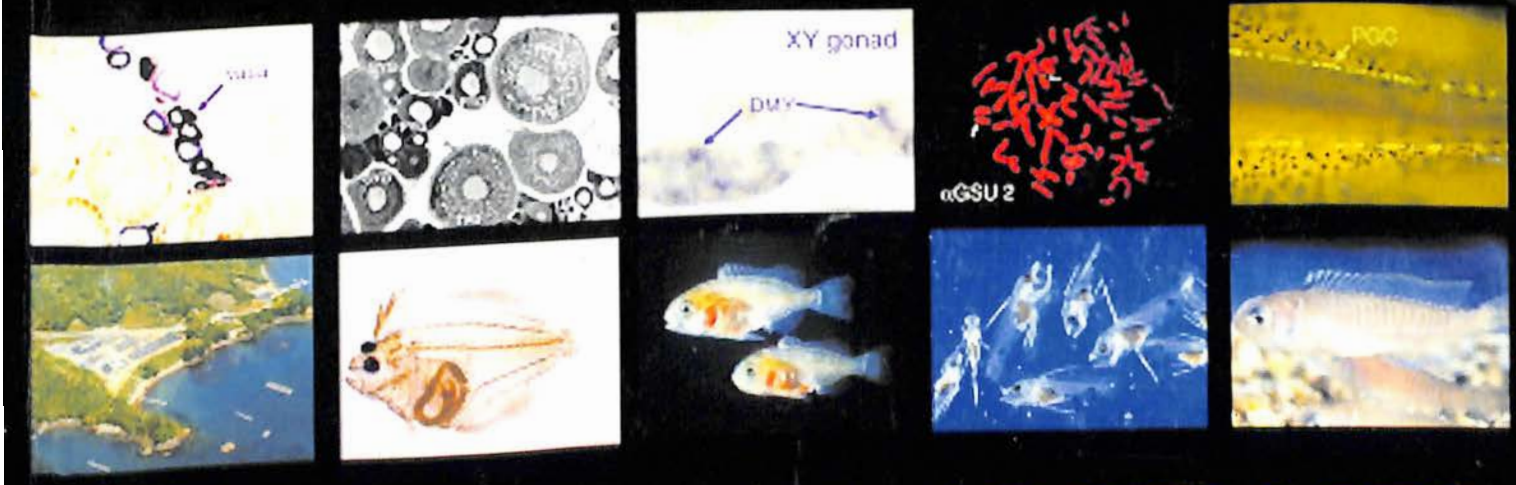
7th International Symposium on Reproductive Physiology of Fish

May 18-23, 2003 in Mie, Japan



PROGRAM AND ABSTRACTS

Sponsors

Ministry of Agriculture, Forestry, and Fisheries
Fisheries Research Agency
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Organizing Committee

Symposium Schedule

May 18, Sunday (Day 1)

15:00-19:00 Check-in and Registration at the Hotel MIELPARL ISE-SHIMA

19:00-21:00 Reception (Welcome party)

May 19, Monday (Day 2)

08:00-09:00 Registration

09:00-09:20 Opening Remarks

09:20-10:20 Plenary lecture

10:25-12:15 Session I: Brain/Hypothalamus

12:15-14:00 Lunch

14:00-14:40 Session I: Brain/Hypothalamus (continued)

14:40-17:50 Session II: Pituitary

May 20, Tuesday (Day 3)

09:00-11:25 Session III: Sex Determination and Gonadal Differentiation

11:25-13:00 Lunch

13:00-15:20 Session IV: Gametogenesis I

15:20-15:35 Coffee Break

15:35-18:00 Session V: Reproductive Behavior and Migration

18:00-19:00 *Dinner*

19:00-22:00 Poster Session I

(Posters must be mounted from 8:30 to 12:00 on May, 19 and removed at the end of Session I. Refreshments will be served.)

May 21, Wednesday (Day 4)

10:00-18:00 Excursion: Bus will start from the Hotel Mielparl Ise-Shima at 10:00.

18:00-20:00 Small Party (Toba Aquarium): Party will start at 18:00 at the entrance hall of the aquarium.

May 22, Thursday (Day 5)

09:00-11:55 Session VI: Gametogenesis II

11:55-13:00 Lunch

13:00-16:00 Poster Session II

(Posters must be mounted from 8:30 to 12:00 on May, 22 and be removed by 13:00 on May 23)

16:00-18:55 Session VII: Environmental Influence on Reproduction

May 23, Friday (Day 6)

09:00- 11:55 Session VIII: Biotechnology in Aquatic Science

11:55-13:20 Lunch

13:20-15:25 Session IX: Aquaculture

15:25-15:55 Concluding Remark

18:00-21:00 Banquet (Farewell Party)

Award Ceremony

Poster Sessions will take place in the ROOM "UMI" of the Hotel Mielparl Ise-Shima.
Time for discussion is as follows. (Presenter must stand by own poster.)
Poster Session I:
Posters in Session I-V : 19:00-20:30
Posters in Session VI-IX : 20:30-22:00
Poster Session II:
Poster in Session I-V : 13:00-14:30
Poster in Session VI-IX : 14:30-16:00
Oral Presentation will take place in the ROOM "DAIOU" of the Hotel Mielparl Ise-Shima.
Bring your slides or OHP sheets to the desk at least 30 min before your session starts. Preview your slides and ensure they are labeled with the author's name and are numbered consecutively.
Those who will make computer-based presentation must bring your PowerPoint file on CD to the desk by the previous day of your presentation. Preview your PowerPoint file to ensure that it runs correctly in our computer system. We will open the desk the following time;
May 18, 15:00-22:30; May 19, 20 and 22, 9:00-20:00; May 21, 9:00-10:00 and one hour after the excursion; May 23: 9:00-16:00
Welcome party will be held in the ROOM "UMI" of the Hotel Mielparl Ise-Shima.
Small party will be held at the entrance hall of the Toba Aquarium at the end of the excursion.
Farewell party will be held in the ROOM "DAIOU" of the Hotel Mielparl Ise-Shima.
Excursion: Bus will start from the Hotel Mielparl Ise-Shima at 10:00 (Participants come and get together to the Hotel Mielparl Ise-Shima) and will take you back to the Hotel Resort Inn Isobe, the Shima Central Hotel Socia, and the Mielparl Ise-Shima after the small party.
Lunch and Dinner at Mielparl Ise-Shima will be served between 11:20 and 14:30, and 17:30 and 21:00, respectively. On May 22, dinner will be served until 21:30.

BUS TIME TABLE

(1) Kintetsu Kashikojima station to the Hotel Mielparl Ise-Shima

(5/18)	(5/19～5/24)
10:10	10:10
11:10	11:10
12:10	
13:45	13:45
14:45	14:45
15:45	15:45
16:45	16:45
17:45	17:45
18:45	
20:05	
21:05	
22:05	

(2) The Hotel Mielpearl Ise-Shima to Kintetsu Kashikojima station

(5/18)	(5/19～5/24)
09:30	09:30
10:30	10:30
11:30	
12:50	12:50
13:50	13:50
14:50	14:50
15:50	15:50
16:50	16:50

(3) Resort Inn Isobe - Shima Central Hotel Socia – The Hotel Mielparl

(5/18)	17:30	17:45	18:15
(5/19)	08:00	08:15	08:45
(5/20)	08:00	08:15	08:45
(5/21)	08:30	08:45	09:15
(5/22)	08:00	08:15	08:45
(5/23)	08:00	08:15	08:45

(4) The Hotel Mielparl - Shima Central Hotel Socia - Resort Inn Isobe

(5/18)	21:00	21:30	21:45
(5/19)	18:20	18:50	19:05
(5/20)	22:30	23:00	23:15
(5/21)			
(5/22)	19:25	19:55	20:10
(5/23)	21:30	22:00	22:15

Scientific Program

Scientific Program

May 19, Monday (Day 2)

Plenary lecture

Chair: Y. Nagahama

09:20-10:20 P. Thomas*
Nongenomic steroid actions initiated at the cell surface: Lessons from studies in fish (Plenary)

Session I: Brain/Hypothalamus

Chairs: H. Goos and G. Somoza

10:25-11:05 State-of-art lecture: I.S. Parhar*
Functional diversity of GnRH molecules and receptors (O-I-1)

11:05-11:25 Y. Zohar*, T.T. Wong, K. Kight, C. Steven, N. Zmora, Y. Yashuvi, U. Klenke, and Y. Gothilf
Early establishment of the gonadotropin releasing hormone (GnRH) system in fish with two and three GnRH forms (O-I-2)

11:25-11:45 M. Amano*, K. Okubo, T. Yamanome, Y. Oka, S. Kitamura, K. Ikuta, A. Takahashi, K. Aida, and K. Yamamori
GnRH systems in masu salmon and barfin flounder (O-I-3)

11:45-12:00 K. Okubo* and K. Aida
GnRH gene products downregulate their neighboring genes encoding protein tyrosine phosphatases (O-I-4)

12:00-12:15 U. Klenke* and Y. Zohar
Gonadal regulation of the gonadotropin-releasing hormone - gonadotropin system: a kinetic study in hybrid striped bass (O-I-5)

12:15-14:00 Lunch

14:00-14:20 J.M. Trant*, R. Goto-Kazeto, Y. Kazeto, and A.R. Place
Expression of brain aromatase (cyp19A2) in channel catfish and zebrafish: gene regulation and potential biological significance (O-I-6)

14:20-14:40 K. Joy* and R. Chaube
Catecholaminergic control of LH secretion in female catfish *Heteropneustes fossilis*: E_2 modulation of tyrosine hydroxylase activity (O-I-7)

Session II: Pituitary

Chairs: A. Takahashi and Z. Yaron

14:40-15:20 State-of-art lecture: P. Swanson* and J.T. Dickey
Differential production of FSH and LH in fish: does the pattern of gene expression reflect function? (O-II-1)

15:20-15:40 H. Rosenfeld*, B. Levavi-Sivan, Z. Yaron, and A. Elizur
The FSHβ gene - regulation in fish (O-II-2)

15:40-15:55 Coffee Break

15:55-16:10 H. Ando*, N. Koide, H. Okada, and A. Urano
Regulation of luteinizing hormone β gene expression by salmon gonadotropin-releasing hormone and sex steroids in masu salmon pituitary cells (O-II-3)

16:10-16:30 W. Ge*
Activin regulation of gonadotropin transcription in the goldfish, *Carassius auratus* (O-II-4)

16:30-16:45 G.F.Y. Cheng* and W. Ge
Cloning and characterization of follistatin in the goldfish, *Carassius auratus* (O-II-5)

16:45-17:00 B. Levavi-Sivan*, A. Avitan, J. Aizen and T. Kanias
Cloning and pharmacological characterization of the inhibitory dopamine receptor from the pituitary of two perciform fish: tilapia and grey mullet (O-II-6)

17:00-17:20 K. Gen*, S. Yamaguchi, K. Okuzawa, N. Kumakura, H. Tanaka, and H. Kagawa
Physiological roles of FSH and LH in red seabream, *Pagrus major* (O-II-7)

17:20-17:35 M.A. Rahman*, K. Ohta, A. Yamaguchi, H. Chuda, T. Hirai, and M. Matsuyama
Gonadotropins, gonadotropin receptors and their expressions during sexual maturation in yellowtail, a carangid fish (O-II-8)

17:35-17:50 M. Nozaki*, Y. Oshima, T. Shimotani, and S.A. Sower,
Immunohistochemical detection of gonadotropin-like material in the hagfish pituitary (O-II-9)

May 20, Tuesday (Day 3)

Session III: Sex determination and gonadal differentiation

Chairs: M. Matsuyama and F. Piferrer

09:00-09:40 State-of-art lecture: M. Nakamura*
Role of estrogens in sex differentiation and sex change in fish (O-III-1)

09:40-10:00 D. Baron and Y. Guiguen*
Gene expression during gonadal sex differentiation in rainbow trout (*Oncorhynchus mykiss*): from candidate gene studies to high throughout genomic approach (O-III-2)

10:00-10:15 G Maack*, H. Segner, and C.R. Tyler
Timing and variability in sexual differentiation in the zebrafish (*Danio rerio*) (O-III-3)

10:15-10:30 C.A. Strüssmann*, T. Oikawa, T. Otake, and S. Kasuga
Potential use of otolith microchemistry for field studies of temperature-dependent sex determination and gonadal degeneration in fish (O-III-4)

10:30-10:45 Coffee Break

10:45-11:05 J. L. Du, Y. H. Lee, C. L. He, Y. S. Huang, and C. F. Chang*
Differential plasma levels of luteinizing hormone and expression of gonadal gonadotropin receptors in protandrous black porgy, *Acanthopagrus schlegeli*: the implication of sex change mechanism (O-III-5)

11:05-11:25 M. Matsuda*, Y. Nagahama, T. Kobayashi, S. Hamaguchi, and M. Sakaizumi
The sex determining gene of medaka: a Y-specific DM domain gene (DMY) is required for male development (O-III-6)

11:25-13:00 Lunch

Session IV: Gametogenesis I

Chairs: O. Linhart and R. Schulz

13:00-13:40 **State-of-art lecture**: T. Miura*
Molecular control mechanisms of fish spermatogenesis (O-IV-1)

13:40-14:00 L.R. de França*, D.A.R. Vilela, M.T.D. Peixoto, S.G.B. Silva, and H.P. Godinho
Spermatogenesis in teleost: insights from the Nile tilapia model (O-IV-2)

14:00-14:15 J. Bouma*, J.G. Cloud, and J.J. Nagler
Estradiol-17β causes proliferation of interstitial cells in the pre-spermatogenic rainbow trout (*Oncorhynchus mykiss*) testis (O-IV-3)

14:15-14:30 J. K. Sundaray*, K. Ohta, and A.Yamaguchi, K. Suzuki, and M. Matsuyama
Diurnal rhythm of steroid biosynthesis in the testis of terminal phase male of protogynous wrasse, *Pseudolabrus seiboldi*, a daily spawner (O-IV-4)

14:30-14:45 M. Kusakabe*, I. Nakamura, and G. Young
Enzymatic activity of 11β-hydroxysteroid dehydrogenase in rainbow trout (*Oncorhynchus mykiss*) (O-IV-5)

14:45-15:05 H. Ohtake*
Sperm-activating proteins obtained from the herring eggs (O-IV-6)

15:05-15:20 O. Linhart* , J. Cosson, S.D. Mims, W.L. Shelton, and M. Rodina
Seminal fluid composition of paddlefish (*Polyodon spathula*) and ions effects on motility of fresh and demembranate sperm (O-IV-7)

15:20-15:35 Coffee Break

Session V: Reproductive Behavior and Migration

Chairs: A. Moore and H. Ueda

15:35-16:15 **State-of-art lecture**: N.E. Stacey*
Hormones, pheromones, and reproductive behavior (O-V-1)

16:15-16:30 B. Zielinski*, W. Arbuckle, A. Belanger, L.D. Corkum, W. Li, and A.P. Scott
Evidence for the release of sex pheromones by male round gobies (*Neogobius melanostomus*) (O-V-2)

16:30-16:45 R.F. Oliveira*, L.A. Carneiro, M.S. Grober, and A.V.M. Canario
Androgens, AVT and alternative reproductive tactics in the peacock blenny (O-V-3)

16:45-17:05 E. Plate*, J.G. Eales, C.W. Hawryshyn, and B. Adams
Fluctuations, interactions and stimulations related to GnRH and forms of thyroid hormones in migrating salmon (O-V-4)

17:05-17:20 T. Shoji*, D. Nishikawa, Y. Yamamoto, K. Kurihara, and H. Ueda
Amino acids in stream water are essential for salmon homing migration (O-V-5)

17:20-17:40 P.W. Sorensen*, L.A. Vrieze, and J.M. Fine
A multi-component migratory pheromone in the sea lamprey (O-V-6)

17:40-18:00 W. Li* and M. J. Siefkes
Behavioral and pysiological responses of female sea lamprey (*Petromyzon marinus*) to a male bile acid pheromone (O-V-7)

18:00-19:00 *Dinner*

19:00-22:00 **Poster Session I**
(Posters must be mounted from 8:30 to 12:00 on May, 19 and removed at the end of the Poster Session I. Refreshments will be served.)

May 22, Thursday (Day 5)

Session VI: Gametogenesis II

Chairs: C.F. Chang and G. Young

09:00-09:15 A. Kanamori* and H. Hori
Genes expressed at the onset of oogenesis in medaka (*Oryzias latipes*) (O-VI-1)

09:15-09:30 P.M. Lokman*, K.A.N. George, and G. Young
Effects of steroid and peptide hormones on *in vitro* growth of previtellogenic oocytes from eel, *Anguilla australis* (O-VI-2)

09:30-09:45 B. Campbell*, J. Dickey, B. Beckman, and G. Young, A. Pierce, and P. Swanson
Endocrine changes associated with the growth of pre-vitellogenic oocytes in coho salmon (O-VI-3)

09:45-10:00 A.J. Ibáñez, J. Peinado-Onsurbe, E. Sánchez, and F. Prat*
The role of lipoprotein lipase (LPL) in the incorporation of neutral lipids into the oocytes of the european sea bass (*Dicentrarchus labrax* L.) during gonadal development (O-VI-4)

10:00-10:20 T. Matsubara*, M. Nagae, N. Ohkubo, T. Andoh, S. Sawaguchi, N. Hiramatsu, C.V. Sullivan, and A. Hara
Multiple vitellogenins and their unique roles in marine teleosts (O-VI-5)

10:20-10:35 Coffee Break

10:35-10:50 N. Hiramatsu*, A. Hara, T. Matsubara, K. Hiramatsu, and C.V. Sullivan
Oocyte growth and cytoplasmic maturation of temperate basses: multiple vitellogenins and their receptor (O-VI-6)

10:50-11:10 R. Patiño*, P. Thomas, and G. Yoshizaki
Regulation and mechanisms of oocyte maturational competence (O-VI-7)

11:10-11:25 J. Bobe*, G. Maugars and B. Jalabert
Specific gene expression profiles are associated with follicular maturational competence acquisition in rainbow trout (*Oncorhynchus mykiss*) (O-VI-8)

11:25-11:40 B. Senthilkumaran*, Y. Yoshiura, Y. Oba, C.C. Sudhakumari, D.S. Wang, T. Kobayashi, and Y. Nagahama
Steroidogenic shift is a critical event for ovarian follicles to under go final maturation (O-VI-9)

11:40-11:55 Y. Wang* and W. Ge
Cloning of epidermal growth factor (EGF) and EGF receptor (EGFR) from the zebrafish ovary and the potential roles of EGF in the regulation of ovarian activin/follistatin system (O-VI-10)

11:55-13:00 Lunch

13:00-16:00 **Poster Session II**
(Posters must be mounted from 8:30 to 12:00 on May, 22 and be removed by 13:00 on May 23)

Session VII: Environmental Influence on Reproduction
Chairs: H. Bern and B. Norberg

16:00-16:40 **State-of-art lecture**: N.W. Pankhurst* and M.R. Porter
Cold and dark or warm and light: variations on the theme of environmental control of reproduction (O-VII-1)

16:40-16:55 M.J.R. Porter*, H. Woolcott, and N.W. Pankhurst
The use of additional lighting and artificial photoperiods to recondition early maturing Atlantic salmon (*Salmo salar*) in Tasmania (O-VII-2)

16:55-17:10 H. Migaud*, R. Mandiki, J.N. Gardeur, P. Kestemont, N. Bromage, and P. Fontaine
Influence of photoperiod regimes on the Eurasian perch gonadogenesis, spawning and egg and larvae quality (O-VII-3)

17:10-17:25 A. Davie*, M. Porter and N.R. Bromage
Photoperiod manipulation of maturation and growth of Atlantic cod (*Gadhus morhua*). The effect of timing, period and system on successful application (O-VII-4)

17:25-17:40 Coffee Break

17:40-18:00 G.L. Taranger*, E. Andersson, E. Vikingstad, U. Klenke, I. Mayer, S.O. Stefansson, T. Hansen, Y. Zohar, and B. Norberg
Effects of photoperiod, temperature and GnRHa treatment on the reproductive physiology of Atlantic salmon (*Salmo salar* L.) broodstock (O-VII-5)

18:00-18:20 K. Ikuta* and S. Kitamura
Effects of low pH on reproductive behavior of salmonid fishes (O-VII-6)

18:20-18:40 T. Kitano*, T. Koyanagi, and S.-I. Abe
The mechanism of transcriptional regulation of vitellogenin gene in Japanese flounder (*Paralichthys olivaceus*) (O-VII-7)

18:40-18:55 E. Noaksson*, M. Linderoth, Y. Zebühr, B. Gustavsson, D. Broman, and L. Balk
Reproductive failure and disturbed steroidogenesis in female perch (*Perca fluviatilis*) exposed to toxic refuse dump leachate (O-VII-8)

May 23, Friday (Day 6)

Session VIII: Biotechnology in Aquatic Science

Chairs: A. Elizur and J.M. Trant

09:00-09:40 State-of-art lecture: G. Yoshizaki*, Y. Takeuchi, T. Kobayashi, and T. Takeuchi
Primordial germ cell: a novel tool for fish bioengineering (O-VIII-1)

09:40-10:00 J.G. Cloud*
Cryopreservation and transplantation of sexually immature gonads of rainbow trout (O-VIII-2)

10:00-10:15 Y. Takeuchi*, G. Yoshizaki, and T. Takeuchi
Production of donor-derived offspring by xenotransplantation of primordial germ cells in salmonids (O-VIII-3)

10:15-10:30 Coffee Break

10:30-10:50 H.R. Habibi*
Enhancement of growth in cultured fish using recombinant carp growth hormone (O-VIII-4)

10:50-11:05 M. Kobayashi*, T. Morita, K. Ikeguchi, G. Yoshizaki, T. Suzuki, and S. Watabe
Production of recombinant goldfish gonadotropins by baculovirus in silkworm larvae (O-VIII-5)

11:05-11:20 T. Morita*, G. Yoshizaki, M. Kobayashi, and T. Takeuchi
Production of biologically-active recombinant goldfish gonadotropins in transgenic rainbow trout (O-VIII-6)

11:20-11:35 N. Zmora*, S. Kumar, Y. Kazeto, T.T. Wong, and J.M. Trant
Production of channel catfish (*Ictalurus punctatus*) recombinant gonadotropins using the S2 drosophila cell line (O-VIII-7)

11:35-11:55 K. Kikuchi*, J. Willoughby, J. Wegner, and M. Westerfield
Reverse genetics in zebrafish (O-VIII-8)

11:55-13:20 Lunch

Session IX: Aquaculture

Chairs: S. Adachi and K. Hirose

13:20-14:00 **State-of-art lecture**: C.V. Sullivan*
Induced maturation and spawning: opportunities and applications for research on oogenesis (O-IX-1)

14:00-14:20 K. Mushiake*
Effects of spawning conditions on multiplication of the causative virus of viral nervous necrosis (VNN) in broodstock of striped jack (*Pseudocaranx dentex*) (O-IX-2)

14:20-14:35 C.W. Laidley* and R. J. Shields
Domestication of greater amberjack (*Seriola dumerili*) at the Oceanic Institute in Hawaii (O-IX-3)

14:35-14:50 N. Papanikos*, R.P. Phelps, K. Williams, A. Ferry, and D. Maus
Comparison of egg and larval quality between natural and induced spawns of red snapper *Lutjanus campechanus* (O-IX-4)

14:50-15:05 A. C. Emata*, H. Y. Ogata, E. S. Garibay, and H. Furuita
Effect of nutritional quality of broodstock diets on reproductive performance and arachidonic acid level of mangrove red snapper, *Lutjanus argentimaculatus* (O-IX-5)

15:05-15:25 H. Tanaka*, H. Kagawa, H. Ohta, T. Unuma, and K. Nomura
The first production of glass eel in captivity: Fish reproductive physiology facilitates great progress in aquaculture (O-IX-6)

15:25-15:55 **Concluding Remark**: A. P. Scott

Plenary lecture

Plenary

NONGENOMIC STEROID ACTIONS INITIATED AT THE CELL SURFACE: LESSONS FROM STUDIES IN FISH

P. Thomas*
University of Texas Marine Science Institute, Port Aransas, Texas, U.S.A

Many actions of steroids cannot be readily explained by the classic mechanism of steroid hormone action involving alterations of gene transcription, a relatively slow action initiated by the steroid binding to intracellular receptors belonging to the nuclear receptor superfamily. Extensive evidence has been obtained that steroids can also exert rapid, nongenomic actions involving activation of intracellular signaling pathways that are initiated at the cell surface by binding to specific steroid membrane receptors (mSR). However, lack of information on the molecular structures of any mSRs has impeded further development of this alternative model of steroid hormone action. One of the best characterized models of nongenomic steroid actions is the induction of oocyte maturation in fish and amphibians by progestins. Recently, we discovered a novel gene in a spotted seatrout ovarian cDNA library that has the characteristics of a progestin membrane receptor (mPR) and appears to be the intermediary in progestin induction of oocyte maturation in this species. Structural analyses of the deduced amino acid sequence of the protein as well as functional studies with the recombinant protein in a mammalian cell expression system indicate it is a 7- transmembrane receptor coupled to an inhibitory G-protein ($G_{i/o}$), suggesting it is a G-protein coupled receptor (GPCR). Western blot analysis with mPR antibodies shows the receptor is also present on seatrout sperm. Thirteen structurally-related genes were subsequently identified in other vertebrate species, including three in humans. Several of these mammalian genes also had several characteristics typical of mPRs. The discovery of the molecular structure and likely orientation in the plasma membrane of a new class of steroid receptors, unrelated to nuclear receptors but instead characteristic of GPCRs, provides the first plausible mechanistic explanation of how steroids acting at the cell surface can cause rapid intracellular responses. Membrane receptors for estrogens (mER) and androgens (mAR) have also been characterized biochemically in seatrout and croaker gonads. Several lines of evidence suggest the seatrout mER is also a GPCR. Addition of estradiol-17β to ovarian plasma membranes causes rapid upregulation of adenyl cyclase, suggesting the mER is coupled to a stimulatory G-protein (G_s). In contrast, preliminary evidence suggests the gonadal mAR is not a GPCR. Fish have also proven to be useful for investigating chemical interference with nongenomic steroid actions; the first clear evidence for endocrine disruption by binding to a mSR was obtained with the seatrout mPR. Thus fish are valuable models for investigating nonclassical steroid actions and their interference by environmental contaminants.

Session I

Brain/Hypothalamus

O-I-1
FUNCTIONAL DIVERSITY OF GnRH MOLECULES AND RECEPTORS

I.S. Parhar
Department of Physiology, Nippon Medical School, Tokyo, Japan

Gonadotropin-releasing hormone (GnRH = LHRH) coordinates the complex physiology of reproduction and reproductive behavior in vertebrate. It was initially assumed that GnRH occurred as a single molecular form, in the septo-preoptic area of vertebrate brain, and that it served a single function. GnRH peptide has evolved to give rise to 16 structurally distinct molecular forms. It has become increasingly clear that all vertebrate species investigated to date possess two or three different GnRH forms. GnRH may have originated as a regulator of reproduction; however, during evolution GnRH variants have also acquired non-reproductive and extra-pituitary functions. For example, within the central nervous system, GnRH acts as a neuromodulator and has effects on reproductive behavior. Outside the central nervous system, GnRH has been shown in the placenta, endometrium, gonads, adrenal glands, mast cells, kidneys and breast tumor cells. Although GnRH subtypes participate in some aspect of reproduction; the precise function of each GnRH form still remains unclear. The development of GnRH and GnRH receptor subtypes in the brain and pituitary provide an interesting avenue to explore the significance of the GnRH forms. In the pituitary, the distribution of GnRH receptors Type IA in gonadotropes, Type IB in prolactin cells and Type III in growth hormone cells provide evidence that the three native GnRH variants in tilapia *Oreochromis* have cognate receptors, each capable of regulating different pituitary endocrine cells. In the brain, the cells of diencephalonic origin (GnRH-I) have been implicated in gonadotropin release, gonadal sex differentiation, gonadal maturation and territorial behavior. Cells of mesencephalonic origin (GnRH-II) might function in the release of prolactin, acid-base balance and as neuromodulators involved in motor-associated reproductive behaviors. The cells of olfactory origin (GnRH-III) might be pivotal for growth hormone release, olfaction, metamorphosis, neuromodulation and the regulation of visual-associated reproductive behaviors. This lecture will try to review recent literature and our own work with special emphasis to tease-out possible functions of GnRH and GnRH receptors in the control of endocrine cells of the pituitary and reproductive behaviors.

O-I-2
EARLY ESTABLISHMENT OF THE GONADOTROPIN RELEASING HORMONE (GnRH) SYSTEM IN FISH WITH TWO AND THREE GnRH FORMS

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The vertebrate GnRH system develops very early during ontogeny, and has been shown to play a key role in the establishment of the endocrine reproductive axis. We have examined the early development of the brain GnRH system in zebrafish, a fish model with two GnRH forms (salmon (s) GnRH and chicken (c) GnRH-II) and in three perciform species, gilthead seabream, hybrid striped bass and European seabass, all having three GnRHs (sGnRH, cGnRH-II and seabream (sb) GnRH). cDNAs encoding all prepro-GnRHs were cloned and characterized from all studied species. The early development of the GnRH system was studied using whole-mount and section in situ hybridization and real-time fluorescence-based quantitative RT-PCR. In zebrafish, additional GnRH localization studies were carried out using transient expression of green and red fluorescent proteins driven by sGnRH and cGnRH-II promoters, respectively. In all studied species, mRNA of all forms of GnRH appeared within the first 30 hours (zebrafish) to 5 days (perciform models) post-fertilization (PF). cGnRH-II-producing cells were detected as early as 23-60 hours PF, first in the hindbrain, a novel site for cGnRH-II expression, and then in the midbrain of all species. While the hindbrain cGnRH-II signal gradually decreased to become undetectable at 10 days PF, the midbrain cGnRH-II expression intensified. In all studied species, sGnRH expressing neurons were first detected at 32-72 hours PF in the forebrain-nasal region, followed by a caudal migration of these neurons along the ventral telencephalon towards the preoptic area. In the 2-GnRH model (zebrafish), sGnRH developed to ultimately become the only form expressed in the olfactory bulb, terminal nerve and preoptic areas. In the perciform models, sbGnRH mRNA levels were very low during the first month of development, and sbGnRH-expressing neurons were first observed at 39 days PF in the nucleus preopticus parvocellularis, thalamus and terminal nerve area. The early development patterns suggest that cGnRH-II neurons arise from a different brain region than sGnRH and sbGnRH. Co-expression of sGnRH and sbGnRH in the same cells suggests that both of these GnRH forms arise from the olfactory placode. In seabream, the early development of the GnRH system was correlated to expression patterns of other key factors of the endocrine system. Three concomitant increases of transcripts encoding the GnRHs, GnRH receptor, gonadotropins, gonadotropin receptors and VASA suggest a coordinated establishment of the GnRH-pituitary-gonadal system.

O-I-3
GnRH SYSTEMS IN MASU SALMON AND BARFIN FLOUNDER

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To date, 14 forms of gonadotropin-releasing hormone (GnRH) have been identified based on primary structure or cDNA sequence in vertebrates. Multiple forms of the GnRH molecule exist in teleost fish. Masu salmon *Oncorhynchus masou* has salmon GnRH (sGnRH) and chicken GnRH-II (cGnRH-II). sGnRH but not cGnRH-II was detected in the pituitary. sGnRH neurons were scattered from the olfactory nerve through the ventral telencephalon (VT) and the preoptic area (POA). sGnRH mRNA levels in the VT and the POA increased during gonadal maturation, indicating that sGnRH neurons in these areas are involved in gonadotropin (GTH) secretion. Neurons expressing sGnRH mRNA were not detected in the brain after the olfactory epithelia lesions. These results suggest that sGnRH neurons are derived from the olfactory epithelium and play different roles according to the location of neurons in masu salmon. A pleuronectiform fish, barfin flounder *Verasper moseri* has sGnRH, cGnRH-II and seabream GnRH (sbGnRH). sbGnRH neurons were located in the POA and sent fibers to the pituitary. sGnRH neurons were located in the ventromedial part of the rostral olfactory bulbs and in the terminal nerve ganglion (TN). cGnRH-II neurons were localized in the midbrain tegmentum. sGnRH-immunoreactive (ir) and cGnRH-II-ir fibers were observed throughout the brain, but not in the pituitary gland. The amount of sbGnRH mRNA per brain significantly increased two months before spermiation and remained at high levels until spermiation. Neither sGnRH nor cGnRH-II mRNA level per brain showed significant changes during experimental periods. Pituitary sbGnRH peptide content significantly increased when fish spermiated. Pituitary sGnRH peptide and cGnRH-II peptide contents were extremely low compared to sbGnRH peptide level and showed no significant changes during experimental periods. These results indicate that sbGnRH is involved in testicular maturation. From the ontogenic study, it is suggested that sGnRH neurons are derived from the olfactory placode and migrate into the brain, while cGnRH-II and sbGnRH neurons originate from the ventricular ependyma and from the POA, respectively. These results suggest that both the origin and function of sGnRH neurons (TN) and sbGnRH neurons (POA) differ in barfin flounder.

O-I-4
GnRH GENE PRODUCTS DOWNREGULATE THEIR NEIGHBORING GENES ENCODING PROTEIN TYROSINE PHOSPHATASES

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Gonadotropin-releasing hormone (GnRH) was isolated originally from the mammalian hypothalamus, and was named for its role as the stimulator of gonadotropin release from the anterior pituitary. In addition, considerable evidence has been accumulated that GnRH, when administered into the brain, promotes reproductive behavior, suggesting a neuromodulatory function for the peptide. The question then arises about the molecular mechanism underlying the neuromodulatory effect of GnRH. Here we have demonstrated using an in vitro medaka (*Oryzias latipes*) whole-brain culture system that GnRH downregulates the gene expression of two members of protein tyrosine phosphatase (PTP), PTPα and PTPε, which have been shown in mammals to suppress neuronal excitability through interacting with voltage-gated potassium channel. This observation indicates that GnRH acts as a novel neuromodulator that inhibits the PTPα/PTPε signaling, and this mechanism may account for the positive behavioral effect of GnRH. It is noteworthy that in addition to the brain, prominent expression of GnRH has been evident in tumor cells of mammals, where it has been implicated in the modulation of cell proliferation, and, like GnRH, PTP/PTPε have been shown to be expressed in tumor cells and involved in cell proliferation. Hence, it seems possible that the proliferative effect of GnRH is also mediated by the PTPα/PTPε signaling, and inhibition of this signaling may be the common molecular mechanism underlying the behavioral and proliferative effects of GnRH. Intriguingly, here we have also found that the PTPα and PTPε genes adjoin the GnRH genes in the medaka genome, and that the adjacent localization of the GnRH and PTP genes has been conserved in vertebrates from teleost to mammal. The physical linkage of these genes might play an important role for the functional linkage of these genes.

O-I-5

GONADAL REGULATION OF THE GONADOTROPIN-RELEASING HORMONE - GONADOTROPIN SYSTEM: A KINETIC STUDY IN HYBRID STRIPED BASS

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Gonadal steroid hormones play a pivotal role in the regulation of the reproductive axis in fish. They influence expression and release of the gonadotropins (GtH), follicle-stimulation hormone (FSH) and luteinizing hormone (LH), either by modifying brain expression and secretion of gonadotropin-releasing hormone (GnRH), or acting directly on the gonadotroph cells in the pituitary. To assess the response over time of the GnRH-GtH system to the removal of gonadal feedback, effects of bilateral gonadectomy and 17β-estradiol (E2) replacement treatment on GnRH and GtH peptide levels, as well as GtH and GnRH-receptor (GnRH-R) gene expression, were monitored. Adult, female hybrid striped bass undergoing mid-vitellogenesis were divided into two gonadectomized (gdx; n = 47) and one sham operated (control; n = 30) groups. One gdx group received E2 (2 mg/kg) via microspheric delivery systems; the other was given vehicle injection. Groups were sampled on days 3, 6, 9, 14, and 28 post-operation. Pituitary samples were analyzed for GnRH and LH protein levels utilizing specific ELISAs; messenger RNA (mRNA) levels of the alpha, FSHβ and LHβsubunits and the GnRH-R were determined using real-time quantitative RT-PCR (TaqMan) normalized to 18S mRNA levels; plasma steroid levels were measured using RIAs. In our study, steroid levels decreased in gdx hybrids, verifying the success of the gonadectomy. Removal of gonadal feedback caused a significant decrease in seabream GnRH (sbGnRH) peptide levels in the pituitary over time, but had no effect on either chicken GnRH II (cGnRH II) or salmon GnRH (sGnRH). E2 replacement maintained sb- and sGnRH peptide levels at control levels, however it significantly decreased cGnRH II over time. Expression of all three GtH subunits rose significantly over the course of the study in the gdx group, whereas E2 replacement dramatically reduced FSHβ and LHβ mRNA levels. The same pattern was seen in GnRH-R expression. The concentration of LH in the pituitary was comparable to control levels over the course of the study, however E2 replacement led to a significant decrease. In conclusion, during mid-vitellogenesis gonadal feedback exerted negative effects on the expression of all GtH subunits, as is also evident by the dramatic decrease of expression after E2 replacement. This effect may be mediated, among multiple factors, through gonadal regulation of sbGnRH secretion and/or turnover in the pituitary.

