituitary/ gonadal axis can be induced by exogenous and synthetic hormones. This is necessary because migratory fish can prepare gonads to reproduction however can not ovulate or spawn in captivity. The most widely used exogenous hormone treatment in fish is the pituitary carp extract (PCE) that shows a synergistic effect to natural gonadotropins. In the other hand, it does not present a quantitative and qualitative standardization. The gonadotropin releasing hormone (GnRH) presents some advantages over PCE, as the way it induce the beginning of the hypothalamic pituitary gonadal axis, stimulating the fish to synthesize their own natural gonadotropin. Deslorelin is a synthetic agonist of GnRH that stimulates endogenous luteinizing hormone (LH), which promotes final maturation of the oocytes during spawn.

Methods: Therefore, the aim of the present study was to evaluate the effect of deslorelin in the induced reproduction of *Colossoma macropomum* (Tambaqui). Five females, weighting from 7.5 to 9kg, were selected by evaluation of their belly, curved and soft to the touch and hyperemic/edematous urogenital papilla. The central position of the germinal vesicle (CPGV) was evaluated during the spawn. Males, weighting from 3 to 4.5kg, that were liberating sperm under soft compression of cealomathic cavity were selected and induced with PCE. The fish were maintained in polyethylene boxes with thermostats that kept water at 27 °C. Female were induced with two doses of deslorelin in concentrations of 1% at first dose and 4% at second dose, in a 12-hour interval, independent of the weight, the total volume used was standardized to 1mL.

Results and Discussion : It was induced five female and three male of Tambaqui. Females started to spawn with 10 hours after first hormone injection of deslorelin. Spawn of tambaqui general iniciated 12 hours after the injection of CPE. A control was induced with CPE and its reproduction happened after females induced with deslorelin. Females extruded in average 422,5 g of oocytes during the spawn, independent of the hormone. Before the induction with deslorelin 60,74% of the oocytes had CPGV after the hormone only 5% of the oocytes had central position. Oocytes in final maturation presents nucleus in periphery of the cell, in turn immature oocytes have central position of it. Females that receive deslorelin presented 70% of the nucleus of the oocytes in peripheric position, indicating that deslorelin induced final maturation of those cells.

Conclusion : We conclude that deslorelin promote an anticipation of the spawn in Tambaqui females and final maturation of the oocytes. It could be an alternative for reproduction of Tambaqui, due its capacity to anticipate spawning in females with a low concentration/volume for induction, independent of the breeder weight. This is the first study that indicates that these synthetic GnRH analogues molecules can be used Tambaqui`s breeding season, more work is being perform to determine a protocol for use of these hormonal preparation in the field.

POSTER 51

PRELIMINARY RESULTS ON THE USE OF DIFFERENT DOSE INTERVALS FOR *Astyanax altiparanae* INDUCED REPRODUCTION

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Introduction

Recent studies developed in our laboratory have shown that the traditional protocol applied for induced spawning in *Astyanax altiparanae* with a single carp pituitary extract (CPE) dose (6mg.kg^{-1}) provokes ovulation in only part of the injected females. Hypophysation is the most used technique for induced spawning, but there are still many issues concerning particularities to achieve spawning efficiency for each species. Thus, within a larger proposal, which covers the reproduction of this species, at this stage we report by the first time the effects of using fractioned doses with different intervals for *A. altiparanae* induced spawning.

Methods

To that, adult *A. altiparanae* females (22,4±6,6g) were distributed into four different treatments: 3, 6, 12 or 24 hours of interval between doses (T-3h, T-6h, T-12h and T-24h respectively). For all treatments 6mg.kg-^{1} CPE was fractionated into 10% (first dose) and 90% (second dose). For each treatment three experimental units (10L plastic box containing 04 females and 08 males) were used. A saline control (SC) and a positive control (PC) (6mg.kg^{-1} CPE single dose) were also applied. All males (10,2±4,4g) received a single dose of 3 mg of CPE.kg^{-1} at the time of the female's second doses. Reproductive parameters (egg volume, fertilization and hatching rates) were measured. The frequency of units in which spawning was observed, and the number of viable embryos obtained per experimental unit was determined. Data were analyzed by the Kruskal-Wallis test at the $p \leq 0.05$ level of significance.

Results and Discussion

Spawning was observed in all replicates of the treatments T-3h, T-6h and T-12h. For the CP group spawning was observed in only two of the three replicates. The number of viable embryos was similar among treatments ($p>0.05$), but the group T-12h had the most consistent results and the lower standard errors for all variables.

Conclusion

Preliminarily, the treatment T-12h was the most trustworthy concerning reproductive performance for this species. From now on we are developing histological analyzis and evaluating the plasma concentration of eicosanoids and gonadal steroids to study the relationship between the different intervals applied with the evolution of the meiotic process (final maturation and ovulation) and mainly establishing the effective number of females that in fact attained spawning in each unit. These results indicated that as for the clear majority of tropical migratory species, *A. altiparanae* can have its spawning performance intensified by a process called "steroidogenic shift", which is associated with successful ovulation, but is only obtained when the treatment is fractioned. This hypothesis is now being evaluated during the continuation of the proposal.

POSTER 52

EXOGENOUS GONADOTROPINS ADVANCE OOCYTE DEVELOPMENTAL STAGE IN PREVITELLOGENIC NEW ZEALAND SHORTFINNED EELS (*Anguilla australis*)

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Introduction

Gonadotropins (Fsh: follicle-stimulating hormone and Lh: luteinizing hormone) are key mediators of gametogenesis in vertebrate animals. Their involvement in cyclical gonad growth, culminating in the production of fertilizable gametes, are well-documented. In fish, however, the function of Fsh and Lh during the earlier stages of oocyte development (previtellogenesis) remain little understood. The objective of this study was to elucidate the response of previtellogenic fish to exogenous purified gonadotropins. For this purpose, we used the eel, *Anguilla australis*, as the animal model to evaluate the effects of administration of recombinant eel follicle-stimulating hormone (rec-Fsh) or the Lh-analogue human chorionic gonadotropin (hCG).

Methods

Eels received weekly intraperitoneal (IP) injections of either phosphate-buffered saline (control group) or of rec-Fsh (20, 100 or 500 $\mu g.kg^{-1}$) or hCG (20, 100 or 500 $IU.kg^{-1}$). Two days after the third, final injection, fish were euthanized (0.3% benzocaine) for tissue sampling. The expression of target genes was measured by real-time quantitative PCR, while plasma sex steroid levels (11-KT and E2) were obtained using radioimmunoassay. In addition, the changes in GSI and oocyte diameters were recorded.

Results and Discussion

Rec-Fsh or hCG induced an increase in GSI. Similarly, oocyte diameters increased from around 85 μm in controls to over 100 μm in rec-Fsh-treated eels. Both rec-Fsh and hCG increased the plasma E2 levels (0.7 ng/ml *vs* 0.1 ng/ml), while only hCG treatment yielded a significant increase in plasma 11-KT levels in comparison with control fish (5.0 ng/ml vs. 2.7 ng/ml). Both rec-Fsh and hCG up-regulated the expression of *fshr* and *lpl* in the ovary. Meanwhile, mRNA levels of *lhr*, *ldlr* and *cyp11b* were not significantly changed by any of the treatment regimes. Both rec-Fsh and hCG decreased pituitary *fshb* mRNA levels, but significant differences between treatments were only found in response to hCG exposure.

Conclusion

Treatment with exogenous Fsh or hCG advanced the developmental stage of ovarian follicles. We contend that this finding is compelling evidence for actual involvement of gonadotropic hormones in oocyte growth during previtellogenesis in eels, and possible, teleost fish at large.

POSTER 53

SPERM EVALUATION OF NILE TILAPIA (*Oreochromis niloticus*) REARED IN BIOFLOC SYSTEM

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Introduction

The biofloc technology (BFT) is an aquaculture system based on limited water exchange, where microbial conversion of nutrient waste into microbial biomass (bioflocs) is maximized and the resulting bioflocs can be utilized back by the cultured organisms as a food source. Effects of BFT on reproduction of aquaculture species have been reported, however, the results described for fish species in this production system were not conclusive. Positive effects of BFT on reproduction of aquatic cultured species have been described, mainly for shrimps. Besides, to our knowledge, there are no studies in the literature describing sperm characteristics of Nile tilapia reared in BFT.

Methods

The experiments were conducted in a greenhouse at the Laboratory of Aquaculture - UFMG. We used two lines originated from Chitralada Nile tilapia. Each animal received a microchip and were acclimatized for two weeks. Males were kept in tanks with 800 L of water and were randomly divided in control and BFT groups. In control system, tilapias were maintained in clear water with renewal rate of 50% per day. The water renewal in BFT was provided only for evaporation replacement. Forty male tilapias were used to compare the semen production of animals reared in BFT or clear water. Semen samples were evaluated for six times, at 8, 15, 22, 36 and 50 days. Microscopic analysis included evaluation of motility (0-100%), vigor (0-5), concentration (n. Sptz / mL) and sperm morphology (% of normal or defective spermatozoa). Distilled water was used to activate the semen. A single trained operator performed all the analysis.

Results and Discussion

The sperm motility was different between treatments and lines. Both Control and Line 1 presented higher values in sperm motility than BFT and Line 2, respectively. However, the values were near the maximum score of 100%, and probably would not affect in vivo fertility. For sperm vigor, we only found differences between lines, with Line 1 showing higher value than Line 2. Once again, the vigor 3 found in Line 2 is still acceptable for good semen fertility. For concentration and sperm morphology, we did not identify effects of treatments, lines nor collection date ($p > 0.05$).

Conclusion

Our results demonstrated that although BFT and Lines can affect sperm production, the differences between treatments and lines are minimal. Thus, the semen of Nile tilapias reared in BFT presents high quality, as well as of the animals reared in clear water.

POSTER 54

ARTIFICIAL AND SEMI-NATURAL REPRODUCTION IN CAPTIVITY OF *Astyanax altiparanae* OUT OF REPRODUCTION SEASON

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Introduction

The yellowtail tetra, *Astyanax altiparanae* (Pisces, Characiformes) is a Brazil native species of Prata River Basin, with large period of reproduction (September at march). This species has great importance for national fish farming, because it can be marketed in the form of bait and meat, however, to be successful the reproduction is a crucial stage and must be studied. In this way, the aim was to evaluate the two techniques used in the process of reproduction of the species (artificial and semi natural) out of reproduction season normally applied in captivity.

Methods

The experiment was developed in May/2017 (autumn), where 200 mature fish (80 females and 120 males) were selected and divided into a randomized experimental composed of two treatments and four replicates, the treatments were the two reproduction forms: artificial and semi natural. For artificial reproduction, the females were induced with 5.5 mg of crude pituitary carp extract (CPCE) kg^{-1} of breeder and after was submitted to manual extrusion and artificial fertilization the dry method. For the semi-natural reproduction, the animals were induced (5.5mg CPCE kg^{-1}) the males and females were allocated together in a box overnight for the reproduction to occur naturally and the eggs were collected in the next morning.

Results and Discussion

The relative fecundity, fertilization rates and larvae estimation were not difference ($P>0.05$, t-student test) by the two reproduction techniques used. So we obtained (Mean ± standard deviation) 15,516 ± 9,954 oocyte g^{-1} of female, 38.2 ± 20.0% of fertilization rate and 6,413 ± 4,862 larvae $fish^{-1}$. The artificial spawning stands out for the ease of manipulation separately of the gametes, avoiding loss and optimizing the use of the reproducers. On the other hand, artificial spawning presents a disadvantage because it gives greater stress to the animals, caused by the constant manipulation of the same ones to obtain the gametes and consequently, leading to the mortality of some fish. On the other hand, the semi-natural reproduction offers some disadvantages, such as the loss of biological material at the time of collection in the boxes where the breeding animals are conditioned, due to the possibility of parental cannibalism and manipulation for collection. However, there is a great advantage with fewer expenses for the production of fingerlings.

Conclusion

Out of reproduction season the semi natural and artificial methods can be applied in the reproduction procedure of the species, and the semi natural presents advantages by the ease of application, less manipulation of the reproducers and less labor employed.

POSTER 55

OBSERVATIONS ON THE USE OF SALMON GNRH ANALOGUE (sGnRHa) IN *Astyanax altiparanae*

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Introduction

In tropical fishes the use of carp pituitary extract (CPE) is still the main protocol to induce spawning. However, this technique shows unpredictability of a successful spawning. Thus, the aim of this study was to achieve knowledge about the use of the sGnRHa as a hormonal inducer for reproduction of lambari, which has been considered a good model for studies on reproduction.

Methods

Experiment 1: Six doses of sGnRHa were tested (1, 10, 20, 40, 80 and 160 µg kg^{-1}) as well as a saline control group (0.9% NaCl) and a positive control group (6 mg CPE kg^{-1}), with three replicates each. All females received a single intraperitoneal injection of 4 mL kg^{-1}. *Experiment 2*: Three doses of sGnRHa were tested (10, 100 and 1000 µg kg^{-1}) as well as a saline control group (0.9% NaCl) and a positive control group (6 mg CPE kg^{-1}), with four replicates each. In this assay all females received two intraperitoneal injection of 4 mL kg^{-1}, being the first one 10% of total dose and 12h later the second with the resolving dose. In both assays were used 10L experimental units, where five females (16.64 ±2.87g) and ten males (9.38 ±2.33g) were allocated. Males received a single dose of 3 mg of CPE kg^{-1}. Reproductive performance was evaluated considering spawning rate, relative fecundity (egg volume/body weight), fertilization and hatching rate. Data were analyzed by one-way ANOVA followed by the Tukey test at the $p \leq 0.05$ level of significance or by the Kruskal-Wallis test, a nonparametric test, depending on normality and variances homogeneity.

Results and Discussion :

Experiment 1: The response to sGnRHa was not in a dose-dependent manner. No ovulation was seen in the 1 µg kg^{-1} and saline group. A low proportion of females receiving higher doses of sGnRHa and CPE treatment ovulated. The spawning rate and relative fecundity were similar and relatively low in all groups that spawned ($p>0.05$). The fertilization and hatching were at least 50% in all groups. Thus, aiming mainly to improve spawning rate we increased and fractioned the doses. *Experiment 2:* The use of two doses improved reproductive performance of females, mainly for the positive control. Ovulation was seen in all groups, including in saline control. Relative fecundity was now higher in CPE treatment than all other groups, except for the highest dose of sGnRHa ($p<0.05$). However, the values of the latter were similar to all other groups. The highest spawning rate was seen in the CPE ($p<0.05$). The fertilization and hatching were similar between groups ($p>0.05$).

Conclusion: Although 1000 µg kg^{-1} fractioned sGnRHa dose has shown some results similar to the CPE, the latter is still better overall. With this preliminaries results, sGnRHa has not been shown to be a good hormonal inducer for reproduction of lambari.

POSTER 56

hCG HORMONAL INDUCTION IN WILD QUEEN DRUM (*Cynoscion albus*) IN CAPTIVITY.

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Introduction

The queen drum, *Cynoscion albus*, is one of the 34 species distributed along the Central América pacific coast, which is captured with a trammel in the Gulf of Nicoya. Because overfishing reports of sciaenid species in Costa Rica, the laboratory start a Sciaenid Reproduction Program to implement the reproduction in captivity. The first specie was weakfish *C. squamipinnis*, which spawning in captivity after 2.5 years of adaptation without hormonal induction. First generation also spawned applying the same protocols. However, wild queen drums captured and transported to the laboratory at the same conditions of weakfish, not respond to the captivity reproduction strategies applied to weakfish, although both species share the same habitats and reproduction behavior. The objective of this study was to evaluate the reproductive response of *C. albus* to hCG hormonal induction in captivity.

Methods

Wild fish (6 females and 6 males) were captured and transported by boat to the laboratory and were maintained in an 18 t external cylindrical fiberglass tank. The spawning conditions were Saran cover (80% shade), aeration (20 PSI), and daily water exchange (80%). Daily, fish were fed with fresh sardines at 2% body weight (BW), and maturity was observed every two months to reduce handling stress. Fish were anesthetized with eugenol (clove oil, 0.1 ml L-1), and ovarian biopsies were taken by inserting a plastic cannula. After 2.5 years, females and males were totally ripens, but not spontaneously spawn occurred. Females (n=3) were injected with a 300 IU/Kg BW, and males (n=2) with a 50 IU/ Kg BW. Fish and tank conditions were observed during latency period, and presence of floating eggs was indication of spawning. Eggs were collected and concentrate in a 2 lts volume, samples were taken (1 mL, n=3) and eggs counted.

Results and Discussion

After 2.5 years in captivity, females were mature at 11.7±1.0 kg BW, and males at12.5±1.0 kg BW. Males with a fluid sperm (1:30 minutes motility time), and female's ovary samples showed oocyte diameter of 500±20 µm, with gray-clear cytoplasm. Latency period after injection was 49 hours, when the eggs were detected in the collector. The first spawn was 522467 eggs, with a fertilization of 85%, and second was 116375 eggs with a fertilization of 80%.

Conclusion

C. albus respond to hormonal stimulation of hCG producing a good quality eggs and sperm. Although females reach the gonadal development in captivity, the final maturation and ovulation not occurred after adaptation period, like weakfish *C. squamipinnis*, which live in the same habitats. The results obtained confirm *C. albus* as strong candidate in possible restocking programs and mariculture.

POSTER 57

INDUCED REPRODUCTION AND SPERMATIC CHARACTERISTICS OF *Leporinus friderici* (Anostomidae, Teleostei) UNDER CAPTIVITY CONDITIONS

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Introduction

L. friderici is one of the most predominant and preferred fish species in the Colombian Amazon and require hormonal induction for it reproduction in captivity. The over-fishing of this species has affected the food security of its inhabitants, especially to the indigenous communities. Therefore, the Vaupés Governorate financed this macro project aimed to understand its reproductive biology (Special Cooperation Agreement 0032-2013, Sistema General de Regalías, Colombia).

Methods

Sixty broodstock of *L. friderici* from the Vaupes River were induced with three hormonal treatments: carp pituitary extract (CPE), Ovaprim® and CPE+ Ovaprim®. The fertilization rate, fecundity, relative fecundity and hatching rate were determined. In males the sperm concentration, gross sperm mobility and sperm activation time were assessed.

Results and discussion

The pre-induction oocyte diameter was 1151 ± 56 μm and post-induction diameter of 1365 ± 44 μm, the fertility rate for females induced with CPE was 84.5 ± 9%, in females induced with Ovaprim was 69.2 ± 11% and with CPE+Ovaprim was 79.0 ± 14% ($p<0.05$). The relative fecundity of *L. friderici* fluctuated between 28 to 157 g oocyte/kg and the absolute fecundity between 15.133 to 422370 oocytes/female. The hatching rate in fish induced with CPE was 46 ± 6.0%, with Ovaprim was 32.4 ± 1.5% and with CPE+Ovaprim was 40.2 ± 2.0% ($p<0.05$). The average seminal volume was 3.1 ± 2 ml. The higher sperm concentration was observed in males injected with CPE (28.632.000 of spermatozoa/ml) followed by CPE+ovaprim (26.040.000 spermatozoa/ml); however, males injected with ovaprim had the lowest sperm concentration (21.180.000 spermatozoa/ml, $p<0.05$). The sperm motility percentage in males induced with CPE was 81 ± 9%, with Ovaprim was 78 ± 10% and the combination CPE + Ovaprim 84 ± 5%; The average activation time was 47 ± 24 sec.

Conclusion

Although *L. friderici* responded effectively to hormonal protocols, the best results were obtained in fish induced with CPE. These results contribute to the reproductive knowledge of this species, information necessary to motivate its introduction in the regional aquaculture.

POSTER 58

MEIOSIS RESUMPTION AND EARLY MITOSIS AFTER SOMATIC CELL NUCLEAR TRANSFER IN NON-ENUCLEATED GOLDFISH OOCYTES.

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Introduction

The cryopreservation of genetic resources is of major interest for the security of biodiversity and for the sustainability of agronomic industry. Cryopreservation of somatic cells, which carry the genome of both parents, is one mean to regenerate functional breeders, but this requires the mastering of nuclear transfer. In mammals, somatic cell nuclear transfer consists in injecting the somatic nuclei into an enucleated mature recipient oocyte, in order to obtain clone which bear the genome of interest from the donor animal. However, in fish species, the oocyte structure makes the enucleation step difficult to achieve (presence of the chorion and bulk nutritive reserve), so a non-enucleated oocyte is often used for nuclear transfer. To obtain a real clone and not hybrid embryos, the oocyte chromatin must therefore be spontaneously excluded from the developing embryo. This phenomenon has been described in several studies but the mechanism involved is still unknown. Deciphering this phenomenon requires a high understanding of the first developmental steps that are meiosis resumption and early mitosis. The aim of the study is to understand the interplay between the maternal chromatin and the injected one upon nuclear transfer during these entire critical steps, in order to identify and use means to improve the success rate of nuclear transfer in fish.

Methods

Fin somatic cell and mature oocytes were obtained from 2 years old goldfish (*Carassius auratus*) females. For nuclear transfer, the whole fin cell was injected under the micropyle. The egg activation was induced 30min after cell injection. Somatic and maternal chromatin during meiosis resumption and early mitosis was characterized by immunofluorescence (hoechst and vybrant labelling for chromatin identification and α-tubulin for spindle organization).

Results and Discussion

We observed that a high proportion of clone underwent a normal polar body extrusion after activation (60% vs 96 % for the controls). We hypothesize that in most cases, maternal chromatin achieved correctly its meiosis resumption and that the maternal chromatin behavior was not disturbed by nuclear transfer. However, these observations do not augur early mitosis fitness in clone. Indeed, we observed several spindle defects in clone cells: metaphase misalignment, abnormal segregation, and multicentrosomal spindle, associated with DNA fragmentation.

Conclusion

Oocyte non-enucleation for SCNT procedure did not drastically alter the meiosis resumption process, but it could be involved in the perturbation of mitosis during clone early development.

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OOGENESIS
AND
VITELLOGENESIS

POSTER 59

ULTRASOUND AS A NON-INVASIVE TOOL FOR MONITORING REPRODUCTIVE PHYSIOLOGY IN FEMALE ATLANTIC SALMON (*Salmo salar*).

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Introduction

For maturation monitoring, methods requiring the sacrifice of fish (e.g. gonadosomatic index (GSI), gonad histology, gene expression) or invasive sampling (e.g endoscopy, blood samples) are usually used in research and for population management in both wild and commercially produced fish. We hypothesize that ultrasound can be used as a reliable non-invasive tool for monitoring Atlantic salmon reproductive physiology. We therefore compared ultrasound measurements with traditional invasive methods for maturation monitoring (i.e. GSI, sex hormone analysis and histology).

Methods

Female Atlantic salmon were examined regularly one year before their expected. Once a month, GSI was estimated and blood and ovary samples collected from 20 females. Ovary length was estimated, and a cross sectional image taken using a MyLab Alpha ultrasound unit (Esaote, Italy). Plasma concentrations of sex hormones (i.e. oestradiol, testosterone, 11-ketotestosterone, follicle stimulating hormone, luteinizing hormone and maturation inducing hormone were analysed using enzyme-linked immunosorbent assay (ELISA) kits from Cayman chemical (Ann Arbor, MI, USA). Ovary sections were H&E stained and oocyte development stage was determined according to Taranger *et al.* (1999) and Andersson *et al.* (2009).

Results and Discussion

Ultrasound-based length measurements of ovaries correlated strongly with GSI in females of Atlantic salmon and was used to established ultrasound-based GSI measurements to monitor maturation without sacrificing females ($R = 0.91$, $p < 0.01$). Ultrasound-based GSI measurements correlated with sex hormone levels ($R = 074\text{-}0.81$, $p < 0.01$) and cross-section ultrasound images revealed a visible change in oocyte size related to the progress of vitellogenesis confirmed by histology. This way of monitoring maturation offers a possibility to reduce stress and improve animal welfare in broodstock management of both wild and farmed Atlantic salmon populations.

Conclusion

Ultrasound technology is well suited to substitute traditional invasive methods during sexual maturation monitoring in Atlantic salmon broodfish populations.

POSTER 60

MIR-202 REGULATES FEMALE FECUNDITY BY CONTROLLING EARLY STEPS OF OOGENESIS IN THE MEDAKA OVARY

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Introduction

In fish, the female fecundity is closely linked to the proper completion of oogenesis that takes place in the ovary. An increasing number of studies in mammals have recently provided evidence that small non-coding microRNAs (miRNAs) play an important role in the regulation of this cellular process. However, data related to the role of miRNAs in the fish ovary remain rather scarce and *in vivo* functional evidence of the role specific miRNAs in the fish ovary has never been provided to date. In the present work, we aimed at studying the role(s) of *miR-202*, a miRNA specifically expressed in the vertebrate gonads, in the medaka ovary.

Methods

We first used fluorescent *in situ* hybridization to determine the precise cellular expression of *miR-202* in the medaka ovary. We then generated a mutant fish line (CRISPR/Cas9) to analyze its role in female fecundity and oogenesis. Phenotype of mutant females was analyzed at both cellular and molecular levels. Fluorescent imaging (2D and 3D) and image processing strategies were developed to analyze the cellular modifications. A genome-wide transcriptomic analysis (microarrays) was performed on mutant ovaries to analyze gene expression modifications.

Results and Discussion

Our results showed that *miR-202* is highly and specifically expressed in granulosa cells of ovarian follicles at all stages. Analysis of mutant females revealed that the inactivation of *miR-202* drastically reduced the fish fecundity (reduced number of spawned eggs) and impaired the egg quality. Further image analyses of mutant ovaries showed that early steps of follicular growth are affected, leading to an accumulation of small follicles and to a lower number of larger follicles. While data obtained by transcriptomic analysis were consistente with this cellular phenotype (*i.e.* down-regulation of genes encoding key steroidogenic enzymes such as *cyp19a1a* and *cyp17*), it also revealed the down-regulation of key genes expressed in early meiotic oocytes within the germinal cradle, including *foxl3* and *sycp3*. Finally, results shed light on novel molecular players that are dysregulated in mutant females, such as *setd4*, *npr1b* and *srgap3*.

Conclusion

Here, we provide evidence that *mir-202* is a key mircoRNA necessary for female reproductive success in medaka. Our results indicate that *mir-202* is necessary for the early steps of oogenesis. Thus, this study largely contributes to elucidate the regulatory mechanisms that control the early steps of oogenesis, in particular the follicle recruitment that remains poorly understood to date.

POSTER 61

SEPARATE AND COMBINED TREATMENT EFFECTS OF SIMULATED REPRODUCTIVE MIGRATION AND HORMONAL STIMULATION ON SEXUAL MATURATION IN EUROPEAN EELS.

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Introduction

In nature, European eels (*Anguilla anguilla*) sexually mature during and/or after their ~6,000 km reproductive migration. Experimentally, maturation can be induced by weekly injections with pituitary extract (PE; hypophysation) over a long period of 12-24 weeks. However, this long trajectory treatment compromises egg quality. In our studies, broodstock is conditioned from the early juvenile glass eel stage onwards by feminisation and simulated migration to advance maturation and reduce the hypophysation period. Here we present the results of the separate and combined treatment effects of simulated migration and hormonal stimulation on sexual maturation in eels of different background.

Methods : In a first experiment, groups of farmed, feminized and wild silver eels were subjected to simulated migration to investigate the effects on sexual maturation. A fourth group of wild silver eels was not subjected to simulated migration but received a 17-methyltestosterone (17MT) implant to compare treatment effects. In a second experiment, groups of farmed, feminized and wild silver eels were each split and did or did not receive a single PE injection after which they were all subjected to simulated migration to investigate whether more advanced maturation could be induced by the combined treatment. Simulated migration (2 months swimming covering ~3,000 km under mimicked photothermal conditions) was executed in a 3,600 L swim gutter. Before and after, eels were measured to determine changes in the eye index (EI); sampled for blood to measure plasma 17β-estradiol (E2) and 11-ketotestosterone (11KT) levels, and gonadosomatic index (GSI) and liver area (LA) were non-invasively determined by applying ultrasound.

Results and Discussion : Both experiments showed that simulated migration enhanced early maturation (higher EI and GSI). The 17MT implants had similar but stronger effects and increased GSI up to values of 4, known for eels that started vitellogenesis which was supported by increased LA. Eels that received a PE injection showed more advanced maturation after simulated migration (higher GSI) than non-injected eels but still below GSI values indicating yolk deposition. Feminised eels were more sensitive to treatment (higher GSI, plasma E2) than farmed eels. The hypophysation period was shortened after treatments. N=77 females fully matured, N=39 egg batches were fertilized, N=6 batches gave embryos and in N=3 cases larvae were obtained that survived up to 8 days.

Conclusion: Simulated migration and 17MT implants provide useful tools to enhance early maturation in eels and to reduce the hypophysation period which supposedly increases egg quality and reproductive success.

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POSTER 62

DE NOVO ASSEMBLY AND CHARACTERIZATION OF THE OVARIAN TRANSCRIPTOME IN SWORDFISH *Xiphias gladius* (LINNAEUS, 1758): FOCUS ON GENES DRIVING VITELLOGENIC PROCESS.

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Introduction:

The swordfish *Xiphias gladius* (Linnaeus, 1758) is a large, highly migratory mesopelagic species with a global distribution and seasonal migrations. An important swordfish fishery has developed over the last 20 years but catches started to decline from the 1980s. To date, information on swordfish reproduction is lacking and does not provide comprehensive insights into gonadal development, which are necessary to determine their reproductive output. Knowledge on female gonadal development is required to establish the duration of the spawning season, the size and age of maturity and spawning patterns. The aim of the present project was a *de novo* assembly and annotation of the transcriptome of X. gladius. Moreover, a differential expression analysis was performed in order to compare pre-vitellogenic and vitellogenic ovaries, highlighting all genes driving vitellogenic process in swordfish.

Methods: The Illumina HiSeq 2000 paired-end sequencing platform was adopted to sequence RNA isolated from 3 pre-reproductive and 3 reproductive ovaries. After quality check and trimming of low quality reads the transcriptome assembly was performed with Trinity v2.5.1. Then, the overall quality of the assembly was assessed by means of the BUSCO3 pipeline. Next, quantification of the assembled transcripts and identification of differentially expressed genes were achieved through the software Kallisto and the package NOISeq (False Discovery Rate: <=0.01), respectively. Finally, a Gene Ontology Enrichment Analysis (GOEA) and a Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway Analysis was performed. To validate the results, qPCR was carried out for a pool of genes of interest.