O-I-6

EXPRESSION OF BRAIN AROMATASE (CYP19A2) IN CHANNEL CATFISH AND ZEBRAFISH: GENE REGULATION AND POTENTIAL BIOLOGICAL SIGNIFICANCE

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Aromatase (cytochrome P450 aromatase; CYP19) is the enzyme responsible for the conversion of androgens to estrogens and represents a critical regulator of sex steroid synthesis. This enzyme is not only found in the ovary of all vertebrates, but it is expressed in a variety of extra-gonadal tissues. In teleosts, it is highly expressed in the brain although its regulation and physiological function has not been characterized. Unlike the single gene found in all other vertebrates, teleosts have evolved two different genes for the expression of aromatase in the gonads (CYP19A1) and in extra-gonadal tissues (CYP19A2). These two genes have markedly different regulatory elements in the 5' flanking region of their respective genes, different tissue-specific expression, and different patterns of expression during development and reproductive activity. In fact, expression of CYP19A2 in the brain of female zebrafish correlates better than the ovarian gene with the cyclic reproductive physiology of this multiple spawner. Correlates to the male patterns of expression have not been identified. Additionally, there appears to be sex-specific patterns of expression of the brain form of aromatase in developing zebrafish larvae although there seems to be environmental influences and important differences between strains. Alternately, the seasonally spawning channel catfish has a very different reproductive strategy and a very different pattern of CYP19A2 expression. Transcript abundance of the catfish CYP19A2 is much lower than in zebrafish and we have not seen a clear association of its expression with reproduction. The CYP19A2 gene of both species is highly responsive to estrogens, including xenoestrogens like nonylphenol. Additionally, the zebrafish CYP19A2 gene is responsive to multiple non-estrogenic xenobiotics such as benz- α-pyrene. This report characterizes the CYP19A2 gene and its promoter, describes its pattern of expression associated with reproduction and endocrine disrupting chemicals, and contrasts the differences in zebrafish and catfish.

O-I-7

CATECHOLAMINERGIC CONTROL OF LH SECRETION IN FEMALE CATFISH *Heteropneustes fossilis*: E_2 MODULATION OF TYROSINE HYDROXYLASE ACTIVITY

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In the catfish *Heteropneustes fossilis*, catecholamines (CA: dopamine and noradrenaline), are major regulators of basal or GnRH-induced LH (gonadotropin-II secretion (Senthilkumaran and Joy, Gen. Comp.Endocrinol. 1995, 97, 121-134; 1996, 101, 220-226). During ovarian recrudescence, catecholaminergic activity increases but in a differential manner: dopamine activity decreases and noradrenaline/adrenaline activity increases up to spawning. The pattern is reversed in non-breeding fish with a dominant dopaminergic activity throughout. These CA patterns are determined by environmental photoperiod and temperature, and by E_2 feedbacks. Tyrosine hydroxylase (TH) which catalyses the rate limiting step in CA synthesis shows significant annual variations with activity and kinetics increasing with gonad recrudescence up to spawning and decreasing thereafter. Long photoperiod and high ambient temperature stimulate and short photoperiod / total darkness and low ambient temperature inhibit TH activity. Ovariectomy of the catfish produced biphasic effects on enzyme activity: short- term duration (week 1 and 2) did not influence, but long- term (week 3, 4 and 5) ovariectomy inhibited it. E_2 replacement in 3-week ovariectomized fish also produced biphasic effects depending on dosages. Low dosages of E_2 (0.05 and 0.5ug/g body weight) stimulated and high dosages (1.0 and 2.0ug/g body weight) inhibited enzyme activity. Biphasic effects of E_2 were also demonstrated under in vitro conditions, incubation with low concentrations of E_2 ($1x\ 10^{-12}$ and $1x\ 10^{-9}$ M) stimulated, and high concentrations ($1x\ 10^{-3}$ and $1x\ 10^{-4}$ M) inhibited enzyme activity. The stimulatory or inhibitory effects on TH activity could be correlated with the affinity of the enzyme towards substrate and cofactor. The results indicate that tyrosine hydroxylase is a crucial step in CA synthesis influenced by feed back actions of E_2.

P-I-1

EFFECT OF PHOTOPERIOD MANIPULATION ON THE DAILY RHYTHMS OF MELATONIN AND REPRODUCTIVE HORMONES IN CAGED EUROPEAN SEA BASS (*DICENTRARCHUS LABRAX*)

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Photoperiod is probably the most powerful factor that entrains animal rhythms, including the reproductive cycle. In addition, rhythmic hormone secretion provides internal synchronization for several physiological functions such as seasonal breeding. The aim of the present research was to investigate the effects of photoperiod manipulation on daily rhythms of melatonin and reproductive hormones (pitupituitary and plasma LH, testosterone [T] and 11-ketotestosterone [11KT]) in juvenile sea bass, kept in net cages under farming conditions in winter. Metal halide lamps were used to supply an 18h light: 6h dark cycle. Artificial lights suppressed circulating melatonin, and the duration of the nocturnal melatonin rise was longer in the control group than in the group exposed to the artificial photoperiod. The daily rhythm of plasma LH was significantly affected by photoperiod, the highest levels being found in the dark phase in both groups. Moreover, the highest LH plasma levels appeared 5 hours after the beginning of the dark phase in both groups. However, the daily pituitary LH content was not altered by photoperiod manipulation. Sexual steroids rhythms were less influenced by light, T levels showing sinusoidal patterns with an acrophase around sunrise. The number of immature fish was significantly higher in animals exposed to artificial lights than in those under natural photoperiod. In summary, our results provide the first evidence for a daily pituitary and plasma LH rhythm in sea bass. In addition, long photoperiod significantly affected the daily rhythm of LH release. Further research is required to elucidate the functional mechanisms of the pineal-pituitary-gonadal axis. Research supported by MCYT projects no. 1FD97-1699 and AGL2001-0593-(03-01) to Dr. F.J. Sánchez-Vázquez and Dr. S. Zanuy. (#) Both authors have contributed equally in the preparation of this work.

P-I-2

CHARACTERIZATION OF THREE GnRH cDNA SEQUENCES IN THE PEJERREY FISH DONTESTHES BONARIENSIS (ATHERINIFORMES)

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Gonadotropin-releasing hormone (GnRH) is the master neuropeptide that coordinates and regulates reproduction in all vertebrates. Sixteen GnRH forms have been isolated and characterized from brain tissue of different species. The brain of the pejerrey fish was shown to contain three forms of GnRH: two of them, chicken GnRH-II (cGnRH-II) and pejerrey GnRH (pjGnRH), were isolated and sequenced. However, the third one, salmon GnRH (sGnRH), was only characterized by immunological and chromatographic properties. Immunostaining of pejerrey brain showed that sGnRH is expressed in neurons of terminal nerve ganglion, pjGnRH in neurons of the preoptic area, and cGnRH-II in neurons of the midbrain tegmentum. Interestingly, of these three forms, pjGnRH was the only form present in the pituitary. In the present work we have isolated, cloned and sequenced cDNAs fragments of: pjGnRH (344 bp), sGnRH (365 bp) and cGnRH-II (363 bp) preprohormones, by 3'RACE-PCR using primers designed by sequence alignment of the same molecules described in other teleost fish species. This characterization definitely confirms the presence and expression of the three forms in brain tissue of this multiple-spawner fish and offers an important molecular tool for the determination of their neuroanatomical pattern. But, only sGnRH was demonstrated by 3'RACE-PCR and immunocytochemistry with specific antisera in pejerrey gonads. Sequence analysis revealed that the partial pjGnRH cDNA from the pejerrey fish, shares a higher identity with sbGnRH cDNA from *Verasper moseri* (Pleuronectiformes) than to pjGnRH (=mdGnRH) from *Oryzias latipes* (Beloniformes, Atherinomorpha). However full length sequences should be obtained in order to support this statement. This work constitutes an initial step in the study of the physiological role of GnRHs during the sexual differentiation, maturation and reproduction of pejerrey.

P-I-3

EXISTENCE OF MULTIPLE ISOFORMS OF GnRH LIGAND AND RECEPTOR IN THE DWARF GOURAMI, *COLISA LALIA*

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GnRH plays a pivotal role in the regulation of reproduction. GnRH was found out originally as a hypothalamic decapeptide, gonadotropin-releasing hormone, which stimulates pituitary gonadotropes to synthesize and release gonadotropins. In many single species, multiple GnRH isoforms and multiple types of GnRH receptor are distributed in a wide range of tissues. Thus, GnRH is supposed to have diverse physiological functions, including neurotransmittion, neuromodulation, immunomodulation, autocrine/paracrine regulation and the regulation of other pituitary hormones in addition to gonadotropins. The dwarf gourami, *Colisa lalia*, has been used for an ideal model for studying the cellular physiology and morphology of GnRH neurons. This species has experimental advantages over other vertebrates; its GnRH neurons are large and make a tight cell cluster without intercalating glial cells just beneath the meningeal membrane, so that one can readily record the activities of a single GnRH neuron in a semi-intact whole brain. To study the molecular and cellular mechanisms of GnRH, we have identified three forms of GnRH and three types of GnRH receptor in the dwarf gourami. To know the sites where GnRH might be synthesized and consider its potential functions, the spatial expression pattern of GnRH ligands and receptors were analysed using RT-PCR-sequencing method. Almost all of the examined tissues and organs expressed at least one type of both ligand and receptor. This widespread expression is consistent with the previous demonstrations in several species, though the roles of GnRH outside the hypothalamus-pituitary-gonadal axis remain largely unknown. In conclusion, these remarkable expressions of GnRH ligand and receptor suggest that this species would be effective in investigating putative various functions of GnRH.

P-I-4

THE EXPRESSION AND LOCALIZATION OF CORTICOTROPIN-RELEASING HORMONE AND UROTENSIN I TRANSCRIPTS IN THE JAPANESE EEL

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Involvement of corticotropin-releasing hormone (CRH) family peptides has been suggested for the inhibitory effect of stress on reproduction in teleosts. Under rearing conditions, the Japanese eel (*Anguilla japonica*), never spontaneously reaches sexual maturity. As a first step toward elucidation of the suppressing mechanisms on reproduction in the eel, here we investigated gene expression patterns of CRH family peptides, CRH and urotensin I (UI), in the eel. Northern blot analysis showed the presences of a single transcript for CRH in the brain, a major transcript and two minor transcripts for UI in the brain and a single transcript for UI in the gonad. The localizations of CRH family transcripts within the eel brain were determined by RT-PCR and in situ hybridization. The CRH and UI transcripts were abundantly expressed in the medial and ventral division of the paraventricular area, respectively, and low levels of CRH family transcripts were widely distributed in the brain. Interestingly, these expression loci of CRH family transcripts were inconsistent with the loci reported previously on the other teleosts, the nucleus preopticus and nucleus lateral tuberalis. It is of interest to clarify whether CRH and UI cells project to the pituitary and GnRH cells in the brain. The genetic and histological information on stress-related hormones in this study is of use for inquiring the reciprocal actions between stress and the reproductive system of the eel.

P-I-5

EVIDENCE FOR TWO DISTINCT GnRH RECEPTORS ISOFORMS EXPRESSED IN TELEOSTS

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The Gonadotropin releasing hormone-related peptides (GnRHs) are expressed not only in the brain but also in peripheral tissues, and have been involved in different functions including regulation of cell proliferation/apoptosis and reinitiation of oocytes meiosis. Different GnRH receptors have been identified in fish species including goldfish, astatotilapia, african catfish, sea bass, eel, and medaka. To better understand the function of the GnRHs peptides in salmonids, we recently isolated a cDNA encoding a rainbow trout GnRH receptor (GnRHR) that was expressed mainly in the central nervous system and other tissues including gonads but not in pituitary. This suggested that at least another GnRH receptor remained to be identified. In order to determine the number of GnRH receptor types in fish, we have carried out an *in silico* analysis of the Fugu rubripes genome. The computer-based analysis identified five distinct genes encoding GnRHRs. The phylogenetic analysis demonstrated that these receptors segregated into two classes and each class included two to three subtypes. To date the presence of two different GnRHR types was only described in the perciforms fish medaka and astatotilapia. In addition, the presence of different subtypes corresponding to only one class was demonstrated in the goldfish and catfish. To confirm the *in silico* data, the presence of two distinct GnRHR types in trout was investigated by RT-PCR and PCR on genomic DNA using degenerate primers. A partial cDNA encoding a second trout GnRHR type, belonging to a second class, was isolated. Moreover, two different genomic DNA fragments corresponding to this second GnRHR type were amplified. Nucleotide sequence and splicing boundaries analyses showed that these fragments shared 94% homology in the coding regions but only 84% in the intronic region. This suggests that these DNA fragments may correspond to genes encoding different forms of the second rtGnRHR. The tissue distribution of the different GnRHR types and subtypes is under investigation. Taken together our data, along with those of the literature, demonstrate that two different GnRHR belonging to two main classes are expressed in Teleostei. Supported by the European Union Q5RS-2002-01801 PUBERTIMING

P-I-6

MOLECULAR TOOLS FOR STUDYING PUBERTY IN THE GREY MULLET, *MUGIL CEPHALUS*: CLONING OF KEY cDNAS THAT REGULATE REPRODUCTIVE FUNCTION

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We are using the grey mullet as a model to study puberty in fish. The grey mullet is considered a late maturing species, reaching first sexual maturation in 3-4 years. The species is of particular importance for coastal fisheries, aquaculture, and bioremediation purposes. Using reverse transcription and polymerase chain reaction (RT-PCR), we have cloned the precursor of three forms (salmon, seabream, chicken-II) of gonadotropin-releasing hormones (GnRHs) from the brain of juvenile female mullet. The amino acid (aa) sequences showed highest identities of the salmon form (91 aa) with the red seabream (78%); the seabream form (95 aa) with tilapia (74%), and the chicken-II form (86 aa) with the tilapia and European seabass (76%). Preliminary tests by PCR on cDNAs derived from the pituitary and gonad suggest differential GnRH expression. We have also cloned other pituitary cDNAs that may be involved in pubertal development. Precursors for the mullet growth hormone (205 aa) showed highest identity with orange-spotted grouper, red drum and gilthead seabream (81%); prolactin (213 aa) with Mozambique tilapia (76%), and somatolactin (233 aa) with Japanese flounder (85%). Partial cDNA sequences have also been obtained for the aromatases from the brain and the gonad, and insulin-like growth factor-I and II from the liver. We are currently developing quantitative PCR to measure expression levels of these key genes at different stages of gonadal development.

P-I-7

MODULATION OF GnRH SUBTYPES BY SOCIAL STRESS AND AGGRESSIVE BEHAVIOR

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We examined how in a social setting, molecular variants of gonadotropin-releasing hormone (GnRH) are influenced by stress and dominance. Territorial males of tilapia *Oreochromis niloticus* were matched with two other almost equal size males and were put together in a neutral experimental tank. Gravels were provided, which allowed the dominant males to establish territories. Each tank had one clear dominant male. Twenty-four hours from the start of the experiment, the aggressive territorial male and a subordinate stressed male were sacrificed. Another stressed male was allowed to recover for 24 h before sacrifice. Fish taken from a large community tank served as control. In another experiment, we stereotaxically placed intracerebral injections of antibodies against three GnRH isoforms and observed the social status of these fish. Another group of fish were intraperitoneally injected with cortisol to observe changes in GnRH mRNA levels. At the time of sacrifice, blood was collected to determine sex steroid and cortisol levels. Using immunocytochemistry we examined the neuronal numbers, neuronal size and optical density of staining. Real time quantitative PCR and *in situ* hybridization techniques were used to measure GnRH mRNA levels. Of the three distinct GnRH molecular variants in tilapia, aggressive interactions had no effect on preoptic-seabream GnRH and midbrain chicken II GnRH cell numbers, cell profile area and cell optical density of staining when compared between unstressed controls, stressed, stress recovered and aggressive males. On the contrary, terminal nerve-salmon GnRH cell optical density but not the cell numbers or the cell profile area were significantly decreased in stress compared to aggressive males. Cortisol injections significantly decreased salmon- and chicken II-GnRH but had no effect on seabream-GnRH mRNA levels. Furthermore, the mRNAs of all three GnRH variants were significantly decreased in socially stressed males. Aggressive encounters were significantly reduced following injections of anti-salmon GnRH. From these results, we hypothesize that extreme social stress influences all three GnRH molecular variants, and terminal nerve-GnRH neurons can mediate aggressive behavior.

P-I-8

NEUROANATOMICAL DISTRIBUTION AND PARTIAL SEQUENCE OF cDNA ENCODING BRAIN CYTOCHROME P450 AROMATASE (P450aromB) IN PEJERREY *ODONTESTHES BONARIENSIS*

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The cytochrome P450 ovarian aromatase, encoded by the CYP19 gene, is an important steroid-metabolizing enzyme responsible for the conversion of androgens to estrogens in different tissues. Teleost fishes are characterized by having a higher level of aromatase than any other vertebrate group in the brain. Recently, another aromatese encoded by a novel CYP19 gene, has been found preferentially expressed in the brain of many bony fish species. This work describes the partial (840 bp) molecular cloning of a cDNA encoding the pejerrey brain cytochrome P450 aromatase (P450aromB), differing from the ovarian aromatase (P450aromA) previously reported on this species. The pejerrey brain aromatase form share higher identity to other brain aromatases from different species than with their respective ovarian counterparts and the self ovarian aromatase. Tissue-specific expression for brain and ovarian aromatase was examined in adult pejerrey by RT-PCR. P450aromA was expressed in ovary, testis and brain tissues. However, P450aromB only was detected in brain and pituitary tissues. Aromatase-containing cells and fibers were detected in the brain, but not in the ovary and testis, of adult pejerrey by immunocytochemistry using a teleost-specific aromatase antibody. The Western-blot showed only one band of approximately 57.2 KD in microsomes from brain extracts but not from ovary extracts. Aromatase immunoreactive (arom-ir) cells and fibers were found around the ventricular surface of the telencephalon and in the optic tract. Diencephalic arom-ir cells and fibers were also detected around the surface of the third ventricle. In the midbrain, arom-ir cells and fibers were labeled on periventricular regions of the fourth ventricle and tectum. The forebrain has the greatest number of arom-ir compared with the other regions.

Session II

Pituitary

O-II-1

DIFFERENTIAL PRODUCTION OF FSH AND LH IN FISH: DOES THE PATTERN OF GENE EXPRESSION REFLECT FUNCTION?

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The duality of fish gonadotropins has been clearly established, but the functions and differential regulation of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) in fish are not well understood, in part because of the lack of FSH for biological studies and specific assays for FSH in nonsalmonid fish. Substantially more information is available on gonadotropin subunit cDNA structures and transcript levels during various phases of reproduction in a variety of fish species. Initial studies in salmonids suggested that FSH was important during early phases of gametogenesis while LH regulated final oocyte maturation, ovulation and spermiation. However, data on expression of FSH and LH subunit genes in nonsalmonid species reveal a different pattern, and brings into question the physiological relevance of FSH. Part of this question may stem from the large gap between gene expression and hormone function. Pituitary gene expression does not necessarily reveal a great deal about hormonal regulation of the gonad. The biosynthesis of glycoprotein hormones is complex and regulation can occur at a multitude of steps including: transcript synthesis, processing and degradation; translation, precursor processing, and glycosylation; subunit association and oligosaccharide processing; degradation, packaging and vesicle docking; secretion; and clearance. Furthermore, biological activity is also determined by the nature and distribution of receptors in target tissues. In this paper, expression of FSH and LH subunit genes during the reproductive cycle in representative fish species will be reviewed as well as information on factors that potentially regulate FSH and LH differentially in fish. Examples from both fish and mammals will be used to illustrate situations where it may or may not be appropriate to infer physiological function of gonadotropins from pituitary subunit transcript levels.

O-II-2

THE FSHβ GENE - REGULATION IN FISH

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The full sequence of tilapia FSHβ (tFSHβ) gene was isolated from a genomic library of the mozambique tilapia (*Oreochromis mossambicus*). A 1.7 kb fragment of the tFSHβ 5' flanking regions (5'FR) fused to the firefly luciferase (LUC) gene has directed an efficient basal expression in primary culture of tilapia pituitary cells. Sequential deletion analysis confined its minimal promoter within the 600 bp upstream to the CAP site, and identified two more active regions critical for basal expression. The first (-800 to -1200 bp) encompasses one or more enhancing elements whereas the second (-1200 to -1400 bp) includes silencing elements. It is suggested that three AP1 motifs and a single cAMP response element (CRE) are involved in directing the expression of tFSHβ gene under basal condition. Despite the notable restricted cell type expression of the FSHβ gene, the tFSHβ/LUC constructs were transiently expressed with relatively high basal activity in a multitude of cell lines including COS7, TO2 and αT3-1. This would indicate that the tFSHβ promoter, at least up to 1.7 kb of its 5'FR, does not absolutely require gonadotrope-specific factors to direct significant basal expression. Nevertheless, analysis with deleted mutants of the reporter constructs showed a unique pattern for each of these cell types, which differs from the pattern observed in the homologous pituitary cells. This would implicate dissimilar repertoire of basal transcription factors operating in the heterologous cell lines and the homologous pituitary cells. The 1.7 kb tFSHβ promoter successfully directed GnRH-induced transcription in a dose-dependent manner with a maximal 1.5-2 fold increase in LUC activity. The response to GnRH changed according to the reproductive state of the fish. Our data also indicate that the tFSHβ promoter responds to the three native forms of GnRH with cGnRH-II and sbGnRH being more potent than sGnRH. Deletion analysis revealed that the region between -800 to -1200 bp, which contains putative AP1 and CRE motifs, is essential for GnRH stimulation. Furthermore, forskolin and PMA rapidly induced tFSHβ promoter activity in αT3-1 cells. Taken together, these observations suggest the involvement of cAMP-PKA and PKC (probably *via* the JNK cascade) in mediating GnRH induction of tFSHβ promoter activity. Future studies will characterize the effect of selective alteration within the tFSHβ promoter region to define the exact role of cis-acting motifs such as AP1 and CRE in regulating basal as well as GnRH stimulated gene expression.

O-II-3

REGULATION OF LUTEINING HORMONE β GENE EXPRESSION BY SALMON GONADOTROPIN-RELEASING HORMONE AND SEX STEROIDS IN MASU SALMON PITUITARY CELLS

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Roles of salmon gonadotropin-releasing hormone (sGnRH) and sex steroid hormones in regulation of luteinizing hormone (LH) β subunit gene expression were examined in the primary pituitary cultures of masu salmon (*Oncorhynchus masou*) at different reproductive stages. Pituitaries were taken from fish initiated to mature and expected to spawn in autumn, every two months from March to September. The level of LH β subunit mRNA in the pituitary cells was measured by real-time PCR. It was considerably increased by testosterone (T, 10 ng/ml) in the cultures from males and by estradiol-17β (E2, 10 ng/ml) in those from females in March and May, when the level was increasing in the control cultures. The level was increased by sex steroids to the lesser extent in July, when it reached its maximum in the control cultures. In September, the spawning season, sex steroids showed no effect on the levels in both sexes. sGnRH (100 nM) tended to increase the level only in March, whereas the combination of E2 and sGnRH increased the level in cultures from males in May. To further clarify the role of sGnRH, we examined effect of sGnRH on LH β gene transcription using primary cultures which were transfected with 5' flanking region (3.4 kb) of chinook salmon LH β gene fused to a chloramphenicol acetyl transferase (CAT) gene. After transient expression for 24hr with or without sGnRH, cells were harvested for CAT assay to measure LH β transcriptional activity. In May, sGnRH stimulated the basal activity 1.8 fold, whereas it suppressed the basal activity to 69% in July. sGnRH tended to stimulate LH β transcriptional activity again in September. These results indicate that sGnRH exerts biphasic regulatory action on LH β gene transcription, depending on the reproductive stages. sGnRH and sex steroids differentially regulate LH β gene expression at different reproductive stages in the pituitary of masu salmon.

O-II-4

ACTIVIN REGULATION OF GONADOTROPIN TRANSCRIPTION IN THE GOLDFISH, *CARASSIUS AURATUS*

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Follicle-stimulating hormone (FSH) and luteinizing hormone (LH) are two gonadotropins produced in the pituitary that play critical roles in controlling vertebrate gonadal development and function. Activin, a dimeric growth factor initially identified in the gonads, is important in the differential regulation of the two gonadotropins in mammals. Using goldfish as a model, we have been investigating the regulation of FSHβ and LHβ expression by activin and the mechanisms involved. When tested in vitro, both recombinant human activin A and goldfish activin B stimulate goldfish FSHβ but suppress LHβ expression. The novel inverse effects of activin on the two gonadotropin β subunits can be abolished by follistatin, a specific activin-binding protein. Furthermore, follistatin alone significantly suppresses the basal expression of FSHβ but stimulates that of LHβ, which is opposite to the effects of activin. This strongly suggests that the pituitary produces activin that may act as an autocrine/paracrine factor to regulate FSH and LH biosynthesis. This hypothesis is supported by our demonstration that activin and its entire signaling system are expressed in both the intact goldfish pituitary and the cultured cells, including activin βB subunit, activin type IIA, IIB and type IB receptors, intracellular activin signaling molecules Smad2 and Smad3, as well as follistatin. We have further demonstrated that the goldfish FSHβ promoter is functional in a mouse gonadotrope cell line, LβT2 cells, and it drives the expression of the reporter gene SEAP (secreted alkaline phosphatase) in response to activin. Similar to that in the cultured pituitary cells, the activin-stimulated FSHβ promoter activity in the LβT2 cells can also be blocked by follistatin. The FSHβ promoter activity is significantly enhanced by overexpression of goldfish activin type IIA receptor in the LβT2 cells and suppressed by a dominant negative mutant of the receptor. Similarly, overexpression of goldfish Smad2 or Smad3 in the cells significantly increases the FSHβ promoter activity and its response to activin. Preliminary promoter analysis using the LβT2 cells shows that there may be multiple activin-responsive elements (AER) on the goldfish FSHβ promoter. Further characterization of these cis-regulatory elements responsible for activin stimulation is now under way in our laboratory. [The work was substantially supported by grants (CUHK4176/99M, CUHK4150/01M and CUHK4258/02M) to W. Ge from the Research Grants Council of the Hong Kong Special Administrative Region.]

O-II-5

CLONING AND CHARACTERIZATION OF FOLLISTATIN IN THE GOLDFISH, *CARASSIUS AURATUS*

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Activin is a potent regulator of gonadotropin (GTH) biosynthesis in the vertebrate pituitary. The activity of activin is fine-tuned by its binding protein follistatin (FS) that bio-neutralizes activin by forming an activin-FS complex. In the goldfish, a full-length cDNA of FS has been cloned from the ovary, and it shows 74% sequence identity with that of mammals. To confirm its functionality, the cloned cDNA was transfected into the Chinese hamster ovary (CHO) cells and stable clones selected. Recombinant goldfish follistatin in the conditioned medium of the cloned CHO cells significantly blocked activin-induced F5-5 differentiation in a dose-dependent manner.

Goldfish FS is expressed in a wide range of tissues including the brain, pituitary, ovary and testis. Using primary pituitary cell culture and a semi-quantitative RT-PCR, we have demonstrated that the expression of FS mRNA in the pituitary is regulated by a variety of factors. Treatment with recombinant goldfish activin B for 24 hrs significantly up-regulated goldfish FS expression in the pituitary, suggesting a closed feedback loop in the pituitary. In agreement with this, incubation of the pituitary cells with recombinant human FS (50 ng/ml) down-regulated goldfish FS expression, which is likely due to the neutralization of endogenous activin. Examination of FSHβ and LHβ expression in the same samples supports the role of activin and FS in the differential regulation of FSH and LH as previously demonstrated in our laboratory. Similar stimulatory effects on FS have also been observed with gonadal steroids estradiol and testosterone. To confirm the effects of gonadal steroids, we performed ovariectomy in the goldfish followed by measurement of FS mRNA levels in the pituitary. In one experiment, the expression of FS in the goldfish pituitary significantly decreased 2 weeks after ovariectomy. Seasonal variation of FS mRNA expression in single pituitaries was also examined during a 12-month period with samples collected every month. The temporal expression profile of goldfish pituitary FS will be compared with those of FSHβ and LHβ in the same animals. Our preliminary results also showed that forskolin, an activator of adenylate cyclase, stimulated FS expression, suggesting a role for cAMP-dependent pathway in the regulation. These studies will provide clues to the potential physiological role of FS in the regulation of gonadotropin biosynthesis. [The work was substantially supported by grants (CUHK4176/99M, CUHK4150/01M and CUHK4258/02M) to W. Ge from the Research Grants Council of the Hong Kong Special Administrative Region.]

O-II-6

CLONING AND PHARMACOLOGICAL CHARACTERIZATION OF THE INHIBITORY DOPAMINE RECEPTOR FROM THE PITUITARY OF TWO PERCIFORM FISH: TILAPIA AND GREY MULLET

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The nucleotide sequence of the tilapia (*Oreochromis niloticus*) and the mullet (*Mugil cephalus*) dopamine receptors, cloned by RT-PCR followed by 5 • and 3 • RACE, encodes seven TM-spanning domains; show 93.6% identity to each other, 84% homology to the D2 receptor of pufferfish and 72% to the mouse D2 receptor. A phylogenetic analysis of the known dopamine D2-like receptor sequences places those of the tilapia and the mullet with known vertebrate D2 receptors. Tissue distribution analysis of the dopamine receptor revealed that the receptor is transcribed in the brain, retina, pituitary and kidney. In order to pharmacologically identify the dopamine receptor we used various specific agonists or antagonists to the different receptor subtypes. Dopamine D2 and D3 agonists inhibited GnRH-stimulated LH release from tilapia pituitary cells more efficiently than D4 agonists. Furthermore, D2 and D3, antagonists were more efficient in increasing LH release than D4 antagonists. However, no significant differences were observed between D2 and D3 drugs activity. Transfection of COS-7 cells with tilapia D2 dopamine receptor resulted in the inhibition of forskolin-stimulated cAMP accumulation. Moreover, the tilapia D2 dopamine receptor inhibits adenylate cyclase via coupling to pertusis toxin-sensitive G proteins of the Gi/Go family. The cloning of the tilapia D2 dopamine receptor subtype will promote further study on its role in regulating pituitary function.

O-II-7

PHYSIOLOGICAL ROLES OF FSH AND LH IN RED SEABREAM, *PAGRUS MAJOR*

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The gonadotropins (GTHs), follicle-simulating hormone (FSH) and luteinizing hormone (LH), are crucial modulators of gametogenesis and gonadal steroidogenesis in vertebrates. Like their mammalian counterparts, two distinct GTHs have been isolated and characterized in many teleost species. Although the physiological roles of GTHs have been extensively studied in salmonids, very little is known about their biological functions in non-salmonid fishes. In this study, the expression profiles of GTH subunit genes during sexual maturation were analyzed in both male and female red seabream to elucidate the roles of FSH and LH in reproduction,. Additionally, *in vitro* studies were carried out to examine the effects of FSH and LH on steroid hormone production and cytochrome P450 aromatase (P450arom) expression in red seabream gonads. During sexual maturation, FSHβ mRNA levels in males increased during gonadal development, reached a peak in the spawning season, and then sharply declined in the regressed phase. Conversely, FSHβ mRNA levels in females remained low throughout sexual development. Transcript levels of LHβ in both male and female red seabream were maintained at high levels from early gametogenesis until the spawning season, and declined in the regressed phase. From *in vitro* studies using purified red seabream GTHs, FSH had a similar potency to LH in stimulating 11-ketotestosterone production from testicular slices. In female red seabream, however, the biological activity of FSH was much lower than that of LH with regard to *in vitro* estradiol-17β production by vitellogenic follicles. Furthermore, P450arom mRNA was induced by LH, but not FSH, in ovarian follicles *in vitro*. FSH was also ineffective in inducing germinal vesicle breakdown. These results suggest that, unlike salmonids, FSH may play an important role during gametogenesis in male, but not female, red seabream, while LH may be involved in regulation of early and late gametogenesis in both sexes.

O-II-8

GONADOTROPINS, GONADOTROPIN RECEPTORS AND THEIR EXPRESSIONS DURING SEXUAL MATURATION IN YELLOWTAIL, A CARANGID FISH

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Fish reproduction is highly regulated by glycoprotein hormones, gonadotropins (GtHs: FSH and LH) and their receptors. The yellowtail is extensively cultured in Japan. To study the physiological roles of gonadotropins in this species, the cDNA encoding each GtH subunit (GtHα, FSHβ, and LHβ) and their receptors (FSHR and LHR) was isolated from the pituitary gland and gonads using the polymerase chain reaction (PCR), and 3'- and 5'-rapid amplification of cDNA ends (RACE). In addition, thyrotropin receptor (TSHR) was isolated from the ovary and testis. Using these gonadotropin subunits and their receptors as molecular probes, the changes in the mRNA levels of each subunit were determined at different stages of maturation. The isolated cDNAs of GtHα, FSHβ, and LHβ were 662, 545 and 595 bp long, respectively, and consisted of a 5'-untranslated region (UTR), an open reading frame and a 3'-UTR, which contained a putative polyadenylation signal and a poly (A) tail. The respective amino acid sequence identity of the yellowtail GtHα, FSHβ, and LHβ subunits was 85-63, 68-33, and 93-65% compared with other fish species. Northern blot analysis showed that GtHα and FSHβ were expressed significantly in early vitellogenic stage and during spermatogenesis, whereas LHβ was expressed significantly in the late vitellogenic stage and in both spermatogenesis and spermiation. Full-length cDNAs encoding FSHR, LHR, and TSHR were obtained from the ovaries and testes of the yellowtail. These receptors belong to a sub-family of glycoprotein hormone receptors, in which the receptor has three major subdivisions: a large extracellular domain, seven transmembrane domains, and a short cytoplasmic domain. The FSHR cDNA encodes a protein of 680 amino acids. It has the highest identity with the FSHR of the tilapia (76%), followed by those of the salmon (66%), catfish (58%) and human (52%). The LHR cDNA encodes a protein of 702 amino acids and has the highest identity with LHR of the tilapia (84%), followed by the catfish (62%), salmon (50%), and human (53%). The TSHR cDNA encodes a protein of 778 amino acids and has the highest identity with the TSHR of the striped bass (95%), followed by the salmon (79%), and mammals (60-62%). It contains a TSHR-specific insertion in the extracellular domain, as seen in mammalian receptors. Northern blotting and RT-PCR analyses indicated that all these receptors are expressed abundantly in the ovaries and testes of sexually mature fish. The different seasonal patterns of these three receptors indicate that they have different functions in regulating steroidogenesis and other physiological processes in the gonads.

O-II-9

IMMUNOHISTOCHEMICAL DETECTION OF GONADOTROPIN-LIKE MATERIAL IN THE HAGFISH PITUITARY

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The hagfish is considered the most primitive vertebrate known, living or extinct. It remains enigma whether adenohypophysial hormones similar to those of more advanced vertebrates are present in the hagfish pituitary or not. The present study aimed to detect immunoreactive gonadotropin (GTH)-like material in the pituitary of the Atlantic and the Pacific hagfish, *Myxine glutinosa*, *Eptatretus burgeri* and *Paramyxine atami*. In all three species, GTH-like cells were observed in the adenohypophysis, which were stained with several antibodies to LH-related GTHs, such as human LHβ, ovine LHβ, bullfrog LH, and salmon GTHIIβ. To obtain further insights into the functional significance of GTH-like material in the hagfish pituitary, the relationship between the amount of GTH-like material and gonadal conditions was examined in *Paramyxine atami*. The adenohypophysis of adult animals exhibited well developed features, and consisted of many clusters of cells, embedded densely in the connective tissue. In those animals, more than half of the adenohypophysial cells were stained intensely with anti-ovine LHβ. On the other hand, the adenohypophysis of juveniles exhibited less developed features, and no or a few adenohypophysial cells were stained with anti-ovine LHβ. Following intraperitoneal administration of estradiol benzoate in juvenile animals (once 65 mg / 0.1 ml every third day, in total nine times), intensities of GTH-like material in the adenohypophysis increased significantly when compared to those of controls. These results suggest that GTH is present in the hagfish pituitary.

P-II-1

MOLECULAR CLONING OF GONADOTROPIN SUBUNIT cDNAS IN THE MANCHURIAN TROUT (*BRACHYMYSTAX LENOK*)

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Two gonadotropins (GTHs; FSH and LH) are produced in the pituitary of teleost fishes and their molecules are comprised of common α and a distinct β subunit. In salmonid species, FSH mediates vitellogenesis and spermatogenesis, and LH regulates oocyte maturation and spermiation. One objective of the present research is to obtain cDNAs of common α, FSHβ and LHβ subunits in a exterminating salmonid species, Manchurian trout (*Brachymystax lenok*), in Korea. Polymerase chain reaction (PCR) was used to amplify the cDNAs of GTH subunits prepared from maturing fish, and the obtained PCR products were subcloned and sequenced. The deduced amino acid sequences of GTH subunits showed high homology to masu salmon GTHα (74%), FSHβ (92%) and LHβ (97%), respectively. Next, we examined *in vivo*short-term effects of estradiol-17β (E2) and nonylphenol (NP) on GTH subunits expression in juvenile masu salmon as a model fish of Manchurian trout using semi-quantitative RT-PCR method. In this experiment, the mRNA levels of FSHβ and LHβ subunits were increased by E2 (5 mg/kg) and NP (10 mg/kg) treatment (72 hours). We are also attempting to produce recombinant FSH and LH *in vitro* using a eukaryotic expression system. The understanding of Manchurian trout GTH subunits gene expression and the production of hormones will be helpful to improve the reproductive control system of exterminating Manchurian trout in Korea.

P-II-2

DIFFERENTIAL IMPACT OF RECOMBINANT HUMAN ACTIVIN ON LH SECRETION IN MALE AND FEMALE COMMON CARP DURING WINTERING AND SPAWNING PERIOD

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Activins play an important role in the regulation of synthesis and secretion of gonadotropins in vertebrates. In mammals these factors were shown to influence mainly follicle-stimulating hormone (FSH), while in fish activins have also an impact on the synthesis and seceretion of luteinizing hormone (LH). The aim of this study was to evaluate the influence of recombinant human activin at two doses (10 and 50 ng/ml) on basal as well as sGnRH-stimulated secretion of LH from dispersed pituitary cells of male and female common carp during wintering and during spawning period. Pituitaries were dispersed enzymatically using incubation with collagenase and placed onto microplates. After 60-hour period of preincubation the dispersed pituitary cells were rinsed and incubated with recombinant human activin and/or different doses of synthetic sGnRH for 24 hours. After incubation, the medium was subjected to the analysis of LH levels using a specific ELISA method. In females during wintering activin at a dose of 10 ng/ml decreased basal LH secretion, having no impact on sGnRH-stimulated release of this hormone. On the other hand during preovulatory period activin at higher dose stimulated basal LH secretion. Moreover smaller activin at a dose of 10 ng/ml stimulated LH secretion in response to sGnRH at a dose of 10^{-9}M. The secretion of LH in male common carp during wintering was increased after incubation with activin at a dose of 50 ng/ml. At this stage activin increased also sGnRH-stimulated LH release. Contradictory to the above activin failed to modify basal as well as sGnRH-stimulated LH secretion in males during spawning period. These results suggest the differential and sex-dependent influence of activins on LH secretion in common carp.

P-II-3

CLONING AND SEQUENCING OF FSH-ß AND LH ß-SUBUNITS AND CHANGES IN THEIR EXPRESSION DURING THE REPRODUCTIVE CYCLE IN THE THREE-SPINED STICKLEBACK, *GASTEROSTEUS ACULEATUS*

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The three-spined stickleback has a marked seasonal reproductive cycle, in which spermatogenesis and the development of androgen-dependent secondary sexual characters are separated in time. In teleost fishes, like in other vertebrates, the gonads are stimulated by two gonadotropic hormones, luteinizing hormone (LH) and follicle-stimulating hormone (FSH). To achieve a better understanding of the role of gonadotropins (GTHs) in the three-spined stickleback we have cloned the full-length cDNAs for FSH- β and LH- β subunits. The complete cDNA of FHS- β was 540 bp long, encoding a protein of 122 amino acids and LH- β cDNA was 568 bp long, encoding a protein of 148 amino acids. Compared with other GTHβ -subunits published so far, the highest degree of similarity was found with perciform fishes, with amino acid similarities of 50-58% for FSH- βand 71-76% for LH- β subunits. The cloned cDNAs were used as probes to analyze LH- β and FSH- β mRNA expression during the annual cycle in male and female three-spined sticklebacks and compared with the development of the gonads and secondary sexual characters. In male sticklebacks the kidney hydrophies during the breeding season, to produce a glue used when building nests. This secondary sexual character, measured as kidneysomatic index (kidney weight/body weight x 100), reached a peak in May. The gonadosomatic index (GSI) in females started to increase in April, and peaked in May as well. In late summer, after the breeding season, these features declined. In females, LH- β expression followed the GSI values very closely, levels were low during winter and early spring, increased to a peak in late May and declined again in July. FSH- β expression peaked earlier, in January and declined slowly over spring. In males, LH- β expression reached a peak in May while during June-September, when spermatogenesis was active, LH- βlevels were very low. FSH- β expression peaked in January, and lowest levels were observed in July. Thus, when spermatogenesis started at the end of summer, the expression of both GTH- β mRNAs displayed their lowest levels.

P-II-4

GONADAL DEVELOPMENT AND EXPRESSION PROFILES OF GONADOTROPIN GENES IN WILD SEA CONGER (*ARIOSOMA MEEKI*)

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Reproductive physiology of the Japanese eel remains unclear because fully mature eels have never been caught from the wild, and because they have never gone through gametogenesis spontaneously when maintained in captivity. Although gonadal development can be stimulated by hormonal treatment in this species, there are increasing argument about whether such consequences are normal or not. In contrast, wild mature sea conger, which also belong to anguilliformes, are available in Miyazaki, Japan. This species, thus, enables us to adopt comparative approaches to assess the question concerned with reproduction of the Japanese eel. One hundred fish (male 20, female 80) obtained from local fishermen were used in this study. Histological observation revealed that the spawning season of the sea conger is between July and August, and that their oocytes show synchronous development. We cloned the cDNAs encoding the gonadotropin subunits (GTH-α,FSH- and LH-β), and the abundance of their transcripts in the pituitary glands was measured using real-time quantitative RT-PCR. In immature females, which possess previtellogenic oocytes in their ovaries, mRNA levels of all GTH subunits were very low. mRNA levels of GTH-α and LH-β subunits increased markedly in accordance with ovarian development, whereas that of FSH-β subunit remained high during vitellogenic stages. In males, transcripts of GTH-α and LH-β subunits were very few when the fish were immature, and they increased when the fish matured. In contrast, FSH-β subunit mRNA levels showed no significant changes during spermatogenesis, although tendency to increase was observed. After spawning, transcripts of all GTH subunits decreased in both sexes. Present results were inconsistent with our previous results obtained in female Japanese eels, where they had oocytes with asynchronous development, and their mRNA levels of FSH-β subunit decreased after injection of salmon GTH. However, these results may be an artifact and the Japanese eel may have the similar gonadal development and expression profiles of GTH genes as in the sea conger in natural conditions.

P-II-5

ANDROGEN SECRETION ACTIVITY OF RECOMBINANT FOLLICLE-STIMULATING HORMONE OF JAPANESE EEL, *ANGUILLA JAPONICA* IN IMMATURE AND MATURING EEL TESTES

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In the Japanese eel, *Anguilla japonica*, the administration of exogenous gonadotropin (GTH) is necessary for the artificial induction and completion of gonadal maturation due to its GTH deficiency under captive conditions. The isolation of native eel GTH has not been accomplished, which has made it difficult to fully elucidate the biological functioning of the two GTHs (follicle-stimulating hormone; FSH and luteinizing hormone; LH) in teleosts, particularly that of FSH. In this study, we attempted to investigate the androgen (testosterone; T and 11-ketotestosterone; 11-KT) secretion activities of eel FSH in the testis using recombinant Japanese eel FSH (rjeFSH) produced in methylotrophic yeast, *Pichia pastoris* in order to gain more understanding of the biological features of FSH in this species. rjeFSH was examined in terms of androgen secretion activity using immature (GSI; 0.12-0.35%) and maturing (GSI; 3.8-6.5%) eel testes. Sexually maturing male eels were obtained by weekly injection (5 times in total) of human chorionic gonadotropin (300 international unit/fish/week) into immature male eels. Histological observation showed that immature testis contained only non-proliferated spermatogonia, whereas maturing testis contained proliferated germ cells; spermacytes and spermatid. In *in vitro* bioassay, the fraction containing rjeFSH significantly stimulated the release of T and 11-KT, the spermatogenesis-inducing steroid in eel, from immature testis in a dose dependent manner, while maturing testis did not respond. These results suggest that FSH in eel promotes the early stages of spermatogenesis via sex steroid production, and the sensitivity to FSH changes depending on the degree of sexual maturity of the fish.

P-II-6

EFFECTS OF GONADOTROPIN-RELEASING HORMONE AGONIST AND TESTOSTERONE ON GONADOTROPIN SUBUNIT GENES EXPRESSION IN FEMALE RED SEABREAM (*PAGRUS MAJOR*)

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The regulation of synthesis and release of two gonadotropins, FSH and LH has not been fully understood yet. In the present study, we investigated the effects of GnRH agonist (GnRHa) and testosterone (T) on gene expression of gonadotropin subunits (glycoprotein (GP) α, FSHβ and LHβ) and release of LH in immature female red seabream. Sham operated (Sham) or ovariectomized (OVX) fish were implanted with a cholesterol pellet containing GnRHa or silicon capsules filled with T or vehicle alone (Cont). Three weeks after implantation, fish were sampled. In OVX/Cont fish, serum estradiol-17β (E2) levels were significantly decreased, although T levels were relatively low from the beginning and unchanged. The levels of seabream (sb) GnRH mRNA in OVX/Cont fish significantly increased compared with those of sham/Cont fish. Moreover, the levels of both GPα and LHβ mRNA and serum LH were significantly increased, and FSHβ mRNA also tended to increase in OVX/Cont fish. In Sham/GnRHa and OVX/GnRHa fish, levels of both GPα and LHβ mRNA and serum LH were dramatically increased compared with those of Sham/Cont fish, whereas no marked changes were observed in FSHβ mRNA levels. In Sham/GnRHa fish, serum T and E_2 levels were drastically increased, and ovarian development and ovulation were induced. In OVX/T fish, OVX induced increase of sbGnRH, GPα, LHβ mRNA and serum LH levels were completely depressed. In contrast, T treatment did not suppress the OVX induced increase of FSHβ mRNA levels. In T treated fish, serum T levels were increased and reached to same levels as those of mature fish. In addition, serum E2 levels were also increased may be due to the conversion from exogenous T to E2 by inherent aromatization. These results suggest that the expression of both GPα and LHβ mRNAs and release of LH, but not expression of FSHβ mRNA, are stimulated by GnRH. On the other hand, synthesis and release of LH are inhibited by sex steroids (T and/or E2) via negative feedback regulation to sbGnRH synthesis. Moreover, expression of FSHβ mRNA may be inhibited by ovarian factor(s) other than T and E2.

P-II-7

CLONING OF SMAD PROTEINS IN THE GOLDFISH AND THEIR INVOLVEMENT IN ACTIVIN REGULATION OF FSH β TRANSCRIPTION

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Follicle-stimulating hormone (FSH) is a glycoprotein consisting of a common α subunit and a unique β subunit, and it is an essential regulator of gonadal development and function in vertebrates including teleosts. FSH is regulated by a variety of neuroendocrine and endocrine factors and its biosynthesis is primarily determined by the expression of the β subunit. Previous in vitro studies from our laboratory demonstrated that activin stimulated FSHβ expression in both the primary culture of goldfish pituitary cells and the mouse gonadotrope-derived LβT2 cells transfected with goldfish FSHβ (gfFSHβ) promoter-reproter constructs. However, little is known about the signal transduction pathway involved in the transcriptional activation of this gene by activin. As activin signals by interacting with a heteromeric receptor complex and intracellular signaling proteins Smad2 or Smad3, we investigated the involvement of these Smads in the activin-stimulated goldfish FSHβ expression in the present study. To study the participation of Smads in regulating the gfFSHβ promoter, we first cloned full-length cDNAs for goldfish Smad2 and Smad3 from the pituitary, which show 86% amino acid sequence identity with their counterparts of mammals. Co-transfection of Smad2 or Smad3 cDNA with the reporter construct of gfFSHβ promoter-SEAP (secreted alkaline phosphatase) into the LβT2 cells significantly enhanced the basal and activin-stimulated SEAP expression. Interestingly, the effect of over-expressing Smad3 is much higher than that of Smad2. The activin-stimulated SEAP activity was completely blocked by follistatin, an activin-binding protein. To determine the presence of any Smad-responsive elements (SREs) on the gfFSHβ promoter, Smad2 or Smad3 cDNA was co-transfected with SEAP reporter constructs containing gfFSHβ promoter of different lengths. Preliminary results showed multiple SREs might exist on the gfFSHβ promoter region. Studies are now underway to characterize these putative SREs. We have also cloned goldfish Smad4, a common Smad that associates with Smad2 or Smad3 after their activation by activin receptors, and Smad7, an intracellular inhibitory Smad that blocks activin signaling. Their involvement in the activin-stimulated gfFSHβ expression will also be analyzed. [The work was substantially supported by grants (CUHK4176/99M, CUHK4150/01M and CUHK4258/02M) to W. Ge from the Research Grants Council of the Hong Kong Special Administrative Region.]

P-II-8

MOLECULAR CLONING OF cDNAS ENCODING FSH-ß AND LH-ß SUBUNITS IN THE PEJERREY FISH, *ODONTESTHES BONARIENSIS*

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Gonadotropins (GtHs) are pituitary hormones that regulate sexual maturation and the reproductive process. It is generally accepted that GtHs in fish are structurally related to the tetrapod follicle-stimulating (FSH) and luteinizing (LH) hormones. GtHs are heterodimeric glycoproteins consisting of the non-covalent association of a common α–subunit and a ß–subunit that confers hormonal specificity. The pejerrey Odontesthes bonariensis is a freshwater atherinid fish from South America that is considered a promising alternative to commonly aquaculture freshwater fish species. Pejerrey is a multiple-spawning fish which spawn several times during their reproductive season. In this species it was also suggested that GtHs are related with the sex determination/differentiation process. Therefore, it is important to study the function of GtHs in this species. As an initial step the partial fragments of the cDNAs encoding for FSH-ß (427bp) and LH-ß (236 bp) subunits were obtained by using RT-PCR with consensus primers, cloned and sequenced. A comparison of this sequence with several gonadotropins from teleost fishes was performed. The present study also provides a proof of the existence of GtH duality in the pejerrey fish as has been recently shown in other teleost species.

P-II-9

GONADOTROPIN ALPHA (α)-SUBUNIT IN TWO SALMON GONADOTROPES (GTH I-, GTH II-CELLS): TRANSCRIPTION, TRANSLATION AND SECRETION

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Salmon pituitary produces two distinct gonadotropins (GTH I and GTH II) separately in two different gonadotropes (GTH I- and GTH II-cells). Salmon gonadotropins are structurally and functionally similar to tetrapod FSH and LH, and heterodimers composed of an α and a unique β subunit. This study asks whether the pattern of transcription, translation and secretion of gonadotropin subunits (α and β subunits) in each individual gonadotropes (GTH I- and GTH II-cell) correlates with sexual maturity. In the maturing females, the GTH II-cells demonstrated a coordinated increase of α- and II β-subunit mRNA levels. However, intracellular protein levels of the II β were much higher than that of the α-subunit. The Cell immunoblot assay (CIBA) detected immunoreactivity for α-protein extracellularly around GTH II-cells. This may support secretion of α-subunit in its free form. In GTHI-cells, however, mRNA levels for α-subunit selectively remained very low, although I β transcription and translation elevated markedly. Furthermore, the elevated plasma levels of GTH I by RIA and also marked immunoreactivity by CIBA raise a question of whether GTH I-cells release the I β-subunit in its free form. These findings suggest that in the salmon pituitary, transcription, translation and secretion of α-subunit are differentially regulated, in a cell specific manner, in two types of gonadotropes, GTH I- and GTH II-cells.

P-II-10

DIFFERENTIAL EFFECT OF INSULIN-LIKE GROWTH FACTOR I ON *IN VITRO* GONADOTROPIN SUBUNITS EXPRESSION IN ATLANTIC SALMON

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In Atlantic salmon, males may mature either as small parr in freshwater or later at large size after having spent one or several years in the sea. Early sexual maturation at age 1+, observed both in wild and hatchery reared populations, is influenced by somatic growth and/or energy stores. There is increasing evidence that metabolic hormones and growth factors may act directly at different levels of the hypothalamus-pituitary-gonadal axis to promote the onset of sexual maturation in various vertebrate species. Recent data suggest that insulin-like growth factor I (IGF I) may play a role as a link between growth and puberty. In the present study a serum-free primary culture of pituitary cells was used to study the direct effect of IGF on the gonadotropin gene expression in immature Atlantic salmon. IGF I increased the expression of the luteinizing hormone (LH) β subunit in a dose-dependent manner. IGF I and IGF II had similar potencies but insulin was less effective in stimulating LH-β subunit expression. On the other hand, IGFs or insulin had no effect on the expression of the follicle-stimulating hormone (FSH) β-subunit. The effect of IGF I on the glycoprotein alpha subunits expression was only significant at the highest concentrations tested (10^{-7}M).

P-II-11

MOLECULAR CLONING OF GONADOTROPIN cDNA IN SEVENBAND GROUPER, *EPINEPHELUS SEPTEMFASCIATUS*

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The sevenband grouper is one of the most important fish in the aquaculture industry of Japan. However, spontaneous spawning has not occurred in the cultured female sevenband grouper. We carried out the artificial maturation and ovulation and result have been successful using LHRHa treatment in sevenband grouper. However, egg quality was very low and reliable supply of larvae was also unstable. In many endocrine factors regulating gonadal development and maturation, gonadotropin (GtH) is the most major and indispensable hormone. Studies on GtH synthesis in particular, and its function in gonad are necessary to understand maturational process in target species. We performed molecular cloning of GtH to obtain the information of sevenband grouper GtH. Full-length cDNA sequences encoding glycoprotein hormone (GPH) α subunit, β-subunits of the FSH (GtH I) and LH (GtH II) were isolated, cloned, and sequenced from sevenband grouper pituitary using RACE PCR. DNA sequence analysis of the cloned PCR products confirmed the presence of the predicted complete coding region as well as 5′ and 3′ untranslated sequences. The deduced amino acid sequences of α and β-subunits from the sevenband grouper were compared to GtH sequences from a number of teleosts. Our results indicate that the nucleotide sequences of the GPH α subunit, FSH β and LH β are 622, 525 and 580 bases long, encoding peptides of 351, 360 and 441 amino acids, respectively. Sevenband grouper GPH α, FSH β and LH β share an average sequences identity of 95%, 69% and 94% at the amino acid level with other teleosts. These results show that sevenband grouper has two different types of gonadotropins FSH (GtH I) and LH (GtH II), as in other teleosts. Further study is necessary to measure GtH gene expressions and to establish the assay system for measurement of GtH protein during the oocyte development.

P-II-12

IMMUNOCYTOCHEMICAL IDENTIFICATION OF GONADOTROPHS (FSH CELLS AND LH CELLS) IN VARIOUS PERCIFORM FISHES USING ANTISERA RAISED AGAINST SYNTHETIC PEPTIDES

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Endocrine mechanism that controls reproductive events may considerably vary among teleost species which belong to wide taxa and have diverse reproductive strategies. However, the respective roles of gonadotropins, follicle stimulating hormone (FSH) and luteinizing hormone (LH), in various reproductive events are not well understood in multiple spawners. Knowledge is especially poor for the function and expression profiles of FSH in non-salmonid fishes. This study is intended to identify two types of gonadotrophs (FSH cells and LH cells) of perciform fishes using immuno-cytochemistry with possible universal antisera; and then to make a basis to evaluate activities of gonadotrophs in various fish species. Pituitaries were taken from adult fishes which belong to order Perciformes but different families, and were immunostained with antisera against synthetic fragment peptides corresponding to conservative regions of gonadotropin β subunits (mummichog FSH β 50-60 and mummichog LHβ 91-106). Both immunoreactive FSH cells and immunoreactive LH cells were successfully identified in these fishes, such as whiting (Sillaginidae), wrasse (Labridae), rabbitfish (Siganidae), croaker (Sciaenidae), blackfish (Girellidae), *etc.* Both types of the cells distributed mainly in the proximal pars distalis, and the distributing patterns were considerably different each other. Duality or multiplicity of gonadotrophs as well as duality of gonadotropins were therefore considered to be common in many perciform fishes; although further examination on the possibility of the co-localization of FSH and LH in same cells will be needed to confirm precise distribution of gonadotropins in the pituitary. Extensive diversity in the abundance of the FSH cells among fish species was noticed, and the meaning of such diversity is discussed.

P-II-13

MOLECULAR CLONING OF cDNAS FOR FOLLITROPINß AND LUTROPINß IN MILKFISH: EVOLUTIONAL IMPLICATIONS

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In most tetrapods, two gonadotropins (GTH), the follitropin (FSH) and the lutropin (LH) have been identified. Also in ray-finned fish and cartilaginous fish, FSH and LH are present, which were originally named GTH I and II, respectively. Thus, the duality of GTH might have established in a common ancestor of gnathostomes. Milkfish, *Chanos chanos*, is a member of early group, order gonorhynchiformes, of euteleosts, which is an important aquaculture species in Southeast Asia. The present study was undertaken to clone FSHβ cDNA and LHβ cDNA. This will allow us to develop the tools (gene probes) as the first step toward understanding milkfish reproduction. Nucleotide sequence was determined using cDNA prepared from milkfish pituitary glands by reverse transcription-polymerase chain reaction and rapid amplification of cDNA end method for 3' and 5' region. The open reading frame of milkfish (mf) FSHβ cDNA encoded preFSHβ consisting of 127 amino acids (aa). Sequence comparison with other teleost FSHβ showed that N-terminal 16 aa sequence is a signal peptide and mfFSHβ is composed of 112 aa. mfFSHβ showed the highest sequence identity to goldfish FSHβ (70%) and followed by eel and salmonid FSHβ. The mfFSHβ contains 13 Cys residues and one N-glycosylation site whose positions are identical to those in goldfish FSHβ. The open reading frame of mfLHβ cDNA encoded preLHβ consisting of 138 aa. Sequence comparison with other teleost LHβ showed that N-terminal 21aa sequence is a signal peptide and mfLH is composed of 117 aa. mfLHβ showed the highest sequence identity to goldfish LHβ (93%) and followed by salmonid and eel LHβ. The sequence contains 12 Cys residues and one N-glycosylation site whose locations are identical to those in other vertebrate homologues. Sequence comparison and phylogenetic tree constructed by neighbor-joining method show that both fish FSHβ and LHβ are classified into two groups: one is the primitive group containing milkfish, goldfish, salmon, and eel and the other is advanced group containing flounder, bass, tuna, and killifish. Based on the tree, the evolutional rate of fish FSHβ is much faster than that of fish LHβ. For example, the evolutional distance between milkfish and goldfish FSHβ is five times greater than that between their LHβ. Similar difference is also observed in advanced group; the distance between tuna and flounder FSHβ is two times greater than that between their LHβ. Thus, FSHβ has weaker functional constraint than LHβ in fish. The biological function of FSHβ may have diverged according to the evolution of fish, while LHβ may have common function throughout fish.

P-II-14

REGULATION OF GONADOTROPIN SUBUNIT GENES EXPRESSION BY 11-KETOTESTOSTERONE DURING EARLY SPERMATOGENESIS IN MALE RED SEABREAM, *PAGRUS MAJOR*

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Gonadotropins (GTHs), follicle-stimulating hormone (FSH) and luteinizing hormone (LH), are regulators of steroidogenesis and gonadal development in teleosts, as in other vertebrates. In mammals, biosynthesis and secretion of GTHs are regulated by feedback mechanisms of gonadal steroid hormones. In many teleosts, considerable information is available on the regulation of LH synthesis by gonadal steroid hormones. However, the feedback regulation of FSH biosynthesis is still controversial. In order to understand the regulatory mechanisms underlying biosynthesis of GTHs at molecular levels, we investigated effects of castration and replacement of 11-ketotestosterone (11-KT) on GTH subunits (FSH β, LHβ, and glycoprotein α; GPα) genes expression during early spermatogenesis in male red seabream. After castration or sham-operation, fish were implanted with silicone capsules filled with or without 11-KT. One month after implantation, pituitaries were collected following decapitation, and total RNA was extracted from each individual separately. GTH subunits mRNA levels were analyzed by ribonuclease protection assay. FSH β mRNA levels were significantly increased by castration and the increase of FSHβ mRNA was suppressed to near sham-operated fish levels by 11-KT treatment. Furthermore, FSHβ mRNA levels in sham-operated fish were also significantly decreased by 11-KT implantation. In contrast, LHβ mRNA levels were changed by neither castration nor 11-KT replacement. Interestingly, 11-KT significantly decreased LHβmRNA levels in sham-operated fish. GPα subunit mRNA levels were not affected by any treatments. These results suggest that 11-KT is one of the gonadal factor which has negative feedback effect on FSHβ gene expression during early spermatogenesis in male red seabream. In addition, 11-KT exerts negative effect on LHβ gene expression via other gonadal factor(s).

Session III

Sex Determination and Gonadal Differentiation

O-III-1

ROLE OF ESTROGENS IN SEX DIFFERENTIATION AND SEX CHANGE IN FISH

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In teleost fish, as well as in other vertebrates, gonadal primordia are formed during ontogenesis. Subsequently, they differentiate into the ovary or the testis. Recent experiments with genetically controlled all-female and all-male tilapia have provided information about the role endogenous sex hormones play in sex differentiation. In this presentation, I will discuss the morphological characteristics of sex differentiation in fish, the differentiation of steroid-producing cells, the expression of steroidogenic enzymes accompanying sex differentiation, and the effects of estrogen antagonists on sex differentiation. The data indicate that endogenous estrogens play an important role in ovarian differentiation and that a deficiency of estrogen is important for testicular differentiation. More recently, I found that long-term treatment with non-steroidal aromatase inhibitor fadrozol induced testicular differentiation in the developed ovary long after ovarian differentiation, even in the mature ovary, though androgen treatment did not bring about testicular differentiation. These facts indicate that germ cells in the developed ovary retain their plasticity long after sex differentiation and that estrogen also plays an important role in the differentiation of germ cells in the ovary after sex differentiation. In addition to gonochorism, various types of hermaphroditsm are known: protandrous hermaphrodites, protogynous hermaphrodites, and multiple sex changing fish. We recently succeeded in inducing females to change to males in the protogynous wrasse and grouper by treating them with aromatase inhibitor. In contrast, combination treatment with aromatase inhibitor and estradiol prevented sex change. These results show that estrogen also plays an important role in sex change in fish. I will review the overall importance of estrogens on gonadal sex differentiation and sex change in fish.

O-III-2

GENE EXPRESSION DURING GONADAL SEX DIFFERENTIATION IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) : FROM CANDIDATE GENE STUDIES TO HIGH THROUGHOUT GENOMIC APPROACH

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Steroids have been widely used in aquaculture for sex control and generally androgens drive testis differentiation and estrogens ovarian differentiation. However, even if these effects are well documented, the mechanism of action of these molecules still remains poorly understood. Past studies looking at only a few candidate genes in all-male and all-female populations of rainbow trout, have confirmed the clear implication of steroid enzymes in gonadal differentiation in this species. We more recently switch to an intermediate approach by looking at gene expression of roughly 100 putative mediators or targets of steroid action. These genes belong to the following families : transcription factors (SF1, WT-1, SOX9, DAX1, DMRT1...), germ cells proteins (Vasa, Germ cell less, POG, ...), steroidogenic enzymes (3βHSD, 11βHSD, P450scc, P450c11,P450aro, ...), growth factors (TGFβs, IGFs, ...), hormones (LH, FSH, AMH, ...), apoptosis regulators (Caspases, BCLX, BAX, Survivin, ...) and hormone and growth factor receptors (receptors for LH or FSH, androgens and estrogens, IGFs,...). Trout homologues were searched in public (EMBL, GenBank) and private (AGENAE-INRA trout ESTs and Genome-BC Salmon ESTs Projects) databases and primers were chosen for expression studies using a real-time quantitative PCR approach. All these genes have been looked during natural or steroid-induced gonadal differentiation and this data was completed by gonadal histology for each experimental point. The results of this study bring a lot of new interesting data on gene expression during rainbow trout gonadal differentiation with especially some gene clusters that are clearly up or down regulated either during natural or steroid-induced differentiation. This real-time quantitative PCR approach is now completed by a more conventional genomic high throughout approach using array technologies.

O-III-3

TIMING AND VARIABILITY IN SEXUAL DIFFERENTIATION IN THE ZEBRAFISH (*DANIO RERIO*)

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The zebrafish undergoes juvenile hermaphroditism, where all fish first develop immature 'ovaries' before the gonadal sex is subsequently fixed and individuals become either male (in which the immature ovaries transform into testes) or female. Sexual differentiation in fish is sensitive to disruption by chemicals that interfere with sex hormone synthesis/function, and gender assignment has been adopted as a biomarker for chemical disruption. Despite this, there is only very limited information on the timing and variability in the sexual differentiation process within and between strains of fish. In this study, using gonad histology, we assessed the timing and variability in sexual differentiation in a strain of zebrafish with a high level of heterozygocity (the WIK strain, that is only one generation removed from the wild type collected from India) and a strain that had been inbred for nearly 10 years. There was a high variability in the timing of sex differentiation within a specific strain, but there were no obvious differences between the two strains. Transformation of the immature fish into males started in week 5 post fertilization (pf), with sexually distinct males appearing first in week 6/7 pf and completion of sexual differentiation for all fish in the population by week 11-12pf. The size of the fish containing transforming gonads in the WIK strain and homozygous strain ranged from 12 to 23 mm, and from 13 to 22 mm total length (TL), respectively. A threshold size of 8 mm (TL) appeared to be a pre-requisite before development progressed from an undifferentiated gonad. A size-dependent relationship with the subsequent timing/rate of gonad development, however, was not apparent for either strain. A lack of any clear size-dependency for sexual development in the zebrafish populations studied might suggest that stocking density and/or social factors play a key role in determining the timing of sexual development for individuals within a population.

O-III-4

POTENTIAL USE OF OTOLITH MICROCHEMISTRY FOR FIELD STUDIES OF TEMPERATURE-DEPENDENT SEX DETERMINATION AND GONADAL DEGENERATION IN FISH

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A growing number of fish species is being shown to have temperature-dependent sex determination (TSD) or at least the overriding of putative genetic sex factors under extreme temperatures. A typical example of TSD is the formation of more females at low temperatures and more males at high temperatures. High temperature has been implicated also in gonadal degeneration, loss of germinal elements and subsequent sterility. Thus, atypical water temperature patterns, including those induced by human activities such as discharge of industrial heated effluents and global warming, might have serious effects on the reproduction of natural fish populations. The possible outcomes include skewed sex ratios and reduced fecundity, with obvious implications for the stability of stocks and maybe for the very survival of the most temperature-sensitive species. Nevertheless, field expression of TSD may be difficult to establish because sex determination often occurs when fish are already freely swimming larvae or juveniles whose thermal experience cannot be adequately monitored. A cause-effect relationship between high temperature and reduced fecundity or sterility in the wild may be also difficult to ascertain because the past thermal history of the fish is unknown. In this study, we examined the possibility of reconstructing past thermal history based on the analysis of otolith elemental composition and using this information to study the field expression of temperature-dependent sex determination and high temperature-induced gonadal degeneration in fish. We used the pejerrey (*Odontesthes bonariensis*), an atherinid fish with well-documented TSD and heat-induced germ cell loss, as a model in this study. We first examined the growth and elemental composition of the otoliths of fish reared at different temperatures by electron probe microanalysis as well as the time, body- and otolith size at sex determination. Strontium content of the otolith was found to accurately reflect rearing temperature. Preliminary field studies were conducted in the Lake Kasumigaura (Ibaraki, Japan) and showed a good correlation between the phenotypical (gonadal) sex and the temperature estimated from the strontium content of the otolith region corresponding to the time of sex determination. Thus, this technique might be a valuable tool to study the expression of TSD and temperature-induced reproductive dysfunctions in wild fish populations.

O-III-5

DIFFERENTIAL PLASMA LEVELS OF LUTEINIZING HORMONE AND EXPRESSION OF GONADAL GONADOTROPIN RECEPTORS IN PROTANDROUS BLACK PORGY, *ACANTHOPAGRUS SCHLEGELI*: THE IMPLICATION OF SEX CHANGE MECHANISM

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Black porgy, *Acanthopagrus schlegeli*, is a marine protandrous hermaphrodite fish. The testicular and ovarian tissue was separated by connective tissue in the bisexual gonad. The ovarian tissue became dominant during the non-spawning season. During the first and second spawning season, the testicular tissue was dominant with only a small portion of primary oocytes, which made the fish functional males. About 30-50% fish will change to female after the third year. Estrogen receptors (ERα, ERβ), androgen receptor (AR), aromatase, follicle-stimulating hormone (FSHR), and luteinizing hormone receptor (LHR) were cloned. ERα, AR, FSHR and LHR transcripts were significantly higher in bisexual testis than bisexual ovary in 1^+- and 2^+-year-old fish. ERα, ERβ and AR transcripts decreased in the functional testis of 3-year-old fish (after sex change). Similar levels of ERβ and AR were detected in the ovary of sex-changed females and functional testis of males in 3-year-old fish. Significantly decreased AR transcripts were found in testicular tissue of bisexual and functional male and female gonad in 3-year-old fish compared to 1- and 2-year-old fish. In contrast, high ERα and aromatase but not ERβ transcripts were detected in sex-changed ovary. Aromatase inhibitors further blocked natural sex change in 3-year-old fish. Higher levels of plasma LH was found in males compared to sex-changing females during the non-spawning season. Our present data on the differential expression of ERα, AR, FSHR, and LHR in bisexual testicular and ovarian tissue suggest that testicular tissue is probably more responsive than ovarian tissue in terms of the competition for tissue development under the stimulation of same endocrine signals. LH may be one of the endocrine signals to differentially regulate the development of testicular or ovarian tissues. These findings provide the valuable concepts to understand the complicated process of sex change mechanism in protandrous black porgy.

O-III-6

THE SEX DETERMINING GENE OF MEDAKA: A Y-SPECIFIC DM DOMAIN GENE (DMY) IS REQUIRED FOR MALE DEVELOPMENT

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Although the sex-determining gene *Sry* has been identified in mammals, no comparable genes have been found in non-mammalian vertebrates. To identify such a sex-determining gene, a positional cloning approach is suitable. For this approach, the medaka, *Oryzias latipes*, is a good model animal, because this speciese has male heterogametic sex-determining syetem and has two major advantages for genetic research: a large genetic diversity within the species and the existence of several inbred strains. To clone positionally the sex-determining region, we generated a Y congenic strain to highlight the genetic differences between the X and Y chromosomes from inbred strains of medaka. The Y congenic strain has a sex-determining region derived from the HNI-strain Y chromosome on the genetic background of an Hd-rR strain. Using this strain, we had previously constructed a genetic map of the medaka sex chromosome and constructed a BAC (bacterial artificial chromosome) genomic library. We used recombinant breakpoint analysis and deletion analysis of the Y chromosome of a congenic XY female to restrict the sex-determining region to 250-kb stretch of the Y chromosome. Shotgun sequencing of this region predicted 27 genes. Three of these genes were expressed during sexual differentiation. However, only one gene *PG17* was Y specific. The full-length cDNA sequence of *PG17* encodes a putative protein of 267 amino acids, including the highly conserved DM domain. We thus named it *DMY*. To establish a role for *DMY* during sexual differentiation, we screened wild medaka populations for naturally occurring *DMY* mutants. Two XY females with distinct mutations in *DMY* were found in separate populations (Awara and Shirone). The first heritable mutant - a single insertion in exon 3 and the subsequent truncation of DMY - resulted in all XY female offspring. Similarly, the second XY mutant female showed reduced *DMY* expression with a high proportion of XY female offspring. Furthermore, during normal development, *DMY* is expressed only in somatic cells of XY gonads. These findings strongly suggest that the sex-specific *DMY* is required for normal testicular development and is a prime candidate for the medaka sex-determining gene.

P-III-1

SEASONAL CYCLES OF PLASMA SEX STEROID LEVELS AND GONADAL DEVELOPMENT OF GROUPER FISH *EPINEPHELUS COIOIDES* (FAMILY *SERRANIDAE*) IN THE PERSIAN GULF WATERS

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The protogynous serranid Epinephelus Coioides one of the important groupers of Persian Gulf. Blood samples and oocytes at all stages of development were taken from *Epinephelus Coioides* from May to September in 2001 and 2002. Plasma 17-β oestradiol (E2) levels increased in April, reached their highest in May in ripe females whose gonads contained both cortical alveoli and vitellogenic oocytes. Testosterone (T) peaked in female during vitellogensis and maturation. Plasma concentrations of 17,20 BP were significantly elevated in females with ovaries undergoing final oocyte maturation (FOM). Female exhibited synchronous ovarian development. Gonad were ripe from early March until April and spawning occurred from April until June. A few *E.coioides* with either perinucleolar or cortical alveoli stage oocytes were undergoing sex change after the spawning period and involving regression of ovarian tissue and proliferation of testicular tissue in the gonad.

P-III-2

AROMATASE INHIBITOR INDUCES COMPLETE SEX CHANGE IN PROTOGYNOUS HONEYCOMB GROUPER (*EPINEPHELUS MERRA*)

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Protogynous hermaphrodite fish change sex from female to male at the certain stages of life cycle. The endocrine mechanisms involved in gonadal restructuring throughout protogynous sex change are not clearly understood. In the present study, we implanted maturing female honeycomb groupers intra-peritoneally with nonsteroidal aromatase inhibitor, Fadrozole (AI; 0, 1 and 10 mg/fish) and examined changes in gonadal structures and serum levels of sex steroid hormones three months after implantation. Ovaries of control females had oocytes undergoing active vitellogenesis, whereas AI caused females to develop into functional males. These males had testes, which were indistinguishable in structure from those of normal males, but bigger in size, and completed all stages of spermatogenesis including accumulation of large amount of sperm in the seminiferous tubules. AI significantly reduced the serum levels of estradiol-17β (E2) and increased levels of testosterone (T), 11-ketotestosterone (11-KT) and 17α,20β-dihydroxy-4-pregnen-3-one (DHP). Further, AI suppressed in vitro production of E2, and stimulated the production of T and 11-KT in the ovarian fragments of mature female. In honeycomb grouper, suppression of bothin *vitro* and *in vivo* production of E2 and degeneration of oocytes by AI suggests that AI induces complete sex change through inhibition of estrogen biosynthesis, and perhaps, subsequent induction of androgen function.