Results: A total of 100.869 sequences with N50 of 2.037 bp were generated from cDNA libraries of pre-vitellogenic and vitellogenic ovaries and a GO annotation was assigned to 30.398 transcripts. On the other hand, we identified 25.151 unigenes of which 22.433 and 2718 were annotated in Trembl and Swissprot database, respectively. The differential expression analysis between pre-vitellogenic and vitellogenic ovaries revealed a total of 6.501 transcripts differentially expressed, with 2494 predicted to be up-regulated in pre-vitellogenic ovaries. Furthermore, GOEA highlighted that the most differentially expressed transcripts within the category “Biological Process” were related to RNA/DNA processing, cell cycle regulation, endosome organization and transport and lipid metabolism. In addition, 2862 unigenes were mapped to 361 pathways in the KEEG database revealing that the most affected pathways are RNA/DNA processing, ovarian steroidogenesis, autophagy and apoptosis processes and lysosome formation/maturation.

Conclusions: This study provides the first *de novo* transcriptome analysis currently available for X. gladius, and identify many important functional genes, GO terms and KEGG pathways involved in swordfish oocyte quality. Results of the present study will facilitate future studies on swordfish reproduction.

Funding : This work was supported by Ministry of Agriculture, Food and Forestry Policies (MIPAAF), note 6775, Art.36 Paragraph 1 Reg (UE9 n 508/2014) to O.C.

POSTER 63

ANNUAL OOGENESIS OF FEMALE SWAMP EEL, *Monopterus albus* (Zuiew, 1793) BROODSTOCK, KHON KAEN, THAILAND

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Introduction

Swamp eel, *Monopterus albus* (Zuiew, 1793), is a protogynous freshwater fish species that belong to class Actinopterygii, order Synbranchiformes and family Synbranchidae. *M. albus* has a relatively high economical value among freshwater fishes in Thailand. Its yield is currently based on captured freshwater fisheries. Artificial breeding and culture of *M. albus* by imitating natural environment has been successfully demonstrated, but it was still limited in a small scale. Based on previous studies, breeding and reproductive biology of female *M. albus* broodstock in Thailand were not well understood. Gonadosomatic index (GSI) and fecundity were often reported, but annual oogenesis and developmental stages of oocyte have been over-looked. Thus, this study was aimed to characterize annual oogenesis and oocyte development for a precise identification of sex maturity of female *M. albus* broodstock and to define its breeding season.

Methods: A total of 60 female *M. albus* broodstock with 60 to 200 g body weight (BW), were obtained monthly from a local market in Khon Kaen, Thailand during December 2015 to November 2016. The female broodstock were acclimatized in concrete ponds (1.2x1.2 m^2) with about 150 liters of water for a week after that 5 of female broodstock were randomly taken. Individual BW was recorded and the ovaries was removed and weighed for calculating the GSI. Then ovaries were kept in Bouin's solution until tissue processing. Three segments of ovary (anterior, middle and posterior regions) were collected for gonadal histology analysis. In brief, the ovaries was embedded in paraffin and sectioned at 4 μm. The transverse sections were stained with haematoxylin and eosin stain (H&E stain). Oogenesis and oocyte development determination of each individual was examined.

Results and Discussion: Oocyte development of female *M. albus* broodstock were divided into 4 stages include perinucleolar, cortical alveoli, vitellogenic and maturation stage. In December to January, the dominant oocytes were perinucleolar and cortical alveoli oocyte, while in February to April, remaining oocytes were in cortical alveoli and vitellogenic oocyte. Oocyte diameter and GSI value gradually increased from 0.06$\pm$0.02 mm and 0.24$\pm$0.13 to 1.88$\pm$0.04 mm and 1.42$\pm$1.08 in December to April, respectively. Besides, last vitellogenic oocyte and mature oocyte were found at the beginning of April to August. This period, April to August, mature oocyte diameter vary from 1.58$\pm$0.11 mm to 2.00$\pm$0.32 mm which was close to the ripe eggs with a diameter of 2.50-3.00 mm. The biggest oocyte diameter and highest GSI value were 2.00$\pm$0.32 mm and 3.43$\pm$2.39 in May, respectively. Then, in September to November, oocytes turned into perinucleolar and cortical alveoli stages. Based on the stage of oocyte development, oocyte diameter and GSI value, this could be concluded that sex maturity and breeding season of female *M. albus* broodstock reared in captivity is from April to August

Conclusion: Female *M. albus* broodstock showed highest value of oocyte diameter and GSI from April to August suggested its breeding season.

POSTER 64

11-KETOTESTOSTERONE INDUCES OOCYTE GROWTH, BUT DOES NOT AFFECT OOCYTE CYTOLOGY IN PREVITELLOGENIC CAPTIVE GREAT STURGEON, *Huso huso*

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Introduction

Female captive great sturgeon usually fail to reach sexual maturity. The female maturation failure reflects a prolonged blockade of ovarian maturity at the prepubertal, previtellogenic stage. Recently, a role for the fish-specific androgen, 11-ketotestosterone (11-KT) in previtellogenic oocyte growth has been described in eels, salmon, and Atlantic cod. 11-KT can influence oocyte growth by facilitating lipid uptake, at least in eels. However, this mechanism does not seem to operate in more derived fishes, such as in the perciform wreckbass, the hapuka. High levels of 11-KT have been detected in the serum of female silver eels and vitellogenic sturgeon. It suggests that 11-KT is not only quantitatively, but also functionally important in eels and sturgeon. Therefore, this study investigates the effects of 11-KT treatment *in vivo* on ovarian growth, hormonal and biochemical changes, and ovarian lipidogenesis-related protein mRNA levels in female captive previtellogenic great sturgeon.

Methods

Twelve female captive previtellogenic great sturgeon (4-year old, mean body weight 5580 ± 680 g) were randomly divided between two groups. Fish received an intraperitoneal sustained slow-release implant of either 11-KT (2.5 mg 11-KT) or the compressed matrix (placebo). Ovarian biopsy was done to obtain pre-treatment baseline data on histology and target gene expression. The expression of lipidogenesis-related genes was measured by real-time quantitative PCR. Blood sampling was carried out to detect serum sex steroid levels using radioimmunoassay and to determine triacylglycerides and cholesterol by colorimetric assays. Ovarian biopsy and blood sampling were repeated three weeks after commencement of treatment.

Results and Discussion: After three weeks of treatment, the 11-KT treated sturgeon showed an increase in serum 11-KT levels from 2.2 to 83 ng/mL. However, 11-KT implants reduced the E2 serum levels in female great sturgeon from 0.09 to <0.07 ng/mL. 11-KT implantation, not that of empty pellets, increased oocyte diameters of fish after three weeks (from 259 to 309 µm). Regardless of the increase in oocyte size, ovaries remained in the previtellogenic stage, mostly presenting with late perinucleolar oocytes although few early cortical alveoli stage oocytes were found in some 11-KT implanted females. 11-KT treatment did not yield significant changes in serum levels of triacylglycerides and cholesterol. Meanwhile, at the molecular level, the expression of lipidogenesis-related transcripts (lipoprotein lipase, very low density lipoprotein receptors, apolipoprotein E, low-density lipoprotein receptor-related protein 8-like) were significantly up-regulated after 3 weeks. Taken together, these findings indicate the different fish taxa respond differently to treatment with 11-KT.

Conclusion: Administration of 11-KT increased 11-KT serum levels, oocyte size and expression of genes mediating lipid uptake. However, it was unable to advance ovarian development.

POSTER 65

RECOMBINANT FOLLICLE-STIMULATING HORMONE INDUCES VITELLOGENESIS AND SPERMATOGENESIS IN FLATHEAD GREY MULLET (*Mugil cephalus*)

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Introduction

The flathead grey mullet (*Mugil cephalus*) is a catadromous fish that does not spawn spontaneously in captivity. In Mediterranean aquaculture, few males produce flowing milt and females have dysfunctions at the early stages of vitellogenesis and oocyte maturation and ovulation. Research has focused on and hormonal therapies to address final maturation and spawning, but has been less successful in gametogenesis. Recently, promising results with the use of recombinant gonadotropins in finfish species have induced gametogenesis. Therefore, this study aimed to induce vitellogenesis and spermatogenesis in grey mullet using homologous recombinant follicle-stimulating hormone (mugil-rFSH), produced in Chinese Hamster Ovary (CHO) cells.

Methods : Two groups of 12 immature grey mullet (9♀ and 3♂, mean weight 990 ± 212g) received weekly intramuscular injections either of CHO cells medium (control) or mugil-rFSH at a dose of 15 µg.kg^{-1}. Approximately every two weeks blood and gonadal samples (with cannulas or abdominal pressure) were taken for analysis. Ovarian development was evaluated *in situ* examining the diameter of the most advanced vitellogenic oocytes (n=20) and in samples fixed for histology. The presence of milt was assessed and motility measured. Plasma levels of 11-ketoteststerone and 17β-estradiol (E_2) were measured in plasma throughout the experiment. Females with oocytes greater than 350 µm and rFSH-treated males received exogenous luteinizing hormone (LH) or human chorionic gonadotropin (hCG) (*Solea senegalensis*-rLH, hCG, hCG+Progesterone) in different consecutive weeks depending on the ovarian response. Statistical comparisons between groups were made with the Student's t-test.

Results and discussion: Initial significant increases ($P<0.05$) in oocyte diameter and E_2 levels were observed in the r-FSH-treated group. In six weeks, oocytes grew from 96.25±26.31 µm to 306.75±116.81 µm and E_2 levels increased from 37.90±29.47 pg.mL^{-1} to 267.34±120.78 pg.mL^{-1}. In the control group oocyte diameter and E_2 levels did not change from 92.62±17.16 µm and 33.86±16.25 pg.mL^{-1} respectively. Administration of just r-FSH induced the development to late-vitellogenic oocytes with a maximum diameter of 450 µm. Small quantities of viscous milt with high motility was obtained in rFSH-treated group, whilst no sperm was obtained in control group. The mugil-rFSH treatment induced gametogenesis. However, *Solea senegalensis*-rLH, hCG and hCG+Progesterone did not complete oocyte growth, maturation and spermiation.

Conclusion: The present study demonstrated the efficacy of mugil-rFSH in inducing E_2 secretion and vitellogenesis and spermatogenesis, but additional hormonal stimulus seems to be required to complete oocyte growth and maturation.

The project received funding from the European Union 7FP (GA603121, DIVERSIFY).

POSTER 66

OVARIAN HISTOLOGY AND REPRODUCTIVE STRATEGY OF THE INVASIVE SPECIES *Loricariichthys platymetopon* (LORICARIIDAE) ON A NEOTROPICAL FLOODPLAIN

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Introduction: *Loricariichthys platymetopon* was originally distributed in the lower Paraná River. In 1982, after the formation of Itaipu Reservoir, this species colonized the upper Paraná River and became one of the most abundant. Thus, this study i) describes the germ cell stages of *L. platymetopon* females; ii) recognizes the reproductive phases through the microscopic characteristics of the germ cells; iii) determines relative fecundity; iv) estimates relative fecundity for standard length and total weight and v) determine the reproduction sites during the period of investigations on the upper Parana River floodplain.

Methods: The samples of 252 individuals were collected on the upper Paraná River floodplain in March, June, September and December 2013, 2014 and 2015. Sampling was carried out in rivers (Paraná, Baia and Ivinheima), open lagoons (Patos, Guaraná, Garças and Ressaco do Pau Veio) and closed lagoons (Fechada and Ventura). The gonadal development phase was attributed macroscopically, considering the size and shape of the gonad, occupation in the abdominal cavity, evidence of vascularization, color, visualization of the oocytes and flaccidity. A sample from each gonad was fixed in Bouin solution for histological studies and a sample from a spawning capable ovary was fixed in 10% buffered formalin to estimate fecundity. The ovaries dehydrated in ethanol and embedded in Historesin were cross-sectioned and stained using Periodic Acid-Schiff/hematoxylin/metanil yellow. A 0.3g sample was removed from the ovary to estimate fecundity. Relative fecundity was estimated using gravimetric analysis based on the relation between ovary weight and the oocyte density in the ovary. The description of the main reproduction sites of the species was done using the female abundance in each reproductive phase found in each sampled site.

Results and Discussion: The oogenesis of *L. platymetopon* was characterized by the development stages oogonial proliferation, primary growth oocytes, secondary growth [vitellogenesis] oocytes and oocyte maturation. Different phases of atresia were observed in the secondary growth follicles. The germ cell types and their abundance in the ovarian lumen were used to characterize the reproductive phases: Development, Spawning capable, Regression and Regenerating. The relative fecundity varied from 2658 to 9942 oocytes (mean = 5704). The estimate of relative fecundity by weight was 25 to 46 oocytes g^{-1} (mean = 34). The relation between relative fecundity and length was 114 to 349 oocytes cm^{-1} (mean = 210). Relative fecundity is positively correlated with the total weight and the standard length, so that the larger the individual, the higher its fecundity. The frequency of individuals in different reproductive phases per sampling site revealed reproductive success in Guaraná and Fechada lagoons and the Baia River, followed by Patos and Garças lagoons and Ivinheima River.

Conclusion: We conclude that evaluation of fecundity by weight and length, associated with information about reproduction areas based on oogenesis, is an efficient management tool to evaliate the reproductive cycle. The individuals of this species are sedentary, possess a detritivorous feeding habit, predominate in lentic environments and generally reproduce there. These ecological characteristics permit their occupation and reproductive success on the floodplain, especially in the lagoons.

POSTER 67

GAINING INSIGHTS INTO THE INITIATION OF VITELLOGENESIS BY COMPARING THE EUROPEAN EEL (*Anguilla anguilla*) AND THE SHORTFIN EEL (*A. australis*)

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Introduction : A negative correlation exists between the maturation stage at the start of the oceanic reproductive migration and the migration distance to the spawning grounds for the various eel species. The European eel migrates up to 6,000 km and leaves in a previtellogenic state. The shortfin eel *A. australis* migrates 2-4,000 km and leaves in an early vitellogenic state. In this study, we compared previtellogenic European silver eels (*Anguilla anguilla*) with immature yellow eels, and early vitellogenic silver shortfin eels with yellow eels, to gain insights into the initiation of vitellogenesis.

Methods : Immediately after being caught and at the catch site, measurements were performed and eels (N=6 yellow and N=6 silver for each species) were sampled for blood and tissues. Eye index (EI), gonadosomatic index (GSI) and hepatosomatic index (HSI) were calculated. Plasma 11-ketotestosterone (11KT) and 17β-estradiol (E2) levels were measured by specific radio-immunoassay (RIA). Pituitary, liver and ovaries were dissected for quantitative real time PCR analyses (pituitary dopamine D2 receptor *d2br*, gonadotropin-releasing hormone receptors 1 and 2 *gnrhr1* and *2*, growth hormone *gh* and follicle-stimulating-hormone-β *fshβ*; liver estrogen receptor 1 *esr1*; gonad follicle-stimulating hormone receptor *fshr*, androgen receptors α and β *ara* and *b*, vitellogenin receptor *vtgr* and P450 aromatase *cyp19*). For each species, fold-change expression was determined of silver *vs*. yellow eels and these were compared between species.

Results and Discussion : GSI values of 3.0 ± 0.2% in silver shortfin eels reflected a vitellogenic maturation state while GSI values of 1.4 ± 0.1% indicated previtellogenesis in European silver eels. Plasma 11KT levels were much higher in shortfin than in European silver eels (82.3 ± 11.3 vs. 1.2 ± 0.3 ng mL^{-1}), whereas plasma E2 levels were higher in European silver eels (3.1 ± 0.5 vs. 1.5 ± 0.1 ng mL^{-1}). In the pituitary of shortfin eels, expression of *fshβ*, *gnrhr1* and *2*, and *d2br* was up-regulated in the silver-stage compared to yellow-stage females, in the gonads, *fshr*, *ara* and *b* expression. For the European eel, *fshβ*, *gnrhr1* and *2*, *d2br*, *fshr*, *cyp19*, *ara* and *b* expression did not show any change between yellow and silver eels. Expression of *esr1* in European eels was low while *esr1* expression was up-regulated over 100-fold in silver shortfin eels.

Conclusion : Comparison between the silvering European and shortfin eels suggests that pituitary dopaminergic signaling (*d2br*) is increased, whereas the brain-pituitary-gonad reproductive axis (*gnrhr1* and *2*, *fshβ*, *esr1*, *fshr*, and *ara* and *b*) is stimulated during the initiation of vitellogenesis.

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POSTER 68

PROGRESSION OF 'NATURAL' VITELLOGENESIS IN WILD-CAUGHT LONGFINNED EEL, *Anguilla dieffenbachii*, FROM SOUTHERN NEW ZEALAND

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Introduction

Study of the reproductive physiology of freshwater eels has been hampered by the prolonged offshore migration that these fish undertake. The longfinned eel, *Anguilla dieffenbachii*, is in a relatively advanced stage of gametogenesis as it departs New Zealand's shores, providing opportunities to gain insights that have hitherto remained obscure. Indeed, as oocyte diameters may reach 0.4 mm by the time the animals emigrate, information on progressive change in the ovary can be collected. Here, we report on the change in physiological state (oocyte cytology, ovarian gene expression, blood chemistry) of female longfins in southern New Zealand (Lake Manapouri) between February–May (austral summer-autumn); in keeping with global concerns for eel stocks and with the value of eels to New Zealand's indigenous Maori people, samples were collected using non-terminal approaches.

Methods

Female longfins were collected as part of Meridian Energy's trap-and-transfer programme. Fishers selected fish that were deemed most advanced (closest to migratory readiness) on the basis of eye size, head shape and general appearance. Within several hours of capture, biometrical measurements were made and a blood sample (caudal vein) and ovarian biopsy (needle aspiration) were collected for analysis by histology, target gene quantitative PCR and steroid radioimmunoassay.

Results and Discussion

Fish presented with previtellogenic or very early vitellogenic ovarian follicles in February (~ 0.24 mm). Oocytes increased in diameter in a seemingly linear fashion (~ 0.05 mm per month), reaching 0.40 mm in May in the most advanced specimens. The increase was accompanied by extensive accumulation of yolk granules and globules in the cytoplasm. Biochemical and molecular analyses are in progress. Our data indicate that onset of puberty in these fish occurs as early as mid-summer, earlier than may be expected on the basis of the time of out-migration. Data on rate of oocyte growth may be of interest for captive breeding programmes.

Conclusion

Notable growth of ovarian follicles, up to the midvitellogenic stage at a size of at least 0.40 mm, occurs in longfinned eels during a period that appears to extend over around 3 months.

POSTER 69

EXOGENOUS GONADOTROPINS ADVANCE OOCYTE DEVELOPMENTAL STAGE IN PREVITELLOGENIC NEW ZEALAND SHORTFINNED EELS (*Anguilla australis*)

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Introduction

Gonadotropins (Fsh: follicle-stimulating hormone and Lh: luteinizing hormone) are key mediators of gametogenesis in vertebrate animals. Their involvement in cyclical gonad growth, culminating in the production of fertilizable gametes, are well-documented. In fish, however, the function of Fsh and Lh during the earlier stages of oocyte development (previtellogenesis) remain little understood. The objective of this study was to elucidate the response of previtellogenic fish to exogenous purified gonadotropins. For this purpose, we used the eel, *Anguilla australis*, as the animal model to evaluate the effects of administration of recombinant eel follicle-stimulating hormone (rec-Fsh) or the Lh-analogue human chorionic gonadotropin (hCG).

Methods

Eels received weekly intraperitoneal (IP) injections of either phosphate-buffered saline (control group) or of rec-Fsh (20, 100 or 500 $\mu g.kg^{-1}$) or hCG (20, 100 or 500 $IU.kg^{-1}$). Two days after the third, final injection, fish were euthanized (0.3% benzocaine) for tissue sampling. The expression of target genes was measured by real-time quantitative PCR, while plasma sex steroid levels (11-KT and E2) were obtained using radioimmunoassay. In addition, the changes in GSI and oocyte diameters were recorded.

Results and Discussion

Rec-Fsh or hCG induced an increase in GSI. Similarly, oocyte diameters increased from around 85 μm in controls to over 100 μm in rec-Fsh-treated eels. Both rec-Fsh and hCG increased the plasma E2 levels (0.7 ng/ml *vs* 0.1 ng/ml), while only hCG treatment yielded a significant increase in plasma 11-KT levels in comparison with control fish (5.0 ng/ml vs. 2.7 ng/ml). Both rec-Fsh and hCG up-regulated the expression of *fshr* and *lpl* in the ovary. Meanwhile, mRNA levels of *lhr*, *ldlr* and *cyp11b* were not significantly changed by any of the treatment regimes. Both rec-Fsh and hCG decreased pituitary *fshb* mRNA levels, but significant differences between treatments were only found in response to hCG exposure.

Conclusion

Treatment with exogenous Fsh or hCG advanced the developmental stage of ovarian follicles. We contend that this finding is compelling evidence for actual involvement of gonadotropic hormones in oocyte growth during previtellogenesis in eels, and possible, teleost fish at large.

POSTER 70

DISSECTING THE MOLECULAR AND CELLULAR MECHANISMS OF OOCYTE ATRESIA IN EUROPEAN HAKE (*Merluccius merluccius*)

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Introduction

Reabsorption of oocytes, in a process termed atresia, is a physiological energy-saving process during fish ovarian development and folliculogenesis. However, this phenomenon could become pathological under contaminant exposure or after prolonged starvation. The study of fish oocyte quality is a crucial requirement for scientifically based estimations of stock fecundity in fisheries or for aquaculture management. Extended oocyte atresia is associated with decreased fecundity, thus, understanding how this process is molecularly controlled under different circumstances would help developing early warning biomarkers of reproduction failure in fish. In this study an important commercial species for European fisheries hake (*Merluccius merluccius*) was selected as an indeterminate fecundity species showing episodes with high rates of oocyte atresia.

Methods

Females hakes (150) were collected during April-June 2016 and March-May 2017 in the Bay of Biscay (23 to 30 samples per month) and ovaries were sampled for histology, apoptosis identification by the TUNEL-assay, and qPCR quantification of transcript levels of apoptosis (*caspase-3, p53, mdm2, bclX2, fshr*) and autophagocytosis (*beclin-1, PTENb*) related genes.

Results and Discussion

Most of the hakes were in previtellogenesis, but individuals were also identified with ovaries also at cortical alveoli, and at (late)-vitellogenic stages. Atresia was histologically detected in around 25% of the samples. Atretic previtellogenic oocytes were TUNEL-positive with stained nuclei. Labelling was also conspicuous in hypertrophied granulosa cells. At more advanced developmental stages theca cells and granulosa cells were TUNEL-positive, but not oocytes. Transcription levels of *caspase-3*, *p53* and *beclin-1* were significantly higher in ovaries displaying atretic follicles in previtellogenesis, cortical alveoli and vitellogenesis stages. On-going western blot and immunohistochemicals analysis of p53 and mdm2 levels will tell us the relevance of apoptosis at the cellular level in hake ovaries.

Conclusion

The results hereby indicate that oocyte-atresia in hake is regulated by both autophagocytosis and apoptosis, depending on the oocyte developmental stage. Such mechanisms could be studied to anticipate scenarios of massive atresia and skipped spawning so as to correct fecundity estimations during evaluation of fish stock dynamics. **Funded:** Basque Government (IT810-13), UPV/EHU (UFI 11/37), Spanish MINECO and EU-FEDER/ERDF (AGL2015-63936-R).

POSTER 71

PRODUCTION OF DONOR-DERIVED EGGS AFTER OVARIAN GERM CELL TRANSPLANTATION INTO ADULT GONADS OF GERM-CELL DEFICIENT HYBRID CROAKERS

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Introduction : Spermatogonial transplantation directly into the testes of adult fish, pioneered in tilapia by Lacerda, França and colleagues, enabled donor spermatogenesis and the production of donor-derived offspring. In this study, we applied this approach to produce donor-derived eggs after ovarian germ cell transplantation into the germ-cell deficient gonads of a pelagic-egg spawning marine teleost.

Methods : Interspecific hybrid offspring obtained from a cross between blue drum, *Nibea mitsukurii*, females and white croaker, *Pennahia argentata*, males possessed diminutive gonads without germ cells. Direct transplantation of testicular germ cells into the adult gonads of the hybrid recipients resulted in the production of functional sperm in a short time period, indicating that the sterile hybrid fish was an ideal recipient for germ cell transplantation. However, all of the germ-cell deficient gonads of the hybrid fish had a testis-like morphology and showed a male-specific gene expression pattern. Thus, in order to obtain germ cell-less gonads with ovary-like characteristics, chromosome set manipulation was applied to fertilized hybrid eggs to induce triploidization. The gonads of the triploid hybrids were examined histologically and subjected to RT-PCR analysis with female-specific gene primers. In addition, ovarian germ cells were collected from homozygous pHSC-GFP transgenic (GFP +/+) blue drum and transplanted into the gonads of triploid hybrid croakers through the urogenital papilla or oviduct. After 9 months, the transplanted recipients were crossed with wild-type blue drum (GFP -/-) and the F1 offspring were analyzed by PCR with GFP-specific primers.

Results and Discussion : Thirty one percent of the triploid hybrids possessed germ cell-deficient gonads having an ovarian cavity. *cyp19a1a* and *foxl2* were expressed in these gonads, while *dmrt1* and *vasa* were not expressed, suggesting that ovary-like germ cell-deficient gonads developed in the triploid hybrids. Another 26% of the triploid hybrids possessed testis-like germ cell-deficient gonads that were similar to the diploid hybrids. Among 6 triploid recipients that survived 9-month post transplantation, 1 female and 1 male produced eggs and motile sperm carrying GFP-specific DNA sequences, respectively. Progeny tests revealed that all of the F1 offspring from these recipients possessed GFP-specific DNA sequences, suggesting that these triploid hybrid recipients produced only donor-derived eggs and sperm. Histological observation confirmed the occurrence of gametogenesis in the recipient gonads.

Conclusion : The present study demonstrated the production of donor-derived eggs in sterile hybrid triploid recipients through transplanting ovarian germ cells directly into the gonads of adult fish. The findings indicate that the microenvironment of the germ cell-deficient gonads of the triploid hybrid recipients supported the oogenesis of donor germ cells. The mechanisms underlying ovarian differentiation of the germ cell-deficient gonads observed in triploid hybrids are currently under investigation.

POSTER 72

MORPHOLOGICAL CHARACTERISTICS OF OVARIAN DYNAMICS OF *Leporinus friderici* (Anostomidae, Teleostei) IN VAUPÉS RIVER, COLOMBIA.

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Introduction

Leporinus is a representative fish genus of the Amazon region being a great source of protein for the indigenous communities of the Vaupés state, Colombia; however, it has been affected by high fishing pressure by the indigenous communities inhabiting the region surrounding the Vaupés River, causing a decrease in this fish population. Therefore, the Vaupés Governorate financed this macro project aimed to understand its reproductive biology (Special Cooperation Agreement 0032-2013, Sistema General de Regalías, Colombia). In Amazonian species such as *Leporinus friderici* there is still a lack of information on biological aspects that allows the determination of its reproductive characteristics and gonadal morphology.

Methods

In order to evaluate the gonadal dynamics of *Leporinus friderici* females (known as warakú tres puntos) during one hydrobiological cycle, ovaries were collected monthly for 12 consecutive months from the Vaupés River, Colombia. The gonads were fixed and processed with routine histological techniques, stained with hematoxylin-eosin and examined under the optical microscope. The stages of gonadal maturation, the hepatosomatic and gonadosomatic indices and the oocytes morphometry were determined histologically. In addition, the hepatosomatic and gonadosomatic indices were correlated with the hydrobiological cycle.

Results and Discussion

The ovaries of *L. friderici* are even and elongated symmetrical sacs located ventrally to the swim bladder which merge into a single oviduct. The ovaries coloration changed according to the maturation. Oocyte development is characterized by six oocytes stages: oogonia (82.25 µm), early perinuclear (89.28 µm), late perinuclear (149.1 µm), previtellogenic (389.09 µm), vitelogenic (722.79 µm) and atretic. The scale of gonadal maturation consists of 5 stages: A, immature or virgin; B, in maturation; C, mature; D, empty or in recovery and E, in rest. The gonadosomatic - GSI (fluctuates from 0.1 a 9.5 %) and hepatosomatic - HSI index expressed the highest levels in the months of March, April and May, when the females of *L. friderici* were in maturation and mature stage.

Conclusion

The oocyte development, the gonadal maturation scale, the GSI and HSI of *Leporinus friderici* females were determined showing that the reproductive stage of the females was during the months of March, April and May, related with rainy season and high temperatures, which are characteristics directly associated to the gonadal maturation season of fish from the genus *L. friderici*.

OVULATION
AND
EGG QUALITY

POSTER 73

THE IMPACT OF POST-OVULATORY AGEING ON THE DEVELOPMENT OF DIPLOID AND TRIPLOID ATLANTIC SALMON (*Salmo salar* L.).

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Introduction

A common observation in domesticated triploid salmon populations is the larger spread of growth in fry and parr, and increased mortality in early egg (i.e. pre-eyeing) stages compared to diploid siblings. Many of the suggestions that stem from earlier results point to early life stage developments which have yet to be explored. Good egg quality is the fundamental biological requirement for the adequate development of an individual. One factor that may influence egg quality is post-ovulatory ageing i.e. the length of time between ovulation and manual stripping of the broodstock. Post-ovulatory ageing can contribute to decreased egg quality which could be a likely factor contributing to this phenotypic difference. Ageing of the eggs post-ovulation may alter the composition to a "sub-optimal" status.

Under routine commercial practice in Atlantic salmon aquaculture, females may be stripped up to 21 days post-ovulation with no deleterious effects on offspring in "normal" diploid production protocols. However, egg-ageing effects need to be explored to determine what impact this has on egg quality and development in diploid and triploid Atlantic salmon and whether different "ageing thresholds" can / should be defined for commercial practice.

Methods

Females (n = 7) will be partially stripped over 5 day increments to generate populations of eggs with varying ages post-ovulation (0, 5, 10, 15, and 20 days). Egg quality indicators will be investigated in all groups. To assess the maternal influence on egg quality, all populations will be fertilization from the same male. Diploid (2N) and triploid (3N) Atlantic salmon eggs will be incubated in parallel and grown to ~5g. At this final stage, a final assessment will be conducted to assess the response of ageing on development in both ploidies.