P-III-3

MOLECULAR CLONING OF THE THREE GONADOTROPIN SUBUNITS AND ITS POSSIBLE INVOLVEMENT IN EARLY SEX DIFFERENTIATION IN THE NILE TILAPIA, *OREOCHROMIS NILOTICUS*

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It is well known that gonadotropins (GTHs) are the main regulators of gametogenesis and steroidogenesis. Expression of the ovarian type aromatase at very early stages only in female gonads confirms the importance of estradiol-17β in ovarian sex differentiation of non-mammalian vertebrates. However, it is unclear whether GTH is involved in the sex differentiation of vertebrates. In chicken, lower expressions of both luteinizing hormone receptors (LHR) and follicle stimulating hormone receptors (FSHR) were found in the undifferentiated gonads, and later the expression intensified in female gonads. In the Nile tilapia, FSH and LH receptors were found expressed in gonads as early as 5 and 10 days after hatching (dah) by RT-PCR followed by Southern hybridization. Later at 15-20dah, FSHR expression increased transiently in males while LHR elevated in females. Therefore, it is reasonable to assume that GTH might be involved in the control of estrogen production and early sex differentiation in fish. For this purpose, we cloned the GTH α, FSH β and LH β cDNAs from the pituitary of the Nile tilapia by RT-PCR and RACE. Cloned GTH α, FSH β and LH β subunit cDNAs are 445bp, 383bp and 462 bp encoding peptides of 117, 113 and 146 amino acids (aa), which show 84~91, 55~99 and 76~86% similarities, respectively to its counter parts from perciforms. However, the similarity between tilapia FSH β and LH β is only 31.6% in nucleotides and 21.9% in aa, respectively. Northern blot analysis using the total RNA obtained from the pituitary of adult female at post-vitellogenic stage detected bands of around 900bp, 700bp and 800bp for GTH α, FSH β and LH β respectively with stronger expression of GTH α and LH β, but weaker signal for FSH β. Preliminary studies during the ontogeny indicate that FSH β subunit expressed as early as 5dah in the fish pituitary, however, more detailed analysis are under way to understand the role of two subunits and all together GTHs during the sex differentiation of the Nile tilapia.

P-III-4

MOLECULAR CLONING OF SOX9 AND DMRT1 cDNA PARTIAL SEQUENCES IN THE PEJERREY FISH *ODONTESTHES BONARIENSIS* (ATHERINIFORMES)

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In bony fish the process of sex determination/differentiation is controlled either by genetic or environmental factors. The pejerrey fish is a differentiated gonochorist, at least at intermediate temperatures and presents a strong Temperature Sex Determination (TSD). In mammals the Sry gene initiates the sex determination process, however no homologue of this gene has already been found in non-mammalian species, although, a sex specific gene DMY is a candidate for a sex male determining gene in the medaka fish. Other genes involved in the mammalian sex-determining pathway have already been identified in different vertebrates: Sox9 (SRY-related HMG box gen), Dmrt1 (Doublesex and Mab-3 related transcription factor) and Dax1 (Dosage-sensitive sex-reversal, adrenal hypoplasia congenital, X chromo-some, gene 1) were found to be expressed at the onset of gonadal development in mammals, birds, reptiles and teleost fishes. Different clones were obtained by RT-PCR amplification from cDNA obtained from different tissues using consensus primers. These clones were then sequenced and verified to be Sox9 (either in testis and ovaries) and Dmrt1 (only testis). The next step is determine their expression pattern in the temperature sensitive period (TSP) in order to study the importance of these genes in the sex determination/differentiation pathway in a bony fish species with a truly remarkable thermolabile sex differentiation.

P-III-5

SEX CHANGE STRATEGY AND THE AROMATASE GENES

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Sequential hermaphroditism is a common reproductive strategy in many teleost fish. As a first step toward understanding the physiology behind the sex change process, a molecular strategy was used to analyse the role of aromatase encoding CYP19 genes in representative sex changing fish, *Lates calcarifer* (protandry), *Cromileptes altivelis* (protogyny), *Gobiodon histrio* (bi-directional) and the gonochoristic *Mugil cephalus*. A polymerase chain reaction (PCR) cloning approach was used to isolate complementary DNAs encoding two distinct CYP19 isoforms in the four fish, CYP19A1 and CYP19A2. Structural analysis of the deduced amino acids of both CYP19A1 and CYP19A2 revealed that functional regions and physiochemical alignment show high homology with all other reported CYP19 genes. Following the isolation of aromatase isoforms, CYP19A1 and CYP19A2, a PCR based genomic walking was used to isolate the 5' flanking regions from these genes with the premise to determine putative transcriptional elements influencing gene expression. 5' flanking regions are currently being isolated from the 8 CYP19 genes, and putative transcriptional response elements identified and analysed. A working model is proposed for the hierarchal arrangement of CYP19A1 and CYP19A2 and possible endocrine interactions in sex changing fish.

P-III-6

ROLE OF STEROID HORMONES IN SEX CHANGE OF PROTOGYNOUS WRASSE

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Although steroid hormones are potent inducers of sex reversal in early juvenile fish, adult fish usually does not show such flexibility. On the other hand, there are considerable number of hermaphrodite fish species, naturally changing sex from female to male or vice versa, in adults. The physiological mechanism underlying the hermaphroditism is largely unknown. In protogynous wrasse, social cues stimulate sex change of their behavior, body coloration and gonad which completes total transformation within a few weeks. The transformation is accompanied with rapid decline of serum estradiol (E2) levels and gradual increase of serum 11-ketotestosterone levels. However, it is still uncertain whether the changes in the steroid levels actually trigger sex change. We report here that a non-steroidal aromatase inhibitor (AI), fadrozole, can stimulate sex change in the threespot wrasse, *Halichoeres trimaculatus*. Adult females were fed with diets containing different amounts of AI (0, 100 and 500 mg/kg) alone or with estradiol (0 and 100 mg/kg). After two or six weeks, gonads were removed and fixed for standard histological analysis. AI at concentrations of 100 and 500 mg/kg diet resulted in complete sex reversal of all individuals. They showed typical body coloration of terminal phase males, and had functional testis with all stages of spermatogenesis. Addition of E2 in the same diets prevented fish from changing sex. Moreover, the inhibitory effect of E2 on sex change was observed when androgens such as 11-ketotestosterone and 17alpha-methyltestosterone were administrated in combination with E2. These results suggest that in the protogynous wrasse, rapid decline of serum E2 levels is an initial trigger of sex change.

P-III-7

TRANSGENIC CELL LINES WHICH STABLY EXPRESS PROGESTOGEN RECEPTORS (PRS) α AND ß AND THE PR-RESPONSIVE REPORTER GENES

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Two subtypes (α and β) of progestogen receptors (PRs) were isolated from the testis of the Japanese eel. Amino acid homology of the open reading frames between PR α and PR β is about 40%, but the DNA- and ligand-binding domains show high homology between two subtypes. Tissue distribution of both subtype mRNAs was different. Protein interaction between two PRs was demonstrated *in vitro* by a GST pull-down assay. They exhibit similar transactivation properties with native hormones at 100 nM, but there were some minor differences; for example, 20β-S and S induced weak transcriptional activity in PR α, whereas PR β was not significantly stimulated by these steroids. It is expected that like ARs, both PR subtypes show different affinity for other progesterone-related substances (medroxyprogesterone, melengesterol, danasol, etc.). Thus, transactivation analyses were performed for various progesterone-related substances at various doses using transgenic cell lines which stably express PRs and the PR-responsive reporter genes. Median effective concentrations (EC_{50}) with 17α, 20β-dihydroxy-4-pregnen-3-one, progesterone, 20β-hydroxyprogesterone and 11-deoxycorticosterone were 1.61, 67.1, 0.16 and 7.94 nM, respectively, for PR α, and 6.10, 13.5, 71.0 and 130 nM for PR β. Norethisterone, a mammalian PR agonist, was a strong inducer for transactivity through PRs. Interestingly, two mammalian PR agonists (medroxyprogesterone and melengesterol) induced very weak transcriptional activity in PRs. Mifepristone (a mammalian PR antagonist) was not effective in either subtype. Danasol exhibited antagonistic effects for both subtypes, but induced weak transcriptional activity at 10 μM. Unexpectedly, mibolerone (dimethylnortestosterone), a synthetic androgen, induced transactivity in a dose-dependent manner in both cell lines, although methyltestosterone was not effective in either line.

P-III-8

ESTROGEN-INDUCED AND SUPPRESSED GENE EXPRESSION DURING XY SEX REVERSAL IN A TELEOST FISH, TILAPIA

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To clarify the importance of endogenous estrogens during sex differentiation in a teleost fish, the Nile tilapia, we examined the target events for endogenous estrogens and their role during gonadal sex differentiation. *P450arom* (*CYP19a*) expression precedes any morphological gonadal sex differentiation. Further to these findings, the treatment of XX females with non-steroidal aromatase inhibitor (AI), Fadrozole, from 7 to 14 days after hatching caused complete sex reversal to functional males. The XX-sex reversal induced by AI was rescued completely with simultaneous estrogen treatment. We also found that XY fry treated with 17α-ethinylestradiol, before the appearance of morphological sex differences, caused complete sex reversal from males to females. Taken together, these results suggest that endogenous estrogens are required for ovarian differentiation. Further, to identify the down-stream gene products of estrogen during ovarian differentiation, we performed subtractive hybridization using mRNA derived from normal and estrogen treated XY gonads. The clones obtained after subtractive hybridization screening were re-screened by reverse-Northern analysis. Finally, seven up-regulated gene products and three down-regulated gene products were obtained. In situ hybridization showed that two of up-regulated gene products (EU-2, -3) were expressed in germ cells but not in somatic cells. One of down-regulated gene products (ED-2) showed that specific signals were observed in interstitial cells and gonadal epithelium on the surface of ventral side of XY fry during gonadal differentiation.

P-III-9

GONADAL SEX DIFFERENTIATION AND SEX CONTROL IN RED SEA BREAM, *PAGRUS MAJOR*

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In Japan, the aquacultural yield of red sea bream, *Pagrus major* is the second highest and the number of juvenile red sea bream sold as aquacultural seedlings was the highest among marine fish species. Thus red sea bream is the most important species as a target of breeding. In the spawning season, male red sea bream show dark coloration of the body and the market value decreases. Therefore, all-female populations are preferable for aquaculture of red sea bream. In order to develop the sex control technics in the fish, we studied the gonadal sex differentiation and investigated the suitable conditions for induction of all-male groups.
The process of gonadal sex differentiation of red sea bream of a selected strain was histologically studied using fish reared in the laboratory. Central cavities were observed in gonads in all of the fish aged 3 and 4 months. The gonads in 34 of 40 fish aged 7 months had oocytes at the peri-nucleolus stage. From 8 months to 12 months after hatching, about half of the fish had bisexual gonads. Gonads of fish from 1 year to 1 year and 4 months old were ovaries or bisexual gonads, while those of the 1-year-old fish after September and 2-year-old fish were ovaries, bisexual gonads or testes. Bisexual gonads were not seen in 3-year-old fish. Therefore, the pattern of gonadal sex differentiation in this selected strain may be summarized as follows. Differentiation to ovaries progresses in all fish until 7 months after hatching. Then half of fish continues to develop their ovaries, while the rest half starts to develop testis instead until 1 to 2 years old. The necessary dose and growth stage for oral administration of 17α-methyltestosterone (MT) suitable for induction of all-male groups were investigated in red sea bream. Oral administration of MT (0.01 – 1.0 mg MT / kg BW / day) for 16 weeks to 281-day-old meiotic gynogenetic diploids resulted in 100 % functional males in the following spawning season. MT treatment (0.1 mg / kg BW / day) to fish of different ages (55, 141, and 893 days after hatching) for 16 weeks induced males, and testicular tissue was observed in the gonads of all MT-treated fish. While functional sperm were obtained from the fish treated with MT from 141 and 893 days-of-age, no sperm was produced in fish treated from 55 days-of-age.

P-III-10

GERM CELLS DURING GONADAL SEX DIFFERENTIATION IN THE TELEOST

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In Nile tilapia, a teleost fish, primordial germ cells (PGCs) migrated and then became localized into gonadal anlagen 3 days after hatching (dah). First morphological sex difference is regarded as the number of germ cells between sexes. Second sexual dimorphism in germ cells is the timing for entry of germ cells into meiosis. In general, although sexual dimorphism in germ cells was examined morphologically, the differences at molecular levels remained unclear. Recently, we found that two isoforms of *vasa* mRNA and protein are present in tilapia. One (*vas*-s) lacks a part of the N-terminal region found in the other isoform (*vas*). Both isoforms are expressed in oocytes through the embryonic stage when primordial germ cells (PGCs) localize in the lateral plate mesoderm. After PGCs localized in the gonadal anlagen, *vas*-s expression increased and *vas* expression became undetectable. Expression of both isoforms was observed again after morphological gonadal sex differentiation, irrespective of genotypic sexes. These changes are coincident with sex differences in number of gonial germ cells during this period, suggesting that molecular sexual dimorphism occurs in germ cells between both sexes during this period. Recently, we also found another germ cell-specific gene product, which was cloned by subtractive hybridization screening, during gonadal sex differentiation (Kajiura-Kobayashi *et al.* in this meeting). The dynamics of these germ cell specific genes during gonadal differentiation will also be discussed.

P-III-11

DEVELOPMENT OF DNA MARKERS LINKED TO THE SEX-DETERMINING LOCUS IN AN ATHERINID FISH, THE PATAGONIAN PEJERREY (*ODONTESTHES HATCHERI*)

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Sex determination in teleost fish may be brought about by genetically inherited sex determining factors, environmental factors, or a combination of the two. In the pejerrey (*Odontesthes bonariensis*), a South American atherinid species with marked temperature-dependent sex determination (TSD), all fish exposed to temperatures below 19°C during the critical time of gonadal sex differentiation become females whereas those exposed to 29°C during the same time become male. Sex ratios at intermediate temperatures decrease gradually from all-female to all-male and are balanced (ratio male:female, 1:1) only in a narrow (c.a. 1°C) range that lies within 24-26°C. In contrast, sex ratios of a closely related species, the Patagonian pejerrey (*O. hatcheri*), are only slightly female- and male-biased at temperatures below 15°C and above 25°C, respectively, whereas intermediate temperatures produce strictly balanced sex ratios. Thus, gonadal sex in *O. hatcheri* appears to be under stronger genotypic sex control (GSD) than in *O. bonariensis*. The existence of closely related species with such contrasting modes of sex determination provides a valuable tool to elucidate the genetic factors underlying GSD and TSD in atherinid fish. In the first step of this analysis, we performed linkage analysis using amplified fragment length polymorphism (AFLP) and microsatellite markers in order to develop a DNA marker linked to the sex-determining locus in *O. hatcheri*. For this purpose, the eggs of one female were inseminated with the sperm of one male and the offspring was reared from hatching to the juvenile stage at 21°C. The sex ratio in the two families was 1:1. One AFLP marker (ACG/CAA-217) tightly linked to the sex-determining locus (n=46, recombination rate = 0%, Lod score = 13.8) was identified in the progeny of one cross. This sex-determining locus-linked AFLP marker is currently being used to screen other families of *O. hatcheri* and *O. bonariensis*, to amplify possible male specific DNA fragments, and as a genetic sex marker in temperature-induced sex reversal experiments.

P-III-12

A COMPARISON OF AROMATASE INHIBITORS FOR THE SEX REVERSAL OF FEMALE ATLANTIC SALMON (*SALMO SALAR* L)

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The efficacy of immersion treatment with the aromatase inhibitors (AI) 1,4,6-androstatrien-3,17-dione (ATD), 4-hydroxy-4-androstene-3,17-dione (OHA), 1-[2,4-dichloro-β-([2,4-dichlorobenzyl]oxy)-phenethyl]imidazole (miconazole), 3-[p-aminophenyl]-3-ethyl-piperidine-2,6-dione (aminoglutethimide) and 4-(5,6,7,8-tetrahydroimidazo[1,5-α]pyridin-5-yl)benzonitrile monohydrochloride (fadrozole) for the sex inversion of all female Atlantic salmon (*Salmo salar* L.) was assessed in two experiments. Only treatment with ATD resulted in significant changes in the sex ratio. Significant levels of sex inversion were induced by treatment with ATD at doses of 500 μg L-1 and 5 mg L-1. There was no significant difference between the effects of single immersion in 500 μg L-1 ATD at 14 days post median hatch (DPMH) and repeated immersion at 14 and 28 DPMH. However, treatment with 500 μg L-1 ATD at 7 and 14 DPMH, or with 5 mg L-1 ATD at 7 and 14 or at 14 and 21 DPMH was significantly more effective than treatment with 500 μg L-1 ATD at 14 and 21 DPMH. Immersion treatment with the androgen 17α-methyldihydrotestosterone (MDHT) (500 μg L-1) was equally effective at 7 and 14 or 14 and 21 DPMH, indicating that the labile period determined by androgen treatment may differ from that for low dose ATD treatment. Fertility of neomales produced by immersion treatment with ATD was not significantly different to untreated males. The uptake and retention of different AI varied substantially, and this may explain, in part, the poor efficacy of AI other than ATD.

P-III-13

EFFECTS OF WATER TEMPERATURE ON THE GONADAL DEVELOPMENT AND EXPRESSION OF STEROIDOGENIC ENZYMES IN THE GONAD OF RED SEABREAM, *PAGRUS MAJOR*

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The present study examined the effects of water temperature on the gonadal development, expression of steroidogenic enzymes, and serum levels of steroid hormones in red seabream during the period of juvenile hermaphroditism. Nineteen-month-old red seabream were divided into 4 groups (n=30) and each group was reared for 2 months (September to December) at 15, 20, 25 °C and ambient water temperature (25~14 °C), respectively. Sampling was done after 4 and 8 weeks. Histological observation revealed that about 80-90% fish had gonads shown hermaphroditism, and remaining 10-20% had testes in all experimental groups during the experimental period. After 4 weeks, oocytes at the oil stage was observed in about 14-33% hermaphroditic gonad in 15 °C, 20 °C, and ambient water temperature groups, but not in 25 °C group. After 8 weeks, oocytes at the oil stage were observed in hermaphroditic gonads of 15 °C and ambient water temperature groups. However, in 20 and 25 °C groups, only oocytes at the perinucleolus stage were observed. Spermatogenesis was observed in 33-40% fish, but in remaining 60-67% hermaphroditic gonad had only spermatogonia. Significant morphological differences of testicular portion of gonads were not observed among experimental groups during experimental period. Expression of cytochrome P450 aromatase mRNA was suppressed in 25 °C after 4 weeks and 20 °C and 25 °C groups after 8 weeks in comparison with other groups. Expression of 11β -hydroxylase mRNA exhibited no temperature dependence at 4 weeks, whereas it was repressed in 20 °C and 25 °C groups after 8 weeks. No significant differences were observed in serum steroid hormone (estradiol-17β testosterone, 11-ketotestosterone) levels among experimental groups during experimental period. These results suggest that high water temperature suppresses gonadal aromatase activity through aromatase expression and inhibits oocytes development. Expression of 11β-hydroxylase mRNA was also suppressed by high water temperature.

P-III-14

EFFECT OF TEMPERATURE ON THE EFFICIENCY OF FEMINIZATION OF MEDAKA (*ORYZIAS LATIPES*) BY HORMONAL (ESTRADIOL) MANIPULATION

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In many fish species, the phenotypic (gonadal) sex of an individual is predetermined by genetic factors at the time of fertilization. Even in these species, however, the gonads remain bipotential until histological sex differentiation and the development of the male or female gonadal architecture. Thus, functional sex reversal can be achieved easily by the timely administration of exogenous steroid hormones during the early stages of gonadal differentiation. The process of sex differentiation in fish can be affected also by many environmental factors. For instance, low and high temperatures can induce functional feminization or masculinization in many species. Recent studies on the molecular basis for hormonal- and temperature-induced sex differentiation suggest that both kinds of factors affect the endogenous steroid biosynthesis. Thus, temperature conceivably has synergistic/antagonist effects with exogenously administered steroid hormones during sex differentiation. In order to examine this possibility, we conducted a series of sex reversal experiments at different temperatures (14, 18, 22, 26, 30, and 34°C) and estradiol-17β (E2) concentrations (0, 1 and 10 ppb) using Japanese medaka (*Oryzias latipes*) of the d-rR strain. In this strain, which was employed by T. Yamamoto in his pioneer studies on hormonal sex reversal in fish, genetic males and females are orange-red and white, respectively, due to the presence of a sex-linked color gene. Medaka also has secondary sex characteristics (sexually dimorphic fin morphology) that enable discrimination of phenotypic sex. Fertilized eggs were incubated under different E2 and temperature conditions between 6 days post-fertilization and hatching and then raised to sexual maturity at 26°C. Determination of the genetic and phenotypic sex of all individuals revealed the occurrence of sex-reversed (orange-red) females in an E2- and temperature-dependent manner. The percentage of sex reversal varied from 100% at 14-18°C to 0% at 34°C for 10 ppb and from 33% at 14°C to 0% at 26-34°C for 1ppb. These results suggest a marked synergism between E2 and low temperature for the induction of feminization in medaka and conversely an antagonistic effect by high temperatures. These results may be relevant for the screening of compounds with estrogenic activity (=xenoestrogens) and the study of their mode of action. Also, judicious choice of temperature during hormonal manipulation of sex may help reduce the hormonal load required for successful sex control of farmed fish.

P-III-15

GONADAL SEX DIFFERENTIATION IN PROTANDROUS ANEMONE FISH *AMPHIPRION CLARKII*

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Sequencial hermaphroditism involves males becoming females, or females becoming males, or bi-directional sex change. They are classified as protandrous if they mature first as males, or protogynous if development first occurs as female. A large literature exists describing hermaphrodite fish gonad development, behavior and mating strategies however, the mechanism of gonadal sex differentiation in those species are still largely unknown. In protandrous amemone fish, gonad in female phase is characterized by presence of only oocytes, but gonad in male phase possess both immature oocytes and active spermatogenic germ cells. The mechanism of development of ovotestis in male anemone fish is still unknown. In the present study, we investigated the process of sex differentiation in the protandrous anemone fish, *Amphirpion clarkia* histologically. F1 generation was produced, and the process of sex differentiation was observed until 214 days after hatching (dah). In undifferentiated gonad, primordial germ cells appeared on the dorsal peritoneal wall at 1 dah, while at 40 dah, single germ cells were enclosed by somatic cells. At 61 dah, some meiotic germ cells for oogenesis appeared initially, and somatic tissue from proximal and distal area began elongating laterally, which at 92 dah fused to form the ovarian cavity and oocytes were at the perinucleolus stage. At 122 dah, ovarian cavity formation was complete, later at 153 and 183 dah, somatic cells increased on the outer periphery of ovarian cells. Interestingly, at 214 dah, spermatogenic germ cells appeared initially around oocytes at the perinucleolus stage, which might be the initiation of male characteristics. Thus present results suggest that, in anemone fish, ovarian differentiation takes precedence over testicular development, and testicular differentiation conforms only after five months of ovarian development. Hence, the development of interspersed gametes in the testes of protandrous anemone fish could be an interesting phenomenon in sex differentiation.

P-III-16

INFLUENCE OF TEMPERATURE AND GENETICS ON SEX DIFFERENTIATION AND GROWTH IN THE EUROPEAN SEA BASS (*DICENTRARCHUS LABRAX*)

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In European sea bass aquaculture the occurrence of males often exceeds 90%. This presents a problem to the industry, since males exhibit slower growth than females. To examine the influence of water temperature on sex differentiation, larvae of two different populations (Northwestern, NE, and Southeastern, SE, Mediterranean region) were reared at three temperatures (13, 17 or 21°C) during two periods (days 10-50 or 50-90 post hatching, dph). Fish were sampled to monitor growth throughout the study. At 300 dph, gonads from 100 fish per treatment were examined to determine sex ratios. During both periods, there were significant differences ($P<0.05$) in growth among treatments, with higher growth observed in higher temperatures. However, the fish exposed to either 13 or 17°C during 10-50 dph (13-1050 and 17-1050) exhibited compensatory growth during the ensuing period, and by 300 dph there were no significant differences in size among the three groups. On the contrary, fish from the 13-5090 group did not display compensatory growth and their body size at 300 dph was significantly lower than fish exposed to either 17 or 21°C. The effect of temperature exposure was similar between the two populations, with the high temperature inducing masculinization and the low temperatures feminization. Between the two populations, the number of females obtained in response to the temperature treatments differed significantly ($P < 0.05$). The female percentage was consistently lower in the SE compared to the NW. The highest number of females in the SE was observed in the 13-5090 group (51%), whereas the NW subjected to the same treatment resulted in 68% females. On the other hand, the masculinizing effect of high water temperature in the 10-50 dph was stronger in the SE, resulting in a mean value of 8% females compared to 30% in the NW. The study demonstrated that sex differentiation in the European sea bass is influenced by temperature during the period 10-90 dph, with low temperature favouring feminization and high temperatures masculinization. The strongest masculinizing effect was observed during the period 10-50 dph. Although the influence of temperature was similar among the two populations, it seems that the SE Mediterranean one is prone to naturally give a higher number of males, a predisposition which was exacerbated by exposure to high temperatures during larval rearing. Supported by EU grant Q5RS-2000-31365.

P-III-17

DAILY TEMPERATURE SHIFTS DURING THE EMBRYONIC PERIOD DO NOT ALTER THE PHENOTYPIC SEX RATIO OF CHINOOK SALMON (*ONCORHYNCHUS TSHAWYTSCHA)*

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The effect of temperature on sex determination varies amongst fish species. There is limited evidence within the genus *Oncorhynchus* (i.e. Pacific salmons) that temperature has a significant effect on sex determination in these fishes. However, a report of an unusual distribution of sex-linked genetic markers in chinook salmon affected by fluctuating water temperatures because of hydroelectric dam operations on the Columbia River prompted an investigation. The purpose of this study was to determine whether daily-elevated water temperature shifts during the embryonic period in chinook salmon, similar to those observed on the Columbia River, could influence the resulting sex ratio. Three families of chinook salmon (~500 eggs per family) were produced by single-pair matings between three females and three males. Immediately after fertilization the eggs were placed in an incubator at 7.5°C. The next day the eggs from each family were split into two groups, one that remained at 7.5°C (control), and the other which was placed in another incubator at 12°C for 6-8 hours (temperature shifted [TS] group). The TS group was moved between 7.5°C and 12°C each day for 105 days, while the control remained at a constant 7.5°C. After this period each of the groups were raised in separate tanks to a size at which the gonads could be removed and sexed by visual examination. Random samples of 51-76 fish from each of the control and TS treatments from each family were examined. Sex ratios varied between the three families (i.e. male:female - 1:1.3; 1:0.9; 1:1.08), but there were no significant sex ratio differences between the control and TS groups within each family. We conclude that daily temperature shifts of 4.5 °C during the period of gonadal differentiation do not alter the sex ratio of chinook salmon.

P-III-18

EXPRESSION OF DMY AND DMRT1 IN THE VARIOUS TISSUES OF THE MEDAKA, *ORYZIAS LATIPES*

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Two DM-domain genes (DMY, the sex-determining gene of medaka, and DMRT1, a male related gene) expresses in the testis of medaka. In humans, the sex-determining gene (SRY) expresses not only in the embryonic presumptive testis but also in various adult tissues including mature testis. In this study, we have used a specific RT-PCR assay to determine the expression pattern of DMY and DMRT1 in several tissues form embryos, fries and adults of medaka. The RT-PCR analysis has revealed that DMY expressed throughout the developmental stages of XY males, but not in XX females, confirming that DMY is Y specific gene. Very high expression of DMY was evident in the male embryos just before and after hatching. DMY expression declined at 10 days after hatching (dah) and ultimately became weak at 20 dah; the expression was higher in the body portion of the fries containing the ovary than that of the head portion containing the brain. DMRT1 expression does not occur in either male or female embryo until 20 dah and becomes evident in testis when tested at 30 dah. In sexually maturing fish, DMY expression was observed in several male tissues (brain, liver, testis, eye and muscle) with the highest expression in testis. DMRT1 expresses in various male tissues with the highest expression in testis and also ovary. The expression patterns of DMY were similar to those of sexually mature males, except for a high expression in spleen as well. In the case of DMRT1, high expression was evident in testis, ovary and spleen. The detection of transcripts could suggest possible roles of DMY and DMRT1 in various tissues at each of the developmental stages. The link between these gonadal sex regulators with those of tissues development needs further investigation.

P-III-19

BI-DIRECTIONAL SEX CHANGE AND ITS STEROIDOGENESIS IN THE WRASSE, *PSEUDOLABRUS SIEBOLDI*

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The wrasse, *Pseudolabrus sieboldi*, a diandric protogynous labrid fish, is distributed in shallow coastal waters off southwestern Japan, where the bottom is rocky. Sex-changed large terminal-phase (TP) males arise from small initial-phase (IP) females. In captivity, as in nature, daily spawning between pairs of TP males and IP females occurs during the spawning season in autumn, and sex change can also be induced under the appropriate social conditions. In this study, we investigated the social conditions that bring about protogynous sex change in a rearing tank, and succeeded in inducing the reverse sex change, male to female, by controlling the social conditions. This is the first report of a reversed sex change in a sexually dichromatic species. In addition, using this system, we examined the function of the steroids that likely regulate the sex change directly, based on the detailed steroid-biosynthesis pathways in the ovary and testis. In *P. sieboldi*, natural sex change occurs during the prolonged non-spawning season. As protogynous sex change advances, the hue of the anal fin changes dramatically from yellow to red, and is a visual biomarker showing the degree of sex change. When six IP females were kept in a 1000-L tank, the largest fish changed into a TP male. Conversely, when seven TP males were kept in the tank, the smallest male changed into an IP female, and the color of the anal fin changed from red to yellow. This demonstrated that *P. sieboldi* has the ability to change sex bidirectionally in response to social status. More recently, we clarified the complete synthesis pathways of sex steroids in the ovary and testis of *P. sieboldi*. Estradiol-17β(E2) is synthesized from estrone by 17β-hydroxysteroid dehydrogenase (HSD)-I (17β-HSD-I) in ovarian follicles, which produce no testosterone (T). After a sex change, 11-ketotestosterone (11-KT) is synthesized in the testes via T produced from androstenedione under the control of 17β-HSD-III. We implanted T and 11-KT into IP females intraperitoneally using silicone tubes to investigate the role of T and 11-KT during sex change. Both T and 11-KT induced testicular development and TP body coloration. Therefore, we concluded that at least two types of 17β-HSD exist in the gonads of *P. sieboldi*, and activation of 17β-HSD-III is the first step in inducing the morphological and functional change from an ovary to a testis.

P-III-20

GENE EXPRESSION OF ESTROGEN RECEPTORS α AND ß DURING EARLY SEXUAL DIFFERENTIATION IN THE EUROPEAN SEA BASS (*DICENTRARCHUS LABRAX*)

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Estrogens exert a range of effects on diverse physiological functions such as growth, development and cell differentiation. These effects are mediated by estrogen receptors (ER), of which two subtypes α and β) have been identified in mammals as well as in lower vertebrates. In teleost fish, ERs have been found to be expressed very early during ontogeny, suggesting an important function for ERs during sexual differentiation. The aim of this work was to analyze gene expression of ER α, β1 and β2 during sexual differentiation in European sea bass in order to establish potential sexual dimorphic expression profiles. Male- and female-dominant stocks were created by size-grading, taking advantage of the fact that from very early in development larger fish develop as females (L-group) whereas smaller fish develop as males (S-group). At 360 days posthatch (dph) the S-group consisted of 74.5% males, and the L-group of 96.2% females. Samples were taken at 50, 100, 150, 200, 250 and 300 dph. ER expression was analyzed by semi-quantitative RT-PCRs that were set-up for sea bass ERα, β1 and β2 and normalized against ribosomal 18S expression. ER expression was analyzed in heads and tails (at 50 and 100 dph), and in brain, pituitary (pooled), gonads and liver for the remaining sampling days (n=7 for all tissues). Differences in levels of expression between S- and L-group were mainly observed for ERα, where expression was higher in L-group at 250 and 300 dph in the pituitary and at 200 and 300 dph in gonads, tissues in which ERα was predominantly expressed. At 50 dph, ERα expression was also higher in L-group of heads and tails; however expression levels were much lower than in gonads and pituitaries. Levels of expression of ERβ1 were similar in all tissues/body parts, although a slightly higher expression was found in gonads. Expression was higher in heads and tails of L-group at 50 dph, but significantly lower in tails of the same group at 100 dph. ERβ2 expression was found in all tissues, with highest levels in pituitaries and gonads. No differences in expression between S- and L-groups were observed for this receptor. Supported by an EU grant (Q5RS-2000-31365).

P-III-21

EXPRESSION OF CYTOCHROME P-450AROMATASES IN THE SEX-REVERSED NILE TILAPIA

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In fish, gonadal differentiation is not solely determined by the genetic sex as it is in mammals, and can be influenced by several factors ranging from temperature to hormonal manipulations. Consequently, administration of sex steroids induces sex reversal to genetically determined sex populations. In this study, we evaluated the ability of sex steroids (ethinyl estradiol and methyl testosterone) and androgen receptor antagonist, flutamide to induce sex reversal and subsequently changes in aromatase expression were studied. Synthesis of estrogens is under the direct control of the steroidogenic enzyme, cytochrome P-450 aromatase. Two (ovarian and brain) types of aromatases are identified in the Nile tilapia. Both forms of aromatases were found expressed in the ovary while in the brain only the brain form got expressed. Ovarian type aromatase expression was found expressed dominantly (from 15 days after hatching [dah]) in the developing ovary but not in the developing testis of Nile tilapia. Conversely, the expression of brain type aromatase is not much evident from 15 dah ovary but is evident throughout in the developing testis. Administration of ethinyl estradiol and methyl testosterone during 7 to 15 dah induced sex reversal to all genetic males and females, respectively. Sex steroid induced sex reversal is also accomplished with the changes in aromatase expression pattern. Gain of ovarian and loss of brain type of aromatase occurs in the male gonadal tissue after its transformation into female by ethinyl estradiol treatment. The reverse is true for the female gonad after its transformation to male by methyl testosterone treatment. Flutamide treatment neither altered sex nor aromatase(s) expression. Lack of effect of flutamide indicates that it is neither effective to block androgen receptor nor androgen action in the Nile tilapia. Acquiring the ovarian type of aromatase by the male gonad after sex reversal is probably an important process to become a functional female. The appearance of brain type aromatase in normal male and sex reversed female gonad require further studies to understand its role in male gonadal differentiation.

P-III-22

THE INVOLVEMENT OF GONADOTROPIN RECEPTORS IN SEX REVERSAL: EXPRESSION, DISTRIBUTION AND REGULATION OF GONADAL FSH AND LH RECEPTORS IN THE GILTHEAD SEABREAM

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Sex reversal, the transformation of an individual from one sex to the other in adulthood, is one of the most dramatic forms of sexual plasticity in nature. It seems evident that gonadotropins are involved in conducting and controlling the process of sex reversal. However, definitive knowledge of their actions is still scarce, in part because of the lack of information about FSH and LH receptors (R) in the ambisexual gonad during sex reversal. We present here the first report of the cloning and examination of the gonadal expression and distribution of FSHR and LHR in a protandrous hermaphrodite, the gilthead seabream (sb), which spawns in winter and undergoes sex reversal in summer. Using 5' and 3' RACE, we isolated a 3435-base cDNA of sbFSHR encoding 705 a.a., most (75%) identical to tilapia FSHR, and a 2803-base cDNA of sbLHR encoding 720 a.a., most (68%) identical to amago salmon LHR. As measured by real-time quantitative RT-PCR, two expression peaks of ovarian sbFSHR were seen in July (in the ambisexual gonad) and in December (in the ovarian portion of spermiating gonad); two expression peaks of ovarian sbLHR were found in June (in ambisexual gonad) and in December (in pre-spawning ovary). In the testicular portion, higher expression of sbFSHR was detected from May to August and also in November, while the highest expression of sbLHR was found from May through August. During the sex reversal period, transcript levels of testicular sbFSHR were 25 to 35 fold higher than the levels of ovarian sbFSHR and transcript levels of testicular sbLHR were 5 to 8 fold higher than the levels of ovarian sbLHR. Using *in vitro* tissue culture, we investigated the effect of E2, T, 11β-T and 11-KT on the expression of sbFSHR and sbLHR in the testicular portion of the ambisexual gonad. The expression of sbFSHR was significantly elevated by 11T (up to 2 fold increase). The expression of sbLHR was enhanced significantly by 11-KT (up to 2.5 fold increase) and even more dramatically by T and 11β-T (up to 10 fold increase). sbLHR expression, however, was significantly suppressed by E2. Our results suggest that the effects of gonadotropins, with the emphasis on FSH, may act primarily on the testicular portion of ambisexual gonad during sex reversal, thereby enhancing testicular development and androgen production. Positive feedback of the androgens on the expression of sbLHR, increases the receptor sensitivity to LH in the testicular portion and, ultimately, driving male development. In contrast, E2 suppressed the expression of sbLHR, which may block male development and lead to continuous ovarian growth and subsequent reversal of sex.

Session IV

Gametogenesis I

O-IV-1
MOLECULAR CONTROL MECHANISMS OF FISH SPERMATOGENESIS

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Fish spermatogenesis is controlled by the sex steroid hormones. To understand the molecular mechanisms by which spermatogenesis is regulated by steroids, we used a cDNA subtraction to identify genes that show unique expression in eel testis during spermatogenesis. We isolated 35 stage-specific cDNA clones and named them eel spermatogenesis-related substances (eSRSs). Mitotic divisions of spermatogonia can be categorized by spermatogonial stem cell renewal and spermatogonial proliferation. Spermatogonial renewal is regulated by estradiol-17β (E2) through Sertoli cell action. eSRS34 cDNA clone, which has a high similarity with PD-ECGF, has been isolated as the factor up-regulated by E2 stimulation *in vitro* testicular organ culture. Since recombinant eSRS34 induced spermatogonial mitosis *in vitro*, there is a possibility that eSRS34 regulates spermatogonial renewal. The process of spermatogenesis from spermatogonial proliferation to spermiogenesis is controlled by 11-KT through Sertoli cell action. Among the 35 isolated eSRSs, we focused on the roles of eSRS1 and eSRS21 cDNA clones in the regulation of this process. eSRS1 encodes eel activin B. Activin B mRNA were found in Sertoli cells at the initiation of spermatogenesis after hCG injection. Both transcription and translation of activin B were induced by 11-KT treatment in vitro. Although spermatogonial proliferation has been induced by recombinant activin B *in vitro*, meiosis could not be induced. From deduced amino acids sequence, eSRS21 has a similarity with Müllerian-inhibiting substance. Although eSRS21 mRNA transcripts were detected in Sertoli cells before the initiation of spermatogenesis, its expression was completely suppressed by 11-KT treatment. Spermatogonial proliferation induced by 11-KT *in vitro* was suppressed by the addition of recombinant eSRS21. Furthermore, treatment with an anti-eSRS21 antibody induced spermatogonial proliferation *in vitro*. It can be concluded that activin B and eSRS21 play important roles in the initiation of spermatogenesis through the effect of 11-KT, i.e., activin B may initiate spermatogenesis and eSRS21 may prevent the initiation of spermatogenesis. 17α,20β-dihydroxy-4-pregnen-3-one (DHP) plays an important role in sperm maturation; acquisition of sperm motility. The action of DHP is mediated through an increase in seminal plasma pH. eSRS22 has a high similarity with carbonic anhydrase type II (CA II), which is known to play a significant role in acid-base regulation in the body. The protein of CA II was mainly expressed in the membrane fraction of spermatozoa. DHP treatment significantly raised the pH value of seminal plasma through the activation of CA II *in vitro*. These data suggest that CA II is key enzyme for sperm maturation.

O-IV-2
SPERMATOGENESIS IN TELEOST: INSIGHTS FROM THE NILE TILAPIA MODEL

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Different from mammals, in most teleost the arrangement of germ cells in the seminiferous tubules is cystic. Also, testis size and sperm production in fish increase continually during adulthood. Due to its fast growth and relatively small size, and good adaptability to different environmental conditions, the Nile tilapia is an excellent model to investigate testis function in teleost. In this regard, a careful morphometric analysis of serially sectioned testis fragments and the study of Sertoli cell proliferation and the duration of spermatogenesis (after intracelomatic H^3-thymidine injections at different temperatures: 20°C, 25°C, and 30°C), was performed in sexually mature tilapias. The number of Sertoli cells increased gradually from primary spermatogonia to pachytene spermatocyte cysts (~1.4 to 6.1 Sertoli cells per cyst), showing a strong trend toward stabilization thereafter. The number of germ cells per cyst increased dramatically (1 to ~770 cells) from primary spermatogonia to early spermatid cysts, whereas the opposite trend was observed for nuclear diameter (~10 to 2 μm) and cell volume (~2,250 to ~20μm^3). Approximately 130 early spermatids were found per each Sertoli cell and germ cell loss during spermiogenesis was around 30%. Compared with most mammals investigated, Sertoli cell efficiency in tilapia is 10 to 20 times higher, suggesting a higher efficiency for the cystic arrangement of spermatogenesis. Labeled mature spermatids were observed after 6 and 10-11 days post-thymidine injection at 30°C and 25°C, respectively. However, at 20°C germ cells more advanced than pachytene spermatocytes were not observed after 4 days post-injection, suggesting that the meiotic phase of spermatogenesis in tilapias is critically sensitive to mild temperature changes. Also, the data found in the present work suggest that the duration of spermiogenic phase of spermatogenesis was not altered in fish investigated at 25°C and 30°C. Sertoli cells labelled with H^3-thymidine was frequently found, mainly at the distal (blind end) segment of the seminiferous tubules. Although labelled Sertoli cells in spermatocyte and spermatid cysts was observed, the vast majority of these labeled cells were associated with spermatogonial cysts. These data strongly suggest that Sertoli cell proliferation is the primary factor responsible for the testis growth and the increase in sperm production observed in adult fish. Financial support: CNPq/CAPES/FAPEMIG.

O-IV-3

ESTRADIOL-17β CAUSES PROLIFERATION OF INTERSTITIAL CELLS IN THE PRE-SPERMATOGENIC RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) TESTIS

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Estradiol-17β and estrogen receptor-alpha (ERα) play important roles in fish reproduction. It has previously been demonstrated that in the rainbow trout testis ERα protein expression is restricted to the interstitial cells, throughout the annual reproductive cycle. These interstitial cells, which give rise to Leydig cells, are important for steroid synthesis. The purpose of this study was to determine whether estradiol-17β could induce proliferation of interstitial cells in the pre-spermatogenic rainbow trout testis. In order to investigate the role of estradiol-17β in the rainbow trout testis, a floating organ culture system was developed. Testis fragments were cultured for up to six days and appeared viable based on histological appearance. Small pieces of testis (~1x1 millimeter) were treated to various doses of estradiol-17β with or without pretreatment of the tissue with the ERα antagonist ICI 182,780, for two days. On the third day of culture, the S-phase nuclear labeling agent 5-bromo-2'-deoxyuridine (BrdU) was added to the media. Proliferating cells were identified immunohistochemically using an anti-BrdU antibody and quantified. The results show that a dose of 5ng estradiol-17β per ml medium increased interstitial cell proliferation by approximately 70% compared to the control. Pretreatment of the tissue with ICI 182,780 nullifies this proliferative effect. These results suggest a role for estradiol-17β in interstitial cell proliferation in the immature rainbow trout testis before spermatogenesis begins.

O-IV-4

DIURNAL RHYTHM OF STEROID BIOSYNTHESIS IN THE TESTIS OF TERMINAL PHASE MALE OF PROTOGYNOUS WRASSE, *PSEUDOLABRUS SEIBOLDI*, A DAILY SPAWNER

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The wrasse, *Pseudolabrus sieboldi*, is a diandric protogynous labrid fish. Sex-changed large terminal-phase (TP) males arise from small initial-phase (IP) females. During the spawning season, pairs of TP males and IP females spawn between 0600 and 0900 hr daily, and each female and male fish has a regular diurnal rhythm of gametogenesis for egg and sperm production. We have already studied the steroid hormone synthesis pathways in the ovarian follicles of IP females. In this study, we clarified the steroid hormone synthesis pathways in the testicular fragments of TP males, and further investigated the diurnal change of steroid biosynthesis in the testis. The following conversions of radiolabeled steroid substrates were observed in vitro. Pregnenolone (P5) was transformed into 17-hydroxypregnenolone (17-P5), dehydroepiandrosterone (DHEA), androstenedione (AD), testosterone (T), 11β-hydroxytestosterone (11β-T), and 11-ketotestosterone (11-KT). The testes also showed activity of 20β-hydroxysteroid dehydrogenase (20β-HSD), which converts 17-hydroxyprogesterone (17-P) into 17,20β-P and 11-deoxycortisol (S) into 20β-S. In addition, an unusual finding in the testis was the biosynthesis of estradiol-17β- (E2). The pattern of steroid production in the gonads of the wrasse is unique, because there are two types of 17β-hydroxysteroid dehydrogenase (I and III) and P-450 aromatase (brain and ovarian types) activities. From androstenedione (AD), E2 is synthesized via estrone (E1) in ovarian follicles, where no T is produced. Conversely, in the testis, E2 is synthesized via T produced from AD. All the major gonadal steroid metabolites showed diurnal profiles. The plasma levels of the gonadal steroids T, 11-KT, E1, E2, 17,20β-P, and 20β-S were measured and diurnal profiles were also observed. The circulating E2 levels and testicular E2 production increased drastically and reached a peak at 15 hr, suggests spermatogonial proliferation, since the number of B-type spermatogonia and spermatocytes was maximal. The circulating level and in vitro production of 11-KT were highest between 0300 and 0900 hr, when the male is actively spawning. This is the first report of the complete steroidogenic pathway in the gonads of a diandric protogynous species throughout its life, and the diurnal change in testicular steroid biosynthesis in the male.

O-IV-5

ENZYMATIC ACTIVITY OF 11β-HYDROXYSTEROID DEHYDROGENASE IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

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11β-hydroxysteroid dehydrogenases (11β-HSD) catalyze the interconversion of active (cortisol, corticosterone) and inactive (cortisone, 11-dehydrocorticosterone) glucocorticoids. In teleosts, 11β-HSD catalyzes the final step of biosynthesis of 11-ketotestosterone (11-KT), the predominant male androgen. Although a cDNA encoding Japanese eel testicular 11β-HSD has been isolated and characterized, information on the regulation and roles of 11β-HSD in teleosts is limited to this single study. We have isolated a cDNA encoding rainbow trout (*Oncorhynchus mykiss*) 11β-HSD from testes and head kidney and examined the enzymatic activity of the recombinant protein. The predicted amino acid sequence of rainbow trout 11β-HSD (rt11β-HSD) shares higher homologywith mammalian 11β-HSD type 2 (> 40%) than with mammalian 11β-HSD type 1 (> 20%). Rt11β-HSD also shares reasonably high homology with mammalian 17β-HSD type 4 (> 35%). The results of transient transfection assays using HEK293 cells showed that rt11β-HSD has significant 11β-dehydrogenase activity, but no significant 11-oxoreductase activity. Rt11β-HSD does not possess significant 17β-HSD activity. These results demonstrated that the rt11β-HSD cloned in this study is a homologue of mammalian 11β-HSD type 2, being predominantly a 11β-dehydrogenase. Rt11β-HSD transcripts are relatively abundant in classical steroidogenic tissues, including the ovarian follicle, and are found at lower levels in a number of other tissues. Seasonal changes in gonadal rt11β-HSD transcripts showed a pattern similar to that of stress-induced serum cortisol levels. Taken together, the ability of rt11β-HSD to convert cortisol to cortisone, the existence of rt11β-HSD transcripts in a wide range of tissues and the pattern of the seasonal changes in rt11β-HSD mRNA raises the possibility of a role for rt11β-HSD in protecting gonads from cortisol induced suppression, in addtion to its role in 11-KT biosynthesis.

O-IV-6

SPERM-ACTIVATING PROTEINS OBTAINED FROM THE HERRING EGGS

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The activation of sperm motility by the egg is an ubiquitous phenomenon in the animal kingdom, but the molecules by which the egg activates sperm motility have been clarified in only a few invertebrate species. In the Pacific herring, *Clupea pallasii*, mature unfertilized eggs release the sperm-activating proteins which are prerequisite to successful fertilization. Complementary DNA clones encoding herring sperm-activating proteins (SAPs) were isolated from herring ovarian cDNA library and amino acid sequences were deduced. SAP(s) is a secretory product(s) with a strong homology to Kazal-type trypsin inhibitors, such as mammalian acrosin inhibitors. SAPs were globally distributed in the outermost layer of the egg chorion and its gene was found to be expressed in the follicle cells which surround developing oocytes. These results suggest that in the Pacific herring, trypsin inhibitor-like proteins are synthesized in the follicle cells, secreted, accumulated in the egg chorion during oocyte development, and released into the milieu at spawning to activate the motility of spermatozoa at the time of gamete interaction.

O-IV-7

SEMINAL FLUID COMPOSITION OF PADDLEFISH (*POLYODON SPATHULA*) AND IONS EFFECTS ON MOTILITY OF FRESH AND DEMEMBRANATE SPERM

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Concentrations between 0.5 to 5 mM of KCl in the swimming medium (SM) prevented activation of spermatozoa. When transferred into a SM (SM = 20 mM tris, pH 8.2 and 1 mg/ml BSA) with 0.5 mM KCl (combined with 5 mM of NaCl or MgCl2) 80-100% cells were motile with velocity around 120-150 $\mu m.s^{-1}$. MgCl2 significantly improved velocity of sperm at 10 - 60 seconds after activation up to 140 $\mu m.s^{-1}$. Very low concentration CaCl2 (0.125 mM) combined with 0.5 mM KCl allowed initiate motility in 20% of sperm while 100 % sperm were activated at 2 minutes with 0.25 mM CaCl2 in SM. We also demonstrate that potassium (5-15 mM) inhibited demembranated sperm. Thus, movement initiation of paddlefish spermatozoa is under antagonistic control of K^+ and Ca^{2+} ions concentration. The osmolality of seminal fluid was ranging 33.0 - 62.7 $mOsmol.kg^{-1}$. The highest ionic concentration of 11.11 $mmol.l^{-1}$ in seminal fluid was observed for Na^+ ion. Then other concentrations declined on 1.56, 0.52 and 0.36 $mmol.l^{-1}$ for K^+, Ca^{2+} and Mg^{2+} ions, respectively. There were highly positive correlation between velocity of spermatozoa and osmolality of seminal fluid (R=0.57) and also between osmolality of seminal fluid and Na^+ concentration in seminal fluid (R=0.70).

P-IV-1

MOLECULAR EVOLUTION OF ANDROGEN RECEPTORS IN FISH

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Androgen receptor (AR) belongs to the nuclear receptor super-family and they are of particular importance for the mediation of the androgen signal. In fish, this mediation of androgen action is further complicated by the duality of the active androgens involved in reproduction i.e., regular androgens versus 11-oxygenated androgens. Until now only a few AR cDNA sequences have been described in teleosts (trout, Japanese eel, tilapia, Astatotilapia, medaka...). However, based on this available data the putative evolution of these fish AR sequences was rather difficult to analyze. To get further insights into the molecular basis of molecular evolution of ARs in teleost fish we initiated the cloning of as many as possible new fish ARs. We designed degenerate primers in order to clone new AR sequences and we combined this new fish AR sequences with available fish AR sequences from different fish genome or EST programs. 25 new fish AR cDNA fragments have been obtained in nineteen different teleost fish along with a few progesterone receptors sequences. These sequences were obtained in very divergent evolutionary orders: i.e., from Osteoglossiformes to Tetraodontiformes The molecular phylogenetic analysis of these new sequences with the previously published ones clearly demonstrate that there is two highly divergent forms of ARs in fish that we called AR1 and AR2 forms respectively. This duplication appears to be specific of the fish lineage. It is consistent with an ancestral genome duplication in fish, although in some species there are additional duplicates of each receptor (AR1α and AR1β for instance).

P-IV-2

CHANGES IN IMMUNOLOCALIZATION OF STEROIDOGENIC ENZYMES (P450SCC, P450C17, P450AROM) IN GONAD OF JAPANESE EEL

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Gonadal development is controlled by gonadal steroids. Androgens are synthesized from cholesterol by cholesterol side-chain cleavage (P450scc) and 17α-hydroxylase/C17-20 lyase (P450c17), and may subsequently be converted to estrogens by aromatase (P450arom). In our previous study, changes in mRNA levels of these P450s during ovarian development have been analyzed, but neither expression as protein nor immunolocalization have been studied. To address this, we generated antibodies against the above P450s and observed changes in their immunolocalization in ovary and testis during artificial maturation of Japanese eel, *Anguilla japonica*. Male and female eels were induced to mature by weekly injections of human chorionic gonadotropin (200 U/individual) and salmon pituitary homogenate (40 mg/kg-BW), respectively. 6xHis-tagged recombinant proteins of eel P450scc, P450c17 and P450arom were heterologously expressed in *Escherichia coli* and purified using affinity purification. Female rabbits were immunized by those recombinant proteins and sera were collected and characterized. To characterize antisera of P450scc, P450c17 and P450arom, a supernatant of ovarian homogenate was subjected to immunoblot analysis. Each P450 antiserum specifically recognized a protein of approximately 59 kDa. In immature testis, P450scc and P450c17 were immunolocalized in Leydig cells, forming clusters. The number of these immunoreactive cells decreased as testicular development progressed. In immature ovary, a few cells immunoreactive for P450scc and P450c17 were localized as clusters of steroidogenic cells. With advancing ovarian development, the number of P450scc and P450c17 immunoreactive cells increased. These cells were localized in the outer layer of the ovarian follicle until the late vitellogenic stage but disappeared at the migratory nucleus stage. In contrast, P450arom seemed to be consistently localized in the innermost follicle layer and the number of immunoreactive cells seemed to increase after induced oocyte development. In addition, P450scc and P450c17 successfully recognized clusters of steroidogenic cells in head kidney of not only eel but also goldfish.

P-IV-3

IDENTIFICATION OF GROWTH HORMONE RECEPTOR IN THE GONAD OF TILAPIA, *OREOCHROMIS MOSSAMBICUS*

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In addition to it's metabolic actions of growth hormone (GH) / insulin-like growth factor-I (IGF-I) for somatic growth, GH/IGF-I has been implicated to be involved in gametogenesis due to the localization of IGF-I mRNA and its peptides in the gonad. In order to examine a possible role of GH/IGF-I axis in gametogenesis, the present study aims to identify the GH-receptor (GH-R) in the gonad of tilapia, *Oreochromis mossambicus*. cDNA encoding GH-R was cloned and sequenced from the tilapia liver. The predicted GH-R preprotein was consisted of 635 amino acids and contained a putative signal peptide, an extracellular region with a characteristic motif, a single transmembrane region, and a cytoplasmic region with conserved box 1 and 2 domains. Tilapia GH-R shares 36-67 % identities with known GH-R in vertebrates, and 21 % identity with tilapia prolactin receptor. A single mRNA transcript was observed in the liver by Northern blot analysis. Tissue distribution studies found the expression of GH-R gene in all the tissue examined including ovary (GSI; 1.0-2.7 %) and testis (GSI; 0.3-0.7 %) using RT-PCR. In situ hybridization showed GH-R mRNA signals in the nucleus and in the cytoplasm of small oocytes at the yolk vesicle stage. The signals were found in the nucleus of the oocytes at the yolk globule stage, but not in the cytoplasm. The granulosa and theca cells surrounding vitellogenic oocytes also contained the GH-R mRNA signals. In the testis, GH-R mRNA signals were found in the Leydig cells, but no signal was detected in the germ cells. These results suggest that GH/IGF-I axis is involved in the development or maintenance of ovary and testis in a paracrine or autocrine manner.

P-IV-4

EFFECT OF *P*-NONYLPHENOL ON THE MOTILITY OF MEDAKA (*ORYZIAS LATIPES*) SPERMATOZOA

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p-Nonylphenol (NP) is a chemical compound that shows estrogen-like activity. Recent studies have shown that NP induces gonadal abnormalities such as the presence of testis-ova in teleost fish but it is still not clear if NP affects also the motility of spermatozoa and consequently the fertility of exposed fish. This experiment was designed to obtain preliminary data on the effects of NP on the motility of the spermatozoa of Japanese medaka (*Oryzias latipes*). Sexually mature, spermiating males of the Mizumoto strain were reared in pairs in glass beakers at room temperature (25°C) and a 16L8D photoperiod and exposed for 4 weeks to NP at concentrations of 0 [solvent (acetone) and solvent-free controls], 20 and 100 µg/L in the rearing water. Sperm was collected in the beginning of the experiment and after 2 and 4 weeks. Fish were anesthetized, rinsed in 100X diluted medaka sperm diluent, placed onto a hole slide glass containing µL of the same diluent, and gently pressured in the abdomen to release sperm into the surrounding medium. Diluted, motile sperm was immediately collected and loaded into a sperm motility chamber kept at 25°C and videotaped under a microscope. Spermatozoan motility was assessed in terms of the percentage of motile spermatozoa and the velocity of displacement at different times (60, 120 and 180 s from stripping) using computer-aided image analysis. The results of these measurements showed a relatively large within-group variability in these parameters but also indicated that repeated (careful) stripping of an individual had no detrimental effect on sperm motility. The velocity varied between 30-50 µm/s at 60 s to 0-5 µm/s at 180 s regardless of the kind and length of treatment. The percentage of motile spermatozoa typically decreased from 70-100% at 60 s to 0-40% at 180 s. No marked differences between the groups could be observed at 60 s but control groups generally showed higher percentages of motile spermatozoa at 120 and/or 180 s than NP-treated groups at 2 and 4 weeks. A significant fraction of the fish exposed to NP could not be striped of sperm after 2 weeks. Subsequent histological observation of all experimental fish revealed gonadal abnormalities such as testis-ova and cell degeneration only in the gonads of fish exposed to NP. These results indicate that NP not only causes histological abnormalities in the gonads but that it might impair also sperm production and spermatozoan motility in teleost fish.

P-IV-5

APOPTOSIS IN THE TESTIS OF MALE *GADUS MORHUA* HELD ON CONTINUOUS LIGHT

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It is known that photoperiod manipulation and in particular the introduction of continuous light regimes may arrest or partially arrest further gonadal growth in sexually mature specimens such as in salmonids and gadoids. The use of continuous light has been proven to be a big success in on-growth commercial farms dealing with these type of species significantly increasing somatic growth (due to less investments in gonads) and thereby the economic output. Although a frequently used technique supported by several reports on changes in for instance melatonin profiles, the direct effects of the gonads per se, especially in males, are much less understood. In this work prespawning and spawning Atlantic cod were held on natural and continuous light in 5-m circular tanks and the two groups contrasted for testes development taking regular samples from killed animals to be studied in histological sections by the TUNEL reaction. Testes at continuous light were found to be significantly smaller in relative size than at natural light and showed clearly different TUNEL staining properties. The results are discussed in relation to previous findings on other species.

P-IV-6

ROLE OF COX-2 IN THE CONVERSION OF ARACHIDONIC ACID TO PROSTAGLANDIN E2 BY GOLDFISH TESTIS

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Eicosanoids, such as arachidonic acid (AA) and its cyclooxygenase (COX) metabolites prostaglandins (PG), have been implicated in the control of several reproductive processes in fish, including ovulation, steroidogenesis, and reproductive behaviour. COX exists in two forms: a constitutively expressed form,COX-1, and an inducible form, COX-2. In vitro treatment of fish gonadal tissue with AA results in an increased production of steroids as a result of the conversion of AA to its PG metabolites; however, we know very little about the COX enzymes that mediate this conversion in fish gonadal tissues. Studies have not detected the expression of either enzyme in fish testis, and limited work has been done to characterize their expression in fish ovaries. The purpose of this study was to investigate which form of COX may be responsible for the conversion of AA to prostaglandins by goldfish testis by using the selective COX inhibitors, SC-560 (COX-1) and NS-398 (COX-2) and the non-selective COX inhibitor, indomethacin. Goldfish testis pieces were incubated at 18°C for 18hrs in presence of AA (400 uM) and with or without the chemicals (10 ug/ml). The stimulation of prostaglandin E2 (PGE2) and testosterone (T) attained by the AA (400uM) treatment was significantly attenuated (85%) by the indomethacin and NS-398 treatments. The SC-560 treatment resulted in only a 32 and 39% decrease in PGE2 and T production, respectively. These results suggest that the COX-2 enzyme is predominantly responsible for the conversion of AA to PGE2 in goldfish testis. In other studies, we have shown that treatment of goldfish testis by the non-selective COX inhibitors, indomethacin and diclofenac (10ug/ml), two pharmaceuticals recently measured in North American surface waters, results in increased levels of testosterone. The mechanism by which these drugs increase T in goldfish testis is unknown.

P-IV-7

MOLECULAR CLONING, EXPRESSION DURING GONADAL MATURATION AND FULL-LENGTH SEQUENCE OF A PUTATIVE LH RECEPTOR IN ATLANTIC COD (*GADUS MORHUA*)

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Applying an universal approach for the amplification of fish glycoprotein hormone receptors, partial sequences for all three receptor types were obtained from ovaries of Atlantic cod (*Gadus Morhua*). Subsequent 5' and 3' RACE (rapid amplification of cDNA ends) resulted in the cloning of the full-length cDNA encoding a putative gonadal LH receptor (acLH-R). The cDNA coded for a 694-amino acid protein comprising a putative signal peptide at the N-terminus and seven predicted transmembrane domains in the C-terminal half. Homology searches using the deduced amino acid sequence revealed that the acLH-R had the highest identities with tilapia (67%) and Channel catfish (58%) LH receptors. Overall sequence homology with mammalian LH receptors was 52-53%, whereas homologies below 46% were found with other mammalian and piscine glycoprotein hormone receptors. We therefore conclude that we have cloned a full-length cDNA encoding the LH-R of the Atlantic cod. Expression of LH-R mRNA in gonads during various developmental stages was investigated by nucleic acid-based methods.

P-IV-8

INVOLVEMENT OF 17α, 20β-DIHYDROXY-4-PREGNEN-3-ONE ON SPERMATOGENESIS IN JAPANESE EEL (*ANGUILLA JAPONICA*) AND JAPANESE HUCHEN (*HUCHO PERYII*)

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17α, 20β-dihydroxy-4-pregnen-3-one (17α, 20β-DHP) increase dramatically in final maturation and it induces sperm maturation. On the other hand, serum level of this steroid shows small peak in early spermatogenic process. However, the role of 17α, 20β-DHP in this process is unclear. In histochemical observation, progesterone receptor α expressed mainly in Sertoli cells around type A- and early stage of type B-spermatogonia. This finding indicates that 17α, 20β-DHP has some role in early stage of spermatogenesis. By using eel testis organ culture system, the action of 17α, 20β-DHP on spermatogenesis has been investigated. Adding 17α, 20β-DHP to eel testicular explants at low concentration (100 pg/ml) was sufficient to induce all stages of spermatogenesis from the nonproliferated spermatogonia to spermatozoa within 30 days, which is the same as that of 10 ng/ ml of 11-ketotestosterone, which is spermatogenesis inducing steroid hormone (11-KT). Short-time (3 days) treatment with 11-KT is enough for inducing all stages of spermatogenesis, whereas, 17α, 20β-DHP must be available during all period of culture in order to exert its stimulatory effect on sperm production. In Japanese Huchen (*Hucho peryii*), 17α, 20β-DHP also induce the spermatogonial proliferation toward meiosis *in vitro* organ culture. From these results, it appears that 17α, 20β-DHP has the ability to induce all stages of spermatogenesis as well as 11-KT treatment, but its regulatory mechanisms of spermatogenesis seem to be clearly different. Thus, 17α, 20β-DHP was induced all stages of spermatogenesis in Japanese eel and proliferation of spermatogonia in Japanese huchen *in vitro*. But, it is necessary to investigate the different regulatory mechanisms of 11-KT and 17α, 20β-DHP in spermatogenesis at the molecular level in order to verify and elucidate the physiological significance of 17α, 20β-DHP underlying the regulatory mechanism of spermatogenesis in teleosts.

P-IV-9

MOLECULAR CLONING OF SEX HORMONE-BINDING GLOBULIN cDNA IN CARP, *CYPRINUS CARPIO*

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Sex hormone-binding globulin (SHBG) is a plasma glycoprotein able to bind specifically sex steroids. It transports sex steroids in the blood and regulates their metabolic clearance and access to target cells. Therefore, SHBG is one of the key factors that control the effectiveness of sex steroids. Until now, only a few mammalian SHBG were isolated and characterized and little is known about the details of SHBG (structure, function, expression, etc.) in non-mammalian species. In the present study, we isolated and characterized cDNAs encoding SHBG in carp, *Cyprinus carpio,* as an initial step to understand the function of SHBG in teleosts. Two types of fragment (SHBG-I and SHBG-II, 180 base pair (bp) in each) were amplified by the PCR using degenerate primers which was selected from the conserved region between mammalian SHBG cDNAs and SHBG-like sequence in zebrafish genome. Both amino acid sequence closely resemble (95%) and show high identity (about 60%) with the corresponding sequence of mammals. Using these fragments as a probe, SHBG-I and SHBG-II cDNAs containing whole open reading frame were isolated from liver cDNA library. They encode 401 and 388 amino acids, respectively, and share high amino acid identity (90%). Although their amino acid sequence show low identity (about 35%) with those of mammalian SHBG, only steroid-binding site is conserved better than the other part. In addition, the antibody against recombinant protein of these cDNAs, which bind specifically with serum protein contained rich in sex steroids. These results suggest that the two presently isolated cDNAs encode carp SHBG. Northern blot analysis and RT-PCR revealed that both types of SHBG mRNA were mostly transcribed in the liver. The levels of their mRNAs in the liver did not change by either exogenous estrogen or androgen. This result may indicate that SHBG synthesis is correlated with steroidogenesis.

P-IV-10

REPRODUCTIVE CYCLE OF DEVIL STINGER, *INIMICUS JAPONICUS*

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Reproductive cycle of devil stinger, *Inimicus japonicus*, was investigated by histological observation of gonad and measurement of gonadosomatic index (GSI). In addition, plasma concentration of sex steroid hormones, testosterone (T), 11-ketotestosterone (11-KT), 17β-estradiol (E2), 17α, 20β-dihydroxy-4-pregnen-3-one (DHP) and 17α, 20β, 21-trihydroxy-4-pregnen-3-one (20β-S), were measured to obtained basic information on endocrine changes during gonadal development. Devil stinger was collected at intervals of about one month from April 2000 to July 2001 in Shimabara Bay off Arie, Nagasaki, Japan. GSI values of male fish increased from May to July, while spermatocytes, spermatids and spermatozoa appeared in testes. Thereafter, GSI values sharply decreased in August. Plasma concentrations of 11-KT in males were maintained at high levels from April to July, but plasma T was high only in April and May. High concentrations of plasma DHP and 20β-S were detected from February to May. In contrast, GSI of female rapidly increased from March to June, then decreased sharply in July. After that, GSI values were consistently low until February. Vitellogenic oocytes were observed in the ovary in January. In May, tertiary yolk stage and maturation stage ooytes appeared in the ovary, while oocyte diameter reached maximum levels. Plasma concentrations of E2 and T were high during the middle and late vitellogenesis, and spawning season from March to June. Plasma DHP and 20β-S in females tended to increase from February to June. There was no difference in terms of gonadal development of both sex fish. These observations indicate that gonadal development starts between January and February, and spawning in devil stinger occurs in May and June.

P-IV-11

STEROID-BINDING CHARACTERISTICS OF SEX HORMONE-BINDING GLOBULIN (SHBG) IN CARP, *CYPRINUS CARPIO*

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It is known in mammals that sex hormone-binding globulin (SHBG) is a homodimeric glycoprotein able to bind specifically sex steroids. It transports sex steroids in the blood and regulates their metabolic clearance and access to target cells. Therefore, SHBG is one of the key factors that control the effectiveness of sex steroids. However, its function has not been investigated well in non-mammalian species and only a few mammalian SHBG have been isolated and characterized until now. Recently, we finished molecular cloning of the cDNAs encoding carp SHBG for the first time in lower vertebrates. In this study, the binding characteristics of estradiol-17β (E_2) to SHBG was investigated in female carp at various reproductive stages by the combination of FPLC and steroid ELISA techniques, in addition to the specific antibody against recombinant SHBG. First, the correlation between serum E_2 concentration and quantity of E_2 bound to SHBG were examined. Result showed that there was a high correlation between them; almost all E_2 were bound to SHBG in the blood at any reproductive stages. Next, using non-reproductive female fish injected E_2 (1 μg/g body weight), the effect of exogenous E_2 on the total quantity of E_2 bound to SHBG was investigated (*in vivo* test). Result showed that E_2 quantity bound to SHBG in serum was not changed by the E_2 injection. Similarly, the addition of E_2 to normal serum sample did not affect the quantity of E_2 bound to SHBG (*in vitro* test). These results suggest that only endogenous sex steroids are able to bind SHBG *in vivo* and the increase of steroid-binding capacity of SHBG (SHBG synthesis) is correlated with steroidogenic activity.

P-IV-12

IONIC COMPOSITION AND PHYSIC-CHEMICAL PARAMETERS OF THE EUROPEAN EEL (*ANGUILLA ANGUILLA*) SEMINAL PLASMA

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Hormonal methods for the induction of gonad maturation in the European eel (*A. anguilla*) have been developed in previous studies, obtaining significant sperm volumes with a good quality in the case of males (Perez *et al.*, 2000) as well as spawns in the case of females (Asturiano *et al.*, 2003). However, the high sperm density, together with the short spermatozoa swimming time, make difficult sperm manipulation and assessment for quality. The main objective of the present study was to estimate the variation of different physic-chemical parameters, as well as the ionic composition of the European eel seminal plasma in relation with sperm quality through the treatment. Gonad maturation was induced in eel males, recording weekly the sperm volume, density and quality (0-V). pH, osmolality and concentrations of Na^+, K^+, Mg^{2+} and Ca^{2+} in the seminal plasma were recorded as well. Results were related with sperm quality through the treatment. Significative variations on seminal plasma osmolality were observed during the spermiation period. On the other hand, seminal plasma ionic composition showed variations correlated with sperm quality. Results will be discussed with the intention of determining which must be the suitable characteristics of one extender solution for the sperm of this species. Using a extender solution will facilitate sperm manipulation, as well as its short-time conservation under refrigeration conditions. Moreover, this solution has been the starting point for a sperm cryopreservation medium (see Asturiano *et al.*, this Congress). This study was carried out in collaboration with Valenciana de Acuicultura, S.A. Authors want to thank Mr. Andrés Moñino its technical collaboration on fish care and sampling. The study was financiated by a Project from Presidencia Generalitat Valenciana (CTIDIA/2002/117). J.F.A. have a research contract of the Ramón y Cajal Programme, cofinanciated by the Spanish Ministery of Science and Technology and the Universidad Politecnica de Valencia.

P-IV-13

SEA BASS (*DICENTRARCHUS LABRAX* L.) SEX HORMONE-BINDING GLOBULIN (SHBG): CLONING, CHARACTERIZATION AND SITES OF EXPRESSION

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Sex Hormone-Binding Globulin (SHBG) is a plasma glycoprotein that binds and transports androgens and estrogens in the blood of vertebrate species including fish. Several previous reports have indicated that plasma SHBG levels fluctuate during the breeding season in fish. We have also found that plasma SHBG levels in male/female sea bass exhibit seasonal variations coincident with changes in their reproductive status. To study further the expression and characteristics of sea bass SHBG regarding its role in the regulation of sex steroid action during development and reproduction, we have isolated the full length cDNA sequence encoding SHBG from 5' and 3' RACE cDNA libraries prepared using sea bass liver and kidney RNA. The sequence of the sea bass SHBG cDNA encodes a precursor protein of 396 amino acids with a predicted leader sequence for secretion of 30-32 amino acids. The molecular size of the mature sea bass SHBG polypeptide is 41 kDa, it has 3 N-glycosylation sites, and consists of a tandem repeat of laminin G-like domains. Its primary structure displays 25-30% sequence identity with known SHBG sequences from several mammalian species. When expressed in Chinese hamster ovary cells, the sea bass SHBG secreted into the culture medium displayed the same steroid-binding characteristics as SHBG in sea bass blood samples. In order of affinities, sea bass SHBG binds estradiol (1.00) > testosterone (0.60) > estrone (0.38) > dihydrotestosterone (0.34) > androstenedione (0.27) and progesterone (0.02). To determine whether the sea bass SHBG is a monomer or homodimer, we subjected it to gel filtration and this confirmed a size of approximately 110 kDa consistent with the presence of a homodimer. Northern blot analysis of RNA from sea bass liver, kidney, brain, ovary and testis using a sea bass SHBG cDNA as probe, revealed the presence of SHBG mRNA only in the liver. Thus, it appears that SHBG in sea bass blood most likely originates primarily from the liver. More detailed studies are required to define how SHBG expression may vary in sea bass liver and other tissues during early development and throughout the reproductive cycle.

P-IV-14

SERTOLI CELL PROLIFERATION AND FSH-DEPENDENT SIGNALLING IN AFRICAN CATFISH

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Survival and development of germ cells in the testis depend on the structural and functional support provided by Sertoli cells. Since the germ cell supporting capacity of a given Sertoli cell is limited, the number of these cells is a limiting factor for the testicular sperm production capacity. Therefore, regulation of Sertoli cell proliferation is an important factor in male fecundity. In mammals, most Sertoli cells differentiate terminally –and hence stop proliferating –when the first wave of spermatogenesis has reached the primary spermatocyte stage; Sertoli cell differentiation is stimulated by thyroid hormones and by the pubertal increase in androgen production. Also in fish, Sertoli cell proliferation apparently takes place predominantly before meiosis during spermatogonial proliferation. Different from mammals, however, Sertoli cell proliferation may accompany each wave of the cystic spermatogenesis in fish. Unfortunately, there is no information in fish on the regulation of Sertoli cell proliferation. Since follicle-stimulating hormone (FSH) and FSH-activated growth factor signalling systems regulate Sertoli cell proliferation in mammals, and since there is little information on physiological effects of FSH in fish testis in general, we have studied the possibility that FSH-dependent signalling is involved in Sertoli cell proliferation in African catfish testis. For this work, we have used two experimental models, (i) removal of one testis from mature males (unilateral gonadectomy, UG), and (ii) androgen treatment of juvenile, intact males. Four days after UG, FSH receptor (FSH-R) expression was increased 2-fold in the remaining testis. This was associated with a 7-fold increase of Sertoli cell proliferation, as studied by the incorporation and subsequent immunocytochemical detection of bromodeoxyuridine (BrdU) in testis tissue. In juvenile males, previous work showed that 11-ketotestosterone (11-KT) treatment stimulates testis growth, which is inhibited by co-treatment with testosterone (T); typically, testis growth in juvenile vertebrates is associated with Sertoli cell proliferation. We now show that T, alone or in combination with 11-KT, more than halves FSH-R expression in the testis, and suppresses pituitary FSH beta-subunit to about 1/10th of control values. In our view, these data suggest that FSH-R dependent signalling may be of significant relevance to testis growth and development in fish. Since the FSH-R shows a promiscuous ligand binding behaviour (i.e. binds LH as well with a high affinity), the question of ligand availability during the period of initiation of testis maturation is an important factor for future work.