Results and Discussion

A suite of ovarian fluid and egg quality indicators including; fatty acid composition, lipid class, proximate composition, carotenoid content, vitamin and mineral content, and lipid peroxidation assessment will be presented. Differences of compositions and quality between different ages post-ovulation will be compared.

Conclusion

Understanding the impact of post-ovulatory ageing may suggest refinements to the current stripping protocols for production of diploid and triploid Atlantic salmon. Identification of ploidy specific egg ageing thresholds may help prevent negative impacts on survival and growth. Investigating a range of egg quality parameters may highlight particular biomarkers that could be used as real-time indicators to determine the potential of egg populations.

POSTER 74

SPAWNING PERFORMANCE, EGG QUALITY AND PLASMA CONCENTRATIONS OF FOLLICLE-STIMULATING AND LUTEINISING HORMONES AND SEX STEROIDS, IN FARMED AND WILD-CAUGHT FEMALE ATLANTIC HALIBUT (*Hippoglossus hippoglossus* L.)

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Introduction

Atlantic halibut are group-synchronous, periodic spawners and in captivity wild-caught females release 6-10 batches of eggs in the spawning season, which lasts from late February to late April in southwestern Norway. While wild-caught females generally adapt well in captivity, displaying high fecundity with egg batches spawned at regular intervals, hatchery-produced F1/F2 females appear to suffer from a reproductive dysfunction, releasing small batches of eggs at irregular intervals.

Materials and methods

Reproductive performance of wild-caught halibut and farmed (F1) females was compared by strip-spawning and fertilization of eggs. Eggs were photographed for size measurements. For calculation of hatching rate, fertilized eggs were incubated in darkness at 6°C. Newly hatched larvae were photographed. Blood samples were taken from the same wild-caught and farmed females at 3-6 week intervals. After centrifugation of blood, plasma was frozen immediately on dry ice and stored at -80°C. Estradiol-17ß, testosterone, follicle-stimulating hormone (Fsh) and luteinizing hormone (Lh) were analysed by ELISA.

Results and Discussion

Realised fecundity was identical in wild-caught and farmed females, but ovulatory intervals were more irregular, and fertilization and hatching rates were lower in the farmed broodstock. Plasma E2 was elevated during autumn in both groups and reached peak levels in February. E2 remained high during the spawning period, then decreased and remained low for the rest of the sampling period. Plasma T concentrations were low until spawning, when a peak was observed. Farmed females had lower plasma Fsh concentrations during vitellogenesis than wild-caught fish. The individual variation was high, but Fsh concentrations decreased during spawning. This decrease was more pronounced in wild-caught females. Plasma Fsh increased after spawning. Plasma Lh concentrations were relatively high from September to December. Just before spawning, Lh concentrations appeared to decrease. Highest plasma Lh concentrations were seen during the spawning period.

Conclusion

Farmed females of the F1 generation had a somewhat lower reproductive success and lower Fsh concentrations than wild-caught females. Individual variations were high, however, and further studies, as well as careful selection of breeders, are needed to improve reproductive development and spawning performance.

PHEROMONES
AND
BEHAVIOR

POSTER 75

REPRODUCTIVE BEHAVIOUR OF THE SENEGALESE SOLE (*Solea senegalensis*): A REVIEW WITH INDICATIONS THAT REPRODUCTIVE BEHAVIOURS ARE NOT INNATE.

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Introduction

Senegalese sole (*Solea senegalensis*) has good aquaculture potential and a rapidly increasing aquaculture production. However, cultured sole broodstocks (reared from egg) do not spawn viable eggs and production relies on the spawning of captive wild fish. This paper reviews studies that described the courtship in wild caught sole and the reproductive behavioural dysfunction in cultured sole.

Methods

The nocturnal reproductive behaviour of groups of wild and cultured sole was studied using video recording. The broodstocks (1.1±0.1 to 1.7±0.1kg, and sex ratios ≈1:1) were held in natural conditions. An ethogram and a photo-video ID catalogue was used for behavioural analysis. Microsatellite parentage analysis was used to identify the paternity of viable spawns. The behaviour and spawning were registered for different broodstock groups: all wild captive breeders (positive control), all cultured breeders (negative control), wild males with cultured females, cultured males with wild females and fully mixed wild and cultured broodstocks with males and females of both origins. Finally, cultured broodstock groups with different life experiences were formed to compare cultured breeders that had experience of wild fish spawning before and during puberty with cultured breeders with no experience of spawning.

Results and Discussion

Wild sole courtship and spawning was complex and initiated with chasing behaviours, principally involving males. The courtship finished with a female accepting a male to form a pair that performed a coupled swim to the surface to spawn. Generally, a few pairs that show a degree of fidelity dominated the spawning. Cultured breeders exhibited a lower incidence of courtship behaviours and no paired spawning, which confirmed that eggs were not fertilised. Wild males and cultured females completed courtship and spawning, whilst cultured males and wild females produced unfertilised spawns. Therefore, the behavioural reproductive dysfunction was a specific cultured male problem. In the mixed broodstocks of males and females of both origins, the cultured males increased participation in the courtship over the years and a cultured male spawned with cultured females in seven spawns over two years. Similarly, a cultured male spawned eight times with a cultured female in the cultured broodstock that had experienced spawning before and during puberty. Control groups with no experience of successful spawning produced unfertilised eggs.

Conclusion

Cultured male breeders appeared to learn the reproductive behaviours, which suggested that reproductive behaviour and spawning was not innate. However, the low participation of breeders, both wild and cultured frustrates solid conclusions. Funded by INIA-FEDER RTA2014-0048.

POSTER 76

DIETARY L-TRYPTOPHAN MODULATES AGONISTIC BEHAVIOR IN MALE DYADIC ENCOUNTERS AND BRAIN SEROTONIN IN A SOUTHAMERICAN CICHLID FISH

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Introduction

Certain neurotransmitters, such as serotonin (5-HT), seem to play a central role on agonistic behavior. We can take advantage of the fact that l-tryptophan (Trp) is a precursor of 5-HT, and indirectly manipulate brain concentrations of the latter neurotransmitter. Most researches on dietary Trp effects on fish agonistic behavior have focused on juvenile but not adult agonistic repertories. In the present work we set out to study agonistic behavior between adult males of the South American cichlid fish Cichlasoma dimerus, in dyadic encounters held in a novel context after being or not fed during two weeks with a Trp enriched diet. We also registered the response of brain serotonergic system in four anatomical areas.

Methods

During the first 3 days of isolation, we fed males with commercial pellets. For the following 14 days, they either continued with a control diet (CTL protocol) (n= 24) or received an 8 times supplemented Trp one (n= 24).Once the dietary period ended, both males were placed in a novel aquarium and video recorded (1 h) to evaluate their agonistic repertories. Three different combinations of dyads where tested: CTL/CTL (CC), CTL/TRP (CT), and TRP/TRP (TT) (n = 8 for each combination). At the end of video registrations, we immediately anesthetized both fish and euthanized them by decapitation. We rapidly dissected brains to posteriorly evaluate 5-HT and 5-HIAA (5-HT's main metabolite) concentrations in four brain regions: telencephalon (Tel), preoptic area/hypothalamus (Poa/Hyp), optic tectum (Ot) and brainstem (Bs).

Results and Discussion

Males within TT dyads took twice as long to perform the first attack with respect to CT and CC ones. The relative proportions of bites and passive copings were lower than expected (CC) for TT dyads. Regarding inter-individual behavior, TRP dominant males performed 3 times less bites, whereas subordinate males confronted to TRP males showed 2.5 times less passive copings. The diet had no clear effect on which male resulted to be DOM; instead, opponents' body size differences determined who resulted DOM and SUB. Regarding serotonergic outcomes, SUB males were characterized by higher [5-HIAA/5-HT] at their Ot. On the other hand, TRP fed males showed higher [5-HIAA/5-HT] at their Tel and Poa/Hyp.

Conclusion: In the present research, we showed that dietary Trp reduced the motivation to attack, and modulated both aggressive and submissive behaviors, the latter of which have been generally unconsidered. In addition, Trp supplementation produced a switch on males' agonistic repertory. Modifications at the brain serotonergic accompanied these behavioral outcomes.

POSTER 77

CHEMICAL COMMUNICATION MODULATES REPRODUCTION IN THE SAILFIN MOLLY

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Introduction

Chemical communication is poorly understood in live-bearing fish. Previous behavioral studies in the sailfin molly, *Poecilia latipinna*, have found that this fish can identify conspecific females using only chemical signals. However, the characterization of chemical signals, release routes and olfactory detection of chemical cues, together with the associated reproductive behavior, has not been established. In the present study, we used behavior, endocrinology, electrophysiology and histology to characterize chemical communication in sailfin molly.

Methods

To allow visualization of urine pulses, females were injected with blue dye. Then one mature female and two males were placed together and mating behavior was video recorded for 10 minutes to quantify reproductive behaviors. Before and after each behavioral trial, fish were placed in 5 L of clean water for three hours to collect waterborne sex steroids. Sex steroids were analyzed by solid-phase extraction followed by Liquid Chromatography-Mass Spectrometry. Brain, gonad, gill, nose, and plasma were also collected for biochemical and histological analysis. Additionally, males were placed in a two-choice arena, then female extracts were released in one side of the arena for 5 minute trials. The latency and time spent in each side was measured.

Results and discussion

In our experiments, we characterized mating behaviors in *P. latipinna*. Dye treatment compromised mating behavior; mating only occurred in 54% of dye trials, compared to 100% in control individuals. Moreover, there was no significant correlations between male behaviors and female urine pulses. However, preliminary two-choice arena experiments indicated that male *P. latipinna* are attracted to female extracts. Thus, females release chemical signals that can be detected by olfaction. It is unclear if these signals are urinary. The olfactory epithelia were a paired organ with a single lamella, located anterior to each eye, within a large cavity in the nares. The largest surface area of the lamella is approximately 3 mm. Nose sections revealed a sensitive epithelium with mucus cell clusters accompanied by ciliated cells. Preliminary electro-olfactogram recordings showed a sensitive olfactory epithelium. We anticipate a differential release of sex steroid between male and female sailfin molly during mating. The identified compounds will be tested for olfactory sensitivity by electro-olfactogram.

Conclusion

P. latipinna has sensitive olfactory epithelia and use chemical cues to modulate reproductive behavior.

POSTER 78

DO CULTURED MALE SENEGALESE SOLE (*Solea senegalensis*) HAVE TO LEARN REPRODUCTIVE BEHAVIOUR?

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Introduction

Cultured male Senegalese sole (*Solea senegalensis*) do not complete courtship and fertilise eggs liberated by females. The objective of the present study was to determine the effect of the presence of wild sole with reproductive success on the participation in courtship and reproduction of male cultured sole.

Methods

Two experiments were conducted with wild and cultured broodstocks. In the first experiment, three groups were used, two mixed origin groups: M1 and M2 with wild and cultured breeders and one control group with only cultured breeders. In the second experiment four groups all formed of cultured sole were used, two groups (Exp1 and Exp2) with fish that experienced cohabitation with successfully spawning wild fish during the juvenile stage, and two control groups, one that experienced cohabitation with cultured breeders that did not successfully spawn and a second that was reared in isolation (normal aquaculture practice). A range of parameters were measured and analysed in each group: locomotor activity, counts of courtship behaviours, identification of fish participating in courtship behaviours, number of spawns, spawn quality and paternity of fertilised spawns (microsatellites analysis). Statistical tests ANOVA and t-test were used to compare amongst groups for each experiment.

Results and Discussion

Groups M1, M2, Exp1 and Exp2 produced more spawns and a higher fecundity than control groups. The only fertilised spawns obtained were from groups M1, M2 and Exp1. No fertilised spawns were obtained from the three control groups. Paternity analysis indicated that four spawns from group M2 and eight spawns from group Exp1 involved cultured males. The presence of spawning wild fish either during the reproductive season or during rearing in the juvenile stage appeared to increase the participation of cultured males in the spawning. This was partly confirmed as in the first experiment, cultured males in groups M1 and M2 exhibited significantly more involvement in courtship behaviours compared to cultured males in the control group. However, paternity analysis indicated that just two cultured males were involved in the fertilisation of the spawns.

Conclusion

Two cultured males that experienced cohabitation with spawning wild fish participated in courtship and spawning to fertilised eggs. It would appear that cultured males need to experience and learn the courtship behaviour to participate in successful spawning.

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POSTER 79

DOMINANCE BEHAVIOURS AND SPAWNING IN SENEGALESE SOLE (*Solea senegalensis*) BROODSTOCKS

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Introduction

The hierarchy of dominance regulates social interactions and reproductive capacity. Juvenile Senegalese sole (*Solea senegalensis*) that dominated feeding also dominated place preference and some social behaviours. Senegalese sole breeders present dominance of reproductive success with a few wild breeders dominating spawning, whilst cultured breeders (reared from egg) do not participate in spawning. This paper aimed to explore the relationship between dominance of spawning, feeding and place preference.

Methods

Fish from two broodstocks were classified by reproductive success into three groups: fish that spawned, fish that participated in courtship and fish that neither spawned or participated in courtship. These breeders (n=24) were given paired dominance tests for feeding and place preference (sand area). The pairs were placed in a 400L tank and maintained separated by a wall. After one acclimation night, the wall was removed and feed was introduced two hours later. The fish were video recorded for 24 hours. The following parameters were registered: first fish "feeding"; five behaviours for each fish, "approaches" by one fish to another, "swimming" over another fish, "resting the head" (RTH) on another fish, "displace" another fish and "burying"; and position in the sand area, especially during the "last" two hours of the test, which coincided with the morning resting period. Finally, blood samples were taken to determine plasma 11-Ketotestosterone and 17β-Estradiol. The t-test was used to compare means, Chi-squared to compare proportions of the behaviours associated to dominance and a Principal Component Analysis (PCA) to group variables.

Results and Discussion

The PCA did not group the dominance parameters: "approaches", "RTH", "feeding" and "last" (position in the sand) as observed in juvenile sole. The proportion of fish that dominated the sand area "last" was significantly higher in fish that spawned (75%) compared to fish that did not spawn (31%), whilst fish that dominated the "feeding" was significantly lower in fish that spawned (13%) compared to fish that did not spawn (44%). Fish that participated in spawning and courtship exhibited significantly ($P<0.05$) more "approaches" and "displace" behaviours compared to fish that did not spawn. There were no differences in the proportions of dominate fish for the dominance measure "RTH" and "approaches" or behavioural counts for "swimming", "RTH" and "burying".

Conclusion

Broodstocks present a complicated dominance relationship between behaviours. Fish that dominated spawning and courtship presented spatial dominance and more behaviours in relation to approaching and displacing other individuals. Funded by INIA-FEDER RTA2014-0048.

POSTER 80

HIGH TEMPERATURES REDUCE AGGRESSIVENESS AND STIMULATE REPRODUCTIVE BEHAVIOR IN *Hypancistrus zebra*

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Introduction

Hypancistrus zebra is an endemic species of the Xingu River (Amazon Basin), coveted ornamental fish that suffers from clandestine fishing and the destruction of its habitat due to the construction of a hydroelectric plant. In this context, aquaculture appears as an alternative to avoid extinction of the species. Considering the role that temperature plays on fish's metabolism and the lack of information on the species, we aimed to describe the behavioral repertoire exhibited by *H. zebra* and to generate information to support its well-being and reproductive success under captivity conditions.

Methods

Sixty animals distributed in groups of 4 subjects at a 2:2 sexual ratio were exposed to three temperature ranges (26-27.5°C, 29-30.5°C and 31-32.5°C) and filmed for two periods of 15 days, being the behavioral observation performed by *Ad libitum* and Focal sampling methods with continuous recording.

Results and Discussion

It was possible to identify nineteen behavioral units arranged in four categories: agonistic, locomotion, feeding and reproduction, being all of them described by means of an ethogram. The lower temperature made the animals more aggressive and evidenced males as responsible for most hostile actions (lair defense, displaying, confrontation and chasing); however, this aggressive behavior was minimized over time. Our study confirms the territorial behavior of the species, demonstrating that the grouping time influences the hierarchical organization of the population whereas the beginning of the reproductive behavioral actions depends on the agonistic actions. The finding of ripple behavior and its relation to reproduction in *H. zebra* is described here for the first time.

Conclusion

H. zebra should be reared in a temperature range between 29 and 30.5°C, respecting the grouping time and establishment of a social hierarchy, which will eventually lead to manifestation of the reproductive behavior.

MIGRATION/REPRODUCTION OF NEOTROPICAL FISH

POSTER 81

DESCRIPTION OF PEJERREY *Odontesthes bonariensis* GONADAL STAGES DURING SPAWNING SEASON IN COCHICÓ SHALLOW LAKE (PAMPAS REGION, ARGENTINA). RELATION WITH SEX STEROIDS PLASMA LEVELS AND TEMPERATURE

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Introduction

In this study it was analyzed the reproductive status of pejerrey (*Odontesthes bonariensis)* population during the spawning season in relation with plasma sex steroids levels and air temperature in Cochicó lake. This lake belongs to a System of *Pampas* lakes that extends between the parallels 36° 30 "and 37 ° 30" S and meridians 61 ° 00 "and 63 ° 30" W, not studied yet and surrounded by an intense agricultural activity.

Methods

Pejerrey of both sexes were caught using gill nets in Cochicó lake in the beginning of spawning season (August), peak of the spawning (October) and at the end of the spawning (December) of 2015. Fish were measured, and a piece of a gonad of each fish was dissected and processed by histology techniques to assess gonadal stage. Also, blood samples were taken to measure plasma levels of testosterone (T) in males and estradiol (E_2) in females by ELISA. Air temperature data was recorded by The Argentinean Meteorological Service (Coronel Suarez Weather Station, close to the lake).

Results and Discussion

Pejerrey gonadal development, the gonadosomatic index (GSI) and the plasma levels of T and E_2 fluctuated in the same pattern and in relation with temperature variation. In August, practically all the females were found at cortical alveoli stage with a few, beginning the vitellogenesis. For males, 20% were at spermatogenesis stage and the rest were releasing sperm showing that males mature before the females. The air temperature (° C) in the day of sampling was: Max: 13.3, Mean: 7.8, Min: 2.4; being permissive to pejerrey maturation. In October, the highest values of IGS and sex steroids levels for both sexes were recorded. A high proportion of ovulated females were found, and all males were spermiating. The temperature was: Max: 19, Mean: 12.9, Min: 6.8; showing that they were optimal for pejerrey spawning. In December, 75% of the females were at vitellogenic stages (new reproductive cycle), 15% were ovulated and 10 % were arrested. For males, 80 % were spermiating and 20% were arrested. That day the temperature was: Max: 26.1, Mean: 18.3, Min: 10.6 demonstrating the ending of reproduction. It is important to mention that in August sampling, 2 males with testis-ova were found.

Conclusion : These findings, confirmed that temperature changes modulate pejerrey maturation and spawning. The identification of pejerrey with testis-ova it is a warning signal due to the use of agrochemicals in this region.

POSTER 82

REPRODUCTIVE CYCLE OF TRIPLETAIL FISH, *Lobotes surinamensis*, (PERCIFORMES: LOBOTIDAE) IN NATURAL ENVIRONMENT: A FIRST APPROACH

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Introduction

The tripletail fish, *Lobotes surinamensis,* inhabits tropical and subtropical seas. The studies of this species are mainly on occurrence reports, and the reproductive physiology has not been characterized so far. This study aimed to characterize the reproductive cycle of *L. surinamensis* in natural environment by evaluating the plasma level of gonadal steroids and gonadal morphology throughout the year. The data will be important to gain some expertise on the reproductive biology of this species and to enhance our understanding to conservation actions.

Methods

A total of 48 males and 36 females specimens were collected in Paraty Bay (Rio de Janeiro, Brazil) by trap net and fishhook line during the four seasons (spring, summer, fall and winter). The specimens were anesthetized and the blood was collected to measure plasma level of gonadal steroids such as estradiol-17β (E2), testosterone (T) and 11-ketotestosterone (11-KT) by ELISA immunoassay, with commercial kits. The gonads were processed under histological routine method, stained with hematoxylin-eosin (HE) and the animals were classified in different reproductive stages, based on the description of gonadal maturation stages.

Results and Discussion : In females, the level of plasma E_2 was higher (P=0.037) during spring/summer (88.9±15.10 pg/mL) when the ovaries were classified as “in development” or “spawning capable” phases, compared with fall/winter, when E_2 levels decreased (36.7±11.20 pg/mL) and the ovaries entered into the “regressing” or “regenerating” phase. 11KT levels in females showed an inverse profile, tending to higher values during fall/winter period (39.6±10.94 pg/mL) than during spring/summer (19.1±4.25 pg/mL) but without statistical significance (P=0.068). This profile suggests the role of 11-KT in pre-vitellogenic oocyte growth, recently discussed for other teleost species. In males, T levels did not change throughout the year, during spring/summer, plasma levels were 23.5±3.82 pg/mL and the animals were, as well as females, “in development” or “spawning capable” phases. Plasma T level were 7.7±3.59 pg/mL during fall/winter period, but the maturation stage of testes was different, they were in regressing or regenerating phases. 11-KT profile in males also did not change during the year. During spring/summer, 11- KT levels in males were 27.8±5.86 pg/mL and during winter/fall period, 13.6±12.23 pg/mL.

Conclusion: Based on gonadal morphology and plasma steroids, we conclude that the reproductive period of *L. surinamensis*, in southeast of Brazil, occurs during the spring/summer period. During fall/winter the gonads enter into a regressing phase and then resume the cycle again. The definition of the reproductive period is important to guide public policy to conservation actions.

POSTER 83

GENE EXPRESSION OF *lhβ, cyp19a1a,20βhsd, ptgs2* AND *ptges* IN THE PACU (*Piaractus mesopotamicus*) DURING HORMONAL INDUCTION.

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Introduction

Pacu is a migratory fish, when in captivity, requires exogenous hormonal stimuli to achieve final maturation and ovulation. In granulosa cells the 20β-hydroxysteroid dehydrogenase (20β-HSD) enzyme converts 17α-hydroxyprogesterone (17α-OHP) into17α-20β-dihydroxy-4-pregnen-3-one (DHP), which is the most potent ovulation inducing substance known for teleost fish. Thus, in routine hormonal induction protocols, it is considered that the luteinizing hormone (Lh) in the carp pituitary extract (CPE) is responsible for triggering final maturation and ovulation, since Lh is known to control the synthesis and expression of *20bhsd*. In this study, we investigated the expression, regulation of *lhβ, cyp19a1a, 20bhsd, ptgs2* and *ptges* in the ovary and its respective function in the ovulatory process in pacu.

Methods

Sixteen females were subjected to hormonal induction by hypophysation. Females received two doses of CPE (0.6 and 5.4mg / kg), with a 24 hour interval between doses. Ovary samples were collected in two periods: before the first hormonal dose and at the time of ovulation to evaluate the expression of *20bhsd*, *cyp19a1a*, *ptgs2* and *ptges2*. Total RNA from ovarian fragments was extracted using the RiboPure ™ kit. Sample cDNAs were used as template DNA for the polymerase chain reaction (PCR).

Results and discussion

Non-spawned females showed greater expression of the *cyp19a1a* in relation to the other groups. It is known that CYP19A1A converts testosterone (T) into 17β-estradiol (E_2), which is a substance not directly associated with ovulation. Therefore, higher levels of *cyp19a1a* for unspawned females at the time of ovulation may be associated with higher levels of E_2, but this hypothesis still needs to be investigated. Higher *ptgs2* levels at the time of ovulation compared to females sampled before the first dose indicate a strong association of the eicosanoid pathway with ovulation, however, as the expression was similar between spawned and unspawned females, it is possible that the other pathways, in addition eicosanoids, directly interfere with this process. The expression of the *lhβ* and *20bhsd* genes were similar during the entire hormonal induction process, with no clear and obvious association with ovulation success.

Conclusion

We observed that the expression of *ptgs2* is increased along the induced ovulation process and such increase was associated with the successful spawning.

POSTER 84

PLASMA LEVELS OF GONADAL STEROIDS OF HYPOFISED PACU, *Piaractus mesopotamicus*, FEMALES

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Introduction

The hypophysation method started in the late 1930s in Brazil, which consist to induce ovulation of oocytes and spawning in the ripe fish through injection of pituitary gland. In this context, the pacu (*Piaractus mesopotamicus*) still presents obstacles in induced spawning, like the occurrence of females that fail to spawn their eggs even after hypophysation. It is known that steroid hormones play a major role in the control of female reproductive processes, and that the steroidogenic changes during induced spawning are associated with successful spawning. Thus, to better understand and enhance the pacu induced spawning, the aim of this work was to analyze the profiles of gonadal steroids during hypofisation.

Methods

Sixteen ripe pacu females were divided in the hypophysed and control group (injected with saline solution). Blood samples and ovarian biopsies were collected at different times of induction: at first (A1) and second hormonal dose (A2), two, six, and 8 hours post-induction (HPI). Them the fish were separated into three groups: control (C), spawned (SP) and unspawn (UN) females. The plasma levels of 17β-estradiol (E_2), 17α, 20β-dihydroxy-4-pregnen-3-one (DHP) and testosterone (T) were quantified by ELISA.

Results and Discussion

The E_2 mean levels of SP females were significantly higher than C at 6HPI, concomitant with the DHP peak; meanwhile, a higher frequency of GVBD was observed for both SP and UN in relation to C, so it means that the higher levels of E_2 found in the present study did not inhibit the resumption of meiosis. Nevertheless, the DHP levels of SP females, different from the UN, were significantly higher than C already at 4HPI. Thus, hypophysation promoted increased DHP in both SP and UN, but to UN this increases occurred slowly. In this context, it is known that the delay to reach the peak of DHP may lead to larger numbers of UN females with low percentage of fertility, corroborating with the results founded in the present study. In relation to T, it has a similar profile to DHP throughout the process of hormonal induction. The T pattern changes were probably due to a change in the steroidogenic pathway that led to DHP production from 17α-hydroxyprogesterone, explaining the similar levels found in these hormones.

Conclusion

This study demonstrated that in the pacu the E_2 did not impair the meiosis resumption, moreover, the E_2 and DHP levels during pacu females hypophysation did not have a clear stereoidogenic shift associated with successful spawning like in the carp.

POSTER 85

UNCOVERING THE OXIDATIVE STRESS IN TAMBAQUI (*Colossoma macropomum*) SEMINAL PLASMA

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Introduction

Colossoma macropomum is a native Brazilian fish which has been considered important for the development of fish farming in the Amazon region, mainly due to its high performance in captivity, economic value and acceptance by the consumer market. However, there are still gaps in the breeding area of this species regarding to semen quality, especially about the antioxidant system of seminal plasma. Thus, we aimed to evaluate the parameters indicative of oxidative stress in tambaqui seminal plasma during the reproductive season.

Methods

Males (n = 60) were sampled in November, December (2016) and January, February and March (2017). Semen was centrifuged twice at 1163 x g for 10 min. The supernatant (seminal plasma) was stored in liquid nitrogen until analysis. We measured Reactive Oxygen Species (ROS), Thiobarbituric acid reactive substances (TBARS), Total antioxidant capacity (TAC) and thiols.

Results and Discussion

There was no difference in seminal plasma TAC (0.54 to 0.62 mM of TROLOX) and Thiols (0.18 to 0.26 $mmol.L^{-1}$) values during the period of study. On the other hand, seminal plasma ROS average value was higher in March/2017 (7.0 ± 3.3 µmol H_20_2 $Equi.L^{-1}$) than in November/2016 (2.78 ± 0.9 µmol H_20_2 $Equi.L^{-1}$). TBARS average value was also higher in March/2017 (0.71 ± 0.24 $\mu mol.L^{-1}$) when compared to the other months (0.22 to 0.37 $\mu mol.L^{-1}$). Our study is the first to describe the parameters indicative of oxidative stress (OS) in tambaqui seminal plasma. Uncontrolled production of ROS that exceeds the antioxidant capacity of the seminal plasma leads to oxidative stress, which is harmful to spermatozoa. Oxidative stress condition can be caused by either increased ROS formation or decreased activity of antioxidants in a biological system. Probably the increasing ROS and TBARS values are associated to the decline in semen quality towards the end of the reproductive season.

Conclusion

We identified oxidative stress in tambaqui seminal plasma at the end of the reproductive season. Our findings may help understanding how the antioxidant system works and its relation to semen quality of tambaqui breeding males.

POSTER 86

REPEATED USE OF FEMALE TAMBAQUI (*Colossoma macropomum*) Breeders In The Same Cycle

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Introduction

The tambaqui (*Colossoma macropomum*) is an Amazonian characid widely exploited commercially in Brazil and neighboring countries. The species occupies the second position in the national rank, with 28.1% of the total fish production. The reproduction of tambaqui starts in August/September and lasts until February. Like any reophilic species, in captivity the tambaqui spawns only by hormonal induction. Today we are aware that these protocols do not optimize the use of broodstock, since each breeder is used only once in each cycle/year and then separated to "rest" until the next cycle. The objective of the present study was to evaluate the possibility of a continuous use of tambaqui females in the same cycle, contributing to the development of new technologies for the production of the species.

Methods

Six females (3.5 years old; 5.16 ± 0.81 kg body weight-BW) were used in this study. The external reproductive features were observed (hyperemic urogenital papilla and bulging abdomen) and a small sample of oocytes were collected by cannulation. Then, the were induced by crude carp pituitary extract (6% BW) for the first time and after 60 days. The eggs of each female were fertilized by the milt of the same male in both inductions. The spawning index (SI) or relative fecundity, number of oocytes per g of egg (No/g), fertilization index (FI) and hatching index (HI) were evaluated. The mean values of the first and second spawning were compared by the *t* test.

Results and Discussion

All six females responded positively to the two hormonal inductions. The SI (10.9% and 12.3%), No/g (1,662 and 1,538), FI (89.8% and 84.4%) and HI (92,2% and 92%) were not different between the first and second inductions (respectively). Moreover, one female had an SI of 12.2% and 19.7% in the first and second stripping, respectively, values higher than the average reported for tambaqui. The number of oocytes per g of egg was also superior to other studies. The average of hatch index of both spawns was above 92%. Altogether, these results show that females tambaqui can be induced and stripped at least within 2 months apart without affecting the quantity or quality of the eggs obtained.