P-IV-15

CIRCULATING INSULIN-LIKE GROWTH FACTOR-I AND ITS BINDING PROTEINS IN MATURING SALMON

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Insulin-like growth factor-I (IGF-I) is a potent mitogen that exerts its actions in endocrine, paracrine and autocrine manners. Biological activity of IGF-I is tightly regulated by a family of high-affinity IGF-binding proteins (IGFBPs). Six IGFBPs have been identified in mammals and they differently modulate IGF-I actions. Increasing numbers of studies indicate that paracrine/autocine IGF-I, in concert with IGFBPs, play a significant role in gonadal development, but the contribution of endocrine IGF-I and IGFBPs to gonadal development is not clear. Moreover, in fish little is known about changes and regulation of circulating IGF-I and IGFBPs during maturation. In salmon, at least three IGFBPs with molecular masses of 41, 28 and 22 kDa exist in the circulation. The 41-kDa IGFBP may be IGFBP-3, a main carrier of circulating IGF-I, since its size and regulation are similar to mammalian IGFBP-3. We have recently purified the 41 kDa IGFBP from serum and developed a radioimmunoassay. By use of specific immunoassays, we measured circulating IGF-I and 41-kDa IGFBP levels in maturing male and female chinook salmon. In males, plasma IGF-I and 41-kDa IGFBP levels were higher than in females and showed peaks during maturation. In contrast, plasma IGF-I in maturing females remained unchanged while 41-kDa IGFBP decreased. We next examined possible effects of androgens on plasma IGF-I and 41-kDa IGFBP. Two-year-old coho salmon were injected once either with testosterone or 11-ketotestosterone. Two weeks after injection, plasma IGF-I and 41-kDa IGFBP levels were increased in a dose-dependent manner in both treatments. This result suggests that androgens are involved in the regulation of circulating IGF-I and 41-kDa IGFBP in salmon.

P-IV-16

MOLECULAR CLONING AND GENE EXPRESSION OF THE RIBOFLAVIN BINDING PROTEIN IN THE NILE TILAPIA, *OREOCHROMIS NILOTICUS*

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Riboflavin is a water-soluble B vitamin required by the body for health, growth and especially for reproduction. The deposition of many water-soluble vitamins into the eggs of birds and reptiles or into the developing mammalian fetus is facilitated by vitamin-binding proteins and riboflavin-binding protein (RfBP) is one of them. RfBP has been cloned from the female sex of chicken, turtle, toad and frog, and pregnancy-specific RfBPs have also been found in rat, mouse and human plasma. The RfBP gene expression has been shown to be in an estrogen-dependent manner. In this study, tilapia riboflavin-binding protein cDNA clone was unexpectedly picked up after three rounds of screening of the testis cDNA library with a short-chain dehydrogenase/reductase (SDR) probe, as RfBP and partial SDR probe have more than 50% homology of nucleotide sequence in a small region. It is a full length clone with 177bp 5'UTR, 840bp ORF encoding a 279 aa peptide, and 1064bp 3'UTR with poly A tail. Even though it shows only 31~35% similarity to the reported RfBPs in aa sequences, tilapia RfBP is conserved in amino acid residues that are known to be essential for its protein function: eighteen cysteine residues that form nine disulfide bridges, two asparagine glycosylation sites, as well as 5 of the 6 tryptophan residues that are thought to be involved in ligand binding. Both RfBP and folate-binding proteins belong to the folate receptor family which contains conserved cysteine residues that form nine disulphide bridges for the former while eight for the latter. Tissue distribution analysis by RT-PCR revealed that tilapia RfBP is exclusively expressed in the brain, pituitary and gonads of both sexes, with the highest expression in the female pituitary and gonads. Northern analysis detected a band around 2.1 Kb in the ovary, testis and brain of both sexes with higher expression in females. The expression of RfBP during the spawning cycle (14days) increased in parallel with oocyte growth. The exclusive expression in the brain-pituitary-gonadal axis suggested a possible role of RfBP in fish reproduction. To our knowledge, this is the first report that RfBP cDNA clone was obtained from a teleost fish.

P-IV-17

GROWTH PATTERNS AND PLASMA LEVELS OF TESTOSTERONE, 11-KETOTESTOSTERONE, AND IGF-1 IN MALE ATLANTIC HALIBUT (*HIPPOGLOSSUS HIPPOGLOSSUS*) FROM JUVENILE STAGES THROUGHOUT SEXUAL DEVELOPMENT

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Atlantic halibut is a prime candidate for aquaculture. It shows a pronounced dimorphic growth pattern, where females generally reach sizes manyfold larger than males. During intensive rearing, a large proportion of the males enter puberty with a resulting decrease in growth and flesh quality. We therefore initiated a study to establish basal data on growth patterns, as well as hormonal regulation of growth and maturation throughout development in male Atlantic halibut. The study covered the whole life cycle from juvenile stages through the first two reproductive cycles (from 2-6 years of age). Body growth was slow but steady until the fourth winter of life when growth was temporarily arrested. This arrest was accompanied by elevated plasma levels of testosterone (T) and 11-ketotestosterone (11-KT). Sexually mature males were not observed at this point. All the males reach puberty during their fifth winter of life, and all males were sexually mature also the following winter. Body growth was reduced or negative from September and throughout spawning season (January-March) both years. This could not be adjusted for by the high growth rate observed in the months following spawning. Plasma T and 11-KT showed rapid increases from September, reaching maximum levels between January and March. Plasma levels of IGF-1 are in the process of being analysed. The elevated steroid levels observed one year prior to first sexual maturation, indicate that male Atlantic halibut, like several other teleost species, may undergo one or more dummy runs in the years preceding testicular growth and maturation.

Session V

Reproductive Behavior and Migration

O-V-1
HORMONES, PHEROMONES, AND REPRODUCTIVE BEHAVIOR

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Individuals of sexual species synchronize the timing of their reproductive behavior with the maturity of their gametes by using reproductive hormones as endogenous signals between reproductive tract and brain. In the relatively short time since Kjell Doving and Lorenzo Colombo respectively provided the first theory and evidence that these hormones might also serve as sex pheromones in fish, it has become clear that the reproductive hormones (steroids and prostaglandins) used by fish as endogenous synchronizing signals also are commonly used (along with their precursors and metabolites) as exogenous signals (hormonal pheromones) to synchronize gamete maturation and/or spawning interactions between and among conspecifics. This dual function for hormonal products not only extends our traditional concepts of sex hormone actions being limited to reproductive synchrony within the individual, but also implies that we are unlikely to achieve a comprehensive understanding of reproductive function in any fish without knowledge of both the endogenous and exogenous actions of its hormones and related released compounds. Although such knowledge is beginning to accumulate for several species (e.g. goldfish, Atlantic salmon), even here it is far from complete. Moreover, evidence from olfactory studies showing that acute sensitivity to water-borne hormones is widespread among major freshwater taxa raises fascinating questions about species specificity of hormonal pheromones that are only beginning to be explored. Hormonal pheromone studies have focused on oviparous gonochorists with relatively simple reproductive strategies, with the result that we know nothing about the potential hormonal pheromone functions of the numerous species with sequential hermaphroditism or alternative male strategies, or the possible changes in pheromone function associated with the numerous transitions from oviparity to viviparity. Given the significant insights we have gained from studies of traditional species, it seems certain that expanding hormonal pheromone research to non-traditional species exemplifying the diverse nature of fish mating systems will similarly enrich our understanding of fish reproductive function.

O-V-2
EVIDENCE FOR THE RELEASE OF SEX PHEROMONES BY MALE ROUND GOBIES (*NEOGOBIUS MELANOSTOMUS*)

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We report supporting evidence for the hypothesis that nesting round goby parental males release a sex pheromone (or pheromones) to attract females, and initial investigation into identity of the compound(s). We assessed pheromonal communication through physiological (electro-olfactogram, EOG) and behavioral responses of females to water conditioned by males. Reproductive females (RFs) responded with larger amplitude EOGs than non-reproductive females (NFs) to water conditioned by reproductive males, non-reproductive males, and to amino acids. The RFs reacted by directed movement to water conditioned by reproductive males compared to untreated water, but not to water from non-reproductive males or females. Overall, females of different maturation stages preferred water conditioned by reproductive males and females. Putative pheromones were collected from conditioned water by solid phase extraction on octadecylsilane cartridges. Extracts were fractionated by reverse phase HPLC. The RFs showed strong EOG responses to a subset of HPLC fractions collected from water conditioned by reproductive males, but not to non-reproductive male fractions; and the reproductive male fractions were not stimulatory to NFs. A previous discovery that olfactory epithelium of the round goby is sensitive to sex steroids (and especially to etiocholanolone; Murphy *et al.*, 2001, J. Chem. Ecol. 27: 443-470), led us to identify steroids produced by the testes of mature males through in-vitro incubation with tritiated substrates. At least six metabolites were formed from androstenedione. One of these (albeit in a low proportion) was recognized as etiocholanolone. However, the elution positions of this steroid (and all but one of the unidentified metabolites) on HPLC did not show a good match with the elution position of maximal EOG activity. There are several interpretations of these data: the male pheromone is unlikely to be an androgen; if it is an androgen, then it is likely to be conjugated to a glucuronide or sulfate group (and furthermore, the conjugation possibly take place after it is secreted from the testis); the male pheromone is not a steroid at all and/or does not originate from the testis. We are continuing with the identification of round goby sex pheromones by investigating the stimulatory properties of isolates from conditioned water and organotypic culture.

O-V-3

ANDROGENS, AVT AND ALTERNATIVE REPRODUCTIVE TACTICS IN THE PEACOCK BLENNY

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In the peacock blenny, *Salaria pavo* (Blenniidae) two sexually male types are present: a) older and larger males that defend nests to which they attract females to spawn, and b) younger and smaller males that try to sneak fertilizations, during spawning episodes. In a Portuguese population of *S. pavo* that occurs in a coastal lagoon with limited nest sites, nest-holder males rarely leave their nests to court females due to the high risk of nest-take over by other males. This results in a sex-role reversal in breeding behaviour, with females playing an active role in courtship. Sneaker males mimic female courtship behaviour and nuptial coloration and lack male secondary sex characters (SSC). We have investigated the role of androgens and of the neuropeptide arginine vasotocin (AVT) in the control of the alternative reproductive tactic. Administration of 11-keto-testosterone (KT) to sneakers inhibits the expression of female courtship behaviour but does not promote the expression of male courtship behaviour. Moreover, exogenous KT promotes the expression of some male traits in sneakers, namely the differentiation of the anal gland that secretes a sex pheromone and the differentiation of an accessory gland of the gonads (testicular gland) that is the main site of steroidogenesis in the male gonad. Systemic administration of AVT induces the expression of female courtship behaviour and nuptial coloration in sneakers and females, but fails to induce male courtship behaviour in both male types. The number and size of AVT-ir neurons (ICC) in the forebrain is larger in both male types than in females but the AVT mRNA expression on a per cell basis (ISH) is higher in both sneakers and females than in nest-holder males. These results, suggest an involvement of androgens in the expression of the bourgeois tactic and of AVT in the control of the female-like behaviour of sneaker males.

O-V-4

FLUCTUATIONS, INTERACTIONS AND STIMULATIONS RELATED TO GNRH AND FORMS OF THYROID HORMONES IN MIGRATING SALMON

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Based on results obtained from behavioural experiments with three year old, mature sockeye salmon, *Oncorhynchus nerka*, we determined that a combination of thyroxine (T4) and 3,5,3 triiodothyronine (T3) stimulated olfactory imprinting in the same fish at a juvenile stage. Immature fish from the same cohort and at the same age reacted to the imprinting odorant only after treatment with a Gonadotropin-releasing hormone analogue (GnRHa). Subsequently we found that GnRH also increased the frequency of jumping behaviour in three year old sockeye salmon that were presented with a waterfall. These behavioural responses commonly related to salmon migration, led us to investigate the effects of GnRH on the activation and deactivation pathways of thyroid hormones in sensory tissues that are believed to guide homing in salmon. We found GnRH to increase the deactivation of physiologically active thyroid hormone in the retina. Currently we are examining the natural fluctuations of thyroid hormones and their activation and deactivation pathways in sensory tissues of migrating salmon in nature. Thus we are hoping to relate our laboratory results to natural conditions experienced during salmon homing.

O-V-5

AMINO ACIDS IN STREAM WATER ARE ESSENTIAL FOR SALMON HOMING MIGRATION

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The upstream homing migration of salmonid fishes depends on their olfactory systems as shown by many behavior experiments. Mature fish are guided to their home stream for spawning by home stream odors. However, these odor substances of home river are still unknown. Three experiments were carried out to determine home river substances. First, we analyzed concentration of amino acids, bile acids, and inorganic cations in home streams for salmonids. Amino acids are typical potent odor for fishes, and bile acids are the candidates of home river substances. Secondly, we prepared artificial stream waters based on the quantitative analyses of stream waters, and applied them to masu salmon olfactory organ. Only the artificial stream waters containing amino acids induced large olfactory responses that closely resembled those to the natural stream waters. From these results of preliminary experiments, we concluded that amino acids dissolved in the home stream water were possible home river substances. Finally, we carried out behavior experiments to test whether amino acids mixtures have attractive effects on fish movement or not. Behavior experiments were conducted in 15 m long two-choice test tank consisted of two 12 m long water inlet arms and 3 X 1.8 m pool, which has one outlet at the end of tank. Homing chum salmon and two group of sockeye salmon to the Osaru River, Toya Lake Station and Lake Shikotsu Hatchery respectively were used for test fish. The salmon were placed in the pool of downstream area. Each amino acid mixture was adding to the water inlet of left or right arm for 9 hours in the same concentration as natural water. Of 44 chum salmon tested, 28 fish were found in one of the choice arm and 24 (86%) fish of those were in the arm flowing their artificial home stream water containing amino acid mixture. In sockeye salmon, of 12 fish tested, 8 fish showed upstream movement and 7 (88%) of those were found in the arm flowing their artificial stream water. In contrast, the results of tests using mature sockeye salmon captured in Lake Shikotsu showed that artificial home stream water for Toya Lake Station had no evidence of preference. These results strongly support the hypothesis that amino acids dissolved in stream waters are home river substances for salmonids.

O-V-6

A MULTI-COMPONENT MIGRATORY PHEROMONE IN THE SEA LAMPREY

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The sea lamprey, *Petromyzon marinus*, is a fascinating yet problematic cartilaginous fish which lacks a jaw and is thus ancestoral to modern fishes. Sea lampreys are anadromous; that is, they reproduce in freshwater streams but distribute themselves into oceans or large lakes where they parasitize other fish before maturing and returning to streams to reproduce. Like other anadromous species, sea lampreys locate spawning streams using their sense of smell. However, unlike salmon which learn the odor of natal streams early in life, migratory adult sea lampreys locate streams using a pheromone released by stream-resident larvae. This cue presumably functions as an instinctually recognized indicator of habitat suitability. It is also potent. Behavioral assays demonstrate that individual larval lamprey activate at least 300 liters of stream water in an hour. Removing larvae from streams frequently causes dramatic shifts in adult distribution in the American Great Lakes, a finding which has now been mimicked in the laboratory. A variety of evidence demonstrates that the larval pheromone is comprised of a unique bile steroid, petromyzonol sulfate, and at least one other compound. The unknown component is also derived from cholesterol, has a sulfate group and a molecular weight of 704 daltons. As measured by electrophysiological recording, both compounds are potent, specific odorants with picomolar detection thresholds. Both have also now been measured in natural stream waters and found to evoke strong behavioral responses, especially when tested together as a mixture. The actions of larval odor are further enhanced in a synergistic manner by unknown odors found in natural stream water. Efforts are presently underway to elucidate the complete identity of this multi-component pheromone so that it might eventually be applied to control sea lamprey populations in the Great Lakes where the sea lamprey represents a significant threat to fisheries. Supported by the Great Lakes Fishery Commission and the Minnesota Agricultural Experiment Station.

O-V-7

BEHAVIORAL AND PYSIOLOGICAL RESPONSES OF FEMALE SEA LAMPREY (*PETROMYZON MARINUS*) TO A MALE BILE ACID PHEROMONE

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We recently demonstrated that male sea lamprey (*Petromyzon marinus*) release through their gills two 5α-bile acids, 3 keto-petromyzonol sulfate and 3 keto-allocholic acid, upon spermiation. These compounds induce preference behavior and locomotion in ovulating females under controlled laboratory condition, and may serve as a pheromone that attract the mature females to the nests of mature males. It has yet, however, been demonstrated that a synthetic copy of these bile acids stimulate the olfactory sensory epithelium and induce characteristic behaviors in ovulating females. To provide unequivocal evidence that these compounds function as a sex pheromone, we first used electro-olfactograms (EOG) to characterize their potency. EOG results showed that the synthetic compounds are detected at approximately 10^{-12} M and have a wide range of response dynamics. Cross-adaptation experiments suggest that these two compounds are discriminated from each other, and from the two structurally similar bile acids released by larval sea lamprey. When synthetic 3keto-petromyzonol sulfate was introduced into a section of a spawning stream section to reach a final concentration of 10^{-12} M, it guided ovulating females to search for and stay at the exact site of introduction. In laboratory conditions, 3keto-allocholic acid in combination with 3keto-petromyzonol sulfate induced in mature females behaviors characteristic of those in response to natural pheromones. We conclude that 3keto-petromyzonol sulfate and 3keto-allocholic acid are two essential components of the male sex pheromone. This research is supported by the Great Lakes Fishery Commission.

P-V-1

STEROIDS IN STURGEON'S MIGRATION REGULATION

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In diadromous Russian sturgeon (*Acipenser gueldenstaedti* Br.) serum steroids profiles were elucidated in the sea period of life (North Caspian Sea) and at anadromous migration to river Volga. Cortisol (C), testosterone (T), 11-ketotestosterone (11-KT) (ng/ml) were measured by ELISA in fishes with various gonads state in different seasons. In sea during nutrition period the gonads are far from maturity (early previtellogenesis in females, only spermatogonia in male testis). Serum C and androgens levels in these fishes are low. At the beginning of gametogenesis and preparation to anadromous migration in sea, C, T and 11-KT levels are elevated, differences with the previous period (for male) are significant for T at $p<0,001$. At the beginning of river period of anadromous migration in the spring form sturgeon near to maturity in spring and in the winter form, migrating to river also in spring, but with gonads far from mature stage, high serum concentrations of C and T were determined. For the spring form male C - 126,41±7,85, T - 184,75±22,7; female C - 94,6±18,36, T - 105,20±30,35. For the winter form male C - 94,9±13,29, T - 64,00±26,71; female C - 115,1±24,71, T - 40,97±11,8. No significant differences in serum C levels were observed in spring and winter forms at migration in spite of different gonad CT state. After exception of river period of anadromous migration for the winter form female, maturation is possible in the next spring after long-term reservation (10 - 11 months) at the fish farm. Cortisol profiles in these fishes are lower than during migration (C - 12,71±2,20, $p< 0.001$). The data demonstrate the possible role of C and T in migration regulation in sturgeons.

P-V-2

WINTER SPAWNING OF ALASKA POLLOCK IN THE NORTHERN BERING SEA

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Alaska Pollock (*Theragra chalcogramma*) the most abundant representative of the family Gadidae, is the most important commercial species in the Bering Sea. Total catch exceed about 4 million tons in 1988 and the historical maximum level in northern Bering Sea reached 0,9 million tons in 1981. Study of reproductive biology and migration of Alaska pollock was conducted more than 50 years. The data collected in scientific (1979-1984) and commercial fishery (2001-2003) cruises in December-February. In December prespawning females occurs in the middle and outer shelf (100-175 m), in the first decade of January prespawning females was caught in trawl on the outer shelf and slope (120-400 m). Ripe female was occurred in the mid of January, on the depth 240 m, bottom temperature 1,3 C. At the end of January-middle of February pre spawning females habitat on slope (240-400 m) in warm water (3,3-3,4 C). After spawning females return to the upper slope and outer shelf in the end of February and habitat in cold temperature (1,4 C). Thus, winter spawning of Alaska Pollock took place off shelf zone of northern Bering Sea in January-February.

P-V-3

DEPTH DISTRIBUTION AND TEMPERATURE CONDITION OF SPRING SPAWNING OF WALLEYE POLLOCK IN THE EASTERN BERING SEA

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The main concentrations of Walleye Pollock (*Theragra chalcogramma*) eggs is indicator of spawning grounds. Our research conducted in eastern Bering Sea in 1976-1992. In the cold 1976 in Unimak area spawning took place on the depth 140 meters (bottom temperature 2,7 C), but usually (1980-1992) - 77-98 meters (1,3-3,3 C). The maximum concentrations of eggs are follows: 828 (1976), 3120 (1983), 20376 (1989), 23314 (1990), 3112 (1992) numbers/square meter (No/sq m). Near Pribiloff Islands spawning grounds in cold 1976, 1984 located on the bottom 117 and 95 meters (bottom temperature 1,2 and 1,7 C, respectively), usually -58-83 meters (1,4-2,1 C). The maximum concentrations of eggs are follows: 72 (1976), 9400 (1984), 10360 (1989), 15829 (1990), 120 000 (1991), 6916 (1992) No/sq m. In St. Mattew Island area in cold 1976, 1984, 1990 spawning grounds founded on the outer shelf -133, 126, 175 meters, respectively (surface temperature- 1,4, 1,4, 0,7 below zero C, bottom temperature - 1,0-2,2 C), usually - 117-123 meters (bottom temperature-1,6-2,4 C). Historical maximum level was 960 (1986), 526 (1989), 308 (1984) No/sq m.

P-V-4

OLFACTORY AND ENDOCRINE RESPONSE TO STEROID HORMONES IN AN AFRICAN CICHLID FISH, *HAPLOCHROMIS BURTONI*

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In many fish, steroid and prostaglandin hormones released by conspecifics are detected by the olfactory system and induce a variety of behavioral and physiological responses. Despite evidence that a number of freshwater fish have evolved such*hormonal pheromone*systems, little is known about the pheromones of cichlid fishes (Family *Cichlidae*; Order *Perciformes*). Cichlids appear to be ideal subjects for investigating hormonal pheromone function because they readily reproduce in captivity, their rich behavioral repertoires should facilitate the development of behavioral pheromone bioassays, and their biodiversity and varied reproductive tactics provide numerous opportunities for comparative studies. Electro-olfactogram (EOG) studies using more than 150 synthetic steroids and prostaglandins show that the African rift lake cichlid, *Haplochromis burtoni*, does not detect prostaglandins but does detect a variety of conjugated (sulfated or glucuronidated) 18-, 19-, and 21-carbon steroids with great specificity and sensitivity (0.01-1 nM olfactory detection threshold). EOG cross-adaptation and binary mixture experiments indicate that these steroids act through five independent olfactory receptor mechanisms. Behavioral bioassays involving exposure to single steroid odorants provided no evidence of behavioral response; behavioral bioassays have not been conducted with odor mixtures. Endocrine bioassay involving exposure to a mixture containing steroids acting on all five olfactory receptor types (testosterone sulfate; estradiol-17-glucuronide; estradiol-3,17-disulfate; dehydroepiandrosterone-3-sulfate; 5 β-pregnan-3α,17 α-diol-20-one-3-glucuronide) significantly increased male serum testosterone levels within 60 min; experiments to identify the active steroid(s) are in progress. These results are the first to demonstrate hormonal pheromone activity in *Haplochromis*, and suggest hormonal pheromones could provide novel approaches for examining the explosive speciation of African rift lake cichlids.

P-V-5

NEWLY DEVELOPED ODOR-STIMULATED LABELING METHODS OF OLFACTORY RECEPTOR NEURONS OF SOCKEYE SALMON USING AGMATINE

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Approach of analyses to investigate an olfactory responsiveness with high resolution is essential to understand many olfactory functions in connection with imprinting and homing phenomena of salmonid fishes. The present study was conducted to develop the methods for odor-stimulated labeling of olfactory receptor neurons in the olfactory epithelium and brains of sockeye salmon (*Oncorhynchus nerka*) using a cationic molecule, 1-amino-4-guanidobutane (agmatine; AGB). First, the ability of the olfactory receptor cells (ORCs) to response to an odor stimulus was examined with 10mM AGB or 10mM AGB plus 1mM alanine stimulus to the olfactory epithelium (OE) during smoltification of sockeye salmon. It was demonstrated that ORCs were labeled in OE during stimulation with AGB as a control stimulus, suggesting AGB is olfactory stimulus for sockeye salmon. Moreover, alanine increased the percentage of labeled olfactory cells compared to the AGB control. Alanine sensitive ORCs were located superficially in OE, and immuno-electron microscopy demonstrated that the labeled ORCs were predominately microvillous ORCs. Secondly, the ability of central nervous system to response an odor stimulus was examined by a continuous perfusion of 10mM AGB dissolved in artificial spinal fluid in the forebrain simultaneously stimulating to the olfactory rosette by alanine stimulus. It was revealed that AGB immunoreactive cells were identified mitral cells in the olfactory bulb and neurons in the telencephalon. These results reveal that AGB can be used as an activity-dependent label for olfactory receptor neurons in sockeye salmon and microvillous ORCs are capable of detecting alanine odor.

P-V-6

CHANGES IN GONADOTROPIN-RELEASING HORMONES LEVELS IN THE BRAIN AND PITUITARY GLAND DURING MIGRATION OF SALMONID FISHES

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Gonadotropin-releasing hormone (GnRH) are produced in the various brain regions, and might be involved in many physiological functions of teleost life cycle. In order to clarify GnRH roles on various aspects of salmonid life history and migration, measurements of two molecular types of GnRH, salmon GnRH (sGnRH) and chicken GnRH-II (cGnRH-II), were conducted using specific time-resolved fluoroimmunoassay (TR-FIA) systems. We examined the correlations between six regions of brain and pituitary GnRH levels and migratory behavior during both downstream migration of smolt sockeye salmon (*Oncorhynchus nerka*) and spawning migration of adult chum salmon (*O. keta*). During smoltification of sockeye salmon, sGnRH levels in both female cerebellum and male telencepharon and medulla oblongata were higher in May than in April, whereas only cGnRH-II in medulla oblongata of both sexes increased in May. These changes in GnRH levels coincided with serum thyroid hormone levels. Changes in GnRH levels of adult chum salmon were also investigated during spawning migration from the Bering Sea to the Chitose River. sGnRH levels in the pituitary elevated from the Bering Sea to the coastal sea or estuary of the Ishikari River consistent with a peak of ovarian GTH-I levels and pituitary GTH-II levels in both sexes. Additionally, sGnRH levels in the olfactory bulb and telencepharon increased at the coastal sea and the branch point of the Chitose River from the Ishikari River, respectively. cGnRH-II levels in male optic tectum increased at the pre-spawning ground. These results suggest that sGnRH in the pituitary play a role in gonadal maturation of chum salmon, and sGnRH and cGnRH-II in various brain regions might have different roles in smoltification and spawning migration of salmonid fishes.

P-V-7

ENERGETICS OF REPRODUCTION AND SPAWNING MIGRATION FOR PACIFIC SAURY (*COLOLABIS SAIRA*)

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Pacific saury is a small (up to 35 cm SL) pelagic fish, feeding actively in the food-rich sub-arctic water (10 – 12 °C) during summer (June – August) and spawning in the food-poor sub-tropical water (20 – 22 °C) during winter (December – April). This fish is supposed to experience one or two spawning seasons in its lifetime. Changes in protein, lipid, and energy content for 30 cm standardized fish from July to February were examined. Protein content remained approximately constant, 21.4 – 23.8 g, during study period. In contrast lipid content drastically decreased from 37.6 g in the feeding season (July) through 20.9 g around the middle of spawning migration (October – November) to 1.4 g in the spawning season (February). Neutral lipid as an energy source in February was only 0.53 g (21.1 kJ), indicating that most of the allocated energy to egg production was derived from the energy content of food consumed during spawning season. A batch of 2,400 eggs (20.8 kJ, 4.8 g wet weight) was spawned at an interval of 3.7 days, thus a daily amount of energy requirement for egg production was estimated to 5.6 kJ. This value was as much as 35.6 % of an estimated daily acquisition of energy by feeding (15.8 kJ, 4.9 g wet weight per day), indicating that feeding condition during the spawning season would strongly influence the reproductive activity.

P-V-8

CHANGES IN NEUROSTEROID BIOSYNTHESIS DURING SEXUAL MATURATION OF LACUSTRINE SOCKEYE SALMON

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Steroid hormones synthesized from cholesterol in the brain and pituitary gland of vertebrate are generally defined as neurosteroids. In teleost fish, the gonad and adrenal gland are the main sites for steroid biosynthesis, while little is known about the neurosteroid biosynthesis in the brain and pituitary gland. The present study was conducted to investigate the correlations between neurosteroid biosynthesis and their possible roles on sexual maturation of lacustrine sockeye salmon (*Oncorhynchus nerka*). The ability of salmon brain and pituitary gland to synthesize and metabolize cholesterol was examined by in vitro thin-layer chromatography method with radioactively labeled cholesterol during sexual maturation. It was demonstrated that pregnenolone (P5) and 17α-hydroxypregnenolone (17α-OH-P5) were identified as cholesterol metabolites in the brain and pituitary gland. The radioactivity of 17α-OH-P5 band was higher than that of P5 and greatly exceeded those of the other cholesterol metabolites. The conversion of P5 to 17α-OH-P5 in the brain and pituitary gland showed a clear regional difference, and tended to be higher in the diencephalon and pituitary gland than in the other brain regions. Moreover, the 17α-OH-P5 productivity in the ovary and testis increased during sexual maturation and peaked at the final stage of gonadal maturation, whereas that in the brain and pituitary gland peaked at the pre-spawning period in both sexes. These data reveal the evidence of the P5 and 17α-OH-P5 productivities in the brain and pituitary gland suggesting the existence of cytochrome P450 side-chain cleavage enzyme and cytochrome P450 17α-hydroxylase/c17,20-lyase enzyme in the brain and pituitary gland of lacustrine sockeye salmon. The activities of these steroid converting enzymes in the brain and pituitary gland might be involved in brain region-dependent roles on sexual maturation.

P-V-9

OLFACTORY RESPONSES TO PUTATIVE STEROIDAL PHEROMONES IN ALLOPATRIC AND SYMPATRIC SPECIES OF *SYNODONTIS* CATFISH

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Many fish exhibit olfactory, behavioral and physiological responses to waterborne prostaglandins, steroids and their metabolites, responses assumed to reflect pheromonal functions of hormonally derived products released by conspecifics. Because previous studies demonstrate widespread olfactory detection of similar hormonal products among lower taxa (i.e. within genera and among related genera), and because the hormones from which putative hormonal pheromones are derived exhibit limited chemical diversity, it is unclear if or how fish hormonal pheromones exhibit species specificity. We approached this question by studying olfactory detection of steroids in a widely distributed and speciose (<100 species) genus of African catfish (*Synodontis*; family Mochokidae), to compare patterns of detection within and among sympatric and allopatric congeners. We used electro-olfactogram recordings (employing >150 synthetic steroids and steroid conjugates) to assess olfactory detection in 19 species from northern rivers (Chad, Niger, Nile and Volta; N = 6 " species") and the mid-continental Zaire River (N = 13 " species"). All 19 species detected only unconjugated C19 and C21 steroids and all detected a similar suite of C19 steroids: etiocholanolone (5β-androstan-3α-ol-17-one) and the related 5β-androstan-3α,17β-diol and 5β-androstan-3α-ol-11,17-dione. In contrast, northern and southern species detected different C21 steroids. Northern species detected 5β-pregnan-3α,17,20β-triol and 5 α-pregnan-3α,17,20 β-triol, whereas southern species detected only compounds lacking the 20β-hydroxyl (5β-pregnan-3α,17,21-triol-11,20-dione; 5β-pregnan-3α,17,21-triol-20-one; 5β-pregnan-3α,17-diol-20-one. Assuming the detected steroids subserve pheromonal functions, the observed patterns of detection suggest simple geographical divergence of northern and southern species rather than active selection for species specificity. It remains to be determined whether the apparent absence of species specificity within northern and southern species might result from factors that would reduce selection for specificity, such as small active space of the putative pheromones and spatial or temporal microhabitat isolation during reproduction.

P-V-10

PERIPHERAL CODING OF SEX PHEROMONE INFORMATION IN THE GOLDFISH OLFACTORY EPITHELIUM

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Sex pheromones are one of the most important environmental signals used by fish to synchronise their reproductive behavior and maturation. These pheromones and other odors are detected by olfactory epithelium which contains three morphologically different types of olfactory receptor neurons (ORNs): ciliated ORNs (cORNs), microvillous ORNs (mORNs) and crypt cells. Very little is known about the tuning properties of these cells. In fish, a patch-clamp study of rainbow trout ORNs has shown that while many cORNs are "" which respond to a wide variety of odorants, mORNs are "" specific to amino acids. Notably, the cell types and tuning specificities of pheromone-sensitive ORNs have not yet been identified in any vertebrate. Our study sought to determine the specificity of goldfish ORNs to identified pheromones and other biologically relevant odorants. We examined the molecular receptive range of goldfish ORNs to food related odors (amino acids, nucleotides and polyamines), social aggregants (bile acids) and sex pheromones (prostaglandins and sex steroids) using *in vivo* extracellular single-unit recording. Spike activity was recorded from 103 single-units while fish were exposed to six odor mixtures, each comprised of 1-4 odorants from a single class of odorants. A total of 57 (55.3%) units responded to either one (46.6%), or more than two odor classes (8.7%). Amino acids sensitive units were the most common (34.0%), whereas only a few units responded to either prostaglandins or sex steroids (8.7% for each). All generalist units responded to amino acids. In contrast, sex steroid sensitive units responded to just one steroid and then with high-sensitivity (less than 10^{-8}M). ORNs tended to respond to odor mixtures with excitation (72.1% of all responses), although suppression (19.1%), and oscillation (8.8%) were also noted. These results demonstrate that while the group of amino acid sensitive ORNs includes generalist units which detect multiple odors, those ORNs which detect sex steroid pheromones are all highly-tuned. We conclude that sex pheromone information in the goldfish olfactory epithelium is coded by the activation of specific and unique sets of ORNs. Supported by NIH/DC03792.

P-V-11

METHYLTESTOSTERONE INDUCES MALE SENSITIVITY TO BOTH PRIMER AND RELEASER PHEROMONES IN THE URINE OF OVULATED FEMALE MASU SALMON

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In salmonid fishes, mature female urine has been known to act as sex pheromones having primer and releaser effects. It was reported that responses to a releaser pheromone, which induces specific behaviors in males, are controlled by androgens. In this study, we investigated the optimum term of 17 α-methyltestosterone (MT) -treatment for behavioral test and changes of plasma sex steroids levels in MT-treated yearling immature male masu salmon, *Oncorhynchus masou* parr. The experiments were conducted using immature males treated with MT and the control immature males between May and July. Their behavioral responses to the urine of ovulated or immature females, or well water were observed in an experimental trough. After 5 min of behavioral tests, plasma testosterone (T), 11-ketotestosterone (11-KT) and 17,20 β-dihydroxy-4-pregnene-3-one (DHP) levels were measured by RIAs in same individuals. MT-treated immature males showed specific behavioral responses only to the ovulated female urine from 2 to 7 weeks. In all the MT-treated immature males, the plasma T and 11-KT decreased after the beginning of MT-treatment. MT-treated fish without exposure to the urine showed no clear changes in plasma DHP levels that are known as an indicator for a primer effect in male teleosts. However, plasma DHP levels in MT-treated immature males increased when they were exposed to the urine. These results suggest that MT-treatment induces male sensitivity not only to a releaser effect but also to a primer effect of female sex pheromones in masu salmon.

P-V-12

QUANTIFYING SPONTANEOUS SWIMMING ACTIVITY IN MARINE AND FRESHWATER FISH WITH THE *ETHOVISION® COLOR-PRO* COMPUTERIZED VIDEO TRACKING SYSTEM, A LABORATORY DEVICE BASED ON DIGITAL IMAGING TECHNIQUES

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Thanks to computing power offered by digital imaging techniques (D.I.T.), laboratory studies on fish have now the capabilities to detect and to quantify behavioral parameters like traveled distance, swimming speed which usually cannot be measured by methods of direct observation. After adjusted to fish studies the EthoVision® system, a recent commercial (Noldus) ethometric device based on D.I.T., we conducted experiments concerning several questions relating to effects of various external environmental factors on behavioral patterns in *Chromis chromis* (Mediterranean marine fish) and in the freshwater fish *Carassius auratus*. Since temperature is almost certainly one of the most important abiotic environmental effectors influencing reproductive behavior, we tested in laboratory (aquariums) its effects on swimming freely fishes using the EthoVision® Color-Pro video tracking system for Windows. Four experimental aquariums (L100xW50xD48 cm reduced in arenas of L68xW30xD48 cm) with 1 or 2 fishes in each can be tacked simultaneously by the system with 4 CCD video cameras positioned in front of the arenas recording the scene. The analog video signal is fed into the frame grabber of the computer, which running the EthoVision® software. EthoVision® process 5 digitized images per second, stores them in the memory, extracts a range of image features and writes the moving objects' X-Y coordinates on the disk, ready for tracks display and behavioral parameters computing. So, we measured for each individually identified fish the distance moved (DM), the velocity (V), the social interactions reflected by the distance between the two animals (DO) and the time spent in the defined zones (IZ) of the aquarium (sandy-bottom, middle-water, etc.), according to water temperature, fish density and periods of the day. To track 2 fishes in the same tank, we used fluorescent color plastic pearls (this 1 cm marker has no significant effect on DM, V and IZ) attached under the dorsal fin of damselfish and natural color patterns in goldfish. Presenting the major results of our experiments, we illustrate the new possibilities of this ethometric method to explore the relations between fish and its biotic, abiotic or xenobiotic environments and also its potential interest in aquatic sciences as fish welfare or fish ecotoxicology.

Session VI

Gametogenesis II

O-VI-1

GENES EXPRESSED AT THE ONSET OF OOGENESIS IN MEDAKA (*ORYZIAS LATIPES*)

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The earliest morphological differences between female and male medaka appear at hatching, when female germ cells start to undergo oogenesis. We have identified many genes expressed in female germ cells at these earliest stages of oogenesis by subtractive hybridization. Some of the genes that were identified by their sequences encode ZP (zona pellucida) domain containing egg envelope proteins, oocyte-specific RNA binding proteins, and a transcription factor containing a basic helix-loop-helix motif. The last gene is a homolog of mouse *fig* α, a possible master regulator of oogenesis. To provide insights into the diversity of egg envelope genes in teleosts, we determined the genomic organization and the map position of the medaka egg envelope genes containing ZP domain. The number of teleost ZP genes was increased by extensive gene duplication, which may have caused liver-specific ZP gene evolution in teleosts. Finally, we will briefly discuss our recent results on transcriptional regulation of *fig*α and the ZP genes.

O-VI-2

EFFECTS OF STEROID AND PEPTIDE HORMONES ON *IN VITRO* GROWTH OF PREVITELLOGENIC OOCYTES FROM EEL, *ANGUILLA AUSTRALIS*

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During previtellogenesis, increases in mitochondria and accumulation of lipids and cortical alveoli are the most noticeable changes. Association of the oocyte with somatic cells also occurs during this time. Despite the qualitative and quantitative (at least 5-10 fold increase in diameter) changes of the oocytes during previtellogenesis, the mechanisms controlling their growth remain essentially unknown. A previous study showed that *in vivo* treatment of eels with the non-aromatizable androgen 11-ketotestosterone (11-KT) resulted in increased diameters of previtellogenic oocytes, implicating androgens in regulating previtellogenic growth in this species. Growth hormone and growth factors may also participate in regulating previtellogenic growth since nutrition can affect puberty and fecundity in teleosts. We tested this hypothesis by culturing previtellogenic eel (*Anguilla australis*) ovarian explants for 18 days in the presence or absence of steroid or peptide hormones at a range of doses.

In vitro treatment with 1-1000 ng/mL 11-KT led to a significant increase in oocyte diameters whereas estradiol-17β had no effect. Similarly, oocyte diameters were larger when explants were incubated in the presence of 30 or 1000 ng/mL insulin-like growth factor-I (IFG-I) compared to control incubations or compared to incubations containing insulin, ovine growth hormone or human chorionic gonadotropin. These data provide the first indication of direct effects of 11-KT and IGF-I on growth of previtellogenic oocytes and provide a basis for advancing our understanding of the endocrine control of early previtellogenesis in fishes.

O-VI-3

ENDOCRINE CHANGES ASSOCIATED WITH THE GROWTH OF PRE-VITELLOGENIC OOCYTES IN COHO SALMON

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Salmonid maturation can be profoundly influenced by alterations in growth. It has been hypothesized that there are two periods when growth can alter the decision to mature; thus, for a fall spawning species, an 'initiation' period in the fall is followed by a 'permissive' period for continuing maturation in the spring. One of the major aims of our laboratory is to determine the endocrine mechanism by which alterations in growth affect changes in the reproductive axis. Research using mammalian and avian models have shown that follicle-stimulating hormone (FSH), steroidogenic acute regulatory protein (StAR), insulin-like growth factor I (IGF-I) and 17β-estradiol are key components controlling oocyte growth. In the present study we sampled two-year-old female coho salmon during the proposed fall 'initiation' and spring 'permissive' periods. Histological analyses of the ovaries indicated that by May (6 months prior to spawning) oocytes in many individuals had reached the lipid droplet stage. Both maximum oocyte diameter and the degree of oocyte development were positively correlated to body size. Levels of StAR mRNA in the ovary were positively correlated with plasma 17β-estradiol levels, and both were positively correlated with body size. Furthermore, StAR mRNA levels were positively correlated with FSH receptor mRNA levels in the ovary. Females with oocytes at early stages of cortical alveoli incorporation had significantly lower levels of StAR, FSH receptor and IGF-I mRNA in their ovaries compared to females with ovaries with more advanced oocytes at the oil droplet stage. Further data on the relationship between mRNA expression in the ovary, plasma sex steroid, plasma IGF-I and plasma and pituitary FSH levels, ovary and body growth during the fall and spring critical decision periods will be presented.

O-VI-4

THE ROLE OF LIPOPROTEIN LIPASE (LPL) IN THE INCORPORATION OF NEUTRAL LIPIDS INTO THE OOCYTES OF THE EUROPEAN SEA BASS (*DICENTRARCHUS LABRAX* L.) DURING GONADAL DEVELOPMENT

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In most teleosts, vitellogenin (VTG) is the major precursor of yolk proteins and phospholipids. However, oocytes of perciform fish also accumulate high amounts of neutral lipids, which are stored in the oil droplet. The origin of these lipids is unknown but very low density lipoproteins (VLDL), which are rich in triacylglycerols (TAG) could play an important role in the transport of lipids into the ovary. As for VTG, synthesis of VLDL is stimulated by 17β-estradiol in the liver. A specific enzyme, lipoprotein lipase (LPL), cleaves fatty acids from VLDL TAGs, which are then incorporated into extrahepatic tissues where they can be re-esterified. We have cloned the cDNA encoding LPL and studied its mRNA expression and enzyme activity in the ovary of European sea bass in order to investigate a possible role of this enzyme in the incorporation of neutral lipids into the oocytes. Two identical clones of 3051 bp with an open reading frame of 1554 bp encoding 518 amino acids were obtained from a sea bass ovarian vitellogenic cDNA library. The amino acid sequence was 75.4%, 73.1%, 64.5%, 85.8% and 86.5% similar to LPL in human, chicken, red seabream, zebrafish and rainbow trout, respectively. By Northern blot, a transcript of about 3 kb was observed in adipose tissue, ovary and testis. LPL activity was high in ovaries throughout oogenesis while mRNA expression was only found during the second part of vitellogenesis in ovaries of animals with a GSI higher than 5. Using in situ hybridisation, LPL mRNA expression was detected in follicular cells of mid- and late-vitellogenic oocytes coinciding with the presence of a high number of lipid vacuoles in the cytoplasm. The results suggest that LPL is likely to play an important role in the incorporation of neutral lipids into the oocytes, and that the follicular cells not only participate in steroidogenesis but may also be important for the accumulation of nutrients into the oocytes.
Supported by a Spanish Ministry of Science and Technology grant (AGL2000-1283) to F.P.

O-VI-5

MULTIPLE VITELLOGENINS AND THEIR UNIQUE ROLES IN MARINE TELEOSTS

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Multiplicity of vitellogenin (Vg) gene has been reported in some teleost species based on cDNA or protein analyses. Two distinct types of Vg cDNA have been discovered in mummichog (VgI and II), haddock and barfin flounder (VgA and B). Both of the two types contain yolk protein domains as following arrangement in linear fashion: N-lipovitellin (Lv) heavy chain-phosvitin (Pv)-Lv light chain-beta' component -C-terminal coding region-C. Furthermore, an unusual type of Vg which lacks polyserine Pv domain was discovered in zebrafish and Japanese common goby. We recently found all the three types of Vg in mosquitofish and analyzed their cDNA sequences. In addition, partial sequence analysis of cDNA fragments using primer sets for each type of Vg in seven teleost species demonstrated existence of more than two different types of Vg in the all seven species. Among these Vgs, VgA and VgB have unique roles in regulation of egg buoyancy through a limited proteolysis of Lvs during final oocyte maturation in barfin flounder and walleye pollock. The quantitative ratio of accumulated VgA to VgB in postvitellogenic oocytes was thought to be a key by controlling yielded free amino acids which function as osmotic effectors for oocyte hydration. Estimated composition and quantity of free amino acids calculated from cDNA sequences combined with information from yolk protein analyses coincided well with the results of actual measurement of free amino acids in ovulated eggs. Specification of a protease involved in the maturation-associated proteolysis of the barfin flounder oocyte was conducted using an *in vitro* assay based on the molecular alteration of Lvs with various protease inhibitors. The results suggest the involvement of cathepsin B (like) protease in the limited proteolysis of Lvs. Measurement of pH in oocytes at various stages of oocyte maturation in barfin flounder demonstrated an occurrence of a drastic change of inner pH from 5.9 in postvitellogenic oocytes to 5.0 in oocytes at mid-stage of oocyte maturation. The pH was then neutralized to 6.9 prior to ovulation. Thus, the protease activity is suggested to be controlled by the inner pH of oocytes.

O-VI-6

OOCYTE GROWTH AND CYTOPLASMIC MATURATION OF TEMPERATE BASSES: MULTIPLE VITELLOGENINS AND THEIR RECEPTOR

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Recent gene cloning and biochemical studies confirmed that the presence of multiple forms of vitellogenin (Vg) and its receptor (VgR) might be the norm in oviparous vertebrates, although this remained to be verified in highly advanced perciforms such as *Morone* species. A full-length cDNA (4021 bp) encoding a polypeptide (886 amino acids) with typical characteristic features of a vertebrate VgR was isolated from a vitellogenic white perch (*Morone americana*) ovarian cDNA library. The predicted perch VgR lacked an *O*-linked sugar domain and no trace of a second *O*-linked form of VgR was found in perch ovaries or liver by RT-PCR, unlike the case in all other vertebrates investigated. These results call into question the concept that a splice variant of the VgR, bearing an *O*-linked sugar domain, is normally present in somatic tissues to sequester lipoproteins distinct from Vg. A real-time quantitative RT-PCR assay confirmed that the main site of perch VgR mRNA expression is the ovary. Similar to results from other vertebrates, the highest level of VgR mRNA expression was in ovaries containing pre-vitellogenic oocytes. For the first time in any fish, three immunologically distinct forms of Vg (VgA, VgB, and VgC) were isolated from plasma of estrogen-treated perch. Biochemical analysis and peptide sequencing verified that perch VgA is a homologue of VgA from barfin flounder and haddock, whose major yolk protein derivative (lipovitellin I) is degraded extensively during final oocyte maturation (FOM). Perch VgB is a homologue of VgB from these species, and VgB-derived lipovitellin I generally undergoes only partial proteolysis during FOM. Perch VgA and VgB had identical molecular weights (native/monomer; ~532 kDa/~180 kDa), while perch VgC (~426 kDa/~148 kDa) appears to be a minor but novel form of Vg that is similar to zebrafish Vg3. These findings differ in essence from prior studies of other teleost species and should help to create a new paradigm for investigation of ovarian yolk deposition and utilization in oviparous vertebrates.

O-VI-7

REGULATION AND MECHANISMS OF OOCYTE MATURATIONAL COMPETENCE

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The oocyte ability to resume meiosis (maturational competence) in the presence of maturation-inducing hormone (MIH) as a distinct event of oogenesis, and its relationship with gonadotropins were first described for a few fish and amphibian species almost 30 years ago. Later studies showed this phenomenon to be widespread among fishes. More recently, growing interest in this area of fish reproductive biology has yielded considerable advances in our understanding of physiological, cellular and molecular mechanisms of oocyte maturational competence. The data for teleosts generally indicates that maturational competence develops gradually during late vitellogenic growth or rapidly near the completion of growth, and that it depends on gonadotropin (most likely LH) stimulation of the intact ovarian follicle. Gonadotropin-dependent maturational competence requires protein kinase A activation and de novo transcription and protein production, but not steroid production. Some evidence suggests that activins or insulin-like growth factors mediate the actions of gonadotropin in a paracrine or autocrine fashion. Increased connexin (gap junction protein) mRNA levels and homologous and heterologous gap junction contacts are observed in ovarian follicles of some species, indicating that changes in degree or pattern of communication among granulosa cells, and between granulosa cells and the oocyte, are important for the regulation of maturational competence. Gonadotropin also stimulates oocyte MIH receptor activity, which is the likely functional basis for the acquisition of maturational competence. Finally, there is recent evidence indicating that gonadotropin stimulates ovulatory competence (ability of follicle to ovulate in the presence of MIH) almost coincidentally with maturational competence, but the mechanisms of this stimulation are unknown. Gonadotropin then stimulates MIH production, which sequentially stimulates oocyte meiotic resumption and ovulation. Thus, the regulation of maturation and ovulation may be more closely integrated than previously believed.

O-VI-8

SPECIFIC GENE EXPRESSION PROFILES ARE ASSOCIATED WITH FOLLICULAR MATURATIONAL COMPETENCE ACQUISITION IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

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The joint use of in vitro oocyte maturation and real-time PCR was explored in rainbow trout in order to characterize the expression profiles of Luteinizing Hormone receptor (LH-r), Follicular Stimulating Hormone receptor (FSH-r), Insulin-like Growth Factor 1 (IGF1), Insulin-like Growth Factor 2 (IGF2), Insulin-like Growth Factor receptor 1a (IGF-r1a) and 20beta-hydroxysteroid dehydrogenase (20beta-HSD) during follicular maturational comptence acquisition (FMC). Post-vitellogenic female rainbow trout (Oncorhynchus mykiss) were assayed in vitro for follicular maturational competence (FMC). Ovarian follicles were stimulated in vitro for 60h at 12°C with a range of concentrations of partially purified gonadotropin and the efficient concentration for 50% germinal vesicle breakdown was calculated and used as an indicator of follicular maturational competence. Before in vitro assay, ovarian tissue was sampled in order to quantify mRNA abundance in the ovarian follicle by real-time PCR. In addition, maturation-inducing steroid (MIS, 17, 20beta-dihydroxy-4-pregnen-3-one) and Estradiol (E2) plasma levels were measured by radioimmunoassay. The mRNA expression of LH-r, FSH-r, IGF1, IGF2, IGF-r1a and 20beta-HSD, that are putatively expressed in the preovulatory ovary, was studied in females of varying FMC using real-time PCR. FMC acquisition is characterized by an increase of MIS circulating levels and a concomitant drop of E2 levels. At the ovarian level, no significant variation of LH-r, 20beta-HSD, IGF1 and IGF-r1a mRNA abundance was observed among females of varying FMC. In contrast, FSH-r and IGF2 mRNA levels were significantly higher in females exhibiting high FMC. In addition, correlation analysis showed that IGF2 and FSH-r mRNA levels were positively correlated with FMC. These results indicate that FMC acquisition is associated with an increased expression of these gene products that may be useful markers of FMC.

O-VI-9

STEROIDOGENIC SHIFT IS A CRITICAL EVENT FOR OVARIAN FOLLICLES TO UNDER GO FINAL MATURATION

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The steroidogenic pathway of the granulosa-thecal layers of fish ovarian follicles under goes a distinct shift from the production of estradiol-17β (E_2) to 17α,20β-dihydroxy-4-pregnen-3-one (17α,20β-DP, maturation-inducing hormone) prior to final oocyte maturation (FOM). This is implicated through the enzyme genes that are required for the production of E_2 (cytochrome P-450 aromatase, *P450arom*) and 17α,20β-DP (20β-hydroxysteroid dehydrogenase, *20β-HSD*) and its transcription factors. A search for cis-element motifs in *P450arom*and *20β-HSD* genes indicated the presence of Ad4s and CREs, which prompted us to clone *Ad4BP/SF-1* and *CREB*. Both *P450arom* and *Ad4BP/SF-1*transcripts increase synergistically during vitellogenesis and became undetectable during FOM. Incubation of vitellogenic follicles with hCG, in vitroenhanced the expression of *P450arom* and *Ad4BP/SF-1* while similar treatment for post-vitellogenic immature follicles purged their expression. These results designate *Ad4BP/SF-* as a transcriptional regulator for *P450arom* in fish ovarian follicles. In granulosa cells, rapid expression of *20β-HSD* gene occurs under the influence preovulatory luteinizing hormone surge during FOM. No *20β-HSD* expression is observed in post-vitellogenic immature follicles. The induction of *20β-HSD* gene expression in post-vitellogenic follicles followed by oocyte maturation by hCG and actinomycin D-induced blockade of hCG-induction revealed the involvement and transcriptional regulation of *20β-HSD* in FOM. Series of 5' -deletion mutants of *20β-HSD* gene identified CRE but not Ad4 consensus motif is responsible for the promoter activity. Among several *CREBs*, one shows a synergistic pattern of expression with *20β-HSD* indicating the regulation of former over the latter. Taken together, the triggering of steroidogenic shift by gonadotropins is manifested through subjugation of *Ad4BP/SF-1* expression *vis-à-vis P450arom* followed by the induction of over expression of*20β-HSD* probably via the *CREB*.

O-VI-10

CLONING OF EPIDERMAL GROWTH FACTOR (EGF) AND EGF RECEPTOR (EGFR) FROM THE ZEBRAFISH OVARY AND THE POTENTIAL ROLES OF EGF IN THE REGULATION OF OVARIAN ACTIVIN/FOLLISTATIN SYSTEM

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In the present study, we cloned full-length cDNAs for both epidermal growth factor (EGF) and EGF receptor (EGFR) from the zebrafish ovary, which represents the first one in non-mammalian vertebrates. The EGF cDNA encodes a large membrane-anchored protein of 1114 amino acids with 7 characteristic EGF-like motifs in the extracellular region. The potential mature EGF peptide contains 53 amino acids with significant sequence homology (53-60%) with that of mammals. Zebrafish EGFR is also a large membrane protein consisting of 1191 amino acids, and it shares 63% homology with EGFR from other vertebrates. In the ovary, Northern and RT-PCR analysis demonstrated that both EGF and EGFR are expressed in the follicles of all stages; however, the expression of EGF seems more abundant in the follicles of earlier developmental stages, while EGFR has higher expression levels at later stages. We have previously demonstrated that recombinant human EGF enhances zebrafish oocyte maturation and its effect is likely mediated by the intra-ovarian activin system. To further elucidate the role of EGF in the regulation of ovarian activin system, a primary culture of zebrafish ovarian follicle cells was used to examine the effects of EGF on the expression of activin βA, βB and follistatin (an activin-binding protein). Recombinant human EGF strongly stimulated the expression of both activin βA and βB in the absence or presence of hCG, while it suppressed both the basal and hCG-induced follistatin expression in time- and dose-dependent manners. Although both have been demonstrated to stimulate activin βA expression by our previous studies, hCG and EGF exhibited no additive effect in the present study. Interestingly, although hCG suppressed the basal expression of activin βB, it had no effect on EGF-stimulated βB expression. The present study provides convincing evidence that both EGF and EGFR are expressed in the fish ovary, and that EGF regulates the expression of both activin and its binding protein follistatin in the zebrafish ovary. These results strongly suggest that EGF is a potential autocrine/paracrine regulator of ovarian functions and its actions in the ovary are, at least partially, related to the intra-ovarian activin-follistatin system. [The work was substantially supported by grants (CUHK4176/99M, CUHK4150/01M and CUHK4258/02M) to W. Ge from the Research Grants Council of the Hong Kong Special Administrative Region.]

P-VI-1

MATERNAL mRNA STOCKPILE AND OOCYTE DEVELOPMENTAL COMPETENCE IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

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Developmental competence (egg quality) can be defined as the oocyte ability to be fertilized and to ensure a normal embryonic development. The purpose of this study was to identify molecular markers of rainbow trout (*Oncorhynchus mykiss*) oocyte developmental competence. During oogenesis, large amounts of maternal mRNA are stockpiled in the oocyte and are essential for embryonic development. Therefore, several transcripts were chosen for their putative involvement in early embryonic development and the correlation between the abundance these transcripts in the oocyte and the oocyte developmental competence was investigated. For this purpose, post-ovulatory ageing was used as a tool to generate oocytes of varying developmental competence. Mature female were held at 12°C and periodically checked for ovulation. Oocytes were thus collected from each female at ovulation and 5, 14, 21 days later. For each collected egg batch, fertilization was performed (using a pool of sperm originating from five males), egg development was monitored and the abundance of several mRNAs in the oocyte was analyzed by real-time PCR. Egg quality was estimated not only through embryonic survival at eyed-stage and yolk sac resorption but also by studying the occurrence of specific morphological abnormalities (such as Cyclops, spinal cord torsion, Siamese, spiraloid torsion, no-caudal fin, prognathes) detected at yolk-sac resorption. Our results showed that the abundance of specific messenger RNAs was correlated with either the embryonic survival or the occurrence of malformations. Thus, we report, for the first time in a teleost, a significant correlation between the maternal stockpile of specific mRNAs and the developmental competence of the oocyte.

P-VI-2

THE POSSIBLE INVOLVEMENT OF THE RETINOIC ACID PATHWAY IN THE REGULATION OF REPRODUCTIVE FUNCTION IN ZEBRAFISH

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There have been few studies conducted on the role of retinoic acid (RA) and its metabolites in reproductive processes of fish. Our earlier studies describe the binding characteristics of two types of RA receptors (RAR and RXR) in a variety of fish tissues, including their distribution in ovarian and testis tissue using a radioligand competitive binding assay (Alsop et al. 2001 Gen Comp Endo 123:254-67). The purpose of the present study is to develop a model by which we can assess the role of RA in the development and maturation of zebrafish gonads. Our initial studies focussed on the expression of the RA synthesizing enzyme Raldh2, metabolizing enzyme Cyp26, and RA receptors RARalpha/gamma and RXRalpha/gamma/delta/epsilon by RT-PCR. All transcripts except RXRgamma were expressed in the ovary. Additional studies were conducted in an effort to deplete the storage forms of RA in adult zebrafish by feeding a retinoid deficient diet. By 70 d, zebrafish had lost approximately 95% of the whole body retinyl esters. By 174 d, retinyl ester levels were non-detectable in 7 of 8 fish and retinol had decreased by 88%. The primary retinoid after 70 d was retinal and this was localized in the eye possibly due to its fundamental role in vision. Ongoing studies are examining gonadal development by histological methods in zebrafish collected on 174 d. These initial data provide the impetus for further studies examining the role of RA in reproductive processes of fish.

P-VI-3

EFFECTS OF METHYLTESTOSTERONE ON VTG LEVELS AND BRAIN AND GONAD AROMATASE ACTIVITY IN THE ZEBRAFISH (*DANIO RERIO*)

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Vitellogenin (vtg) is a well known biomarker for testing estrogenic compounds in male fish. However, preliminary result has showed that the production of vtg can also be induced by low concentrations of methyltestosterone in male zebrafish. This phenomenon has previously only been observed in fish exposed for methyltestosterone for long periods or in fish exposed to high concentrations of MT. This has been ascribed the conversion of MT by the aromatase. This study investigates if this induction of vtg can be reflected in the activity of the aromatase in brain and gonads. A dose-response relationship of induction of vtg and aromatase activity in male zebrafish exposed to low concentrations of MT were therefore performed by exposing adult male zebrafish 0, 2.0, 5.0, 10.0, 25.0, and 50.0 ng MT/L for 7 days. Furthermore, the time dependent induction of vtg, and aromatase activity by MT and E2 in adult male zebrafish were also investigated. The fish were exposed to 50 ng MT/L, and 200 ng E2/L for 7 days and fish were sampled at 0, 6, 12, 24, 48, 72, 96, 120, 144, 168 hr after start of exposure. Vitellogenin were measured in whole-body homogenate by ELISA, and the aromatase activity of brain and gonads was measured using an enzyme assay quantifying the release of tritiated water during the conversion of ^{3}H-androst-4-ene-3,17-dione to estrone by aromatase. The results from the experiments will be presented and discussed.

P-VI-4

ESTROGEN BINDING SITES IN THE OVARY OF THE EUROPEAN SEA BASS (*DICENTRARCHUS LABRAX*)

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Several lines of research have clearly established the involvement of estrogens in the process of gonadal sex differentiation in many species of teleost fish. Estrogen effects are mediated through binding to specific nuclear receptors. In this context, estrogen receptor binding affinity and estrogen receptor abundance become important issues in the study of female sex differentiation. Here we describe the characterization of estrogen binding sites in the ovary of the sea bass (*Dicentrarchus labrax*). Saturation analysis followed by a Scatchard plot analysis revealed a single class of high affinity binding site with a Kd of 4.53 nM in cytosolic extracts and 1.13 nM in nuclear extracts. The receptor content was 12.76 pmol/mg protein in cytosolic extracts, while in nuclear extracts it was 6.46 pmol/mg protein. Association kinetics showed half maximum specific binding after 90 min incubation at 4°C. Association and dissociation profiles of these estrogen binding sites were typical of nuclear receptors characterized in other tissues. Regarding specificity, similar steroid binding affinity patterns were found in both cytosolic and nuclear extracts (diethylstilbestrol > estradiol >> estriol > estrone). No affinity for androgens was found at physiological androgen concentrations. For comparison, steroid specificity studies were also carried out with the Atlantic Croaker (*Micropogonias undulatus*) estrogen receptor. A similar steroid-binding specificity pattern was found in ovarian extracts of this species, although affinity for diethylstilbestrol differed in cytosolic extracts. Finally, adult sea bass approaching the reproductive season were primed with a single injection of estradiol at 5 mg/Kg BW. When compared with untreated fish, fish treated with estradiol exhibited an up-regulation of estrogen binding sites, suggesting a positive control mechanism to ensure appropriate response after stimulation. However, no changes in the steroid binding affinity pattern were observed.

P-VI-5

SEX STEROIDS CONCENTRATIONS IN STURGEON (*ACIPENSER GUELDEN-STAEDTI* BR.) SERUM AND COELOMIC FLUID AT FINAL MATURATION

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Final maturation was induced by sturgeon pituitary preparation and GnRH-A in female sturgeon in migrating spring form after short-term reservation and in the winter form after long-term reservation (9 - 10 months) in spring at the fish farm. Testosterone (T), 11 ketotestosterone (11-KT) (ng/ml), estradiol 17β (E2) (pg/ml) were measured by enzyme-linked immunosorbent assay [ELISA]. In the spring form T level in the coelomic fluid at ovulation was lower than in the serum of same fishes (4,98±1,54 and 19,09±6,70 respectively) as well as 11-KT (2,81±0,97 and 28,84±6,72 respectively; $p<0,01$). E2 concentration in the coelomic fluid and serum did not differ significantly (98,82±23,10 and 113,32±22,5 respectively). In the winter form T level in the coelomic fluid was also significantly lower than in the blood (1,11±0,32 and 18,70±3,32 respectively; $p<0,001$) as well as 11-KT (2,78±0,78 and 18,20±4,38 respectively; $p<0,01$). Serum 11-KT concentration was higher in the spring form than in the winter form, but no differences were observed in 11-KT coelomic fluid levels in spring and winter forms. E2 concentration in the winter form was lower in the coelomic fluid than in the serum (89,06±10,08 and 224,86±48,61 respectively; $p<0,01$); E2 level in the blood of the winter form was higher than in the spring form, but no differences were found in the E2 coelomic fluid levels in spring and winter forms. The same regularities in steroids profiles were elucidated in sturgeons after ovulation induction by sturgeon pituitary preparation or GnRH-A. After ovulation the eggs in sturgeons are reserved before and during spawning in the coelomic fluid; definite levels of sex steroids possibly are necessary for maintenance of its normal physiological state.

P-VI-6

INTERACTIONS OF CALCIUM AND CYCLIC AMP SIGNALING PATHWAYS REGULATING STEROIDOGENESIS IN PRIMARY CULTURED THECA AND GRANULOSA CELLS OF ATLANTIC CROAKER

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Previous research has demonstrated a role of both cyclic AMP- (cAMP) and calcium-dependent pathways in mediating ovarian steroid production in Atlantic croaker (*Micropogonias undulatus*) and other vertebrates. However, the interactions between these pathways in the regulation of teleost follicular steroid synthesis are not well-understood. Thus, the purpose of this research was to examine calcium regulation of specific sites in the gonadotropin-activated cAMP pathway including adenylyl cyclase (AC) and protein kinase A (PKA). Primary cultures of theca and granulosa cells were established by enzymatic digestion of ovarian tissue and isolation of cells by density-gradient centrifugation. Treatment of cells with human chorionic gonadotropin (hCG), the AC activator forskolin and the PKA activator db cAMP significantly stimulated testosterone synthesis 8-21 fold. Additionally, hCG-stimulated steroidogenesis was attenuated by the voltage-sensitive calcium channel blocker verapamil, the IP3-sensitive calcium channel blocker TMB-8 and the calmodulin inhibitor W-7. These results demonstrate that primary cultured croaker follicular cells are a viable model for investigating the potential for cross-talk between these two signaling pathways. Steroid production in response to hCG, forskolin and db cAMP was significantly decreased after co-treatment with verapamil indicating that extracellular calcium is required for a complete steroidogenic response to activation of AC and PKA. Some studies have suggested that calcium may have a direct modulating effect on AC function. However, removal or blockage of extracellular calcium influx with EGTA or verapamil had no significant effect on hCG-stimulated cAMP cell content. Additionally, treatment with a calmodulin inhibitor or calcium ionophore was ineffective in altering cAMP levels. These data suggest that the principal site of calcium action on the cAMP-dependent pathway is distal to AC function in croaker theca and granulosa cells. Therefore, current research is investigating the steroidogenic acute regulatory protein (StAR), which is downstream from PKA activation, as a possible regulatory site of both calcium and adenylyl cyclase pathways.

P-VI- 7

OVARIAN ATRESIA IN TEMPERATE BASSES: DETECTION OF HATCHING ENZYMES (CHORIOLYSINS) IN ATRETIC OVARIES

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In female vertebrates undergoing atresia, ovarian follicles are eliminated to prevent ovulation of damaged or inferior eggs and to resorb nutrients from oocytes failing to mature. Granulosa cells of atretic striped bass (*Morone saxatilis*) lose their steroidogenic capabilities and undergo drastic morphological changes coincident with disintegration of the oocyte zona radiata (ZR). These changes include pronounced edema in the granulosa layer, possibly resulting from secretion and hydration of proteolytic enzymes. Destruction of the ZR during atresia resembles that occurring at hatching, which is due to the action of an embryonic enzyme known as choriolysin (CL). These observations led us to search for expression of a comparable enzyme in atretic ovarian follicles. Degenerate primers were designed to target conserved regions of CL genes from other teleosts. Two partial cDNAs (seCL103 and seCL148) were isolated from hatching striped bass embryos by RT-PCR using these primers followed by TA-cloning. The seCL103 clone encodes 103 amino acids (AA) and was found to be ~50% identical to seCL148 and medaka choriolysin L (mCLL), while higher identities (>60%) were found between seCL103 and medaka choriolysin H1 or H2 (mCLH1 and mCLH2) over the aligned AA residues. The seCL148 encodes 148 AA and was found to be 81%, 79%, and 78% identical, respectively, to mCLL, mCLH1 and mCLH2. Two partial cDNAs (paCL103A and paCL103B) also were cloned from atretic white perch (*M. americana*) ovaries utilizing similar techniques. The paCL103B clone was identical to the seCL103 clone. The paCL103A clone encodes 103 AA and was 85%, 65%, and 62% identical to paCL103B, mCLH1 and mCLH2, respectively, while it was less similar (~51% identical) to seCL148 and mCLL as regards deduced AA sequence. In summary, we cloned three distinct cDNAs encoding CL-like enzyme(s) from hatching embryos and atretic ovaries of *Morone* species. One of these (paCL103A) may be a novel ovary-specific CL homologue produced by granulosa cells to breach the ZR antecedent to their entry into and extensive phagocytosis of the ooplasm.

P-VI-8

INVOLVEMENT OF SEX STEROIDS IN FINAL STAGES OF OVOGENESIS IN EURASIAN PERCH, *PERCA FLUVIATILIS*

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This study was designed to specify, in natural habitat, the testosterone (T), estradiol-17β (E2) and 17, 20β-dihydroxy-4-pregnen-3-one (17,20β-P) variations in blood during the pre-ovulatory period in female Eurasian perch, *Perca fluviatilis*, and also to examine the ovarian *in vitro* potential synthesis of 17,20β-P and 17, 20β, 21-trihydroxy-4-pregnen-3-one (20β-S). To do so, five females were captured three times every week at the Lindre Center (Moselle, France) from March 1999 to April 1999 and blood sampled at 10 am. Two females were also collected from the Lindre pond on three dates (February 18, March 14 and 23) for an *in vitro* study in order to examine the ovarian potential synthesis of 17,20β-P and 20β-S. First of all, significant plasma 17,20β-P peaks were measured for the first time in this species reaching about 3.5 $ng.ml^{-1}$ on March 10, April 26 and 28. However, the release of 17,20β-P in blood may not be synchronized with the final oocyte maturational stage, as plasma 17,20β-P could not be related to germinal vesicle migration or breakdown stages. The *in vitro* study showed that ovaries at the pre-ovulatory stage produce some 17,20β-P but no 20β-S from a tritiated precursor (17P-^{3}H) at the three different sampling dates. Three increasing incubation times (30 min, 2 and 4 hours) were tested at 10°C. These showed that 17,20β-P is produced after half-an-hour and is still present in the medium after 2 hours of incubation, but no more detectable after 4h. Some other non-identified compounds were synthesised at this stage suggesting that 17,20β-P was quickly metabolised possibly explaining why it is difficult to measure peak levels in perch blood. Collectively, these results suggest a role for 17,20β-P in the final stages of oocyte development; they also confirm peak levels of E2 and T prior to the spawning period. It is suggested that these two steroids have a role in the ovulation and oviposition stages and possibly in the synthesis of the egg jelly coat, which is specific among Eurasian and yellow perch.

P-VI-9

cDNA CLONING AND EXPRESSION OF ZONA PELLUCIDA PROTEIN B IN MASU SALMON (*ONCORHYNCHUS MASOU*)

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In teleost fish it has been demonstrated that the origin of the major components of the chorion (vitelline envelope) varies among species. In salmonid fish and medaka, liver-derived precursor proteins of chorion (choriogenin) have been identified. By contrast, cDNA clones for chorion components (zona pellucida protein B and C; ZPB and ZPC) have been isolated from the ovary of carp, goldfish and zebrafish, and revealed that their expression is restricted to oocytes. However, recent findings in medaka reveal involvement of both liver and ovary in chorion formation, where liver-derived choriogenins and oocyte-derived ZPs have been identified. In this study, cloning of ZPB in masu salmon ovary was conducted to confirm whether the dual synthesis of chorion proteins is a general phenomenon within teleosts. A cDNA fragment encoding ZPB in masu salmon was obtained from the pre-vitellogenic ovary by RT-PCR using degenerate primers designed from carp and zebrafish ZPB sequence data. A full-length was isolated by RT-PCR, following 5'- and 3'- rapid amplification of cDNA ends. The full-length cDNA clone contained an open reading frame encoding 406 amino acids. The predicted amino acid sequence had a zona pellucida domain corresponding with that of mammalian ZPB and revealed over 45% identity with amino acid of ZPBs in carp and goldfish. From these results, the isolated cDNA clone was identified as ZPB of masu salmon. In addition, RT-PCR using gene specific primers for masu salmon ZPB, revealed that expression of ZPB was isolated to the ovary. Thus, it is suggested that chorion formation in masu salmon involves both liver-derived choriogenins and ovary-derived ZPB.

P-VI-10

GENE EXPRESSION LEVELS OF GROWTH HORMONE RECEPTOR AND INSULIN-LIKE GROWTH FACTOR-I IN GONADS OF MATURING COHO SALMON (*ONCORHYNCHUS KISUTCHI*)

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Growth hormone (GH) and insulin like growth factor-I (IGF-I) have many important physiological roles including maturation in fish. GH affects various tissues directly or indirectly via its receptor, growth hormone receptor (GHR). Association of GH with GHR triggers gene expressions, including IGF-I mRNA. Recently GHR cDNA has been cloned from several teleosts. The analysis of GHR contributes to our understanding of GH effects on fish maturation, however there are only a few reports focused on relationship between GHR and gonadal development, and nothing is known about gene expression levels of GHR mRNA in fish. The aim of this study was to observe expression levels of GHR and IGF-I mRNAs in gonads and liver of maturing coho salmon during June, Aug and just prior to spawning in Nov. Realtime RT-PCR was used to measurement mRNA levels. Acidic ribosomal phosphoprotein P0 (ARP) gene was tested as a housekeeping gene for normalizing data. Without normalization, the GHR mRNA levels in testis decreased from June to Aug, and recovered in November to similar level as in June. The IGF-I mRNA levels in testis showed a reverse profile compared to that of GHR expressions. In male liver, GHR and IGF-I expressions showed similar patterns to each other and to GHR levels in testis. However ARP expression in testis and male liver decreased from June to November. In the ovary, GHR and IGF-I expression increased from June to November with or without correction by ARP. The expression levels of ARP were relatively stable during ovarian development. In female liver, all genes decreased from June to November. The GHR and IGF-I mRNAs showed different gene expression pattern between male and female. Further the levels of IGF-I mRNA (except testis) are seemed to be synchronized with those of GHR mRNA levels. Additional housekeeping gene analyses may provide more precise estimates of GHR and IGF-I expression.