Conclusion

The repeated use of tambaqui breeder in the same cycle is feasible and do not interfere with egg quality. More studies are being developed now to check if this return to fertility is due to a rapid (2 mo) ovarian recrudescence or if in captivity the spawning of tambaqui is in batches/incomplete. In any case, these results are of high value for the tambaqui industry since they show that each female can be induced and stripped at least twice per cycle.

POSTER 87

REPRODUCTIVE CYCLE OF THE ESTUARINE SPECIES *Stellifer stellifer* (SCIAENIDAE, PERCIFORMES), BAY GUARATUBA, PARANÁ

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Introduction

The especie *Stellifer stellifer*, little croaker, exhibit low commercial value however it is one of the species that represents the bycatch of shrimp fisheries on the coast of the Paraná state. As a demersal species, it acts on the balance of the ecosystem by providing the nutrients in the sediment to the system, being essential to maintain it. In this context, the present study aimed to characterize the reproductive cycle of the species.

Methods

Monthly collections where carried out at the Guaratuba Bay estuary between October/2012 and September/2013, in order to obtain abiotic and biological data (using trawl net). Data of length and total weight, weight of the gonads, macroscopic determination of sex and the stage of gonadal development were obtained from the samples. Part of the gonads were destined to the histological process. The abiotic factors, temperature, pH, salinity, dissolved oxygen and depth were used to characterize the environment and to verify the relation with the reproduction of the species. The biological data were used for separated sexes in the determination of the maturation curve, the frequency of developmental stages, the total (K) and somatic (K') condition factor, the length of the first maturation (L50) and the sexual proportion.

Results and Discussion

A total of 687 samples were collected, being 326 females and 361 males with average length of 16.2±3.26 and 15.72±2.58 cm, respectively. It was described five gonadal stages: immature, developing, spawning-capable, partially-spawned and post-spawning. The species had reproduction with partial pawning, during the period from august to December, corresponding to the period with greater values of gonadosomatic index (GSI), higher values of K and period of greater energy investment in the ovaries. The L50 of females was 12 cm and 9.4 cm for males. The sex ratio by size class shown predominance of males in length classes between 10.4 and 18.4 cm. The salinity, pH and depth emphasized an eastbound environmental gradient in the estuary, allowing to infer that the interior of the estuary (west) is a preferred area for the species.

Conclusion

The Guaratuba estuary is considered an environment that include a great diversity of fish species, such as *Stellifer stellifer* that use the estuary throughout the reproductive period. Abiotic factors, especially low salinity and depth, as well as temperature change between late winter and early spring, influence the reproduction of this species.

POSTER 88

USING ESTUARY IN THE REPRODUCTIVE CYCLE OF SPECIES *Genidens genidens* **(ARIIDAE, SILURIFORME)**

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Introduction: The guri catfish (*Genidens genidens*) exhibit low commercial value however it is one of the species that represents the bycatch of shrimp fisheries on the coast of the Paraná state. As a demersal species, it acts on the balance of the ecosystem by providing the nutrients in the sediment to the system, being essential to maintain it. In this ecological context, the present study aimed to characterize the reproductive cycle of the species.

Methods: Monthly collections where carried out at the Guaratuba Bay estuary between October/2012 and September/2013, in order to obtain abiotic and biological data (using trawl net). Data of length and total weight, weight of the gonads, macroscopic determination of sex and the stage of gonadal development were obtained from the samples. Part of the gonads were destined to the histological process. The abiotic factors, temperature, pH, salinity, dissolved oxygen and depth were used to characterize the environment and to verify the relation with the reproduction of the species. The biological data were used for separated sexes in the determination of the maturation curve, the frequency of developmental stages, the total (K) and somatic (K') condition factor, the length of the first maturation (L50) and the sexual proportion. Results: A total of 955 samples were collected, being 471 females and 484 males with average length of 18.7±5.3 e 17.5 ± 4.3 cm, respectively. It was described four gonadal stages: immature, developing, mature and post pawning. The species had reproduction with total pawning, during the period from July to November, corresponding to the period with greater values of gonadosomatic index (IGS), higher frequencies of mature individuals, higher values of K and period of greater energy investment in the ovaries. The L50 of females was 14.4 cm and 10.7 for males. The sexual proportion per size classes shown predominance of males on smaller size classes and females on larger classes. The salinity, pH and depth emphasized an eastbound environmental gradient in the estuary, allowing to infer that the interior of the estuary (west) is a preferred area for the species.

Discussion: The species *G. genidens* use the estuary throughout the life-cycle, being obtained in the analysis period both young and adults in different length classes. The low fecundity and parental care registered in literature support our results, indicating that *G. genidens* is not migratory. The abiotic factors, mainly the low salinity and depth as well as the temperature variation in early winter influence the reproduction of the species.

POSTER 89

THE FIRST CASE OF INDUCED GYNOGENESIS IN A NEOTROPICAL FISH USING THE YELLOWTAIL TETRA (*Astyanax altiparanae)* AS A MODEL

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Introduction

Gynogenesis describes the uniparental or partenogenetic reproduction with exclusively maternal inheritance. This technique generally involves the UV irradiation of spermatozoa leading to no functional contribution of the paternal genome in the offspring. Afterwards, the diploidy is restored using thermal or pressure shocks. Depending of the moment of the shock, gynogeneis can be meiotic (suppression of the meiosis II) or mitotic (by suppressing the mitosis I). Gynogenetic individuals are interesting for aquaculture, since can be used for produce monosex or cloned population and to elucidate sex-determining mechanisms. Hence, the aim of this study was to establish a protocol for induce meiotic gynogenesis in the yellowtail tetra *Astyanax altiparanae* using temperature shock.

Methods : Sperm were collected in modified Ringer's solution and homogeneously (500 µL) distributed on a petri dish (90 mm) and submitted to UV irradiation (80 mj/cm^2; 254 nm). Oocytes were stripped and divided in four portions: 1) diploid control fertilized with non-irradiated sperm; 2) haploid group fertilized with irradiated sperm; 3) triploid control fertilized with non-irradiated sperm and heat shocked at 2 min post-fertilization (40 °C, 2 min); and 4) presumptive diploid gynogenetic group fertilized with irradiated sperm and heat shocked at 2 min post-fertilization. Gametes was activated by adding 5 mL of water and maintained at 26 °C until the heat shock. Survival among development was evaluated and Hatched embryos were selected for evaluation of ploidy status by flow cytometry. Results were also confirmed by microsatellite analysis.

Results and Discussion : In the present study, we developed the first protocol to induce diploid gynogenesis in a Neotropical fish using irradiated spermatozoa. Using spermatozoa from diploid males as control, all individuals from the gynogenetic 1n group were haploid (1C). For the control group, the mean DNA content was equal to that observed for presumptive diploid gynogenetic individuals (2C), which were confirmed as gynogenetic individuals by microsatellite analysis. The larvae from the group fertilized with non-irradiated sperm and submitted to heat shock were triploid (3C). At hatch, the percentage of abnormal embryos were significantly higher in gynognetic 1n group (85.16 ± 13.83%; P = 0.0206) when compared with control (18.52 ± 5.62%). The presence of triploid fish showed that gynogenetic individuals were obtained by suppressing the extrusion of the second polar body (meiotic gynogenesis).

Conclusion : Meiotic gynogenesis was obtained in *A. altiparanae* by inactivation of spermatozoa using UV irradiation and diploidization by heat shock (40°C; 2 min).

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POSTER 90

GENOME-INACTIVATION OF SPERM OF YELLOWTAIL TETRA *Astyanax altiparanae* TO INDUCE GYNOGENESIS

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Introduction

Gynogenesis is a uniparental or partogenetic segregation and can be used for investigation of sex determination mechanisms and production of clonal fish. Artificial gynogenesis in fish are commonly induced by fertilization of eggs with UV- irradiated spermatozoa e posterior diploidization of the haploid zygotes using thermal or pressure shocks. In such procedures, inactivation of the paternal genome is essential and the first step for induction of diploid gynogenesis. Therefore, the aim of this study is to establish a protocol for genome-inactivation of spermatozoa from *Astyanax altiparanae* using UV-irradiation for induction of gynogenesis.

Methods

Sperm (500 μL) were homogeneously distributed on a petri dish (90 mm) and submitted to UV irradiation at 40, 80 and 120 mj/cm^2 (254 nm). At each moment, aliquots of 15 μl of eggs (~100) were fertilized with 20 μl of irradiated sperm. Gametes were activated by addition of water (5 mL). At the end, one aliquot of eggs were fertilized with an intact sperm (control group). Such procedures were performed in triplicate. Survival among development was evaluated, as well hatching, with normal and abnormal larvae counted. At hatching, 20 larvae of each treatment were submitted to flow cytometry analysis. To investigate the efficacy of the UV-irradiation, all samples were analyzed with spermatozoa from confirmed diploid males.

Results and Discussion

This is the first experiment in a Neotropical species focusing on optimization of UV irradiation of spermatozoa, the first and essential step for future application and induction of diploid gynogenesis. In the control group, all individuals were diploid. Excepted for 2 hyperhaploid embryos found in the 40s treatment, all other embryos in the irradiated groups were haploid, with higher percentage of abnormal embryos (40mj/cm^2: 96.30 ± 2.18%; 840mj/cm^2: 86.05 ±12.76% and 120mj/cm^2: 97.30 ± 2.70%) compared with the control group (2.23 ± 1.15%). Therefore, the UV irradiation obtained here was efficient for inactivate the paternal genome and embryos present the characteristic “haploid syndrome”, which has been used to verify the absence of paternal genome in other experiments with gynogenesis.

Conclusion

Therefore, the dose of 80 or 120 mj/cm^2 of UV irradiation (254 nm) could be used to inactivate spermatozoa of *A. altiparanae* and be applied in future induction of diploid gynogenesis.

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BRAIN-PITUITARY SYSTEM

POSTER 91

CLONING AND CHARACTERIZATION OF GONADOTROPIN-RELEASING HORMONE – II PRECURSOR GENE IN THE CATFISH *Heteropneustes fossilis*: EXPRESSION PATTERNS AND ESTROGEN REGULATION

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Introduction

The decapeptide Gnrh exists in multiple forms in teleosts. Gnrh-1 is genus-specific, Gnrh-II or chicken Gnrh-II is ubiquitous in fishes and tetrapods and Gnrh-III is teleost-specific. There is limited information on Gnrh physiology in Ostariophysian teleosts. Hence the present study was taken up in the catfish *Heteropneustes fossilis*, which has a limited distribution in Indian peninsular waters.

Methods

cDNA of Gnrh-II was isolated and characterized by PCR and RACE from *H. fossilis* brain total RNA. Bioinformatics tools were used for nucleotide and protein sequence comparisons and phylogeny relationships. Expression of the gene was studied by qPCR method Regulation of the expression of the gene was studied after ovariectomy and steroid replacement.

Results and Discussion

A full-length *gnrh- II* precursor cDNA of 611 bp long was cloned. The ORF (261 bp) encodes a deduced protein of 86 amino acids. The sequence identity of *hfgnrh-II* is 94% with African catfish (*Clarias gariepinus*) *gnrh-II* (accession no. X78047). The deduced Hfgnrh-II precursor protein clustered with the vertebrate Gnrh-II type. The *hfgnrh-II* transcripts were expressed only in brain and gonads with higher expression in the brain and greater in females than males. In the brain, hypothalamus showed the highest transcript abundance, followed by the posterior brain, telencephalon and pituitary. The transcript abundance of *hfgnrh-II* showed significant seasonal variations with opposite patterns in the brain and ovary. The high transcript abundance of *gnrh-II* precursor gene in the hypothalamus is significant since Gnrh-II is known for its midbrain location. This can be attributed to cross reactivity with the yet to be isolated gnrh-1 precursor gene. However, our efforts with cloning strategy as well as tBlastX procedure using Channel catfish predicted *gnrh-1* and *gnrh-II* precursor sequences and African catfish gnrh precursor gene sequence against a *H. fossilis* deduced transcriptome data base (about 59K contigs) could not retrieve a *gnrh-1* precursor gene in *H. fossilis*. Ovariectomy resulted in a duration-dependent inhibition of *hfgnrh-II* mRNA levels. E_2 replacement (0.5µg/g body weight) in 3- week ovaiectomized fish resulted in an increase in *hfgnrh-II* mRNA levels. Tamoxifen, an estrogen receptor antagonist inhibited the stimulatory effect of E_2 on *hfgnrh-II* mRNA levels.

Conclusion : In *H. fossilis*, a g*nrh-II* precursor gene was cloned and characterized. The transcript level was higher in the hypothalamus in females and showed seasonal variations. E_2 stimulates the transcript levels, which was inhibited by tamoxifen.

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POSTER 92

MORPHOLOGICAL RELATIONSHIP BETWEEN GNIH AND GNRH NEURONS IN THE BRAIN OF THE NEOTROPICAL CICHLID FISH *Cichlasoma dimerus*

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Introduction : The first neuropeptide identified that regulates reproduction was the gonadotropin-releasing hormone (GnRH). After 30 years, a new peptide that inhibits gonadotropin synthesis and secretion was discovered, the gonadotropin-inhibitory hormone (GnIH). Its role in reproduction has been widely studied as well as the interaction between GnRH and GnIH neurons, especially in adults of avians and mammals. In other vertebrate groups, there is little information about the relationship between both systems. In previous works, we characterized the three GnRH variants and GnIH peptide, and demonstrated that GnIH inhibited gonadotropins release in adults of *Cichlasoma dimerus*. Because no innervation was detected at pituitary level in adults, we analyzed the anatomical relationship between neurons expressing GnIH and the three GnRH variants in adults and in addition, we started studying that relationship during the development of *C. dimerus*.

Methods : Reproductive adult's brain with pituitary attached and larvae were fixed in Bouin's solution and processed for immunohistochemistry or double labelling confocal immunofluorescence using heterologous antibodies. Larvae were obtained from 4 independent spawning and samples taken from hatching to 85 days post-hatching (dph). Neurons of both nuclei were quantified and measured.

Results and Discussion : In adults, it was shown no apparent contacts between GnIH and GnRH1, so whether GnIH regulates the expression or secretion of GnRH1, an indirect modulation seems more plausible. There were fiber to fiber contacts between GnIH and GnRH2 in the midbrain and in the *nucleus lateralis tuberis*, suggesting an interaction between both systems. Finally, it was observed co-localization of GnIH and GnRH3 variant only in the *nucleus olfacto-retinalis* (NOR) neurons, and fibers that co-expressed both peptides while others that expressed only one of them, in all brain regions studied. During the development, the two GnIH-immunoreactive (GnIH-ir) nuclei with spatial and temporal differences were detected: one was observed by 3 dph in the NOR and the other by 14 dph in the *nucleus posterioris periventricularis*; as described in adults. Moreover, we showed that only NOR neurons co-expressed GnIH and GnRH3 in larvae, result that is in correspondence with the obtained in adults. Surprisingly, there were GnIH-ir fibers at pituitary level from 14 dph, but the density of these fibers diminished by 37 dph; suggesting a change in the regulation of GnIH over pituitary function from larvae to adult.

Conclusion : The present study provide new clues to investigate the role of NOR cells, possible GnIH and GnRH3 interactions in the modulation of the reproductive network of teleost fish, and also new functions of GnIH during the development.

POSTER 93

CHARACTERIZATION OF MELATONIN RECEPTORS IN ATLANTIC SALMON

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Introduction : Melatonin binds to high affinity melatonin receptors, all belonging to the family of seven-transmembrane-domain (7TM) G-protein-coupled receptors (GPCR). Three receptor subtypes have been identified: MT1 and MT2, which are found in all vertebrates investigated to date, and Mel1c that is present in all vertebrate classes except mammals. Research conducted mainly in mammals suggest that MT1 and MT2 receptors are coupled to different intracellular pathways; the adenyl cyclase/cyclic AMP (cAMP)/ protein kinase A (PKA) pathway, which upon receptor activation is inhibited via members of the G_i-protein family; the phospholipase C (PLC)/protein kinase C (PKC) pathway, which is inhibited via Gq proteins; and finally inhibition of the cGMP pathway. In teleosts, a pharmacological characterization has been conducted only(?) in pike (*Esox lucius*) (P2.6), where activation of the XX receptor resulted in decreasing cAMP, similar to what has been shown in mammals. In the present study, three high affinity melatonin receptors (MT1a, MT1d and MT2b) were cloned and pharmacologically characterized in Atlantic salmon (*Salmo salar*).

Methods : Phylogenetic analyses and protein alignment of available vertebrate melatonin receptors were produced using the software MEGA6 and the online tool Clustal Omega, respectively. Melatonin receptor tissue distribution, circadian and seasonal mRNA expression during pubertal development in the pituitary gland were observed via qPCR. Localization of receptor expression in the pituitary gland was obtained via fluorescent *in situ* hybridization (FISH). To study the intracellular signaling pathways of the cloned receptors, we first transfected COS7 cells with the cloned receptors and a luciferase reporter gene coupled with a promoter sensitive to intracellular cAMP levels (CRE-LUC). Then, we exposed the transfected cells to melatonin (Mel), 2-iodo-melatonin (2im) and a combination of melatonin and Luzindole, an inhibitor of mammalian melatonin receptors. Modulation of intracellular cAMP was measured via light emission trough luciferase activity.

Results and Discussion: The cloned receptors showed all the conserved amino acids known for the proper functioning of melatonin receptors when aligned with other known sequences. Phylogenetic analysis located two receptor in the MT1 group and one in the MT2 group. Tissue distribution studies revealed the presence of receptor expression in different areas of the brain, eyes, optic nerve, gonads and skin. MT1a, the main receptor expressed in the pituitary, showed highest expression levels at 12:00. No significant relationship was shown with maturational stages. Pharmacological characterization showed an increasing cAMP production after exposure with Mel or 2im. Luzindole was effective in decreasing cAMP level after melatonin administration.

Conclusion : In this study, we report the cloning and characterization of three melatonin receptor genes in Atlantic salmon. Pharmacological characterization confirmed their action via the cAMP pathway, but differently from mammals and pike, salmon receptors are stimulatory as shown by the increasing intracellular cAMP levels upon activation.

POSTER 94

CHARACTERIZATION OF THE PRECURSOR ENCODING FOR GNIH AND IMMUNOHISTOCHEMICAL LOCATION OF THIS PEPTIDE IN THE BRAIN OF *Odontesthes bonaeriensis* (ATHERINIFORMES).

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Introduction

Gonadotropin-inhibitory hormone (GnIH) is a member of the RFamide neuropeptide family, originally discovered and characterized in birds. It is known that this peptide is involved in the inhibition of gonadotropin release from the pituitary of birds and mammals, however its function in teleosts has not been clearly established. Pejerrey, *Odontesthes bonaeriensis*, is a native fish from continental waters of the Pampas region of Argentina and has been used as a model for research on reproduction and sex differentiation. In view of the diverse mechanisms involved in the hypothalamic control of pituitary function in fish, and due to the scarce information of GnIH physiology in teleosts, we performed an *in silico* analysis of the GnIH precursor sequence and its position in a GnIH phylogenetic tree, along with the study of its brain-pituitary distribution.

Methods

GnIH putative sequences were sought from the pejerrey genome database, and an *in silico* analysis of the putative translated protein was blasted. A maximum likelihood tree was obtained based on the Jones-Taylor-Thornton model with the MEGA5 software. The neuroanatomical distribution of immunoreactive GnIH (ir-GnIH) was characterized in sections of adult pejerrey brain and pituitary using an anti-bullfrog GnIH antiserum kindly provided by Dr. K. Tsutsui (Japan).

Result and Discussion

A partial sequence of 538 bp of cDNA encoding for the GnIH precursor peptide was identified. The *in silico* translation of this sequence included three putative RFamide-related peptides: two 12 amino-acids peptides: a MPMRFamide (PLHMHANMPMRF) and a MPQRFamide (VPKSSPNMPQRF) and a 11 amino-acid peptide LPQRFamide (EAPSPVLPQRF). The phylogenetic analysis showed that pejerrey precursor is grouped in the same clade as Cyprinidontiformes and Beloniformes with the higher percentage of identity with cyprinodontiform sequences (78-81%), and only 23% and 27% with lamprey and human sequences, respectively. On the other hand, similar to other fish species, ir-GnIH somata were observed in the olfactory bulbs, the *Nucleus Olfacto Retinalis* and the *Nucleus Posterioris Periventricularis* (NPPv) in the preoptic area. Immnuoreactive GnIH fibers were observed in almost all brain areas examined, and also reaching the pituitary gland.

Conclusion: In the present study, the GnIH precursor was analyzed and the neuroanatomical distribution of GnIH in pejerrey fish was described. Both analyses showed that these characteristics are fairly conserved in teleosts. This knowledge can enable us to start working on the effects of GnIH in the control of reproduction and other physiological process in pejerrey.

POSTER 95

OBSERVATIONS ON THE USE OF SALMON Gnrh ANALOGUE (sGnRHa) IN *Astyanax altiparanae*

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Introduction

In tropical fishes the use of carp pituitary extract (CPE) is still the main protocol to induce spawning. However, this technique shows unpredictability of a successful spawning. Thus, the aim of this study was to achieve knowledge about the use of the sGnRHa as a hormonal inducer for reproduction of lambari, which has been considered a good model for studies on reproduction.

Methods

Experiment 1: Six doses of sGnRHa were tested (1, 10, 20, 40, 80 and 160 µg kg^{-1}) as well as a saline control group (0.9% NaCl) and a positive control group (6 mg CPE kg^{-1}), with three replicates each. All females received a single intraperitoneal injection of 4 mL kg^{-1}. *Experiment 2*: Three doses of sGnRHa were tested (10, 100 and 1000 µg kg^{-1}) as well as a saline control group (0.9% NaCl) and a positive control group (6 mg CPE kg^{-1}), with four replicates each. In this assay all females received two intraperitoneal injection of 4 mL kg^{-1}, being the first one 10% of total dose and 12h later the second with the resolving dose. In both assays were used 10L experimental units, where five females (16.64 ±2.87g) and ten males (9.38 ±2.33g) were allocated. Males received a single dose of 3 mg of CPE kg^{-1}. Reproductive performance was evaluated considering spawning rate, relative fecundity (egg volume/body weight), fertilization and hatching rate. Data were analyzed by one-way ANOVA followed by the Tukey test at the $p \leq 0.05$ level of significance or by the Kruskal-Wallis test, a nonparametric test, depending on normality and variances homogeneity.

Results and Discussion : *Experiment 1:* The response to sGnRHa was not in a dose-dependent manner. No ovulation was seen in the 1 µg kg^{-1} and saline group. A low proportion of females receiving higher doses of sGnRHa and CPE treatment ovulated. The spawning rate and relative fecundity were similar and relatively low in all groups that spawned ($p>0.05$). The fertilization and hatching were at least 50% in all groups. Thus, aiming mainly to improve spawning rate we increased and fractioned the doses.

Experiment 2: The use of two doses improved reproductive performance of females, mainly for the positive control. Ovulation was seen in all groups, including in saline control. Relative fecundity was now higher in CPE treatment than all other groups, except for the highest dose of sGnRHa ($p<0.05$). However, the values of the latter were similar to all other groups. The highest spawning rate was seen in the CPE ($p<0.05$). The fertilization and hatching were similar between groups ($p>0.05$).

Conclusion :

Although 1000 µg kg^{-1} fractioned sGnRHa dose has shown some results similar to the CPE, the latter is still better overall. With this preliminaries results, sGnRHa has not been shown to be a good hormonal inducer for reproduction of lambari.

POSTER 96

GnRH ANALOGUE AND THERMAL THERAPY APPLIED TO THE REPRODUCTION OF *Astyanax altiparanae females* (CHARACIFORMES: CHARACIDAE) IN CAPTIVITY, AT THE NON-BREEDING SEASON.

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Introduction: *Astyanax altiparanae* is a migratory species that spawns in spring and summer. Recent studies demonstrated the ability of this species to reproduce in winter, in captivity, which can optimize the production by reducing the off-season period. The objective of this study is to understand the physiological control of *A. altiparanae* spawning in the winter, manipulating the water temperature together with a hormonal therapy.

Methods: One year old females, born in captivity, were placed in 750L boxes under controlled temperature conditions for 21 days during the winter. Twenty animals were used in duplicate in each group and were induced to vitellogenesis with gonadotropin releasing hormone analogue (GnRHa) every 7 days during the experimental period. We established a lower dose (LD), injecting 1.33μg/g and a higher dose (HD), injecting 2.66μ/g of GnRHa to reach a final dose of 4 and 8μg/g, respectively. The experimental groups were: G1- Saline (sham) at 20°C; G2- LD at 20°C; G3- HD at 20°C; G4- Saline (sham) at 27°C; G5- LD at 27°C;G6- HD at 27°C. At the end of the experiment 4 females from each group were induced to spawning with human chorionic gonadotropin (hCG; 5000 IU/kg) and 4 applied with saline under the same temperature conditions. After the reproduction, eggs development and hatching were monitored under the same temperature conditions until the opening of the larval mouth. Plasma was collected to measure the levels of 17α-20β-dihydroxy-4-pregnen-3-one (17α,20β-DHP) the maturation induction steroid (MIS) of most freshwater teleost, using ELISA kit. The reproductive parameters such as fertilization and hatching rate, and estimated larvae number were analyzed.

Results and discussion: 17α,20β-DHP was confirmed as being the MIS for this species due to the increase in the level of this steroid only in the females that actually spawned. The dose of hCG was the same for all groups, therefore the differences in the spawning success were assigned to the previous treatment. Females from all groups, treated with hCG spawned, except for G4, and G1 that spawned but with a low larvae hatching hate. MIS levels from females induced with hCG did not differ among groups, but fertilization and hatching rate, and consequently the estimated larvae number was higher in the groups previously treated with the higher GnRH dose (G3 and G6), and even higher at 27$_o$C.

Conclusion: *A. altiparanae* females were vitellogenic during the winter, allowing the spawning induction. The best spawning indices were obtained in animals induced to vitellogenesis with the higher GnRHa dose and 27$_o$C. This result indicates that GnRHa treatment, combined with a higher temperature, even under the winter photoperiod, improves the reproductive parameters in this species.

POSTER 97

IMMUNOLOCALIZATION OF SECRETONEURIN IN THE BRAIN AND OVARY OF THE CATFISH *H.fossilis* : VARIATIONS WITH SEASONS

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Introduction

The neuropeptide secretoneurin (SN) is a short 31-43 conserved peptide derived from the larger ~600 amino acid SgII precursor protein by prohormone convertase- mediated processing. SN is a functional peptide shown to stimulate luteinizing hormone release in goldfish. SN is found in the large dense core secretory vesicles of a variety of neurons and endocrine cells. Cloning and characterization of its precursor secretogranin IIb in the catfish *H. fossilis* established the amino acid sequence of SN. Various physiological roles have been assigned to SN, including those related to reproduction, neuroinflammation and neurotransmitter release. In the present study we report the distribution of the novel peptide secretoneurin in the catfish brain, pituitary and ovary during different phases of reproductive cycle.

Methods

Sexually mature female catfish (40-60g) were collected during different phases of the reproductive cycle. Resting (January), Preparatory (March), Prespawning (May) and Spawning (July). Brain and ovary tissues were sampled and processed for immunolocalisation study. Immunohistochemistry using a rabbit polyclonal antibody against goldfish SNa was performed according to the Vectastatin ABC Kit with some modifications.

Results and Discussion

In the present study the wide distribution of SN in neuroendocrine neurons and pituitary cells of the catfish *Heteropneustes fossilis* was observed. Immunoreactivity was found in the nucleus preopticus area (NPO) of the hypothalamo-hypophysial neurosecretory system.The neuroendocrine cells are hypertrophied and degranulated in the pituitary PPD region during pre-spawning phase. SN-ir fibres were observed in the ventricular region of the brain, dense innervations and cell bodies were observed in the optic tectum layers. Strong SN-immunireactivity (ir) was observed in the proximal pars distalis of the pituitary having acidophilic and basophilic cells expressing lactotropes and gonadotropes.The SN neurons showed seasonal changes. SN-ir cell bodies are small, scattered and less in numbers in resting phase but with its number and density increased in preparatory and prespawning phases. In the ovary, secretoneurin (SN) immunoreactivity was evident in the granulosa layer of vitellogenic follicles. The yolk granules also showed immunoreactivity. There was no immunoreactvity observed in the thecal layer of the follicle.

Conclusion

Our results have shown that SN immunoreactivity is evident in the catfish brain, pituitary and ovary. This implicates SN in the neuroendocrine regulation of reproduction and behaviour.

(Financial support of CSIR-JRF to SS and BHU, UGC-UPE, DBT-ISLS to RC and NSERC to VLT) is acknowledged).

POSTER 98

CLONING AND CHARACTERIZATION OF GONADOTROPIN-RELEASING HORMONE RECEPTOR- 2 GENE IN THE CATFISH *Heteropneustes fossilis*: EXPRESSION PATTERNS AND STEROID HORMONE EFFECTS

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Introduction

Gonadotropin-releasing hormone (Gnrh) actions are mediated through G-protein-coupled receptors (GPCRs), which are recognized into 3 types (R1, R2 or R3) or 4 subfamilies (Ra1, Ra2, Rb1 or Rb2). In Ostariophysan teleosts, GnrhR studies are scarce with the only report of two R types in the African catfish *Clarias gariepinus* (Bogerd et al. 2002). This has prompted us to investigate the receptors in another catfish, *Heteropneustes fossilis*, a well established fish model and an important edible fish in India.

Methods

A cDNA of *Gnrh- R* was isolated and characterized by PCR from *H. fossilis* brain total RNA. Bioinformatics tools were used for nucleotide and protein sequence comparisons and phylogeny relationships. Expression of the gene across tissues, reproductive stage sex and after steroid hormone treatments was studied by qPCR.

Results and Discussion

A partial cDNA sequence of *hfgnrh r2* of 233 bp long coding the highly conserved region of extracellular loop II, transmembrane domain 5 and intracellular loop III from the CDS region was isolated. The hfGnrh R2 has 96% sequence identity with African catfish Gnrh R2 (accession no AF329894) and 76% with African catfish Gnrh R (accession no X97497.2). The deduced hfGnrh-R2 protein clustered with the vertebrate Gnrh-r2 type along with other teleost Gnrh-r2. *Hfgnrh*-r*2* showed significant sex differences with high expressions in females. The *hfgnrh*-r*2* transcripts were expressed only in brain and gonads with higher expressions in the brain. In the brain, hypothalamus showed the highest transcript abundance, followed by the posterior brain, telencephalon and pituitary.

In male brain and testis, the expression of *hfgnrh*-r*2* was the highest in the preparatory phase and decreased subsequently in the prespawning, spawning and resting phases. In female brain, the expression was significantly high in resting, preparatory and prespawning phases, and low in spawning phase. In the ovary, the expression was low in the resting phase and high in preparatory, prespawning and spawning phase. Ovariectomy (ovx) resulted in a duration-dependent increase in *hfgnrh-r2* mRNA levels. Interestingly, E_2 replacement did not alter the mRNA levels but progesterone and testosterone inhibited the stimulatory effect of ovx. But tamoxifen, an estrogen receptor antagonist, inhibited the stimulatory effect of ovx/E_2 treatment on the mRNA levels.

Conclusion

The g*nrh-r2* gene transcript level showed dimorphic variations in its expression in tissues and brain regions, and in brain and gonad seasonal variations. Steroid hormones showed differential effects on the mRNA levels.

*Financial support of INSA, New Delhi and DST-SERB, New Delhi is acknowledged.

POSTER 99

A MULTIDISCIPLINARY STUDY ON THE RELATIONSHIP BETWEEN BRAIN-PITUITARY-GONADS AXIS AND AGONISTIC BEHAVIOR ON REPRODUCTIVE PERFORMANCE IN CAPTIVITY REARED FEMALE *Steindachneridion parahybae*.

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Introduction : *Steindachneridion parahybae* is a freshwater catfish endemic to the Paraíba do Sul River Basin, Brazil, classified as an endangered Neotropical species. This status and the endocrine dysfunction showed by this species in captivity deserves special attention and urgent action addressing the knowledge of its reproductive physiology, the basic premise for restocking programs. We characterized the brain-pituitary-gonads axis in *S. parahybae* female, evaluating the influence of agonistic behavior during artificial reproduction in captivity.

Methods : Broodstocks that were able to spawn were selected in the pounds at the CESP, and induced to artificial reproduction. After hormone administration until spawning, two female's broodstocks (in triplicate) were placed together into a glass aquarium in the laboratory to facilitate the observation of reproductive behavior. Shortly after the gametes extrusion, the samples (brain, pituitary and plasma) were collected. Gonadotropin-releasing hormone (GnRH), arginine-vasotocin (AVT) and follicle-stimulating hormone (FSH) and luteinizing hormone (LH) cells were visualized by immunocytochemistry, and molecular weight (only for FSH/LH) was measured by western blot (wb). Plasma levels of gonadal steroids were quantified by ELISA.

Results and Discussion : During the artificial reproduction, there was a gradual increase in the animals' activity, including aggressive behavior that even hurt one of the females. One female was chasing the other, trapping it in a corner, and sometimes, these chasing were with physical contact between females, showing some bites and tail hit behavior, which we defined as "dominant" (more aggressive) and "no-dominant" females that were injured. LH cells were characterized by positive immunostaining and wb (19kda) methods, while for FSH only the wb method was possible (18kda). This lack of immunoreaction could be attributed to the protein conformation, which does not allow that this antibody recognize the active site. Catfish-GnRH-neurons were found in the forebrain, extending to the antero-posterior direction, from ventral telencephalon to the caudal, mainly in ventral hypothalamus, including fibers to pituitary. In contrast, chicken-GnRH-II-neurons were restricted to the anterior dorsal midbrain tegument area, close to the ventricular surface and do not reach the pituitary. AVT positive neurons were found only in the preoptic area (parvocellular, magnocellular and gigantocellular nucleus) and fibers also reach the pituitary. No-dominant females showed higher cortisol and 11-ketotestosterone levels than dominant females, while 17α-20β-dihydroxy-4-pregnen-3-one was higher in dominant females.

Conclusion : These results suggest the direct effect of aggressive behavior on *S. parahybae* endocrine axis, since dominant females showed better reproductive performance than non-dominant ones. These results can help us in the management of this endangered species from Paraíba do Sul Basin.

POSTER 100

MORPHOLOGICAL CHARACTERIZATION OF THE GNRH SYSTEM AND PITUITARY GLAND IN THE NEON CARDINAL *Parancheirodon axelrodi*

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Introduction

Parancheirodon axelrodi (Characiforms, Characidae) is a Neotropical fish distributed in Black and Orinoco rivers of South America. In many countries, *P. axelrodi* is widely commercialized as an ornamental species (ie: 34% of imports in Argentine), although there are no successful studies or standardized protocols for its culture. Considering that it is an overexploited species from Amazonian rivers, it is important to deepen the knowledge regarding its reproductive physiology from different approaches. The purpose of this study was to perform the first morphological characterization of the GnRH neuronal system and pituitary gland in this species.

Methods

Adult male and female heads were fixed in a Bouin's solution, dehydrated and embedded in paraplast. For histochemical studies of the pituitary, sagittal and transverse sections were stained with periodic acid-Schiff (PAS) and Masson trichrome. For immunohistochemical analysis, brain, pituitary and gonadal sections were immunostained according to SABC complex (streptavidin-biotin-peroxidase complex) with several heterologous antisera which specificity was corroborated by Western blot and preabsortion tests in several species.

Results and Discussion

Three principal populations of GnRH cell bodies were detected by IHQ: GnRH I (ir-salmon GnRH) in the anterior preoptic area, GnRH II (ir-chicken II GnRH) in the midbrain tegmentum and GnRH III (ir-salmon GnRH) in the caudal olfactory bulbs. Regarding the pituitary gland, in the *rostral pars distalis* ir-ACTH and ir-PRL cells were found forming compact clusters. Fusiform ir-βFSH cells were also found. Within the *proximal pars distalis*, ir-GH cells, and round ir-βFSH and ir-βLH cells were abundant in its dorsoventral portion. We were not able to detect ir-TSH cells with the antisera used. In the *pars intermedia*, ir-MSH and ir-SL cells were found surrounding the neurohypophyseal branches. Ir-salmon GnRH fibers reached the pituitary gland. Finally, ir-PRL, βFSH and βLH cells were detected in previtelogenic oocytes.

Conclusion

This is the first morphological study of the brain GnRH system and pituitary components of the Cardenal Neon, *P. axelrodi*. The results showed the presence of three populations of GnRH cells, and a distribution pattern of pituitary cells similar to other teleost species, only with slight differences in βFSH distribution.

POSTER 101

OBSERVATIONS ON THE USE OF SALMON GNRH ANALOGUE (SGNRHA) IN *Astyanax altiparanae*

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Introduction

In tropical fishes the use of carp pituitary extract (CPE) is still the main protocol to induce spawning. However, this technique shows unpredictability of a successful spawning. Thus, the aim of this study was to achieve knowledge about the use of the sGnRHa as a hormonal inducer for reproduction of lambari, which has been considered a good model for studies on reproduction.

Methods

Experiment 1: Six doses of sGnRHa were tested (1, 10, 20, 40, 80 and 160 µg kg^{-1}) as well as a saline control group (0.9% NaCl) and a positive control group (6 mg CPE kg^{-1}), with three replicates each. All females received a single intraperitoneal injection of 4 mL kg^{-1}.

Experiment 2: Three doses of sGnRHa were tested (10, 100 and 1000 µg kg^{-1}) as well as a saline control group (0.9% NaCl) and a positive control group (6 mg CPE kg^{-1}), with four replicates each. In this assay all females received two intraperitoneal injection of 4 mL kg^{-1}, being the first one 10% of total dose and 12h later the second with the resolving dose. In both assays were used 10L experimental units, where five females (16.64 ±2.87g) and ten males (9.38 ±2.33g) were allocated. Males received a single dose of 3 mg of CPE kg^{-1}. Reproductive performance was evaluated considering spawning rate, relative fecundity (egg volume/body weight), fertilization and hatching rate. Data were analyzed by one-way ANOVA followed by the Tukey test at the $p \leq 0.05$ level of significance or by the Kruskal-Wallis test, a nonparametric test, depending on normality and variances homogeneity.

Results and Discussion: *Experiment 1:* The response to sGnRHa was not in a dose-dependent manner. No ovulation was seen in the 1 µg kg^{-1} and saline group. A low proportion of females receiving higher doses of sGnRHa and CPE treatment ovulated. The spawning rate and relative fecundity were similar and relatively low in all groups that spawned ($p>0.05$). The fertilization and hatching were at least 50% in all groups. Thus, aiming mainly to improve spawning rate we increased and fractioned the doses.

Experiment 2: The use of two doses improved reproductive performance of females, mainly for the positive control. Ovulation was seen in all groups, including in saline control. Relative fecundity was now higher in CPE treatment than all other groups, except for the highest dose of sGnRHa ($p<0.05$). However, the values of the latter were similar to all other groups. The highest spawning rate was seen in the CPE ($p<0.05$). The fertilization and hatching were similar between groups ($p>0.05$).

Conclusion : Although 1000 µg kg^{-1} fractioned sGnRHa dose has shown some results similar to the CPE, the latter is still better overall. With this preliminaries results, sGnRHa has not been shown to be a good hormonal inducer for reproduction of lambari.

POSTER 102

GONADOTROPIN INHIBITORY HORMONE (GNIH) DISTRIBUTION IN THE BRAIN OF THE ANCIENT FISH, *Atractosteus tropicus* (LEPISOSTEIFORMES: LEPISOSTEIDAE)

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Introduction: GnIH is a dodecapeptide that belongs to the RFamide family. This peptide downregulates reproductive function in avians and mammals, but its role in other vertebrates' groups is not clearly established. Immunohistochemical studies have provided data of the distribution of GnIH immunoreactive (GnIH-ir) neurons and fibers in the brain of major teleost fish models. Despite of lepisosteis' phylogenetic position, little is known about the brain distribution of important neuropeptides. Thus, we considered worth it to start investigating GnIH system in the brain of lepisosteids from the viewpoint of comparative neuroanatomy and thus, to infer possible conserved functions. The present study was conducted to elucidate GnIH distribution in the brain of *Atractosteus tropicus*.

Methods: Juveniles were obtained from the aquaculture facility of La Universidad Juárez Autónoma de Tabasco in Villahermosa, México. Brains with pituitary attached, fixed in Bouin's solution, were processed for immunohistochemistry. An antiserum raised against a bullfrog peptide closely related to GnIH and previously tested in teleost species, was used.

Results and Discussion: Three GnIH-ir cell groups were found: a few number of neurons observed in the intersection of the telencephalon (Tel) and the olfactory bulb (OB), other group detected in the preoptic area, and the last one, with high number of GnIH-ir somata, surrounding the third ventricle in the hypothalamus (Hpt). In this last group, two somata populations were shown: the more crowded one in a more anterior and dorsal position, and other detected in a ventral position. The Hpt GnIH-ir cell population seems to be the most conserved characteristic of GnIH system vertebrate's species. GnIH-ir fibers showed a wide spread distribution over all brain regions, except the cerebellum which has the lowest density. Fibers were detected in a ventral position in the olfactory nerve. The central region of the OB-Tel, and the Hpt showed the highest density of fibers, with the thickest fibers in a more ventral position. At Hpt level, a high density of labeled fibers was detected in the median eminence and less number of fibers were observed at pituitary level, providing morphological evidence of possible indirect and/or direct action of GnIH over pituitary function. GnIH-ir fibers were shown in the optic nerve and the optic tectum, innervating the periventricular zone, suggesting that GnIH act as a neuromodulator integrating different sensory modalities as it was proposed in other teleost fish.

Conclusion : The present study described for the first time the neuroanatomical distribution of GnIH in a non teleost actinopterigian fish, where a conserved pattern of distribution of fibers and somata was observed.

POSTER 103

EFFECTS OF GnRH AND GnIH ON FSH AND LH EXPRESSION IN PITUITARY EXPLANTS OF *Astyanax altiparanae* MALES

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Introduction

The hypothalamic-pituitary-gonadal axis exerts control over the gonadal function and reproduction in vertebrates. The hypothalamus secretes the gonadotropin-releasing hormone (GnRH), which stimulates the gonadotropic cells in the pituitary to release follicle-stimulating hormone (Fsh) and luteinizing hormone (Lh). In many vertebrates, including fish, the gonadotropin-inhibitory hormone (GnIH) has been described as inhibitory or stimulatory hormone on gonadotropin synthesis and release. *Astyanax altiparanae* is a Neotropical species, known locally as lambari, with noteworthy characteristics, such as commercial and ecological relevance, and more recent, as useful experimental model. The aim of this study was to evaluate the *in vitro* effects of GnRH and GnIH on *fsh* and *lh* expression in the pituitary explants of lambari males in the spawning capable phase.

Methods

To this study, five animals at the same reproductive phase were used per treatment. The animals were anesthetized, and their pituitary glands were dissected and washed. Each pituitary gland was cultivated in Leibovitz's L-15 containing 3 concentrations of zebrafish Gnrh2 or Gnih -10, 100, 1000 nM for 12 hours at 26°C. After this period, the pituitary glands were collected for the gene expression analysis.

To evaluate gonadotropin (*fsh* and *lh*) expression, real-time quantitative polymerase chain reaction (RT-qPCR) was performed. Forward and reverse primers were designed based on specific *fsh* and *lh* sequences of *A. altiparanae* (Jesus et al., 2017).

Results and Discussion

Pituitary glands treated with 10 nM Gnrh did not exhibit changes in *fsh* or *lh* expression when compared to the control group. However, higher doses of Gnrh (100 nM and 1000 nM) significantly increased the transcript levels of *fsh* and *lh* ,which were similar to the expression values found at time zero. With regards to Gnih, the different doses of this hormone did not affect the gene expression of *fsh* and *lh.*

Conclusion

Overall, it can be concluded that small doses of GnRH are insufficient to stimulate the expression of *fsh-β* and lh-*β*; however, at higher concentrations, GnRH acts as a gonadotropin-stimulatory hormone. In the case of GnIH, its inhibitory or stimulatory capacities could not be determined based on the expression of *fsh-β* and lh-*β* through these methods.

Acknowledgments

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POSTER 104

THE GONADOTROPIN-INHIBITORY HORMONE SYSTEM OF SOLE, *Solea senegalensis.*

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Introduction

Gonadotropin-inhibitory hormone (GnIH) belongs to the RFamide family of neuropeptides and exhibits orthologs in different vertebrate groups, including fish, which inhibits the synthesis and/or release of gonadotropin-releasing hormone and gonadotropins. The Senegalese sole, *Solea senegalensis*, is a flatfish species with increasing interest for Mediterranean aquaculture. However, reproductive problems exhibited by F1 generation of cultured soles are limiting the development of the aquaculture of this species. Therefore, the aim of this study was to obtain pioneer information on this inhibitory GnIH system and elucidate its distribution, function and daily/seasonal regulation in the sole.

Methods

The distribution of GniH in the brain and pituitary of sole was determined by RT-PCR and immunohistochemistry by using specific antibodies generated against sole GnIH-1 (ssGnIH-1), GnIH-2 (ssGnIH-2) and GnIH-3 (ssGnIH-3). Moreover, ssGnIH-2 and ssGnIH-3 were injected intramuscularly (0.1 and 1 μg/g bw) and the effects of both peptides on the expression of *gnrh-1*, *gnrh-2*, *gnrh-3*, *kiss2*, *fshβ*, *lhβ* and *gh* were determined by real time quantitative PCR. Finally, the daily and seasonal expression of *gnih* was determined by real time quantitative PCR.

Results and Discussion

The expression of *gnih* was particularly evident in the diencephalon, but also in the olfactory bulbs/cerebral hemispheres, optic tectum/tegmentum, retina, and pituitary. We showed the presence of ssGnIH-immunoreactive cell bodies in the olfactory bulbs, ventral telencephalon, caudal preoptic area, dorsal tegmentum and rostral rhombencephalon. The ssGnIH fibers innervated the brain and pituitary of sole profusely. Intramuscular injection of ssGnih-3 provoked a significant reduction in *gnrh-3* and *lhβ* expression. Daily rhythms in *gnih* expression were found after the completion of metamorphosis, peaking at the beginning of the night. Seasonal variations were also observed in F1 male and female adult specimens, with high levels in April/May, which corresponded with the maturation and spawning season.

Conclusion

Our results reveal the existence of a functional GnIH system in sole, which could represent an important inhibitory factor, underlying the reproductive dysfunctions observed in this flatfish species. The existence of daily and seasonal variations in the expression of *gnih* could reflect a role of this neuropeptidergic system in the mediation of the effects of environmental factors.

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POSTER 105

THE GONADOTROPIN-INHIBITORY HORMONE MODULATES THE NEUROSTEROIDS-SYNTHESIZING PATHWAYS AND AGGRESSIVE BEHAVIOUR IN MALE EUROPEAN SEA BASS.

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Introduction

Gonadotropin-inhibitory hormone (GnIH) is a neuropeptide that inhibits gonadotropin secretion and regulates vertebrate reproduction. Recent evidences obtained in male quail suggested that GnIH may inhibit aggressive behavior by regulating brain aromatase activity and neuroestrogen synthesis. However, information concerning the role of GnIH in the modulation of brain neurosteroidogenesis and aggressive behavior is lacking in fish. In order to fill this gap, we analyzed in this study the effects of GnIH in the brain expression of the main neurosteroids-synthesizing enzymes in the European sea bass, *Dicentrarchus labrax*. Moreover, to understand the behavioral role of GnIH in this species, we performed a behavioral mirror test as a proxy to measure aggression levels.

Methods

Sea bass GnIH-1 (sbGnIH-1) and GnIH-2 (sbGnIH-2) were intracerebroventricularly (icv)-administered to the fish (doses: 1, 2 and 4 μg/fish). Transcription levels of neurosteroids-synthesizing enzymes and steroid receptors were determined in the brain and pituitary by quantitative PCR. The effects of GnIH in the aggressive behavior were determined by using a mirror test in pairs of animals. Video recordings started after the recovery of anesthesia and extended for 30 min. The video recordings were analyzed using Noldus EthoVision XT software and different parameters related to mobility and mirror interactions were monitored.

Results and Discussion

Our results showed that both sbGnIH-1 and sbGnIH-2 decreased the brain transcript levels of 17β-hydroxysteroid dehydrogenase (*hsd17b*) and 3 β-hydroxysteroid dehydrogenase (*hsd3b*) and increased the expression of brain aromatase (*cyp19a1b*). In the pituitary, sbGnIH-1 also stimulated the *cyp19a1b* expression whereas sbGnIH-2 reduced *hsd17b* mRNA levels. Both stimulatory and inhibitory effects of GnIH on the expression of different estrogen receptor subtypes were found. These results suggest that GnIH could reduce the testosterone levels and increase the neuroestrogen synthesis in the sea bass brain. Behavioral analysis showed that GnIH was able to reduce the relative movement and mirror attack events of male sea bass.

Conclusion

Taken together, our results suggest that central administration of GnIH can inhibit the aggressive behavior of male sea bass by decreasing the androgen production and increasing the neuroestrogen synthesis in the brain of this species. *Funded by MINECO (AGL2013-49027-C3-2/3-R) and EMBRC (OOB–EMBRC FR–AAP2018 n° 2200).*

POSTER 106

EVIDENCES OF ALTERNATIVE SPLICING AS A REGULATORY MECHANISM FOR THE KISSPEPTIN RECEPTORS IN TELEOST, WITH EMPHASIS IN PEJERREY FISH.

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Introduction

Kisspeptin receptors are G-Protein-Coupled Receptors that regulates GnRH synthesis and release and mentioned as a key player in the control of puberty in vertebrates. However, in teleost fish some controversies on this respect appeared in the last years. Several studies carried out in fish showed the presence of alternative isoforms of kisspeptin receptors (*kissrs*). In this context, we report the gene structure of *kissr2* and *kissr3* in pejerrey fish, *Odontesthes bonariensis* and evaluate the potential presence of isoforms.

Methods

TBLASTN algorithm was used to retrieve the genomic sequences of *kissr2* and *kissr3* from the pejerrey genome database, to assess the genomic gene structure analysis using the coding sequences of these genes. Total RNA from different tissues and organs were extracted from adult pejerrey with TRIZOL reagent and retro-transcribed, to then analyze the expression pattern by reverse transcription PCR (RT-PCR) and real-time quantitative PCR (qPCR). The Kissr2 and Kissr3 homology models were generated by using the GPCR modelling server GOMoDo.

Result and Discussion

The genomic analysis revealed a differential gene structure between the receptors *kissr2* with 5 exons and 4 introns for and 6 exons and 5 introns *kissr3*. Expression studies revealed the presence of two alternative variants caused by intron retention for both genes named as *kissr2_v1*, *_v2*, *kissr3_v1* and *_v2* and with differential tissue expression. In *kissr2* the intron retention introduced two stop codons leading to a putatively truncated protein; whereas for *kissr3*, the intron retention produced a reading shift leading to a stop codon in exon 5. Modeling and structural analysis of Kissr2 and Kissr3 revealed that truncation of the proteins may produce non-functional proteins, as the structural elements missing are fundamental for receptor function. The expression pattern of the *kissr2* splicing variants was then analyzed on fish subjected to different diet regimes. Fasting induced an overexpression of *kissr2_v1* in the hypothalamus, a brain region related to the control of reproduction and food intake, with no differences in *kissr2_v2*. On the other hand it did not elicit different expression patterns in testes and habenula.

Conclusion : In summary, in this study, we have obtained the full genomic sequence of *kissr2* and *kissr3* in pejerrey and provided the first evidences of alternative splicing in both paralogous genes. Analysis of the Kissr2 and Kissr3 protein structures by 3D-models suggests that the alternative isoforms should give rise to non-functional GPCRs. Our results, together with similar findings reported in other fish species, suggest that *kissr2* presents a conserved regulatory mechanism of its expression through alternative splicing and could contribute to understand the regulation mechanisms of the Kiss/Kissr-signalling pathways.

POSTER 107

DISTRIBUTION OF GnRH SYSTEM 1, ER AND KISSR IN THE ANTERIOR BRAIN OF *Chirostoma humboldtianum*

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Introduction

Gonadotropin-releasing hormone is traditionally responsible for the release of gonadotropic hormones from the anterior pituitary gland. In turn, GnRH can be regulated by other substances that can stimulate or inhibit its release. Among them, in different species it has been determined that GnRHergic neurons can be influenced by estradiol, kisspeptin, neurokinin B and other molecules. The goal is to determine the distribution of GnRH neurons in system 1, and the alfa estradiol receptors and kiss receptors in the encephalon of *Chirostoma humboldtianum*.

Methods

The fish were collected in the lagoon of Zacapu, Michoacán, Mexico. The organisms were anesthetized and sacrificed. In several fish the brain was removes andThe encephalon was removed and fixed in 2% paraformaldehyde in phosphate buffer, washed and processed for inclusion in paraffin. Successive section were obtain and immunohistochemistry was applied for the detection of the isoform and the receptors for the purposes of the in situ hybridization technique for the detection of the a receptor.

Results and Discussion

System 1 of GnRH was fully identified in the preoptic nucleus by the presence of neurons and in the anterior pituitary gland due to the presence of immunoreactive fibers. The Kiss receptors were located in the areas of the telencephalon, the diencephalon and the mesencephalon, among them: dorsal telencephalon, the preoptic region, anterior periventricular nucleus, ventral lateral spinal nucleus, the longitudinal torus, to name a few, as well as in the anterior pituitary. The estradiol receptors were located with a distribution in the anterior periventricular nucleus, periventricular nucleus of the posterior tubercle and other areas of the telencephalon, diencephalon and middle brain. Although the results obtained for *C. humboldtianum* have been achieved with other methodological approaches, they are consistent with the results obtained for species phylogenetically similar to *Odontesthes bonariensis*, in which the presence of these substances in the brain was reported by quantitative PCR.

Conclusion

There is not evidence taht GnRH system 1 neurons coexpressing kiss receptor in this specie.

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POSTER 108

KISS/KISSR IN THE PROTANDROUS COMMON SNOOK (*Centropomus undecimalis*) UNDER *IN VIVO* HORMONAL TREATMENTS

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Introduction

The Kiss/Kissr system is considered a key factor in the neuroendocrine control of puberty, reproduction and brain sex differentiation. Several studies have demonstrated the actions of kisspeptin at the hypothalamic-pituitary-axis its modulating by sexual steroid feedback. The hypothalamic expression of kisspeptin is reported to be sexually dimorphic in many species and the exposure to sex steroids can affect sexual dimorphism of kisspeptin. The common snook is a protandrous hermaphrodite fish and there is considerable interest in controlling sex ratios by manipulating sex change in aquaculture. In this context, the aim of the present work was to test the effects of 17β-estradiol and methyltestosterone in common snook kisspeptin system.

Methods

A total of 270 undifferentiated juveniles (length 10,3 ± 1.2 cm, weight 9,55 ± 0.2 g) were divided in three groups with 3 replicates of 30 fishes each. Fish from two groups were fed *ad libitum* with diet supplemented either with methyltestosterone (60 mg kg-1) or estradiol-l7 (100mg kg-1) for 45 days. One group was used as control. Sampling was performed at the end of the experimental period (45 days), and at intervals of 4 months during a period of one year. Five animals per treatment were sacrificed and their brain and gonads were removed for gene expression and histological analysis, respectively.

Results and Discussion

The *in vivo* methyltestosterone increased *kiss1* mRNA levels in all investigated windows of development, with exception of the later one. Interestingly, 17β-estradiol also increased *kiss1* mRNA levels but at a specific window of development (45 days to 4 months after treatment). With regards to the Kiss receptor 1 (*kiss1r*), no differences were found among the groups. However, *kiss1r* increased its levels from 45 days to 4 months for the estradiol group. Interestingly, the morphological analysis showed more advanced signs of gonadal differentiation in the testosterone group; while in the control and estradiol treatments the gonads remained undifferentiated.

Conclusion

Our results showed that methyltestosterone influenced *kiss1* primary transcripts in the juvenile common snook brain. In addition, there is an interesting window of development in which the brain is more sensitive to the estradiol. More studies are needed to unravel the role of kisspeptin neurons and its relationships with sex steroids.

Acknowledgments

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POSTER 109

THE NEUROENDOCRINE CONTROL OF FEEDING AND REPRODUCTION IN ZEBRAFISH

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Introduction: Reproduction and feeding are two essential life processes in all vertebrates, the regulations of which involve a complex network of interactions. A relationship between the two systems has been well established in mammals, in which low energy stores and restricted food availability inhibit the reproductive axis. However, this relationship remains poorly understood in other vertebrates, including fish. Fish are the largest and most diverse vertebrate group. They show a wide diversity in ecology and physiology, suggesting the possibility of species-specific variations in the regulating mechanisms of feeding and reproduction. In order to better understand these mechanisms, we examined the effects of nutritional status, gender, reproductive stage on the brain expression of reproductive hormones (GnRH, kisspeptin, GnIH, and neurokinin B – NKB) and appetite regulators (orexin and NPY) using zebrafish (*Danio rerio*) as a model. In order to compare different strains of fish, we used both wild and transgenic Casper zebrafish.

Methods: Wild and Casper zebrafish were each acclimated and divided into four separate tanks. Two tanks were fed once daily to satiation, and two tanks were fasted for 7 days. At the end of the experimental period, fish were sampled and brain mRNA expression levels of reproductive and appetite regulators were assessed using qPCR in males and females of both strains. To assess how reproductive stage affects feeding, female zebrafish at various reproductive stages (based on gonadosomatic index, GSI) were sampled and brain mRNA expression levels were measured using qPCR.

Results and Discussion: In wild type zebrafish, fasting increased brain mRNA levels of GnIH, which is consistent with the inhibitory role of this peptide in reproduction. However, in contrast to what has been observed in mammals, fasting increased brain mRNA expressions of GnRH, NKB, and kisspeptin. Differences in the expression levels of reproductive hormones varied between males and females, and between reproductive stages in females suggesting gender- and GSI-specific effects of nutritional status on reproduction. Gender and reproductive stage did not appear to have major effects on the expression of appetite regulators, as fasting tended to increase the expressions of NPY and orexin in both sexes and in all females, regardless of GSI. In Casper zebrafish, fasting decreased orexin expression in males, but had no significant effect on either NPY expression or the expressions of any of the reproductive peptides examined, with the exception of NKB, which was higher in fasted males compared to fed males.

Conclusion: Our results suggest the existence of a link between nutritional status and reproduction in zebrafish, albeit with some significant differences with what has previously been described in mammals and other fish. Our data also indicates reproductive stage-, strain-, and gender-specific differences in the regulation of feeding and reproduction in wild type zebrafish. Although our studies provide new insights on the endocrine mechanisms regulating feeding and reproduction, it is very difficult to compare between fish species, given the diversity of fish with regards to reproductive cycles and feeding habits. Thus, establishing a general model of these mechanisms is challenging.

PITUITARY-GONAD SYSTEM

POSTER 110

TRANSCRIPTION OF GONADOTROPINS IN THE GONADS AND OF GONADOTROPIN RECEPTORS IN THE PITUITARY OF MALE, FEMALE AND INTERSEX THICKLIP GREY MULLETS (*Chelon labrosus*).

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Introduction

Thicklip grey mullet (*Chelon labrosus*) is a gonochoristic fish species; however, intersex males have been identified in estuaries with high availability of xenoestrogens. We have focused our previous transcriptional studies on the HPG axis, studying genes involved in sex differentiation and gametogenesis during the reproduction in females, males and intersex males. Among others, the transcription levels of gonadotropin hormone α-subunit (*gthα*) and the β-subunit of luteinizing hormone (*lhβ*) and follicle stimulating hormone (*fshβ*) were studied in pituitary while that of both gonadotropin receptors (*lhr* and *fshr*) was studied in gonads. Intersex males showed levels similar to normal males, except for *fshr*, whose transcription was downregulated to values lower than in females. Aiming to elucidate the pleiotropic action of such genes, and to detect any transcriptional alteration in intersex males, their expression pattern was studied outside the common expression tissue, receptors in pituitary and gonadotropins (GTH) in gonads.

Methods

Adult mullets were captured during their gametogenic cycle in the Pasaia harbor (SE Bay of Biscay). Histological analysis allowed gametogenic stage classification and intersex identification. Females were classified as previtellogenic, cortical alveoli, vitellogenic and regressing, and males as early, mid or late spermatogenic and regressing. Transcript levels of previously sequenced target genes were measured by qPCR in pituitary and gonads normalizing data to the cDNA concentration loaded per amplification (Quant it oligreen).

Results and Discussion

As in other fish species, significant gonad transcription levels of *gthα and fshβ* were detected in thicklip grey mullets, while both receptors were also transcribed in the pituitary. There was no variation in *gthα* and *fshβ* transcription pattern during the gametogenic cycle neither in ovaries nor in testes, transcription levels being the highest in males. Intersex testis showed lower levels than normal testis and similar to ovaries. This suggests a reduction in FSH production as a consequence of the intersex condition. Regarding the transcription pattern of the receptors in the pituitary, no differences were observed between sexes or among stages.

Conclusion : The presence of GTH subunits transcripts in the gonads and their receptors in the pituitary of mullets has been shown, suggesting a possible role of the GTH signaling system outside their target organs. In addition, females and intersex males present lower transcription levels of *gthα* and *fshβ* in the gonads than males, indicating a possible function during the differentiation of sperm vs oocyte.

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POSTER 111

SEXUAL DIMORPHIC EFFECTS OF DIETARY TAURINE ON REPRODUCTIVE PREFORMANCE OF RABBITFISH (*Siganus rivulatus*)

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Introduction

Taurine is the most abundant free metabolite in animal tissues accounting for 30–50% of the entire free amino acid pool. In vertebrates, taurine can be synthesized from the amino acids cysteine and methionine and plays a role in a vast array of physiological functions that include reproduction in mammals. Nevertheless, the contribution of taurine in fish reproduction remains unclear and under investigated. This study evaluated the effects of supplemental taurine on reproductive performance, in terms of gonadal histology and hormone production in the omnivore rabbitfish (*Siganus rivulatus*).

Methods

Hatchery-produced one-year old rabbitfish juveniles were stocked (14 /tank) in a flow through experimental system comprised of sixteen 300 L tanks with a continuous supply of ambient sea water (24-25°C; 40 ppt) and maintained under natural photoperiod conditions. The experimental system allowed the testing of 4 supplemental taurine (Tau) treatments (0, 0.5, 1.0 and 2.0 % Dry weight diet) in replicates of 4 tanks per treatment. Fish were fed a ration at 2% of tank wet biomass, distributed over two daily feedings, for 126 days where the biomass was adjusted through monthly weighing. At the termination of the trial, 20 fish per treatment were sacrificed where gonadosomatic index (GSI) was calculated and blood and tissues (pituitary, gonad and liver) were sampled. Treatment effects on reproductive performance were evaluated by gonadal histology, and hormone measurements (gonadotropins, sex steroids).

Results and Discussion

There was a dietary taurine dose dependent response on growth in both females and males. Although the gonadal biomass of males increased with dietary taurine, there was a dose dependent decrease in this parameter in females, indicating a taurine sexually-dimorphic effect. In males, but not in females, circulating LH levels increased in a dose dependent manner reaching a maximum in fish fed the high tau diet. Interestingly, the supplemental taurine negatively affected circulating estradiol levels, and consequently decreased the estrogen/androgen ratio in both sexes. Our results with the rabbitfish males further support the growing notion that taurine enhances the induction of spermatogonia proliferation and is one of the factors mediating the control of spermatogenesis. Nonetheless, our study is the first to demonstrate taurine's negative effect on estradiol secretion and its adverse implications on oogenesis in rabbitfish females.

Conclusion

Supplemental taurine in broodstock diets for rabbitfish enhanced growth. However, taurine was observed to exert species specific sexually-dimorphic effects on gonadal development, which should be carefully verified in the studied species before scaled up implementation.

POSTER 112

IDENTIFICATION AND STRUCTURAL PREDICTION OF PITUITARY GONADOTROPINS IN TREVALLY *(Pseudocaranx georgianus)*.

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Introduction

Trevally *(Pseudocaranx georgianus)* is of significant interest for aquaculture in New Zealand and a selective breeding programme is underway. To enable rapid breeding of this species we seek to 1) shorten generation intervals using recombinant gonadotropins, 2) gain knowledge about gametogenesis under culture condition, and 3) apply this knowledge to control broodstock spawning. The pituitary gonadotropins follicle-stimulating hormone and luteinizing hormone play a fundamental role in the endocrine cascade in vertebrates that enables gametes to be formed and released. Here we characterise and predict the structure of these glycoprotein hormones, examine their phylogenetic relationships with other fish species and describe the promoter sequence variation using a *de novo* genome assembly of trevally.

Methods

We used our newly assembled trevally genome (PE150bp at 60-80x, HiRise Chicago scaffolding, Dovetail HI-C: N50 scaffold 25 Mb) to obtain the i) follicle-stimulating hormone beta *(fshβ)*, ii) luteinizing hormone beta *(lhβ)* and iii) glycoprotein alpha *(gpα)* DNA sequences. Gene sequences were aligned to other fish species (n=50-90, depending on the gene) derived from the NCBI data base using the MAFFT Alignment 1.3.5 (implemented in Geneious 9.0.5) and checked using Gblocks to eliminate misaligned positions and divergent regions. Phylogenetic trees were obtained by Bayesian inference (MrBayes v.3.2) and consensus trees were visualized using FigTree v.1.4.2. To predict the three-dimensional structure of the trevally gonadotropins, the nucleotide sequences will be translated in the I-TASSER server (Iterative Threading ASSEmbly Refinement).

Results and Discussion : We identified and described for the first time the three cDNAs encoding trevally *lhβ, fshβ* and *gpα*. Complete cDNAs within the coding region were obtained for *lhβ* (436 bp) and *gpα* (346 bp), while only a partial sequence has been identified for *fshβ* (285 bp). Upon identification of open reading frames for all gonadotropins, structural predictions will be presented. Consensus trees suggest that the Japanese jack mackerel *(Trachurus japonicus)* is closely related to trevally along with other carangoid fishes (i.e. *Seriola* species and cobia *(Rachycentron canadum)*). Identification and comparison of gene promoter regions is currently in progress.

Conclusion: The identification and structural prediction of trevally pituitary gonadotropins advances our understanding in the reproductive physiology of this species and provides a future basis for the development of recombinant gonadotropins.

POSTER 113

VARIATIONS OF STEROID HORMONES AND FSH LEVELS IN ATLANTIC BLUEFIN TUNA CAUGHT IN THE MEDITERRANEAN BASIN DURING SPAWNING AND POST-SPAWNING SEASON.

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Introduction The Atlantic bluefin tuna, *Thunnus thynnus*, is a highly valued commercial species around the world. In the last decades, interest in the basic biology of this species significantly increased. Recent studies shed light on the correlation between molecular markers related to growth with canonical morphological indexes, advancing the knowledge on the metabolic system of the species and offering new useful information for the aquaculture industry (Api et al., 2018). A complex interplay occurs between growth factors and steroid hormones in the hypothalamus, representing a key factor in the neuroendocrine control of its reproductive function. In teleosts, three sex steroid hormones, 17β-estradiol (E_2), 11-ketotestosterone (11-KT) and 17 α20 β- dihydroxy-4-pregnen-3-one (DHP) are produced in gonadal tissues under the control of pituitary gonadotropins (GTH) and these are essential for gametogenesis.

Methods Pools of plasma from three animals (500 ul) were extracted twice with diethyl ether. Concentrations of Estradiol-17b (E_2) and Testosterone (T) and progesterone (P) were measured by commercially available competitive enzyme-linked immunosorbent assays (ELISA) (Cayman Chemicals, Ann Arbor, MI, USA) which measured the total amount of steroids in the plasma. Circulating FSH levels were determined using a heterologous ELISA developed using a *Seriola lalandi* recombinant FSH, produced in the yeast *Pichia* expression system and validated for tuna. Vitellogenin (VTG) concentration in the plasma was assayed using an ELISA method previously validated and described by Rahman et al. 2000. Gonad samples were fixed in Bouin's solution and prepared for histological examination using standard biological procedures. The fixed tissues were embedded in paraffin and sectioned (4 μm) with a microtome (Leitz 1512). Each gonad was fully sectioned and processed for Mayer hematoxylin-eosin staining and observed under a Zeiss Axioskop microscope. Microphotographs were captured using a high resolution digital camera (Canon EOS 6D).

Results and Discussion The results revealed that changes in plasma levels of steroid hormones, (E_2) and (T) closely correlated with gonadal development and reproductive season. In both male and female fish, E_2 levels significantly varied between spawning (summer) and post- spawning (autumn/winter) seasons, with the highest levels observed in the summer. T levels remained almost constant in males in both seasons. In contrast, a significant decrease was observed in post-spawning females. Concerning progesterone (P) levels, no significant variation was observed in the two sampling periods. The follicle-stimulating hormone (FSH) levels significantly increased at post-spawning season and were associated with significantly lower gonadosomatic index (GSI) values in both sexes. During the spawning season, FSH levels decreased concomitant with increase in GSI. At spawning, vitellogenin levels resulted significant higher in female than in male. In post spawning fish, levels resulted lower than the detection limits. Results of hormonal assays agreed with histological observations showing in summer late vitellogenic/reproductive stage ovaries and spermiating males. In autumn, few vitellogenic oocytes could be observed within the ovaries while males were still fluent.

Conclusion: The results obtained add to the basic knowledge of the reproductive biology of Atlantic blue fin tuna and could be used to enhance the development of artificial breeding technology for this high value species.

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POSTER 114

IMPACT OF GONADAL STEROIDS ON PITUITARY GONADOTROPINS IN MALE EUROPEAN SEA BASS (*Dicentrarchus labrax*)

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Introduction

The follicle stimulating hormone (Fsh) and the luteinizing hormone (Lh) are central endocrine regulators of gametogenesis in vertebrates. In the pituitary of male sea bass both hormones have overlapping profiles suggesting a similar dynamic in the synthesis and accumulation but different secretion control to the bloodstream. Gonadotropin-releasing hormones (Gnrh) are main regulators of the synthesis and secretion of Fsh and Lh, and gonadal sex steroids have a feedback effect modulating their availability, and impacting gametogenesis progression. Previous *in vivo* studies in sea bass showed some of these effects. In this study we aimed to elucidate how this system is organized in the pituitary of sea bass.

Methods

Pituitary cells from male sea bass in stage I (only type A spermatogonia) and in full spermatogenesis (stages III-IV) were cultured and stimulated with Estradiol, Testosterone Dihydroxyprogesterone, a Luteinizing Hormone-Releasing Hormone analogue (LHRHa) and a combination of them; and their influence on gonadotropin synthesis and release was analyzed. Gonadotropins were measured by specific ELISAs. Plasma levels of estradiol were measured by EIA. Gene expression was analyzed by RT-qPCR. Immunohistochemistry of sea bass pituitary sections was done by using specific antibodies against sea bass Fsh, Lh, Gnrh1, and sea bream Esr1, Esr2a and Esr2b

Results and Discussion

Annual expression of the estrogen receptor genes *esr1*, *esr2a* and *esr2b*, in male sea bass pituitary was analyzed in relation with the stages of spermatogenesis, the pituitary gonadotropin content and the circulating levels of sex steroids.

By using the pituitary primary culture, we confirmed the strong effect of LHRHa on sea bass Lh release, both in immature animals and in those in spermatogenesis. The effect of LHRHa on Fsh release in immature animals was very weak, and slightly increased as spermatogenesis advanced. On the other hand, the main evidence of repression for both gonadotropins is due to Estradiol, in animals in spermatogenesis, but we observed no effect in immature ones. In this line, immunohistochemistry studies have been performed to identify the steroid receptors in the gonadotrophs, and their relation with the Gnrh1 fibers.

LHRHa also stimulated expression of Fsh and Lh beta-subunit genes during spermatogenesis and to a lesser extend in immature animals.

Conclusion : Gnrh differentially regulates Lh and Fsh cells in male seabass, while E2 showed an inhibitory effect on the secretion of both gonadotropins. The different expression and distribution of the *esr* in the gonadotrophs suggest that they could inhibit the secretion of Fsh or Lh according to the reproductive stage. Funded by MINECO (AGL 2015-67477-C2-1-R), CSIC (201640E073) and GV (PROMETEO II-2014/051).

AQUACULTURE AND GENOME-ENVIRONMENT INTERACTION

POSTER 115

PRESERVATION OF COMMON CARP GERM CELLS UNDER HYPOTHERMIC CONDITIONS: WHOLE TISSUE VS ISOLATED CELLS

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Introduction

Recent advancements in the germ stem cell manipulation and their application to a wider range of species offered an additional possibility to overcome current bottlenecks in conservation of valuable genetic resources. Although these methods are quite promising, their success highly depends on a few crucial steps: (1) availability of individuals of the donor species at an appropriate life stage, (2) availability of good quality germ stem cells from donor individuals, and (3) availability of individuals of the recipient species at an appropriate life stage. Since most of elements in this process depend on natural processes, short- and long-term storage of gonadal tissue or germ cells can help in synchronization of the cell transplantation procedure.

Methods

Testes and ovaries of three individuals per sex were divided into two groups. Left gonads were cut into small pieces and right ones were dissociated in order to prepare cell suspensions. All test groups were kept at +4 ˚C during the experiments. Cell viability was determined by using trypan blue differential staining. Germ cells were identified by staining for alkaline phosphatase and by immunocytochemistry for the vasa protein. The aim of the first experiment was to determine the optimal sample type for hypothermic preservation of germ cells for 24 h. Ovarian / testicular tissue pieces (first test group) and oogonial / spermatogonial cell suspensions (second test group) were kept in different storage media (L-15 or DMEM) depending of the test subgroup. Viability of germ cells was evaluated at 0 h, 6 h, 12 h, 18 h and 24 h. The second experiment was conducted with germ cell suspensions of both sexes to test whether different storage media (L-15 or DMEM) can affect cell survival and if periodical exchange with fresh media can improve the viability of the cells. Viability of germ cells was evaluated at 24-hour intervals for up to 14 days.

Results and Discussion: During the 24 h hypothermic storage, dissociated germ cells of both sexes stored in cell suspensions showed higher viability compared to the tissue pieces. The possible reason might be the unequal distribution and uptake of the medium by the tissue since cells were directly exposed. For oogonial cell suspensions, the storage medium significantly affected the survival of cells, but it was not the case with spermatogonial suspensions. Since, the two tested media are fundamentally different in their buffer capacities and some other characteristics, given results were expected.

Viability of spermatogonial and oogonial cell suspensions after two weeks was approximately 40% and 25%, respectively.

Conclusion :

All remaining cells at the end of experiments appeared morphologically normal and unchanged compared to the controls. Results of the present study propose cold storage as a cost- and labor- efficient method for maintaining cell suspensions. It can be a valuable alternative if short time storage is needed since viability was similar to that after cryopreservation.

POSTER 116

EFFECT OF SEXUAL PROPORTION ON THE REPRODUCTIVE PERFORMANCE OF LAMBARI (*Astyanax lacustris*) IN SEMI-NATURAL SYSTEM.

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Introduction

Previous analyses conducted in our laboratory have indicated that even by placing several *Astyanax lacustris* couples in semi-natural spawning systems, often only one or few females effectively spawn, providing a number of fingerlings far below the potential of the species. In this concern, the aim of the present study was to establish the appropriate proportion that would generate the best reproductive performance of this species in hatcheries.

Methods

A randomized design with five treatments and two controls with three replicates (7x3) was used to evaluate the male: female ratio on reproductive performance. The following treatments were applied (male: female ratio): G1 (1:1); G 2 (2:1); G3 (3:1); G4 (1:2), G5 (1:3). Control 1 (G6) and 2 (G7) contained 100% females, but in the later females were not hormonally injected. All experimental units received 12 fish each. Females received 6 mg fractioned dose of CPE (carp pituitary extract) kg^{-1} with 12 hours interval. Males received a single dosage (3 mg CPE kg^{-1}). For evaluating reproductive performance, we considering: spawning frequency, volume of eggs obtained and fertility and hatching rates. The surveillance of breeders in each treatment was also evaluated.

Results and Discussion

Spawning were recorded in all treatments during the experimental period except for G7. A similar egg volume was observed among all treatments where spawning was observed, which was satisfactory for the species. The relative fecundity (egg volume/female weight) of G3 (1.4 ml/g) was the highest among all treatments ($p<0.05$). Fertility and hatching rates were similar among treatments ($p>0.05$) (general averages equal to 76.65 ± 14.07 and 66.00 ± 16.01, respectively). Fish survival was similar between treatments ($p>0.05$) and deaths were rarely observed. A proportionally larger number of males guaranteed better reproductive performance (G3) and from now on we will investigate if a higher number of males provoked a more intense ovulation by comparing post ovulatory follicles frequencies in post spawning ovaries among different treatments (male: female proportion).

Conclusion

These preliminary results indicated that the G3 treatment (3 males for 1 female) was the one that provided the best egg volume/breeders weight ratio. Since the dose applied is based on the weight of the fish, these results indicate that this proportion not only provides the best result in obtaining viable embryos (because the fertility and hatchability rates did not vary), but it is also the one that provided the best cost/benefit ratio in relation to the cost of the hormone used/the number of fingerlings obtained.

POSTER 117

SUITABILITY OF HYBRID MACKEREL (*Scomber australasicus* × *S. japonicus*) WITH GERM CELL-LESS STERILE GONADS AS A SURROGATE BROODSTOCK FOR PRODUCTION OF BLUEFIN TUNA.

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Introduction : Our ultimate aim is to establish a small-bodied surrogate broodstock, such as mackerel, which produces functional bluefin tuna (BFT) gametes by spermatogonial transplantation. When reproductively fertile fish are used as recipients, endogenous gametogenesis outcompetes donor-derived gametogenesis, resulting in the recipients predominantly producing their own gametes rather than donor-derived gametes. To address this, we previously used sterile salmonid recipients produced by triploidization or gene knockdown of the essential gene for germ cell survival. However, it has proven difficult to apply these sterilization methods to mackerels because of their high mortality during the larval stage. Therefore, we are now focusing on the use of hybrid recipients as an alternative approach to sterilization. In this study, we assessed the viability and sterility of the hybrid mackerel *S. australasicus* × *S. japonicus*, and its suitability as a surrogate broodstock for the production of BFT gametes.

Methods : Hybrid mackerel were produced by artificially inseminating *S. australasicus* eggs with *S. japonicus* spermatozoa. The fertilization and hatching rates were determined at 2 hours and 48 hours post-fertilization, respectively. The hatched larvae (n = 10) were analyzed by PCR with a species-specific primer that targeted both nuclear and mitochondrial DNA to confirm that they were truly hybrids. The gonads of the hybrids were then examined histologically at 30, 60, and 120 days post-hatching (dph), and the expressions of the *vasa*, *gsdf*, and *cyp11b* genes in the gonads were analyzed by RT-PCR and *in situ* hybridization at 120 dph. To determine whether the hybrid gonads could attract and incorporate donor BFT spermatogonia, BFT spermatogonia labeled with PKH26 fluorescent dye were transplanted into the peritoneal cavity of the hybrid larvae at 10 dph. The hybrid recipients were then observed under a fluorescent microscope at 14 days post-transplantation (dpt) to evaluate the incorporation of the PKH26-labeled donor cells into their gonads.

Results and Discussion :PCR analyses revealed that the F1 offspring carried both paternal and maternal genome. There was no significant difference in the fertilization and hatching rates between the hybrid and non-hybrid embryos. Moreover, hybrid mackerel showed no deformation and grew normally. Surprisingly, histological observation revealed that germ cells disappeared in the hybrid gonads after 120 dph, and this was confirmed by a lack of expression of *vasa*. By contrast, *gsdf* and *cyp11b* remained at the same levels as was observed in the non-hybrid gonads. These results show that the hybrid gonads were germ cell-less sterile but still possessed supporting cells and steroid-producing cells, both of which are indispensable to nurse donor-derived germ cells. Fluorescence observation of the hybrid recipients at 14 dpt revealed that the donor cells had been incorporated into the recipients' gonads.

Conclusion: We found that the hybrid mackerel develops as a germ cell-less sterile fish. Moreover, hybrid mackerel gonads can still support the colonization of transplanted BFT spermatogonia. Thus, this hybrid mackerel shows significant promise for use as a recipient for the production of BFT gametes.

POSTER 118

$PGF_{2\alpha}$ AND DHP PLASMA LEVELS DURING *Piaractus mesopotamicus* HYPOPHYSATION WITH OR WITHOUT EXOGENOUS $PGF_{2\alpha}$ ADMINISTRATION

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Introduction

We have recently showed that the rate of ovulation and frequency of post-ovulatory follicles are superior in *Piaractus mesopotamicus* (pacu) females submitted to hypophysation coupled with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) analogue (Ciosin®) in comparison to hypophysed female (treated only with carp pituitary extract (CPE)). However, some CPE + $PGF_{2\alpha}$ treated females still presented unsatisfactory spawning associated with a long period of latency. Thus, to evaluate the proper timing of application of exogenous $PGF_{2\alpha}$ to further enhance ovulation (100% ovulation), in this study we compared the $PGF_{2\alpha}$ and DHP plasma levels of CPE only with CPE + $PGF_{2\alpha}$ treated - females during induced ovulation. Furthermore we established associations of these profiles with the failure or successful ovulation in this species.

Methods

Twenty-three adult pacu females were distributed in the following five treatments using a 24-hour interval between doses: T1 (saline) + (saline) (control); T2 (saline) + (saline and 2 mL $PGF_{2\alpha}$); T3 (0.6 $mg.kg^{-1}$ CPE) + (saline and 2 mL $PGF_{2\alpha}$); T4 (0.6 $mg.kg^{-1}$ CPE) + (5.4 $mg.kg^{-1}$ CPE); and T5 (0.6 $mg.kg^{-1}$ CPE) + (5.4 $mg.kg^{-1}$ CPE and 2 mL $PGF_{2\alpha}$). Blood samples were collected before the first hormonal dose (A1), one (1H), four (4H), eight (8H) and twelve hours (12H) after the second dose for the quantification of plasma levels of $PGF_{2\alpha}$ and 17α, 20β-dihidroxi-4-pregnen-3-one (DHP) through ELISA method.

Results and Discussion: Female ovulation was only obtained when two CPE doses were applied (T4 (66.67% spawning rate) and T5 (83.33% spawning rate)), regardless of the $PGF_{2\alpha}$ use. An increase (≈ 44-fold) in the plasma levels of $PGF_{2\alpha}$ of T2, T3 and T5 groups was observed at 1H ($p<0.05$). In addition, groups T2, T3 and T5 presented higher levels of $PGF_{2\alpha}$ compared to T1 and T4 groups in the same sampling (1H) ($p<0.05$). These findings revealed that ovulation cannot be attained using only $PGF_{2\alpha}$ and a plasmatic $PGF_{2\alpha}$ peak is observed one hour after exogenous administration. At the expected time of ovulation (12H) we observed that $PGF_{2\alpha}$ levels of T4 and T5 were higher than other treatments ($p<0.05$), indicating that this second $PGF_{2\alpha}$ peak was caused by application two doses of CPE, since a similar peak was not observed in the other treatments. These results suggests that the exogenous $PGF_{2\alpha}$ could be applied closer to expected time of ovulation, since a plasma peak was detected one hour after ovulation.

Conclusion : Pacu females induced only with $PGF_{2\alpha}$ did not present ovulation. The use of CPE coupled with $PGF_{2\alpha}$ increased the spawning rate when compared to the use of CPE only. Peaks of plasma $PGF_{2\alpha}$ caused by administration of exogenous PGF and by application two doses of CPE occur respectively one hour after the second doses and at the expected time of ovulation.

POSTER 119

PGF2α PLASMA LEVELS OF *Piaractus mesopotamicus* TREATED WITH EXOGENOUS PGF2α AT DIFFERENT TIMES DURING HYPOPHISATION.

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Introduction

We have recently showed that the rate of ovulation and frequency of post-ovulatory follicles are superior in *Piaractus mesopotamicus* (pacu) females submitted to hypophysation coupled with $PGF_{2\alpha}$ (Ciosin®) in comparison to hypophised females (treated only with carp pituitary extract (CPE)). However, some CPE-only treated females at the second dose still presented unsatisfactory spawning associated with a long period of latency. Thus, to evaluate the proper timing of application of exogenous $PGF_{2\alpha}$ to further enhance ovulation (100% ovulation), in this study we compared the $PGF_{2\alpha}$ plasma levels of CPE treated females that received exogenous $PGF_{2\alpha}$ at the time of CPE second dose or 7H after second dose.

Methods

Ten adult pacu females were treated with 0.6 $mg.kg^{-1}$ CPE (first dose) + 5.4 $mg.kg^{-1}$ CPE (second dose) with 24-hour interval between doses. Fish were divided into two groups: the first received 2 mL exogenous $PGF_{2\alpha}$ at the time of second dose (2D) and the second, 2 mL exogenous $PGF_{2\alpha}$ 7H after second dose (7H). Blood samples were collected just after of ovulation (or at the predictable time of ovulation for females that did not ovulate) for the quantification of $PGF_2\alpha$ plasma levels using ELISA method. During the whole experiment water was maintained at 27^{o}C. Data were analysed by Repeated measures ANOVA and compared by Tukey HSD.

Results and Discussion

Female ovulation was observed for both treatments, but $PGF_{2\alpha}$ at the time of ovulation was 16 times higher for 7H group then for 2D group. Since in this study females had to be intensively manipulated, we opted not to evaluate the spawning rates that would certainly be affected and would require several different controls (a large number of unavailable fish) to be correctly interpreted. A plasma $PGF_{2\alpha}$ peak ~ one hour after its injection (at the time of ovulation) was observed in 7H treatment (650 ng. ml^{-1} at the time of ovulation). We confirmed that by injecting $PGF_{2\alpha}$, a peak of PGF 16 x higher is obtained at the time of ovulation, but the hypothesis that this higher peak would cause an increase in ovulation rate is still being evaluated.

Conclusion

Plasma levels of $PGF_{2\alpha}$ by administering exogenous $PGF_{2\alpha}$ at the time of the second CPE dose or 7H after the second CPE dose were respectively ~ 40 ng. ml^{-1} and 650 ng. ml^{-1} (16 times higher for the later group) at the time of ovulation. Therefore, it is possible to raise plasma $PGF_{2\alpha}$ levels at the time of ovulation by changing the time of application of exogenous $PGF_{2\alpha}$ from the second CPE dose to 7H after the second CPE dose of hypophisation.

POSTER 120

INCORPORATION OF ESSENTIAL FATTY ACIDS IN THE GONADS OF FEMALES AND EMBRYOS OF *Astyanax altiparanae*.

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Introduction

Highly unsaturated fatty acids (AGI) are considered essential in the formation of viable and symmetrical oocytes, acting on the degree of fluidity and permeability of cell membranes and on the formation of good quality larvae. In this sense, the objective of this work was to evaluate the incorporation and mobilization of AGI in the gonads of *Astyanax altiparanae* females and their transfer to the embryos after being submitted to a diet of marine fish oil.

Methods

For this, 2400 juveniles of *A. altiparanae* were divided into two groups. One group was fed Laguna onivoros 36, 2.6mm (CG) and another fed with the same diet plus 5% of marine fish oil (GO), with high percentages of AGI, more specifically DHA (docosahexaenoic acid) and EPA (eicosapentaenoic acids). In order to evaluate the incorporation of AGI in the gonadal tissue, the ovary samples were collected from 5 females of each group monthly, during seven months, as well as the reproduction of females from both groups, to obtain the embryos. The gonads and embryos were stored at -80ºC for later analysis of the fatty acid profile. The total lipids extracted were separated into the neutral and polar fractions by column chromatography, methylated and analyzed on a gas chromatograph coupled to a flame ionizer.

Results and Discussion

In the GO females gonads, a significant increase of omega 3 fatty acids was observed, notably represented by DHA (C22: 6n3). Analyzing the polar lipids of the embryos, smaller values for C16:0 fatty acid were observed in embryos from CG and a considerable increase in the amount of DHA (Omega 3) in GO embryos. Studies relate the presence of these AGIs (Omega 3) to steroidogenic regulation of gonadal and oocyte maturation, in addition to the formation of eggs of better quality and a higher rate of larval survival. This AGI with a large amount of carbon is considered to be very important for the development of the nervous system and other embryonic tissues, as well as for the maintenance and increase of tissue fluidity and permeability, also helping the development of cryopreservation processes for fish embryos.

Conclusion

We conclude that the inclusion of 5% of marine fish oil in the diet of *A. altiparanae* females facilitated the incorporation of AGI in ovarian tissues and also transferred to the embryos from these females.

POSTER 121

THE INFLUENCE OF SEASONAL PHOTOPERIODIC FLUCTUATION ON REPRODUCTION AND STEROID LEVELS IN SOLE.

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Introduction

The sole (*Solea senegalensis*) is an important fish species for Mediterranean aquaculture, but still needs appropriate techniques to control reproduction. Light is a primary environmental cue for the activation of the endocrine reproductive system, being the steroids hormones the ultimate regulators of gonadal growth, maturation and spawning. This study aimed to study reproductive performance of sole reared with or without the fluctuating photoperiodic signal and determine the concentration of relevant steroids, by a newly developed ultra-high performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) method.

Methods

Soles were reared for five years under natural (controls) or constant photoperiod (12L:12D, "LD"). Spawning was monitored and samples collected at the fifth year end-point, to analyze gonad morphology (histology) and steroids levels in plasma, urine, gonad and muscle, by UHPLC-MS/MS. Steroids included, C21 progestogens (progesterone [P4], 11-deoxycortisol [S], cortisol [F], 17α,20β-dihydroprogesterone [DHP], 17α,20β,21-trihydroprogesterone [20β-S]), C19 androgens (androstenedione [A4], testosterone [T], 5α-dihydrotestosterone [DHT], 11β-hydroxyandrostenedione [11OHA], 11ketoandrostenedione [11KA], 11ketotestosterone [11KT]) and C18 estrogens (estrone [E1], 17β-estradiol [E2]).

Results and Discussion

Gonad maturation and spawning occurred in control and LD broodstock, at similar timing. Gonads of both groups synthesized DHP but not 20β-S, suggesting a role of DHP as maturation-inducing steroid in sole. Gonad, plasma and muscle levels of all 5 androgens were higher in controls than LD males, suggesting inhibited androgen synthesis under LD. Similarly, gonad, plasma, muscle and urine levels of the two estrogens were higher in controls then LD females, suggesting reduced estrogen synthesis. The androgen DHT was detectable in the urine of LD males, providing preliminary novel evidences for a role of DHT in sole.

Conclusion: Sole broodstock reared under no light fluctuation (constant photoperiod) matured and spawned yearly in the spring, indicating the existence of an endogenous reproductive rhythm. However, the reduced levels of critical steroids suggests negative endocrine effects of constant conditions on reproductive performance.

POSTER 122

PRIMORDIAL GERM CELLS (PGCS) DISRUPTION BY USE OF SDF – 1A/CXCR4 SYSTEM ANTAGONISTS

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Introduction: Aquaculture is one of the best strategies to sustainably increase the production of food to supply it increasing demand. One of the best tools for it is the sterilization of fish species. Besides the greater somatic growth, sterile fish avoid genetic introgression of native species in case of accidental escapes and outflow of specific fish farmer's strains. Moreover, obtaining of sterile fish is the bottleneck to the successful achievement of germ cell transplantation approach. In view of the benefits of sterile fish, in this study, we tried the massive sterilization of the sterlet sturgeon (*Acispenser ruthenus*) using chemical antagonistic to SDF-1a/CXCR4 chemoattractant system, which is believed to control PGCs migration to fish genital anlage.

Methods: Sampling of sturgeon gametes was artificially inducted by intramuscular injection of carp pituitary extract. Fertilization was performed at room temperature and after that the chorionic layer of each embryo were removed with forceps aiming to facilitate their manipulation. Next, aiming to follow PGCs migration, FITC-dextran was injected in the vegetal pole of each 2 to 4 cells embryo. The chemicals used in the experiment (2-Methylthio-2-imidazoline hydroide, WZ811, N-BOC-piperidine-3-Meethanol, 97% and N-BOC-2-piperidineethanol) were prepared at 1 Molar and were diluted 1000, 10000 and 100000 times in 1.8 ml $MgCL_2$ + 1.8 ml $CaCl_2$ + 1 L distilled water containing 0.01% of penicillin plus 0.01% of streptomycin. Twenty FITC-dextran labeled embryo were incubated by treatment: T1 = 2-Methylthio-2-imidazoline hydroide; T2 = WZ811; T3 = N-BOC-piperidine-3-Meethanol, 97%; T4 = N-BOC-2-piperidineethanol; T5 = Control (Dilution solution only). Solutions of the different treatments were daily changed to avoid chemical degradation as well as to ensure a cleaning and oxygenated water to the suitable embryo development. The analyses were diary performed by observation of FITC-dextran labeled PGCs during their migration and incoming in the genital anlage by use of a fluorescent microscopy. Furthermore, 24 days after fertilization the animals were anesthetized with tricaine solution, sacrificed and their genital anlage were exposed to the quantification of PGCs in that region. Statistical analyses were performed with one-way ANOVA followed by Tukey test ($P<0.05$).

Results and Discussion: Embryo incubated in the higher concentration of WZ811 solution showed a significant reduction in the number of PGCs that arrived gonadal anlage in comparison to the Control and other treatments. In all the other treatments and concentrations, the number of PGCs in the embryo genital ridge were similar. All the embryos treated with N-BOC-2-piperidineethanol in the higher concentration died before the end of the experiment. The reduction, but not total elimination of PGCs number, in the genital ridge of the embryos, can be considered unsuccessful to fish sterilization, since it is already proved that one unique PGC is able to recover the gametogenesis. However, the use of this technique seems to be promisor, since WZ811 solution seems to cause "mis-migration" of PGCs. Because of this, new experiments are being performed trying fish sterilization. **Conclusion:** In the present study we showed that WZ811 reduced the quantity of PGCs that arrived in the gonad ridge.

POSTER 123

ATLANTIC SALMON BREEDING HASTENED BY INTERGENERIC SURROGATE BROODSTOCK TECHNOLOGY: POTENTIAL TO STREAMLINE DEVELOPMENT OF IMPROVED LINES

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Introduction

Surrogate broodstock technology is emerging as a promising biotechnology for xenogeneic production of gametes of highly valuable fish species. In this study, we produced gametes of landlocked Atlantic salmon using triploid rainbow trout as recipient through germ cell transplantation technique in embryos.

Methods

Spermatogonial cells were obtained from Atlantic salmon juveniles and, after purification and labeling with PKH-26 dye, they were transplanted into the coelomic cavity of 150 mixed-sex triploid embryos of rainbow trout. Four weeks post-transplantation, colonized cells were observed under a fluorescent microscope. Gametes from transplanted males were screened with an Atlantic salmon-specific DNA marker whereas the females were screened based on the presence/absence of oocytes. Milt from positive males were used for artificial insemination of Atlantic salmon oocytes. F1 progeny was screened with the same Atlantic salmon specific-marker for confirming whether the offspring was donor derived.

Results and Discussion

Colonization efficiency assessed in larvae four weeks post-transplantation showed presence of PKH-26-labeled cells in 61.1% of individuals. Analysis in sexually mature adults revealed presence of donor-derived sperm in four transplanted males (10%), whereby one of them reached sexual maturity in the first year. Transplanted females became mature after the second year and donor-derived oocytes were detected in four individuals (12.1%). Genetic analysis of eyed-egg embryos produced with milt from a germ cell transplanted male revealed that progeny was composed by pure Atlantic salmon and therefore were donor-derived.

Conclusion

In conclusion, this study shows that surrogate broodstock can be used to produce oocytes and spermatozoa using species from different genus in salmonids. The production of surrogate gametes using species with a shorter life cycle represents an efficient approach to streamline the development of improved strains in aquaculture industry.

POSTER 124

EFFECT OF SEXUAL PROPORTION ON THE REPRODUCTIVE PERFORMANCE OF LAMBARI (Astyanax lacustris) IN SEMI-NATURAL SYSTEM.

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Introduction

Previous analyses conducted in our laboratory have indicated that even by placing several Astyanax lacustris couples in semi-natural spawning systems, often only one or few females effectively spawn, providing a number of fingerlings far below the potential of the species. In this concern, the aim of the present study was to establish the appropriate proportion that would generate the best reproductive performance of this species in hatcheries.

Methods

A randomized design with five treatments and two controls with three replicates (7x3) was used to evaluate the male: female ratio on reproductive performance. The following treatments were applied (male: female ratio): G1 (1:1); G 2 (2:1); G3 (3:1); G4 (1:2), G5 (1:3). Control 1 (G6) and 2 (G7) contained 100% females, but in the later females were not hormonally injected. All experimental units received 12 fish each. Females received 6 mg fractioned dose of CPE (carp pituitary extract) kg-1 with 12 hours interval. Males received a single dosage (3 mg CPE kg-1). For evaluating reproductive performance, we considering: spawning frequency, volume of eggs obtained and fertility and hatching rates. The surveillance of breeders in each treatment was also evaluated.

Results and Discussion

Spawning were recorded in all treatments during the experimental period except for G7. A similar egg volume was observed among all treatments where spawning was observed, which was satisfactory for the species. The relative fecundity (egg volume/female weight) of G3 (1.4 ml/g) was the highest among all treatments (p0.05) (general averages equal to 76.65 ± 14.07 and 66.00 ± 16.01, respectively). Fish survival was similar between treatments ($p>0.05$) and deaths were rarely observed. A proportionally larger number of males guaranteed better reproductive performance (G3) and from now on we will investigate if a higher number of males provoked a more intense ovulation by comparing post ovulatory follicles frequencies in post spawning ovaries among different treatments (male: female proportion).

Conclusion

These preliminary results indicated that the G3 treatment (3 males for 1 female) was the one that provided the best egg volume/breeders weight ratio. Since the dose applied is based on the weight of the fish, these results indicate that this proportion not only provides the best result in obtaining viable embryos (because the fertility and hatchability rates did not vary), but it is also the one that provided the best cost/benefit ratio in relation to the cost of the hormone used/the number of fingerlings obtained.

POSTER 125

INTEGRATED CONTROL OF REPRODUCTION AND GAMETOGENESIS IN FISH

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Control of gonadal development and gametogenesis is multifactorial, and Involves hormones of brain-pituitary gonadal axis as well as other peripheral hormones such as thyroid hormones. Hypophysial hormones interact with local ovarian and testicular steroids and peptides including gonadotropin-releasing hormone (GnRH) and gonadotropin-inhibitory hormone (GnIH) to control synchronize development of early gametogenesis and final maturation of oocytes and sperms. Thyroid hormones suppress reproductive axis during the period of growth and after a period of gonadal recrudescence following ovulation enhance the ability of liver to start vitellogenesis. Macro regulatory hormones, LH and FSH, interact with local gonadal peptides and steroids to control the unset of apoptosis and promote synchronized development of ovary and testis. Disruption of brain pituitary gonadal axis by environmental stressors can lead to abnormal production of brain-pituitary axis and impairment of ovulatory hormones which can lead to increased apoptosis and degeneration of testis and ovary. The present study provides novel information on the role of gonadal peptides and thyroid hormones as well as mechanisms underlying endocrine and paracrine control of gametogenesis. The overall results based on studies in goldfish and zebrafish illustrates complex multifactorial mechanisms involving pituitary hormones, gonadal and thyroid hormones working in concert to regulate reproduction in fish. Funded by grants to HRH.

POSTER 126

DOES THE *VGLL3* LOCUS INFLUENCE THE AGE OF PUBERTY IN A POST-SMOLT MATURATION MODEL IN ALL-MALE ATLANTIC SALMON?

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Introduction: Atlantic salmon (*Salmo salar*) display a large degree of phenotypic plasticity regarding the age at puberty. In the wild, males may mature as parr during early freshwater life or as anadromous males after one or more seawinters. Although the exact mechanisms are unclear, recent work has demonstrated that the *vgll3* locus in the salmon genome accounts for approx. 35-38% of the variation in the age of puberty in wild fish and provides a genetic target for breeding studies that aim to produce later maturing fish. We already know that the *vgll3* alleles affects time of maturity in farmed salmon, but we have not explored to what the extent the environmental factors within culture systems, such as light and temperature, affects the impact of the alleles. In the current study, we aim to establish the extent to which the *vgll3* alleles explain the likelihood of male salmon to enter puberty following a light and temperature regime that stimulates post-smolt maturation. In addition, we aim to study the maternal and paternal contribution to the *vgll3* locus.

Methods: Initially, mixed sex yolk sac larvae were treated with 17α ethinylestradiol to induce sex reversal. From this, a single XY phenotypic female was found, and it was a hermaphrodite producing both X and Y sperm and eggs. The fish was heterozygous (EL) for the *vgll3* locus. Subsequently, we fertilized half of the eggs with its own sperm to produce YY males that were heterozygous (EL) for the *vgll3* locus, while the remaining eggs were fertilized with UV treated sperm to produce double haploid YY males. Following this, two of the resulting EL YY males from the self-fertilization procedure were crossed with four double haploid (dh) females, two of which were homozygous for the early maturing genotype (EE) whereas the other two were homozygous for the late maturing genotype (LL); the eggs from each female were split in two and fertilized with the two different males. This resulted in 2 half sibling groups from each female, and a total of 8 different family groups. Since the males were heterozygous, there were 2 different genotypes within each family group; EE and EL from the EE dh XX × EL YY crosses, and LL and LE from the LL dh XX × EL YY crosses. A further YY male with an EE genotype produced from the double haploid and UV sperm procedure was crossed with a double haploid EE female. These fish were then reared common garden (180 fish/group) and subjected to a post-smolt maturation regime of 16°C and continuous light for eight weeks. At termination (March 2018), the testes will be weighed to identify those fish that have entered puberty as post-smolts, and DNA will be collected to genotype the fish.

Results and Discussion: This experiment is still ongoing, but if the *vgll3* alleles does influence the probability of entering early maturation following light and temperature stimulation, we expect those fish homozygous for the early maturation genotype (EE) to display a higher prevalence of puberty compared to those fish with the LL genotype, with the EL genotype intermediary.

Conclusion: In the present study, we want to determine to what extent the *vgll3* alleles can be used to reduce early maturation in all-male post-smolt salmon stimulated to enter puberty and to what extent the farming environment controls maturation independent of genotype.

POSTER 127

ANDROGEN SIGNALING IN THE EEL, *Anguilla australis*, DURING THE SILVERING TRANSFORMATION: NON-CLASSICAL EFFECTS VIA THE ZIP9 (SLC39A9) ZINC TRANSPORTER

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Introduction

It is well documented that plasma levels of 11-ketotestosterone (11-KT) drastically rise as eels undergo the yellow-to-silver eel ("silvering") transformation. It is plausible that the classical nuclear androgen receptor is mediating this androgen signal. However, non-genomic androgen actions have been identified in some teleost fish, but evidence for such actions in the eel have remained unexplored. Do they include silvering-related changes? ZIP9 is one of 14 members of the ZIP (ZRT-and Irt-like Protein, SLC39A) family involved in zinc transport and regulation of zinc homeostasis in the cytoplasm and was recently shown to specifically bind androgen and mediate its signal. Here, we seek to study the distribution, function and expression of the *zip9* gene in eel, especially in relation to silvering and androgen-mediated ovarian lipid uptake. As a first step, we retrieved the *zip9* DNA sequence and investigated *zip9* expression in a suite of tissues from yellow and silver New Zealand shortfinned eels.

Methods

We employed Illumina sequencing and interrogated three annotated transcriptomes, derived from pooled ovarian tissue, from liver and from a tissue pool (eye, head kidney, pituitary), using the search string 'Zip9'. Putative *zip9* contigs were aligned and the consensus sequence confirmed after PCR. The distribution of *zip9* transcripts across different tissues was similarly evaluated by conventional PCR in silver and yellow eels. QPCR and transient expression analysis are currently in progress.

Results and Discussion

Putative *zip9* contigs of up to 930 bp were obtained from the ovarian and tissue-pool, but not the hepatic, transcriptomes. BLASTP searching of the deduced Zip9 amino acid sequence yielded a full-length protein of 310 amino acids and 33.3 kDa belonging to the ZIP superfamily; this protein had high identity (~ 85%) to that of a suite of Perciforms. Preliminary analyses suggested that *zip9* is principally expressed in gut (anterior and posterior intestine), brain (fore, mid and hind brain), head kidney, pituitary, liver, ovary and gills. There was no clear difference in gene expression between silver and yellow eels for most of the tissues examined, except for midbrain and anterior intestine, in which mRNA levels appeared to be higher in silver than in yellow eels.

Conclusion

Zip9 in eel encodes a protein of 310 amino acids and it is expressed in a wide range of tissues in the eel, both in pre- and post-silvering stages.

POSTER 128

SEMINAL FREEZING OF COMMON CURIMATÃ (*Prochilodus brevis*) AFTER DIFFERENT COOLING TIMES

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Introduction

P. brevis is a species of rheophilic fish, which presents ecological and economic importance. However, its survival is threatened due to anthropic and climatic factors. For this reason, studies on reproductive biotechnologies, such as seminal conservation, are needed to subsidize *P. brevis* fish farming. The objective of this work was to evaluate the motile and curvilinear velocity (VCL) of the post-thawed semen of *P. brevis*, which underwent different cooling times.

Methods

For this, nine pools were formed and then these were processed as follows: 1- immediate freezing; 2- seminal cooling (6, 12, 24 and 48 h): undiluted, diluted in coconut water powder (ACP-104) or diluted to 5% glucose; After cooling: the samples were added with their respective diluent and dimethyl sulfoxide (DMSO). After 15 days, the samples were thawed and analyzed for motility and VCL. For statistical analysis, a completely randomized design with 3 factors (storage form × cooling time and storage form × diluent) was used. The ANOVA and Dunnett tests were applied to compare the means. There was no triple interaction between the storage form, the diluent and the cooling time.

Resultads and Discussion

Comparing the motility (50.56%) and VCL (31.60 μm / s) of the semen immediately frozen with the other treatments, it was observed that the samples kept cooled and diluted for up to 6 h had the best results. Regarding the storage form (diluted and undiluted) and the cooling time, only the treatments diluted for up to 6 h had the best motility and VCL results when compared to the undiluted ones ($P < 0.05$). Regarding the storage form and the diluents (ACP-104 and glucose), regardless of the cooling time, it was observed that the treatment diluted in ACP-104 presented motility and superior VCL ($P < 0.05$) to undiluted and that subsequently received ACP + DMSO. In addition, when the semen was kept cooled and diluted, the best values of the analyzed parameters were obtained when it was maintained in ACP-104 ($P < 0.05$). It is believed that during the cooling, the glucose diluent caused an osmotic stress in the cell, leading to the rupture of the plasma membrane and also that it may have diminished important components of the seminal plasma. On the other hand, the good results achieved with the ACP-104 diluent were attributed to its rich composition, mainly the presence of indole-3-acetic acid (IAA), an auxin with a proven potential for seminal conservation of other species.

Conclusion: Thus, it is concluded that ACP-104 is the most recommended diluent for the seminal freezing of *P. brevis*, since it maintains the good freezing of the semen that has been cooled for up to 6 h.

POSTER 129

GERM STEM CELL TRANSPLANTATION PROCEDURE FOR THE REGENERATION OF ISOGENIC TROUT LINES.

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Introduction

Isogenic fish lines are clonal families, homozygous at all DNA loci. Isogenic trout lines were generated at INRA and they are currently highly valuable models to investigate the genetic and environmental determinism of traits such as disease resistance, stress tolerance, feed efficiency, sex-ratio or gamete quality. The present study was aimed to evaluate a procedure to regenerate these important genetic resources, which are fragile and, if lost, *cannot be reproduced*.

Methods

Isogenic lines were produced using two rounds of endomitotic and meiotic gynogenesis and are all-female populations. Purified undifferentiated spermatogonia were obtained from isogenic neomales (sex-reversed isogenic homozygous females) by centrifugal elutriation. Spermatogonia were injected in the abdominal cavity of hatching *triploid* embryos (2 exp). Male and female recipients were grown until sexual maturation then crossed to analyze their offspring. To discriminate the progeny derived from the *donor*, a "golden" strain (homozygous for a dominant yellow color variant) was used as *recipient*, while isogenic donors present the wild-type "black" coat. DNA genotyping was performed using a panel of diagnostic microsatellite markers.

Results and Discussion: 55% of the transplanted triploid embryos had survived to hatching. The presence of donor cells inside male and female gonads was confirmed by genotyping (70% of positive recipient). In 7- month-old females, histological evaluations showed that oocytes were blocked in the diplotene stage in triploid fry and developed beyond this stage only in individuals transplanted with diploid germ cells. One year post transplantation, 70% of the males matured and sperms were used individually to fertilize 600 eggs from another wild-type trout line. Interestingly 100% of all their progenies were female (SDY negative) and showed the recessive "black" coat, suggesting that triploid recipients produce *functional* spermatozoa derived from the donor transplanted spermatogonia only. Furthermore, all progenies harbored specific alleles derived from the isogenic donor (n=20 per cross). One year later, 77 % of the transplanted female ovulated (1780+/-640 ovules/kg body weight). Ovules from 12 transplanted females were fertilized with fresh or frozen sperm from the transplanted males. The use of cryopreserved sperms produced fewer offspring. 100% of all progenies exhibited the recessive "black" phenotype of the donor line, showing that crossing gametes obtained from male and female transplanted triploid recipients generated progeny from the isogenic donor line only. The first DNA genotypings on the offspring confirmed that one specific isogenic line was generated.

Conclusion: High rate of isogenic oocytes and spermatozoa production can be achieved by transplantation of purified isogenic spermatogonia into triploid recipients, and their crossing regenerate the donor isogenic line in one generation. (This study was supported by the UE AquaExcel2020 project).

POSTER 130

CRYOPRESERVATION AND INTERGENERIC TRANSPLANTATION OF SPERMATOGONIA AND OOGONIA IN SALMONID SPECIES.

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Introduction

Cryopreservation of gametes and embryos has long been recognized as an efficient tool for the preservation of genetic resources, however, in fish only sperm can efficiently be cryopreserved. Recent advances in the transplantation of germ cells allow the surrogate production of donor-derived gametes by suitable recipients. Combined with cryopreservation, surrogate production opens new possibilities in the conservation of genetic resources of endangered species or varieties of fish. The objectives of this research were to develop cryopreservation methods for early germ cells of salmonid fish and to conduct intergeneric transplantation into related species within the frames of a Hungarian-Slovenia bilateral project.

Methods

Gonads were isolated from subadult individuals of brown trout (*Salmo trutta m. fario*), grayling (*Thymallus thymallus*) and rainbow trout (*Oncorhynchus mykiss*). Testicular and ovarian tissue was cryopreserved using either freezing or vitrification and by testing various cryoprotectants, cryoprotectant concentrations and cooling rates. Spermatogonia or oogonia were isolated from freshly collected gonads or following thawing. Viability of isolated cells was determined following staining by Trypan blue. Suspensions containing the isolated cells were transplanted into non-feeding larvae of the recipient species by microinjection. Triploid and diploid rainbow trout was used as a recipient for the germ cells of the brown trout and grayling while the hybrid tiger trout (*Salvelinus fontinalis* × *Salmo trutta*) served as a recipient for rainbow trout. The success of incorporation was verified by the presence of fluorescence-labeled donor-derived cells in recipient fish 3 months following transplantation as well as using species-specific amplification of the mitochondrial DNA control region (mtDNA CR).

Results and Discussion

Optimization of the freezing procedure resulted in 60-65 viable germ cells in up to 74% in the brown trout. The use of the cryoprotectant dimethyl-sulfoxide in concentrations of 1.3-2 M yielded the highest percentages of viable cells following thawing. Vitrification protocols yielded survival rates of 41% in the brown trout. Successful incorporation of spermatogonia and oogonia was detected in rainbow trout recipients. Positive fluorescent signals were detected in 27-40% of rainbow trout juveniles when brown trout was used as a donor and 24-28% when grayling was used. Analysis of the mtDNA CR further verified the proliferation of donor-derived cells. Following the transplantation of rainbow trout germ cells into 371 tiger trout larvae, 55 were alive three months following transplantation, thus, a decision was made to grow these individuals to sexual maturity instead of sacrificing them for the verification of incorporation success.

Conclusion: In this study, we showed that intergeneric transplantation of early germ cells in salmonids is feasible and verifiable using molecular markers.

POSTER 131

CRYOPRESERVATION AND TRANSPLANTATION OF COMMON CARP GERM STEM CELLS INTO GOLDFISH

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Introduction

Common carp is one of the most cultured fish in the world with many different breeds and plenty of published protocols for sperm cryopreservation. However, no method was developed for the preservation of gonadal tissue congaing germ stem cells – bipotential precursors of gametes. Spermatogonia (Spg) and oogonia (Oog) can be cryopreserved and recovered by transplantation into surrogate host resulting in donor derived gametes production.

Material and methods

Gonadal tissue was obtained from juvenile carps. Protocol for Spg cryopreservation was developed in several trial testing cryoprotectants and their concentrations, freezing rates, sugars, tissue size and equilibration time. Similar trials were performed for Oog cryopreservation. Ovarian tissue was stored temporally in +4 °C and -80 °C. Cryopreserved cells were transplanted intraperitoneally into sterilized goldfish larvae.

Results and Discussion

Highest Spg viability (41%) was achieved in medium containing 2M DMSO, 0.3M Glucose, 25mM HEPES and 1.5% BSA. Cryomedium composed from 1.5M DMSO, 0.3M Glucose, 25mM HEPES and 1.5% BSA resulted in 65% viability of Oog. Positive improvement in viability was observed after prolonged equilibration (1h) in the cryomedium before freezing. Viability of Oog stored in +4 °C remained unchanged for 7 days of storage. Viability of hypothermicaly stored tissue for 5 days after subsequent slow rate freezing resulted in same viability as in immediately frozen samples after the dissection. Samples frozen and stored in -80 °C retained viability above 50% only for first 24h of storage, therefore, immediate transfer into liquid nitrogen is necessary. Cryopreserved and transplanted Spg colonized the genital ridge and started gametogenesis in 40% of hosts. Donor-derived origin of cells was confirmed by molecular markers.

Conclusion

Protocols for common carp Spg and Oog slow rate freezing were developed and cryopreserved cells were afterwards transplanted into sterilized goldfish and started gametogenesis. Results of this study can be used as an alternative method for genetic resources preservation of valuable breeds with possible restoration in goldfish.

POSTER 132

CRYOPRESERVATION AND XENOGENEIC TRANSPLANTATION OF *Arapaima gigas* (PIRARUCU) SPERMATOGONIA

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Introduction

The *Arapaima gigas* (pirarucu) is a primitive fish species with high commercial value and high production costs, and presents late sexual maturity and is difficult to breed in captivity. Therefore, it is important to preserve the genetic stock of this species using biotechnological approaches such as cryopreservation. In addition, this technique can be applied to preserve spermatogonial stem cells (SSC) that can be further used for xenogeneic transplantation, allowing the production of viable gametes in appropriate recipient species. The aims of this study was evaluate the feasibility of Arapaima spermatogonia cryopreservation, using two cryomedia associated with slow freezing method, and the subsequent transplantation of these cells into Nile tilapia larvae.

Methods

Spermatogonia were isolated from juveniles pirarucu testes and cryopreserved ($1x10^7$ cells in each cryotube) with freezing two medium: DMSO (20% dimethyl-sulfoxide, 60% DMEM, 20% FBS] or ethylene glycol 10% (EG; D-glucose 0.09 % and 0.5% BSA in PBS). These cells were maintained for 2 hours at -80°C and then stored in −196 °C liquid nitrogen. Seven months after freezing the cryopreserved cells were thawed and the number of viable spermatogonia recovered was analyzed with trypan blue dye-exclusion. Viability of cryopreserved cells was also investigated 6, 24 and 48 hours after thawing using CellTiter-Blue® Cell Viability Assay. To trace spermatogonial cells before transplantation, frozen-thawed cells were labeled with PKH26 and microinjected into the peritoneal cavities of 5–7 days post-fertilization Nile tilapia larvae. The recipient larvae and gonads were analyzed under fluorescence microscope at 1, 5, 14 and 30 days post transplantation**.**

Results and Discussion

In the DMSO group 90% of the cryopreserved cells were recovered, whereas in the EG group was only 40%. In comparison to fresh cells (FC), the DMSO group presented 96%, 87% and 80% viable spermatogonia while in the EG were 91%, 82% e 74%, respectively at 6, 24 and 48 hours after thawing. Regarding cell proliferation, we observed an increase in the absorbance rate over the 48 hours only in the FC group, indicating a possible increase in cell number. In relation to the fluorescence analysis of the recipient larvae it was observed that the transplanted spermatogonia were able to migrate and colonize the receptor gonad.

Conclusion: Our study demonstrated that pirarucu spermatogonial cells can be successfully cryopreserved using DMSO-based cryomedia associated with the slow-freezing method. Moreover, these spermatogonia maintained their functionality/viability after cryopreservation, being able to survive and migrate to the gonad after transplantation into Nile tilapia larvae.
Financial support: FAPEMIG

POSTER 133

REPRODUCTIVE CONTROL OF MEAGRE (*Argyrosomus regius*) TO OBTAIN FAMILIES FOR GENETIC BREEDING PROGRAMS

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Introduction

The aquaculture production of meagre (*Argyrosomus regius*) has increased rapidly from 859 tons in 2004 to 11,770 in 2014. This increase was related to the ease to develop successful culture protocols, including reproductive control. The next step for production should be the implementation of selective breeding programs, for which the industry needs to control reproduction to mate selected breeders to produce the number of different families required.

Methods

Two methods to mate selected breeders, paired spawning with male rotation and *in vitro* fertilisation, were examined. For paired spawning, individual females were paired with a male and induced (single injection of 15 $\mu g.kg^{-1}$ of GnRHa) to spawn and in subsequent weeks males were rotated to pair and spawn with different females. Rotation and induced spawning continued each week until all paired combinations had been achieved or fish lost maturity status and could not be induced to spawn any further. Twenty-one females (≈16 kg) and 18 males (≈13 kg) were used to form 67 different pairs. For the *in vitro* approach, sperm was stripped and cool stored (4ºC) in modified Leibovitz (1:4). The timing of ovulation and optimal egg quality was determined by making serial *in vitro* fertilisations for 14 GnRHa-induced females from which ova were extracted and fertilised every 2.5 h between 35 and 45 hours after GnRHa application. Optimal sperm:egg ratio for fertilisation was determined by testing ratios from 3,000:1 to 500,000:1.

Results and Discussion

Paired spawning produced a total of 56 families with 87,666 ± 11,244 $eggs.kg^{-1}$ of female. There was a decline in the fecundity and spawning success after consecutive weekly GnRHa injections. However, there were no differences in egg fertilization success, hatching or larval survival. The decrease in fecundity and spawning success was attributed to a loss of maturity observed in the females, which may be related to differences in mate selection strategies between male and female meagre. For *in vitro* fertilisation, sperm quality was maintained for up to 7 hours in Leibovitz. Optimal egg quality was observed at 38-39 hours after the GnRHa injection and the optimal sperm:egg ratio to obtain a high percentage of fertilisation was above 200,000:1.

Conclusion

Two methods were provided to mate selected breeders to produce a large number of different families required for genetic breeding programs. The *in vitro* fertilisation method gives total control for a breeding selection program.

The project received funding from the European Union 7FP (GA603121, DIVERSIFY).

POSTER 134

LARVAplus: A SURVEY OF LATIN AMERICAN AIMS IN FISH REPRODUCTION IN AN INTEGRATIVE RESEARCH NETWORK FOR PROMOTING FISH LARVICULTURE

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Introduction: The FAO reports that in recent years aquaculture production in the Latin American region is progressing at rates above world averages. However, this growth is concentrated in crustaceans and non-native fish species such as salmon and tilapia. LARVAplus is a research network funded by the Spanish Agency for International Cooperation and Development (CYTED, Spanish Government). The network aims to generate a place for the exchange of knowledge and experience for the development of the Ibero-American aquaculture industry. In particular, the production of fish larvae and fingerlings and improving the scientific and technological competitiveness of the aquaculture sector.

Methods: A work group was established in fish reproduction to address problems of obtaining good quality eggs and larvae. The work group consisted of 24 researchers and industry managers from 12 countries and 14 institutions / companies. The skills set amongst the participants covered all aspects of fish reproduction required to ensure the production of good quality eggs such as knowledge and tools in genomics, physiology, reproductive development, behaviour and general broodstock management for a wide range of species. The first task of the work group was to make a survey of the species and associated reproductive problems.

Results and Discussion: All species that were identified with reproductive problems were native species for the centres involved, which indicated that future development can be expected to increase the importance of native fish species in Latin American finfish production. The most common problem was no spawning, which is present in species that do not reach late stages of gametogenesis (Mugilidae: *Mugil cephalus, Mugil incilis*; Osteoglossiformes, *Arapaima gigas*, *Osteoglossum bicirrhosum*; Carangidae: *Caranx crysos*; Siluriformes: *Calophysus macropterus*) or that do not respond to hormonal induction (Centropomidae: *Centropomus ensiferus*; Polyprionidae: *Polyprion americanus*; Characiformes: *Piaractus mesopotamicus*). In some species the spawning problems were related to difficulties to assess maturational development caused by morphology and / or behaviour, such as *Arapaima gigas* and *Polyprion americanus*. Poor spawning has been report (Sciaenidae *Cynoscion albus*, Centropomidae: *Centropomus medius* and Eleotridae: Dormitator latifrons) and work needs to be focused on improving hormone induction protocols. Lastly, a number of species need techniques to improve management by sexing fish (Carangidae: *Seriola lalandei* and *Polyprion americanus*) and obtaining out-of-season spawning (Cichlidae: *Cichlasoma dimerus*).

Conclusion: Species and family working groups have been formed to bring together knowledge / tools and species problems to find solutions that will help develop the culture of these species.

POSTER 135

FREEZING OF PEJERREY EMBRYOS AT -14 AND -20°C WITH MICROINJECTION OF A CRYOPROTECTIVE SOLUTION

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Introduction

The pejerrey (*Odontesthes bonariensis*) is a multiple seasonal spawner with a great economic importance and is considering appropriated for aquaculture. For this reason is necessary to apply biotechnologies to optimize its reproduction in captivity. In this context, the objective of this work was to develop a protocol for freezing pejerrey embryos.

Methods

Pejerrey embryos with non-pigmented optical vesicles were selected from the Hydrobiological Station of Chascomús (Buenos Aires, Argentina). Embryos were exposed to 1ml of cryoprotective solution in cryovials for 10 minutes at 0° C (equilibrium temperature) and subjected to rapid (-6.6°C/min) or slow freezing (from 0°C to -7°C; -2°C/min; plateau of 10 min; -0.5°C/min) until a final temperature of -14 or -20°C. At these temperatures the embryos were stored for 1 hour. Two different solutions were used. Solution 1 (S1) was composed of 10% v/v methanol; DMSO 10% v/v, Sucrose 10% w/v, NaCl 5g/l; and Solution 2 (S2), with the same composition of S1 but without DMSO. Moreover, other assays were done microinjecting embryos in the perivitelline space with 32.2nl of S1 or S2 and using the same freezing protocols described above. The freezing equipment was Cryologic (Australia). The microinjection´s needles were made from glass capillaries using the PC10-Puller (Narishige, Japan) and the microinjection was carried out using the microinjector Nanoject II (Drummond, USA). In all cases, the cryovials were thawed at 37°C for 1 minute 30 seconds, and the embryos were washed 3 times with 5 ml of incubation water. Finally, they were incubated in a programmable incubator (Ingelab, Argentina) at 22°C and photoperiod 12h L: 12h O until hatching. All the assays were done by triplicate with 10-12 embryos per treatment. Embryos survival was evaluated daily as well as hatching rate and larval survival (with starvation condition).

Results and Discussion

High rates of embryo survival post-thawing were obtained in all assays (80-100%). Particularly, at the slow freezing until -14°C and -20°C and at the rapid freezing until -20°C with microinjection of S2 there were no significantly differences between the hatching rates of treatment embryos and the control embryos (-14°C: 86,67±11,55 %; -20°C: 67,93±8,31 %). Even though there were detected different morphological abnormalities in the larvae from frozen embryos, its rate survival were no significantly different with the rate survival of the control larvae (6,62±1,21 days).

Conclusion

These preliminary results show that it is possible to freeze pejerrey embryos and establish the basis for future trials in this field.

POSTER 136

TETRAPLOID INDUCTION BY HEAT SHOCKS IN NILE TILAPIA

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Introduction

A strategy to produce a sterile tilapia population is the developing of interploid triploids, where normal diploid fish are mated with tetraploid fish. Although several studies have tried the formation of tetraploid Nile tilapia, none have had successful. For this reason, the objective of the present study was to develop a generation of Nile tilapias tetraploid that, from mating with diploid individuals, would form a triploid population in order to guarantee the functional sterility in this specie.

Methods

To obtain eggs, we performed a protocol of induced spawning and *in vitro* fertilization during five weeks. Females "ready to spawn" (n = 91) were induced to spawn by administration of two intramuscular injections of human chorionic gonadotropin (hCG) (first dose = 10% of total dose; total dose = 3,000 IU/kg, dose interval = 18h). A pool of oocytes was collected in each week and fertilized with a semen pool. We used nine treatments: control (no shock), one shock at 75, 80, 85 or 90 minutes after fertilization of the eggs; or two shock at 75 and 85 minutes, 80 and 90 minutes, 85 and 95 minutes or 90 and 100 minutes after fertilization of the eggs. The shocks were performed in a water bath at 41°C and had 4 minutes of duration. Each treatment had five repetitions. When the offspring reached the mean weight of 10 g, the fingerlings were submitted to cardiac puncture for ploidy status evaluation in flow cytometry.

Results and Discussion

In total, 1,879 animals were analyzed in this experiment. The results were 1,709 normal diploids, 94 abnormal diploids, 15 triploids, 18 mosaic animals and 43 inconclusive analysis. The percentage of mosaic animals were higher in one shock treatments (1.3%) than in treatments with two shocks (0.17%) by Fisher Exact Test ($p<0.05$). The occurrence of triploid presented opposite pattern, two shocks treatments resulted in 2.0% of triploid individuals, while one shock treatment produced 0.2% of triploid fish. Although the treatments caused different chromosomal alterations (triploid and mosaic cells), none shock application resulted in tetraploid fish.

Conclusion

The type of heat shock treatment promoted different chromosomal alterations. Although we analyzed more than 1,800 animals submitted to eight different heat shock treatments, we found any tetraploid fish.

POSTER 137

REPRODUCTIVE PERFORMANCE AND LIFE HISTORY DIVERSITY IN RECONDITIONED ANADROMOUS FEMALE STEELHEAD TROUT.

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Introduction: Anadromous steelhead trout (*Oncorhynchus mykiss*) (steelhead) are known for their life history diversity. Reconditioning of post-spawn female steelhead (kelts) is a recovery tool used in the Columbia River Basin (western US) where populations are in decline. Wild kelts are captured, held in tanks, fed, and released to spawn a second time increasing the reproductive contribution of each fish. Reconditioning takes advantage of iteroparity in steelhead and significantly reduces the mortality observed with a second anadromous migration. Varied maturation timing results in a range of maturation ages and spawning intervals in steelhead. Maturation decisions are thought to be condition-dependent during critical periods. Individuals falling below energetic thresholds are expected to remain reproductively inactive for the subsequent annual cycle. The purpose of this study was to use plasma estradiol-17B (E2) measurements to determine kelt life history trajectory, to assess changes in growth and energy reserves over time, and to quantify reproductive performance in a kelt reconditioning program.

Methods: Hatchery-origin female steelhead were obtained at Dworshak National Fish Hatchery (Clearwater R., ID) in 2015 and 2016. These maiden fish were spawned, placed in tanks, fed, and sampled every 10 weeks thereafter. Plasma E2 measurements (assayed by ELISA) were used to determine the repeat post-spawning life history trajectory, either immediate entry into another reproductive cycle (consecutive) or delaying for 1 or more years (skip). Changes in growth and energy reserves (measured as lipid content) were related to reproductive performance measures (fecundity, egg size, total egg mass) over time in reconditioned fish.

Results and Discussion: Plasma E2 was bimodally distributed with no overlap after 30 weeks, with the high mode indicating consecutive re-maturation (30% in 2015, 40% in 2016). In the year following maiden spawning: mass specific growth rate (SGR) was elevated over the first 10 weeks; plasma triglycerides (TG) were elevated 10 weeks after spawning; and plasma E2, muscle lipid levels (MLL) and condition (K) were elevated after 20 weeks in rematuring versus non-rematuring kelts. In the year prior to repeat spawning: SGR was elevated over the first 10 weeks; plasma TG, K, and MLL were elevated in the first 10 weeks; and E2 was elevated in the first 20 weeks in skip versus consecutive kelts. Reproductive investment (total egg mass), egg size, and fecundity were greatest in skip spawners, with egg size similar in consecutive and maiden spawners (absolute form); when size-standardized, egg size was decreased in consecutive compared to maiden spawners despite a larger body size.

Conclusion: Clearwater River kelts show two distinct post-spawning life history trajectories, with increased growth and energy reserves evident soon after maiden spawning in consecutive spawners. Consecutive spawners, however, may be disadvantaged by their condensed cycle of simultaneous somatic recovery and gonadal recrudescence, resulting in a reduction in proportional investment per offspring. Appropriate management of both types will maximize the contribution of reconditioned fish to recovery.

POSTER 138

THE MORPHOLOGY OF UROGENITAL PAPILLA CAN BE USED AS A SELECTION CRITERIA FOR FEMALE BREEDERS OF NILE TILAPIA

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Introduction

The Nile tilapia (*Oreochromis niloticus*) is one of the most extensively cultured tropical freshwater species and its culture is expanding at an extremely high rate. To meet the increasing demand of tilapia farmers, the selection of superior individuals for breeding purposes might significantly improve seed output. In this study, we analyzed if the morphology of urogenital papilla can be used as a selection criteria for female breeders of Nile tilapia.

Methods

From a broodstock with 600 females and 150 males, we selected 96 females with normal urogenital papilla (normal group), 96 females with a urogenital papilla different from those described as normal for Nile tilapia (abnormal group), and 96 normal males. The animals were tagged for individual recognition. We performed nine reproductive assays during nine weeks. In each week, we used eight 3.5 m^3 tanks with 8 females and 6 males, totaling 64 females and 48 males in reproduction/week. For each female, there was a resting period of two weeks between each reproductive assay. During the assays, we monitored the water quality and analyzed the reproductive variables. After reproductive assays, the animals were euthanized and weights of body, viscera, gonads and liver were obtained for biological indexes calculation. To evaluate if the abnormality in urogenital papilla could be associated with an infectious diseases, we performed bacteriological examination, where fragments of brain, kidney, spleen and gonads from 8 abnormal females were inoculated onto blood and Macconkey agar.

Results and Discussion

We found a significant difference in the reproductive performance between normal and abnormal females by Chi-square test. While 60.5% of normal females reproduced at least once out of 3 weeks in reproduction, only 23.8% of those abnormal reproduced ($p<0.0001$). Viscerosomatic, hepatosomatic and coelomic fat indexes were not different between the normal and abnormal females. The gonadosomatic index (IGS) was significantly different ($p<0.05$) between the groups by Mann-Whitney test and their medians were 3.29 and 4.08 in normal and abnormal females, respectively. In ovary of abnormal females, we observed a lot of inviable oocytes and liquid accumulation, which explain the higher value of IGS. The bacteriological examination was negative for all samples.

Conclusion: The results indicated females with abnormality in urogenital papilla presented lower reproductive performance. Therefore, the morphology of this external reproductive structure can be used as a selection criteria for female breeders of Nile tilapia. Apparently, this reduced reproductive performance was not associated with a bacterial disease and others possible causes are being investigated.

ENDOCRINE DISRUPTION

POSTER 139

SEMINAL PARAMETERS AFTER 96 H OF EXPOSURE OF *Astyanax altiparanae* TO ALUMINUM AND DIFFERENT TEMPERATURES

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Introduction

Anthropogenic actions in the environment, such as the release of domestic effluents with metals, are interfering with the survival of animals, especially fish. Among these metals, we can include aluminum (Al), considered an endocrine disrupter of fish reproduction. Considering that spermatogenesis is under hormonal regulation, our hypothesis is that Al affects sperm parameters, such as pH and osmolarity that directly influence sperm kinetics. Regarding that teleosts are ectotherms, it is hypothesized also that the effects of Al can be potentiated by the increase of temperature. The objective of this study is to analyze the effect of Al exposure on the seminal pH and osmolarity of *Astyanax altiparanae*, maintained for 96 hours at different temperatures.

Methods

A. altiparanae males (n=180) were divided into nine experimental groups (n = 10, duplicate, Al combination - 0.5 mg/L, pH and temperature) and maintained for 96h: T1 - 20ºC, neutral pH, Al; T2 - 20°C, acid pH, no Al; T3 - 20°C, acidic pH, Al; T4 - 25°C, neutral pH, no Al; T5 - 25°C, acidic pH, no Al; T6 - 25 °C, acidic pH, Al; T7 - 30°C, neutral pH, no Al; T8 - 30°C, acidic pH, no Al; T9 - 30°C, acidic pH, Al. Before seminal collection, the animals were induced with pituitary extract of common carp (5 mg/kg body weight) to stimulate spermiation. An aliquot of 5 μL of semen was withdrawn from each animal and applied to pH strips (Merck). Another aliquot of 20 μL of semen was diluted with distilled water and used to analyze osmolarity in a 5004 MICRO-OSMETTE™. Data were analyzed by two way ANOVA and Bonferroni test ($P < 0.05$). GraphPad Prism 5 software was used.

Results and Discussion

Seminal pH was not altered comparing all experimental groups. pH values varied from 8.5 to 9.5, described as the optimal pH for sperm motility for most teleosts. No interaction between pH and temperature was observed in seminal pH. On the other hand, a significant interaction between the effects of temperature and pH was observed in the seminal osmolarity ($P<0.001$). Both, temperature and pH, affected the results of seminal osmolarity in relation to the neutral pH groups ($P<0.01$). Seminal osmolarity decreased in animals exposed to Al (157mOsm ±8.94) in relation to the control group (215mOsm±8.83). This alteration can affect sperm kinetics and consequently fertilization capacity of the gamete.

Conclusion

A. altiparanae exposed to Al in acidic pH, at different temperatures, for 96 h, did not change seminal pH, but seminal osmolarity decreased, demonstrating an interaction between the effects of temperature and pH in relation to the neutral pH groups.

POSTER 140

BISPHENOL A EXPOSURE DURING DIFFERENT STAGES OF SPERMATOGENESIS IMPAIRS PROGENY EARLY EMBRYO DEVELOPMENT

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Introduction

Bisphenol A (BPA), as an endocrine disrupting chemical (EDC), interacts with estrogen receptors, thus interfering with estrogen receptor-dependent signalling pathways. It is a chemical compound used for manufacturing of domestic (food and beverage packaging) and industrial (epoxy resins) devices. Alterations in reproductive health of humans and wildlife (exposed to this toxic through ingestion, inhalation or even dermal contact) depend on doses, life stage, mode, and timing of exposure. Moreover, it has been proven that BPA causes epigenetic modifications that might be inherited by following generations. Therefore disorders, such as cardiogenesis impairment, observed in F1 could be related to epigenotoxic effects on the paternal germline.

Methods

Zebrafish males were exposed to vehicle (0.014% of ethanol, representing control group), 100 µg/L and 2000 µg/L of BPA during early spermatogenesis (first 14 days) or during both spermatogenesis and spermiogenesis (21 days). Then they were mated with non-treated females to obtain F1 embryo. Endocrine disruptive effects on the progeny were measured by comparing the expression of estrogen receptors in embryos from control and treated males. Moreover, acetylation profile of the progeny was analysed by both whole mount inmunostaining and expression of histone acetyltranferases and deacetylases. Cardiac development was followed at histological and at transcriptional level.

Results and Discussion

Paternal exposure during early spermatogenesis led to several types of cardiac malformations in F1 embryo, whereas the exposure up to the end of spermiogenesis was even more deleterious, since no embryo coming from exposed males reached 120 hpf. Changes in expression of different estrogen receptors (esr2a, esr2b and gper1) were observed in F1 embryos no matter the timing of exposure, this fact being strongly related with the modified expression of epigenetic enzymes (kat6a and hdac6), a sharp increase in acetylation of several histone marks (H3K9, H3K14, H3K27 and H4K12) and an alteration of transcription factors involved in cardiac development (hand2 and gata5).

Conclusion

Paternal exposure to BPA during different stages of spermatogenesis leads to endocrine disruptive effects in F1 embryo and results in a differential histone acetylation profile. This fact could lie behind the rise in the expression of transcription factors crucial in cardiogenesis promoting the observed hearth disorders.

POSTER 141

HEAVY METALS ACCUMULATION AND ENDOCRINE DISRUPTION IN *Prochilodus argenteus* FROM A POLLUTED NEOTROPICAL RIVER

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Introduction

Some metals such as aluminum (Al), cadmium (Cd), copper (Cu), lead (Pb) and mercury (Hg) were classified as metalloestrogens due to their ability to interfere in the mechanism of action of estrogenic hormones. Many biomarkers are used in studies on the influence of EDCs, but the most used are those that respond directly to changes in hormone levels such as vitellogenin (vtg) and zona radiata proteins (Zrp). Species of genus *Prochilodus* have detritivorous/iliophagous habits, feeding sediments in the substrate, and for this reason, these species are considered suitable bioindicators for simultaneous evaluation of contamination in water column and sediments. In this study we evaluate histological and molecular changes in *P. argenteus* caused by chronic exposure to heavy metals in a polluted river.

Methods

The samples were performed in Abaeté river (ABR) - reference site, and Paraopeba river (PBR) during the reproductive seasons (Nov / Dec) of 2010 and 2011. Of all fish biometric data were obtained and fragments of tissues were removed and used in histological, histochemical and immunohistochemical analyzes. For the quantification of histopathological parameters and expression of biomarkers, ImageJ software was used. Microscopic images were obtained with light and fluorescence microscopy. Statistical tests and graphs were performed in Graphpad prism 5.0.

Results and Discussion: All fish analyzed (n= 34) did not present significant differences between body weight, total length, gonadosomatic and hepatosomatic indexes. In addition, the abiotic factors of water obtained by IGAM (2011 and 2012) not show significant variations between rivers. This standardization of abiotic and morphological factors avoids results related to water temperature, pH, season and body size. In Paraopeba river, significant increases were observed in all quantified histopathological parameters in spleens, but in liver significant increase was detected only in melano-macrophagic centers (MMCs) ($p < 0.05$). In general, the sections of liver, spleen and gonads of *P. argenteus* from PBR showed more evident tissue damages in all organs when compared to ABR. Similar to histopathologies, molecular changes were observed in fish from PBR. Elevated expression of metallothionein and endocrine disrupting biomarkers (Vitellogenin and zona radiata proteins) in males were observed, evidencing an endocrine disruprion in fish from PBR.

Conclusion: Although it is difficult to state that the alterations are caused only by heavy metals, the data presented in the present study show that the accumulation of these metals play an important role in endocrine disruption of *P. argenteus* in the Paraopeba river.
Financial support: FAPEMIG

POSTER 142

MULTI-BIOMARKER RESPONSES IN THE SUBANTARCTIC FISH (*Patagonotothen tessellata*) TO ASSESS THE IMPACT OF ANTHROPIC POLLUTION IN COASTAL WATERS OF BEAGLE CHANNEL.

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Introduction

Compounds released into the environment by anthropogenic activities disrupt processes of reproduction and development. Ushuaia city (Tierra del Fuego, Argentina) is located over the coast of the Beagle Channel (54'48 ºS, 68'190ºW) and due to the fast and unplanned urban growth and tourist activities this system receives several substances mainly from untreated sewage and industrial wastewaters. The black southern cod, *Patagonotothen tessellata* (Perciformes, Notothenidae) predominates in the intertidal zone of Patagonia, Subantarctic, and Antarctic waters, and is widespread in the Beagle Channel. It possesses male parental care. This study aims to evaluate differential responses in the endemic fish *P. tessellata* in a multi-stressor context.

Methods

Adult male fish and water samples were collected from five sites (three presumed polluted and two reference sites). A total of 33 animals were captured in autumn, winter and spring. Skin mucus and blood samples were taken before dissection. Somatic indexes were calculated. Testes were histologically analyzed and sex steroids, skin mucus and plasmatic proteins were measured. SDS-PAGE followed by Western blot using heterologous antiserum was done for the detection of vitellogenin (VTG). Water physicochemical and microbiological analyses were performed.

Results and Discussion

Males collected from polluted sites had lower HSI and GSI than those from reference locations. Testes were characterized as unrestricted lobular type and no histopathology or intersex condition was identified. Males from contaminated sites had significantly higher levels of estradiol than control males but no difference was found between seasons. Total plasma proteins showed a positive correlation with GSI whereas the opposite was observed with mucus proteins irrespective of the site. Plasmatic protein levels were higher in males from polluted sites, where VTG was detected. Water analysis revealed differences between sampling sites, with high solid residuals and sulphates in water from polluted locations.

Conclusion: The anthropic activities negatively influence on the coastal environment of Ushuaia city, not only in the water quality but also in fish. The abnormal detection of VTG in blood and altered sex hormone levels in contaminated fish may impact parental behavior. The present study sets baseline data to further studies in this region and provides strong evidences of reproductive disruption due to xenobiotics exposure in the sub Antarctic fish *Patagonotothen tessellata*.

POSTER 143

THE INFLUENCE OF ENVIRONMENTAL POLLUTION IN ANDROGENS LEVELS OF *Astyanax fasciatus* (CHARACIFORMES: CHARACIDAE) MALES.

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Introduction

The reservoirs are permanently subjected to environmental pressures due to the intense anthropic activity that causes biological and environment alteration. In the reservoirs of the Metropolitan Region of São Paulo (RMSP), eutrophication processes, the presence of pollutants, anthropogenic products, among them different types of endocrine disruptors (ED) have been reported. Some studies present the interaction of aquatic organisms with compounds that act as ED and can alter fish reproductive physiology, leading to a decrease in male fertility, or even compromising the integrity of aquatic communities. Lambari-do-rabo-vermelho, *Astyanax fasciatus* can be considered a bioindicator in the reservoirs of RMSP, therefore, the aim of this study is to assess the influence of potential ED present in the reservoirs of the Tietê River basin, but with different anthropic pressure,

Methods

A. fasciatus males were collected during the winter and summer in two different reservoirs of the upper Tietê basin, one with a high anthropic pressure, the Billings reservoir and another reservoir with low anthropic pressure, the Ponte Nova (reference site). In Billings, two different sites were considered for sampling, Bororé and Taquacetuba, that differ by the degree of anthropic impact. Testicular histology, gonadosomatic index (GSI), hepatosomatic index (HSI), plasma testosterone (T) and 11-ketotesterone (11KT) levels were analyzed as biomarkers in adult male fish.

Results and Discussion

GSI, HSI, testicular morphology and T levels did not change comparing seasons and reservoirs. 11KT levels were higher in Taquacetuba (178.17 ± 38.83) than Bororé (14.54 ± 3.26) ($P = 0.017$) and Ponte Nova (54.98 ± 35.59) ($P = 0.025$) during summer. Males sampled in Taquacetuba presented higher 11KT levels during summer (178.17 ± 38.83) when compared with the animals sampled in the winter (29.28 ± 11.00) ($P = 0.05$). 11KT is a teleost-specific androgen with the main function of stimulating spermatogenesis from the proliferation of spermatogonia to spermiogenesis. The increase in 11KT levels in males from Taquacetuba, together with the maintenance of mature conditions of testes, suggests the presence of androgenic compounds and/or the need to adjust the levels of this androgen to reproduce under environmental adverse conditions.

Conclusion:

We conclude that 11-KT is a biomarker that is suggesting the presence of ED, more specifically androgenic compounds in Taquacetuba, considering the difference found in relation to the reference site. Additionally, the endocrine alteration detected is dependent on the season of the year, with more pronounced effects during the summer.

POSTER 144

INFLUENCE OF PAPER MILL EFFLUENT AND BETULINOL ON CALCIUM BALANCE IN ZEBRAFISH TESTES.

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Introduction

Previous studies have shown that pulp mill effluents and its constituent compounds such as the wood extractive betulinol can disrupt endocrine function and in male fish can disturb spermatogenesis/steroidogenesis and contribute to infertility. The aim of this work was to study the acute effect of the pulp mill effluent and betulinol on calcium balance in the testes of zebrafish (Danio rerio).

Methods: Zebrafish were immobilized in ice cold water and euthanized and testes was incubated in vitro with/without 45Ca2+ (0.1 µCi/mL), pH 7.4, gassed with an O2:CO2 mixture (95:5; v/v) in Cortland buffer at 28 ºC in the presence/absence of effluent or betulinol. After incubation for 60 min to allow the added 45Ca2+ to be loaded to the testes and to come to equilibrium, drugs were added the incubation to characterize the ionic channels and ionic exchangers involved in the mechanism of action of these agents. After incubation, extracellular 45Ca2+ was washed off the testes in a LaCl3 solution and homogenate aliquots were used for the determination of total protein and radioactivity. Results were expressed as pmol calcium/ug of protein or as % of control (CEUA/UFSC nº P00968).

Results and Discussion: The time-course for calcium equilibrium was carried out at 5, 30, 60 and 120 min. The steady-state of calcium uptake in the zebrafish testes was observed at 60 min. We then tested the effects of various concentrations of effluent and betulinol on 45Ca2+ uptake and showed that 4% effluent and 1 ηM and 1 ρM betulinol caused a significant reduction in 45Ca2+ influx. In the next experiments we tested the effects of various drugs affecting calcium uptake on the actions of the effluent and betulinol. Co-treatment of the testes with 4% effluent and the extracellular calcium chelator (EGTA) decreased the 45Ca2+ influx whereas the Na+/Ca2+ blocker (KB-R7943) or voltage-dependent channel blocker type L and T (nifedipine and flunarizine, respectively) had no effect on 45Ca2+ uptake. As for the actions of betulinol, the addition of EGTA, KB-R7943 or nifedipine/flunarizine caused a significant reduction in 45Ca2+ influx. These data suggest that the effluent regulates the Ca2+ concentration by the external calcium related mechanism and the betulinol by the activation of voltage-dependent calcium channels.

Conclusion : In the present study, the drugs change the calcium balance in testes of zebrafish by different pathways. Future studies need to consider whether changes in calcium balance mediate the inhibitory effects of pulp mill effluents on testicular hormone production, development and fertility. **Support:** CNPq -PVE. 401410/2014-1; CNPqUni - 472071/2013-0, CAPES-PPG-Biochemistry.

POSTER 145

EFFECTS OF METHOXYCHLOR, A POTENT ENDOCRINE DISRUPTING CHEMICAL ON REPRODUCTIVE HEALTH OF THE STINGING CATFISH *Heteropneustes fossilis*

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Introduction

During the last decade there has been increasing concern at the levels of industrial and agricultural contaminants in the aquatic environment and the possible effects of such pollution on both human health and wellbeing of animal populations. The aquatic environment is the ultimate 'sink' for a large number of chemicals arising out of natural weathering processes and anthropogenic activity in industry, agriculture and medicine. Methoxychlor (MXC) is an organochlorine insecticide or pesticide effective against a wide range of pests encountered in agriculture, households, and ornamental plantings. There is now accumulating evidence indicating that organochlorine pesticides can compromise the integrity of the reproductive system of fish, reptiles and mammals by interacting with the estrogen receptors (ERs) and other regulators of reproduction. Till date there has not been much report and studies on effects of methoxychlor on the reproductive health of economically important stinging catfish *Heteropneustes fossilis.*

Methods

Sexually mature female catfish (40-50g) were exposed to sublethal doses of MXC (6 and 16µg/L) for a period of 45 days during pre-vitellogenic (resting) and vitellogenic (prespawning) phase of the reproductive cycle. After completion of experiment, ovary was collected and fixed for histopathological and steroid hormone level assay study using H/E staining and ELISA.

Results and Discussion

After 45 days of exposure, gonadosomatic index (GSI) was calculated and it showed significant increase in GSI in a dose dependent manner. Tissue and plasma estradiol-17β level elevated. Histology of ovary showed increase in number of stage II and III oocytes in previtellogenic phase and increase in stage IV oocytes in vitellogenic phases. In the present study we reported that MXC exposure to catfish induced gonad development, in a dose dependent manner and resulted in estrogenic effect on the catfish. Thus, our study concludes that MXC is an environmentally endocrine disrupting organochlorine which has significant impact on survival of the catfish.

Conclusion

Thus, with present study it can be concluded that MXC can act as potent endocrine disrupting chemical and alters the reproductive capacity and may be responsible for decline in teleost/catfish in aquatic ecosystem.

*Financial support of UGC, New Delhi is acknowledged.

POSTER 146

SEXUAL DIFFERENCES IN BRAIN EXHIBITS AT NEUROSTEROID SYNTHETIC PATHWAY AND GOVERNS GONADAL DEVELOPMENT: A SPECIAL REFERENCE TO INDIAN SALMON

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Introduction

Neurosteroidogenesis is playing a vital role in governing the physiology of reproduction along with neuropeptides and neurotransmitters. Gonadal development influences steroid synthesis in the central nervous system (CNS), and the CNS also regulates the gonadal steroid production. It is well known that the receptors of estrogen modulate the production of GnRH, and serotonin, dopamine and GABAergic neurons modulate the steroidogenic enzymes. However, the influence of neurosteroids and variations of synthetic pathway towards reproductive cycle is not studied in detail.

Methods

The present study examined the presence of various steroids in specific areas of the brain of Indian salmon, *Eleutheronema tetradactylum*, including the quantitative difference in estradiol (E2), testosterone (T), 11-ketotestosterone (11-KT), androstenedione (A), DHEA, and 21-hydroxyprogesterone (21-P), and the conversion of 5α-DHP to 5α,3α-THProg and 5α-DHT to 5α,3α-THT by 3α-HSD. The quantitative expression of mRNA analysis of 3α-HSD, 3β-HSD, Cyp17, Cyp19 and Cyp21 substantiate the variation in sex and maturation of gonadal stages using RT-PCR analysis.

Results and Discussion

The brains of reproductively active female fish showed high testosterone levels when compared with the male brain. It has been derived from the expression of Cyp19 and Cyp17 are higher than the Cyp21. The steroidal production in the incubated tissues of brain highlights the augmented presence of 5α- or 3α-reductase evidence the elimination pathway. Aromatase indicate the shift in the sex dependent pathway. The sulphated steroids of pregnenalone and DHEA indicate the presence of hydroxysteroid sulfotransferase (HST) in its exclusion pathway.

Conclusion

The study suggests that the sexual modulation can be carried out at the CNS by manipulating the steroids and their receptors, more particularly in the thalamus region of the brain. Planned studies on transcriptomics using NGS with region-specific analysis of the brain will unravel the pathways involved in the integration of steroidal signals in gonadal development.

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