P-VI-11

IN VITRO STEROID METABOLISM DURING FINAL OOCYTE MATURATION IN THE WHITE CROAKER, *MICROPOGONIAS FURNIERI* (SCIAENIDAE)

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Final oocyte maturation (FOM) is a process that involves the shift of post-vitellogenic follicle to pre-ovulated oocyte, necessary for fertilization by spermatozoid. This process is regulated by maturation inducing steroid (MIS) at the follicular level. In some Sciaenidae species, a hydroxylated progestagen, 17,20β,21-trihydroxy-4-pregnen-3-one (20β-S) has been identified. Although *Micropogonias furnieri* is the second fishery resource of Uruguay, basic knowledge of reproduction is still very scarce. The aim of this work was to investigate what steroids are synthesised in vitro by oocytes of *M. furnieri* during the maturation process. Fragments of ovary (1 g) in three stages: post-vitellogenics (PV), maturing (Mtg) and mature (M) were incubated with 1μg.g^{-1} of tritiated progesterone (P) at 30, 60 and 180 min. After extraction with ethanol and dichloromethane, steroid metabolites were purified by TLC and RP-HPLC. Two progesterone derivatives with identical chromatographic properties of 20-S and 17,20β-dihydroxy-4-pregnen-3-one (17,20β-P) were identified. 17,20β-P was clearly detected in Mtg and M stages and confirmed by enzymatic oxidation with enzyme 20β-HSD. 20β-S was also detected but only in Mtg oocytes. These results show that 20β-S is not the major hormone synthezised in FOM as in other Sciaenidae species, however they would suggest that 20β-S is acting as MIS in *M. furnieri*.

P-VI-12

CLONING AND SEASONAL CHANGES IN OVARIAN EXPRESSION OF A TSH RECEPTOR IN THE CHANNEL CATFISH, *ICTALURUS PUNCTATUS*

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Thyroid stimulating hormone receptor (TSHR) mediates the pituitary control of thyroidal development, growth and function. TSHR is a G-protein coupled receptor and belongs to the subfamily of glycoprotein hormone receptors (GpHRs). Recently, the gene expression of this receptor was found in both the ovary and testis of striped bass, gilthead seabream and Atlantic salmon. However, nothing is known about the regulation or function of TSHR in the gonad in any vertebrate species. We have partially cloned a TSHR cDNA (encoding the N-terminal 438 amino acid residues) from the catfish ovary and thyroid. The predicted TSHR peptide is 58% identical to the striped bass and amago salmon TSHR and has low identity with the channel catfish LH receptor and FSH receptor. The catfish clone has the TSHR-specific addition to the distal portion of the extra-cellular domain as compared to the LH- and FSH-receptors. RT-PCR analysis detects catfish TSHR transcripts in the thyroid, ovary, testis, and in various other tissues. Therefore, the expression of TSHR in the gonad may not be a rare feature in teleosts. This would imply a direct role of TSH in gametogenesis which has not been previously demonstrated. Channel catfish is a useful species for this study because we have previously described the annual, seasonal pattern of changes in the expression of steroidgenic enzymes, FSH, LH, FSHR and LHR associated with female reproductive physiology. This report will characterize the receptor, describe its seasonal expression in catfish ovaries and discuss its potential gonadal function in teleosts.

P-VI-13

PARTIAL CLONING AND EXPRESSION OF A GLYCOPROTEIN HORMONE RECEPTOR GENE IN THE OVARY OF THE BULLSEYE PUFFER (*SPHOEROIDES ANNULATUS*)

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The bullseye puffer *Sphoeroides annulatus* is a Mexican a relative of the tiger puffer *Takifugu rubripes*, and has a good potential for aquaculture. Studies on pufferfish reproductive biology and physiology have lead to the analysis of genes involved in gonadal development and maturation. In order to study the mechanisms of oocyte maturation to control reproduction in captivity, the transmembrane domain region of a glycoprotein hormone receptor gene was isolated from the ovary using the reverse transcription polymerase chain reaction (RT-PCR) approach. The gene fragment is 437 bp, which encodes helices II to VI from the transmembrane domain of the receptor, showing similarity to the thyroid-stimulating hormone receptor (TSHR), and the luteinizing and follicle-stimulating hormone receptors (LHR and FSHR respectively). The hormone receptor was expressed in the ovaries of both wild and captive vitellogenic females, showing an increase of expression after treatment with an LH-releasing hormone analog (LHRH-a), particularly in ovaries that had reached final oocyte maturation. Results from mRNA *in situ* hibridization using the 437 bp fragment indicated the presence of the receptor in follicle cells, as expected for gonadotropin receptors, however some signal was also detected in the ooplasm, as reported for the TSHR. These results suggest that the 437 bp fragment is hybridizing LHR as well as TSHR mRNAs, due to the strong similarity of both genes in the transmembrane domain region. It is necessary to clone the full-length cDNA to characterize this glycoprotein receptor and to know whether this gene fragment belongs to TSHR or LHR.

P-VI-14

EFFECTS OF SEVERAL SEX STEROID HORMONES ON EARLY OOGENESIS IN JAPANESE HUCHEN (*HUCHO PERRYI*)

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There is a wide range of information on sex differentiation and oocyte maturation in many vertebrates, including fish. However, little is known about early oogenesis, particularly the control mechanism of early oogenesis. One reason for this is that oogenesis up to prophase of the first meiotic division occurs very quickly in most animal species. However, the Japanese huchen (*Hucho perryi*), whose eggs take about five years to mature, is a very useful for analyzing the control mechanism of early oogenesis because oogenesis in this species occurs comparatively slowly. The present study was carried out using immature female Japanese huchen to determine whether several sex steroid hormones are able to induce *in vitro* oogonial proliferation and the initiation of first meiosis in cultured ovarian fragments using an organ culture technique. At first, an experiment was carried out to determine whether various steroids and hormones are able to induce DNA synthesis of ovarian germ cells. It was found that two steroids, 17β-estradiol (E2) and 17α, 20β-dihydroxy-4-pregnen-3-one (17α, 20β-DHP), are able to induce DNA synthesis of ovarian germ cells. In early oogenesis, DNA synthesis occurs at the time of chromosome replication (S-phase) in the mitotic and meiotic prophases. The nuclei with synaptonemal complexes in oocytes at initiation of first meiosis are larger than oogonia. We therefore compared the nuclear sizes of oocytes in which DNA synthesis occurred and the percentages of oocytes with synaptonemal complexes to determine the difference between the control mechanisms of early oogenesis by E2 and by 17α, 20β-DHP. We found that the abovementioned sizes and percentages of oocytes in 17α, 20β-DHP treated fragments were significantly larger than those in other fragments. From these results, we concluded that endogenous E2 synthesized in the ovary plays a significant role in the proliferation of oogonia and low percentage of oocytes with synaptonemal complexes as evidenced by the incorporation of BrdU into germ cells and that 17α, 20β-DHP plays a significant role in the initiation of first meiosis in early oogenesis as evidenced by the incorporation of BrdU into germ cells and high percentage of oocytes with synaptonemal complexes. Further investigations on the expressions of genes related to mitosis or meiosis during early oogenesis are required to elucidate the roles of E2 and 17α, 20β-DHP in the control mechanisms of early oogenesis.

P-VI-15

GLYCOPROTEIN HORMONE RECEPTOR FAMILY IN MEDAKA (*ORYZIAS LATIPES*)

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In some teleost fish, it had been demonstrated that the two gonadotropins, FSH and LH, are recognized by distinct receptors and play important roles in gametogenesis. We have established degenerate PCR system for glycoprotein hormonereceptor (GPHR) family and identified the two types of gonadotropin receptor cDNAs in several teleost species with different reproductive characteristics. To elucidate the unity and diversity of gonadotropin receptors in fish reproduction, we started comparative study on gene structure and expression profiles among various teleosts. Medaka (*Oryzias latipes*) is a daily spawner with group synchronous oocyte development strictly regulated by photoperiod. Recently, this fish attract attention as a good molecular biological model. In this study, we cloned GPHR family gene from medaka ovary and investigated their expression profiles during the course of oocyte development. Previous RT-PCR system often gave nonspecific products and required further complicated process to obtain receptor cDNAs. New degenerate PCR primer sets were designed on the basis of the consensus sequences of known fish GPHRs. This refined system successfully retrieved four types of GPHR cDNAs from medaka ovary. Their expression levels in separate clutches were estimated by gene specific RT-PCR.

P-VI-16

MULTIPLE VITELLOGENINS IN WHITE PERCH (*MORONE AMERICANA*): PURIFICATION, IDENTIFICATION, AND CLASSIFICATION OF THREE DISTINCT FORMS

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The extent of vitellogenin (Vg) multiplicity in fishes and the details of proteolytic cleavage of Vgs and derived yolk proteins at different phases of oogenesis and embryogenesis have not been fully explored. In the present study, three forms of female-specific plasma protein (FSPP) were purified from blood of estrogen-treated white perch. Purification methods combined several types of ion-exchange chromatography, including a novel, fast flow, strong anion exchanger (POROS media), followed by gel filtration. Purified FSPP-1, FSPP-2, and FSPP-3, had molecular masses corresponding to 532 kDa, 532 kDa, and 426 kDa, respectively, in their native state. The apparent molecular mass of purified FSPP-1 and FSPP-2 after SDS-PAGE under reducing conditions was ~180 kDa, whereas FSPP-3 appeared in SDS-PAGE as a major ~148 kDa band. In double immunodiffusion using anti-total FSPPs (antiserum raised against mature female plasma pre-absorbed by male plasma), each purified FSPP formed one precipitin line that crossed those produced by both other FSPPs. Although all of the FSPPs resembled one another with respect to amino acid composition, each appeared to be immunologically distinct. Rabbit antisera were raised against each FSPP and absorbed with combinations of the other two purified FSPPs to increase specificity. Using the antisera, each FSPP was detected by immunoelectrophoresis in plasma from females or estrogen-treated male or immature fish, but no FSPP was detected in normal male plasma. Endopeptidase digests of each FSPP were subjected to HPLC separation followed by peptide sequencing and mapping. Alignment analyses of the peptide sequences lead us to conclude that white perch FSPP-1, FSPP-2, and FSPP-3 can be classified into three Vg groups identified in prior studies: VgA, VgB, and VgC, respectively. This is the first report, of which we are aware, on isolation of more than two native forms of Vg proteins from any species of vertebrate.

P-VI-17

IDENTIFICATION OF A PROTEIN KINASE WHICH PHOSPHORYLATE α4 SUBUNIT OF THE 26S PROTEASOME IN GOLDFISH OOCYTES

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To investigate the regulatory mechanism for the proteasome in the meiotic cell cycle, we purified the 26S proteasome from immature (in G2-phase) and mature (in M-phase) oocytes, and compared its subunits by immunoblotting. A monoclonal antibody, GC3ß cross-reacted with two bands in the 26S proteasome from immature oocytes, however the upper band was absent in the 26S proteasome from mature oocytes. Their cDNAs were isolated by an immuno-screening method using each monoclonal antibody. The 30-kDa protein was a α4 subunit, which is one of the α-subunit groups of the 20S proteasome, and the 62-kDa protein was a homologue of CCTε, one of the components of eukaryotic molecular chaperones. Phosphatase treatment of the 26S proteasome revealed that a part of the α4 subunit of goldfish 20S proteasome, *α4_ca*, is phosphorylated in G2-phase and dephosphorylated in M-phase. By the assay using recombinant *α4_ca* as a substrate, a kinase was purified by column chromatographs. Amino acid sequence analysis was performed for resulting partial purified fraction. A protein band, which well corresponded to the kinase activity, was identified as Casein kinase-1α (CK-1α). The result suggests that CK-1α phosphorylate α4 subunit of the 26S proteasome in immature oocyte of goldfish.

P-VI-18

MOLECULAR STUDY OF THE GONADOTROPIN RECEPTORS IN ATLANTIC HALIBUT (*HIPPOGLOSSUS HIPPOGLOSSUS*)

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The physiological functions of the gonadotropins are mediated by the G protein-coupled receptors named FSH receptor (FSH-R) and LH receptor (LH-R). This interaction on the surface of target cell is important for the initiation of the hormonal cascade in the modulation of gonadal function. The multiple spawner Atlantic halibut seems to express high levels of both FSH and LH throughout the reproductive cycle, in contrast to the expression patterns in salmonids, in which the oocytes develop synchronously. The asynchronous development of oocytes in multiple spawning fish may be regulated by different spatio-temporal expression patterns of the FSH-R and LH-R. In order to verify this hypothesis the Atlantic halibut FSH-R and LH-R cDNAs were cloned from gonadal tissues by RACE-PCR and expression studies have been initiated.
Based on almost full-length sequences Atlantic halibut FSH-R and LH-R showed the highest homology (about 75 % aa sequence identity) to the respective Nile tilapia gonadotropin receptors, whereas halibut FSH-R and LH-R shared only 50 % identity. In addition, we isolated several C-terminal truncated FSH-R variants lacking transmembrane domains and the cytoplasmic domain of importance for signal transduction. Preliminary results showed abundant expression of FSH-R exclusively in the gonads, while LH-R was also expressed in several extra-gonadal tissues (brain, heart, gills). In situ hybridization studies of their spatial expression in oocytes are in progress. These results will elucidate the involvement of FSH-R and LH-R in the control of gonadal development.

P-VI-19

ENDOCRINE CHANGES DURING THE ONSET OF VITELLOGENESIS IN SPRING IN THE MOSQUITOFISH

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The mosquitofish, *Gambusia affinis*, starts vitellogenesis with the increase in temperature during the spring season. To clarify the endocrine changes during the onset of vitellogenesis, we introduced female fish from an outdoor pond into an indoor warm aquarium in March, and investigated some of their physiological changes. Both the gonadosomatic index and oocyte diameter gradually increased from day 2 or 3 to day 10 after transfer, and reached 3.1% and 1 mm, respectively. The oocytes began to accumulate yolk globules on day 3 after transfer, then continued to grow, and matured on day 10. The hepatosomatic index gradually increased to a peak on day 5 after transfer, and decreased on day 10. The plasma vitellogenin (Vg) level increased rapidly from day 2 to 3, and then maintained a high level until day 10. Although no immunoreactivity of the pituitary against anti-mummichog FSH-β antibody was shown in the initial control, the immunoreactivity gradually increased from day 1 to 3. In vitro production of estradiol-17β (E2) in the oocyte follicles increased from day 1 to 5, and decreased on day 10. These results suggest that FSH induces the onset of vitellogenesis through stimulation of E2 production by follicles, following Vg synthesis in hepatocytes, and incorporation of Vg into the oocytes. The addition of pregnant mare serum gonadotropin (PMSG) into the incubation medium did not promote the production of E2 by the follicles in either fish transferred into warm aquaria or natural vitellogenic fish in the outdoor pond. However, addition of forskolin in the incubation medium promoted E2 production by the follicles in the natural vitellogenic fish in the outdoor pond. These results suggest that sensitivity against GTH in the follicle cells was gradually acquired after fish were transferred into warm aquaria.

P-VI-20

INVOLVEMENT OF ANDROGENS AND GROWTH HORMONE IN THE SYNTHESIS OF VITELLOGENIN IN JAPANASE EEL (*ANGUILLA JAPONICA*)

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Previous studies have demonstrated that the involvement of growth hormone (GH) and prolactin as well as estradiol-17β (E2) in the synthesis of vitellogenin (Vg) in eels (*Anguilla japonica*), indicating the multihormonal control of fish vitellogenesis. However, the mechanism of the multihormonal control for this process has not been fully established. In the present study, therefore, the potentiating effect of androgens, GH and E2 in Vg synthesis was investigated *in vivo*, and *in vitro*using the primary culture of immature eel hepatocytes. In the *in vivo* trials, injection of either GH (1μg/kg body weight, BW) or methyltestosterone (MT, 5 mg/kg BW) alone to immature eels failed to induce *in vivo* Vg synthesis. When the eel hepatocytes were cultured with either E2 (10^{-9}~10^{-5}M), bovine GH (10~100 ng/ml), eel GH (10~100 ng/ml), or MT (10^{-9}~10^{-5}M) alone, no Vg was detected in any of the media throughout the experimental period (12 days). However, cultures with E2 in combination with either MT or GH induced Vg synthesis and the rate of its production gradually increased in a time- and dose-dependent manner until at least 10 days. Treatment of the culture with MT+GH in the absence of E2 did not induce Vg synthesis, whereas treatment with MT+GH+E2 induced far more Vg synthesis than those with MT+E2 or GH+E2. Treatment with either testosterone, androstene or progesterone in the presence of E2 was also effective in inducing Vg synthesis although not as potent as MT+E2, GH+E2 or MT+GH+E2. In addition, in the culture of hepatocytes obtained from immature eels primed by E2 injection, the treatment with either E2, GH, or MT alone did not increase the synthesis of Vg, while the combination of E2 either with GH or MT strongly stimulated Vg synthesis. Addition of tamoxifen to these cultures dramatically reduced Vg synthesis, implying that E2 played a key role in the synthesis of Vg in the experimental cultures. Together, these results suggest that androgens and/or GH increase the effect of E2 on the synthesis of Vg in this species.

P-VI-21

SYNTHESIS AND POSSIBLE FUNCTION OF 11-KETOTESTOSTERONE DURING OOGENESIS IN EEL (*ANGUILLA* SPP.)

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In female teleosts, androgens do not only play a role as substrate for the production of estrogens, but they have also been linked, among others, to hypothalamic steroid feedback. One androgen, 11-ketotestosterone (11-KT), traditionally considered male-specific in teleost fish, has also been found in several female fishes. However, it remains as yet unknown where 11-KT is synthesized in females and where it exerts its physiological role. To address this issue, we have been investigating the control of 11-KT biosynthesis and its physiological in female Japanese eel (*Anguilla japonica*) and New Zealand longfinned eel (*Anguilla dieffenbachii*). 11-KT has been detected in serum of Japanese and New Zealand longfinned eels, and levels tended to increase with sexual maturation. RT-PCR analysis revealed that P450c11β hydroxylase (P450c11β) mRNA, coding for one of the enzymes needed for 11-KT synthesis, was expressed in the brain, pituitary, head kidney and ovary. Among these tissues, the ovary had the greatest capacity to synthesize 11-KT in vitro. Furthermore, both ovarian P450c11β enzyme activity and mRNA levels correlated with serum 11-KT levels. In vitro, the ovary readily converted 11β-hydroxyandrostenedione, 11β-hydroxytestosterone and androstenetrione to 11-KT.We have also examined the role of 11-KT during pre-vitellogenic oocyte growth by implanting this androgen in immature female eel (body weight 75-420g) at 0, 0.01, 0.1 and 1 mg/kg body weight. After one month, the oocyte diameters of eels treated with 11-KT had increased significantly. Histologically, these oocytes were found to have an increased number of oil droplets. Our findings suggest that 11-KT in female eels may be mostly of ovarian origin and this androgen appears to play an important role in controlling pre-vitellogenic oocyte growth.

P-VI-22

REGULATION OF STEROIDOGENIC ENZYME mRNAs IN RAINBOW TROUT (*Oncorhynchus mykiss*) OVARIAN FOLLICLES *IN VITRO*

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Follicle-stimulating hormone (FSH) and luteinizing hormone (LH) both stimulate steroid production by gonadal tissue of various teleosts. Insulin-like growth factor I (IGF-I) has also been implicated in regulating steroidogenesis in mammals and other vertebrates including fish. The precise mechanisms by which these hormones regulate steroidogenesis are unclear. The aim of this study was determine the effects of FSH, LH and IGF-I on expression of steroidogenic enzymes genes and steroid production in ovarian follicles of rainbow trout. We measured mRNAs encoding P450 aromatase (P450arom), 3β-hydroxysteroid dehydrogenase (3β-HSD) and P450 side-chain cleavage enzyme (P450scc), and estradiol-17β (E2) production by ovarian follicles *in vitro*. Ovarian tissue was obtained from two-year-old female rainbow trout. Pre-vitellogenic follicles, or early or late vitellogenic follicles were used for incubation. Follicles were isolated from ovarian connective tissue and then incubated for 18h in the presence or absence of forskolin, salmon FSH, LH or human IGF-I. After the incubation, follicles were collected and total RNA was extracted. We then quantified the P450arom, 3β-HSD and P450scc mRNA levels in cultured follicles by Northern blot analysis. E2 levels in incubation media were measured by RIA. E2 production by follicles at all stages was stimulated by forskolin. FSH and LH stimulated E2 production by vitellogenic follicles. FSH stimulated a significant 2-3 fold increases in P450arom, but only in early vitellogenic follicles. By contrast, IGF-I consistently stimulated 3-7 fold increases in P450arom mRNA in follicles at all stages. 3β-HSD mRNA levels were increased in early vitellogenic follicles incubated with forskolin or with FSH or LH. P450scc mRNA levels were not consistently affected by any treatment. These results indicate that gonadotropins regulate 3β-HSD gene expression, partly through a cAMP/protein kinase A-dependent mechanism. The consistent stimulatory effects of IGF-I on P450arom mRNA levels suggest a role for this growth factor in regulating E2 synthesis.

P-VI-23

GROWTH PATTERNS AND PLASMA LEVELS OF ESTRADIOL-17β, TESTOSTERONE AND IGF-1 IN FEMALE ATLANTIC HALIBUT (*HIPPOGLOSSUS HIPPOGLOSSUS*) FROM JUVENILE STAGES THROUGH SEXUAL DEVELOPMENT

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Atlantic halibut show a pronounced sexually dimorphic growth pattern, where females may grow to sizes manyfold larger than males. Observations of a rapid, prepubertal female growth have frequently been reported by halibut farmers. These observations prompted a study to establish basal data on growth patterns, as well as endocrine regulation of growth and maturation, throughout development in female Atlantic halibut, from juvenile to mature stages. A slow but steady growth during the first five years of life was followed by an exponential increase in body weight during the three years that preceded first sexual maturation. Plasma levels of estradiol-17β (E2) were undetectable until two years before first maturation, when a small but significant peak of ca 0.1 ng/ml was apparent. Plasma E2 levels then increased, with another, around five times higher, peak one year before first maturation. During first maturation, average plasma E2 levels were between 15 and 20 ng/ml, which is similar to previous observations in this species. Plasma testosterone levels reflected sexual maturation, but were generally lower than plasma E2 levels. Data on plasma Insulin-like growth factor 1 (IGF-1) levels in female halibut have not been previously reported and will be presented for the first time.

P-VI-24

PLASMA STEROID LEVELS AND FOLLICULAR DEVELOPMENT IN THE STAGHORN DAMSELFISH (*AMBLYGLYPHIDODON CURACAO*)

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Female staghorn damselfish were captured by SCUBA divers from coral outcrops on reefs around Lizard Island on the Great Barrier Reef, Australia, and immediately blood sampled underwater. Fish were then transferred to the surface and the ovaries returned to the laboratory for dissection and determination of follicle type and size distribution. Plasma samples were solvent-extracted on site for later measurement of 17β-estradiol (E2) and testosterone (T). Ovaries always contained a pool of vitellogenic follicles along with up to three identifiable clutches of vitellogenic follicles. The presence of the largest size class of vitellogenic follicles coincided with approximately semi-lunar cycles of male territorial defence and display. Plasma E2 levels ranged from less than 0.3 - 6.5 $ng.ml^{-1}$ and were highest in fish where all three vitellogenic clutches were present, and low when the smallest class of vitellogenic follicles was absent, or when only the small follicles were present. Plasma T levels ranged from less than 0.3 -7.6 $ng.ml^{-1}$ and followed an essentially similar pattern. The results indicate that ovaries are most steroidogenically active when there is active recruitment of follicle clutches, and that elevated steroid levels are not requisite for maintenance of advanced vitellogenic clutches in the ovary. Similarly, plasma steroid levels are not necessarily good predictors of maximum oocyte diameters present in the ovary.

P-VI-25

CLONING OF A GONADAL FOLLICLE STIMULATING HORMONE RECEPTOR cDNA FROM THE EUROPEAN SEA BASS, *DICENTRARCHUS LABRAX*

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Follicle stimulating hormone (FSH) is a central hormone of vertebrate reproduction. In mammals is necessary for gonadal development and maturation at puberty and for gamete production during fertile phase of life. In contrast, there is little information on the physiological role of FSH-R in fish. FSH acts by binding to specific receptors located in the surface of target cells. The FSH receptor (FSH-R) belongs to the G protein-coupled receptors (GPCRs) family. These receptors share a common structure, characterised by the existence of seven transmembrane (TM) spanning domains that connect the extracellular ligand-binding domain with the cytoplasmic region.
To bring new insights regarding the molecular mechanisms that regulate gonadal development in fish, a FSH-R has been cloned from the sea bass (sbsFSH-R). A 3143 bp cDNA fragment was isolated. It contains an open-reading frame of 2108 nucleotides able to code for a polypeptide of 702 amino acids. This coding region is flanked by 5' and 3' untranslated regions of 215 and 818 nucleotides, respectively. Seven hydrophobic segments, corresponding with the 7 TM domains as well as the presence of a putative signal peptide could be predicted. Sequence database searches showed that, the sbsFSH-R has the highest overall amino acid identity to vertebrate FSH-Rs (75-46%). Phylogenetic analysis, incorporating protein sequences of vertebrate and invertebrate glycoprotein hormone receptors, clearly demonstrate that the sbsFSH-R belongs to the FSH-R cluster. To further confirm the identity of this receptor, PCR analysis of sea bass genomic DNA was performed to determine whether or not the sbsFSH-R gene contains the LH-Rs specific intron. In addition, we studied the eventual presence of introns within the sbsFSH-R TM domain as introns have been observed in this region in related GPCRs genes. Finally, expression analysis on tissues of adult sea bass reveled a preferent expression of sbsFSH-R mRNA in gonadal tissues.
The cloning of the sbsFSH-R, together with the already accomplished cloning of the FSH β subunit of this species, provides important tools that will increase our understanding of FSH roles in fish reproduction. Funded by AGL20011257 and SFRH/BD/6901/2001 grants.

P-VI-26

RAINBOW TROUT FSHR GENE STRUCTURE SHOWS MORE COMPLEXITY THAN THE SALMON ORTHOLOGOUS GENE AND IS DIFFERENTLY EXPRESSED THROUGHOUT GAMETOGENESIS

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It is well established that gonadotropins are required for the control of the reproductive cycle in vertebrates. Two gonadotropins have been isolated in teleost as in mammals. Although both fish gonadotropins have similar in vitro biological activities on gonadal growth and steroidogenesis, they show a different pattern of secretion during the reproductive cycle, suggesting different physiological roles. Gonadotropins actions are mediated by cell membrane receptors that belong to the glycoprotein hormone receptors superfamily. Recently, two distinct gonadotropin receptors have been characterised in some teleost species such as salmon, tilapia and catfish. In the present study, a full length cDNA encoding a rainbow trout gonadotropin receptor homologous to the salmon (sGtH-RI) and mammalian FSH receptor (FSHR) has been cloned. The partial genomic organisation of the corresponding gene has been determined and showed a more complex structure compared to that described in salmon because of the presence of an additive exon/intron boundary in the transmembrane domain. Moreover, a splicing variant leading to a 6 amino acids deletion in the second extracellular loop has been identified. The physiological relevance of this shorter transcript has to be further determined. FSHR mRNA tissue distribution has been investigated by real time quantitative PCR in several tissues. FSHR transcripts were mainly expressed in gonads, poorly detected in brain and undetectable in others tested tissues. Generally, FSHR messengers expression was more abundant in testis than in ovary (four to five fold). The expression pattern was studied throughout the male and female reproductive cycle. In female FSHR mRNA progressively increased from pre-vitellogenic stage to ovulation and then decreased from 5 days till 15 days after ovulation. In male, FSHR messengers were expressed at constant levels from the onset to the end of spermatogenesis but strongly increased about 5 fold during spermiation. In female, our data demonstrated that high FSHR messengers expression is concomitant with the plasma FSH levels elevation that occurs at the final oocyte maturation. This strongly suggests a role of FSH in the oocyte maturation and ovulation process. In male, the 5-fold increase in FSHR mRNA expression at the end of the reproductive cycle suggests that FSHR may be involved in the spermiation process.

P-VI-27

PRIMARY STRUCTURES OF THREE TYPES OF VITELLOGENIN IN MOSQUITOFISH (*GAMBUSIA AFFINIS*), A VIVIPAROUS FISH

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Vitellogenins (Vg) are precursors of egg yolk proteins. Recent analyses of Vg cDNA demonstrated multiplicity of Vg genes in some teleost species. The aim of the present study was purification and characterization of the plural Vgs in plasma, and cDNA cloning of the Vg genes of mosquitofish. Estrogen treated female plasma was subjected to two step chromatographies and two major Vg preparations having moleculer masses of 600 and 400 kDa were obtained. Yolk proteins were partially purified from vitellogenic oocytes to obtain inner amino acid (AA) sequences of these Vgs. Three major peaks corresponding to approximately 560, 400 and 28 kDa were obtained and each peak was then subjected to SDS-PAGE followed by transblotting for N-terminal AA sequence analysis. The N-terminal AA sequences of 70, 60 and 21 kDa bands from the 560 kDa yolk preparation and 19 kDa band from 28 kDa yolk preparation had high sequence similarities to *Fundulus heteloclitus* (Fun) VgI.

Meanwhile, highly dense 112 kDa band from 580 kDa yolk preparation was seemed to be a mixture of N-terminal blocked protein with a protein which had a similar N-terminus to Fun VgII. On the other hand, 33 and 26 kDa bands from 400 kDa yolk preparation showed high sequence similarity to zebrafish phosvitinless Vg (PvlVg). From these results, the mosquitofish is suggested to have three Vgs including two 600 kDa Vgs, namely VgA (corresponding to Fun VgI) and VgB (corresponding to Fun VgII), and 400 kDa PvlVg. Based on the inner AA sequences coupled with previously reported Vg cDNA sequences of other fish, we cloned cDNAs encoding the three Vgs from estrogen-treated fish liver cDNA library. One of these three cDNAs contained complete open reading frame of 5088 bps encoding 1696 AA residues including signal peptides. From comparison of the deduced AA sequence of this Vg with partial AA sequences of yolk proteins, the 1696 AA sequence was thought to be VgA. The other two cDNA clones had incompleate sequences being lack of a few bases in signal peptide region. The longer cDNA (5004 bps) and shorter one (3705 bps) were suggested to be Vg B and PvlVg, respectively. The sequence of the PvlVg had indefinable phosvitin domain and shortened C-terminal coding region after Lv heavy chain domain.

P-VI-28

RTGR-1, A NOVEL MEMBER OF THE TGF-β-SUPERFAMILY, CONTROLS PRIMORDIAL GERM CELL DEVELOPMENT IN RAINBOW TROUT

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Primordial germ cell (PGC) is the progenitor of germ cell lineage and differentiates into either oogonia or spermatogonia. Our understanding of PGC specification and development remains quite rudimentary in fish. Particularly, their molecular mechanisms are still poorly understood. Differentiation and proliferation of PGCs are expected to be controlled by the surrounding genital ridge somatic cells. Therefore, we sought to identify genes, which is expressed specifically in genital ridge somatic cells of rainbow trout by a cDNA subtraction followed by a macroarray screening. First, we performed a subtractive cDNA hybridization using a tester cDNA prepared from genital ridges and a driver cDNA from embryonic bodies devoid of genital ridges. The cDNAs thus derived were further screened with macroarray using mRNAs isolated from various adult tissues in order to eliminate pseudo-positive clones. Through these steps, 42 clones were identified to be expressed predominantly in gonadal tissues. Northern analysis of various adult tissues indicated 9 out of 42 clones were expressed in gonads specifically. One clone showing strong signal in this analysis was designated rainbow trout genital ridge-specific gene-1 (*RTGR-1*) and used for further studies. The homology analysis suggested that this protein belongs to the TGF-β superfamily. However, *RTGR-1* lacked a distinct homology with any member of the TGF-β superfamily, indicating that it is a novel member of this family. Whole mount *in situ* hybridization revealed that this gene was expressed in genital ridge somatic cells of 30 day-post-fertilization (dpf) embryos. *in situ* hybridization against paraffin sections of 30, 40, 50 and 60 dpf embryos indicated that the expression is restricted to somatic cells surrounding PGCs. Further, through a gene-knockdown experiment using antisense morpholino oligonucleotides (MO) we studied the effect of inhibition of *RTGR-1* translation on the PGC development. Embryos injected with *RTGR-1* MO were severely impaired in PGC development leading to a reduced number of cells. These results denote that *RTGR-1*, a novel member of the TGF-β superfamily plays a critical role for maintenance or proliferation of PGCs during embryogenesis.

P-VI-29

OVARIAN STEROIDS DURING FINAL MATURATION IN FEMALE STERLET (*Acipenser rhutenus* L)

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Oocyte final maturation (FM) is under a control of the pituitary gonadotropin that stimulates follicular production of sex steroids including a maturation inducing steroid (MIS). This study was designed to determine the in vitro secretion and the blood changes of testosterone (T), 11-ketotestosterone (11KT), 17,20β-dihydroxy-4-pregnen-3-one(17,20β-P) and 17,20β, 21-trihydroxy-4-pregnen-3-one (20β-S) in sterlet female during FM induced by injection of a pituitary preparation (PP). Sex steroid levels (ng/ml) increased significantly 8 hrs after PP treatment from (T) 18.4±9.68 to 96.5±14.33; (11KT) 8.3±34.06 to 30.5±3.93;(17,20β-P) 0.40±0 to 2.7±1.28 and (20β-S) 0.4±0.01 to 2.13±0.61. 20βS levels increased 16 hrs after PP treatment and reached 3.7±1.32, 11KT levels did not change significantly and T and 17,20β-P levels slightly decreased. All steroids decreased significantly after FM to lower levels than before treatment. In vitro secretion of sex steroids in ovarian follicles taken 8 hrs after PP treatment was (pg/follicle/48hours) 73.3±36.96; 3.5±0.90; 8.30±1.79; 88.1±24.32 (T, 11KT, 17,20β-P, 20β-S, respectively). The data shows that although both 17,20β-P and 20β-S levels rose during FM but 20β-S appears to be produced in vitro in larger quantities.

P-VI-30

ASTACIN-LIKE PROTEASE, ALVEOLIN, IS RELEASED FROM CORTICAL VESICLES AND INDUCES THE CHANGES OF EGG ENVELOPE PROTEINS DURING FERTILIZATION IN MEDAKA (*ORYZIAS LATIPES*) EGGS

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In many fishes, the egg envelope transforms to an insoluble hardened form upon fertilization. This change of the egg envelope is implicated through hyper-polymerization of constituent proteins catalyzed by transglutaminase activity located within the envelope. Upon fertilization, two major protein groups, ZI-1,2 and ZI-3, composing the egg envelope are polymerized to the insoluble form via the temporal formations of 132 kDa and 61-62 kDa intermediate proteins. We have shown that astacin-like protease, 'alveolin' is released from cortical vesicles to perivitelline space after egg activation and induces the initiation of the polymerization of egg envelope proteins. At that time, alveolin catalyzes limited digestion of ZI-1,2 to 61-62 kDa protein prior to the polymerization. To clarify the initial reactions of the polymerization, primary structure of 132 kDa protein was analyzed by mass spectrometry and amino acid sequencing. The 132 kDa was composed of 61-62 kDa and ZI-3 with an isopeptide bound formation between the 412th Lys residue of 61-62 kDa and 43rd Gln residue of ZI-3. These results suggest that the transitional formation of the 132 kDa protein is the first cross-linking step of egg envelope proteins during the activation process after fertilization.

P-VI-31

CORRELATION BETWEEN OOCYTE DEVELOPMENT AND PLASMA LEVELS OF STERIODS AND VITELLOGENIN IN GREENBACK FLOUNDER *RHOMBOSOLEA TAPIRINA*

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The pattern of oocyte development and changes of plasma levels of vitellogenin (Vtg), 17β-estradiol (E2) and testosterone (T) were investigated in maturing female greenback flounder *Rhombosolea tapirina* over the first part of a reproductive season (Feb.- Jun., 2002). There were no significant temporal variations in ovarian development and plasma levels of Vtg and steroids during the sampling period despite some fish showing reproductive maturity at all sampling times. This indicates that reproductive development is not synchronized within the population. However, significant increases in plasma levels of Vtg, E2 and T were observed in vitellogenic fish, and in fish undergoing final maturation. A positive relationship was also found between the growth of the oocyte and plasma levels of Vtg and steroids, although the patterns of increase were different. Plasma levels of Vtg and E2 rose steadily over oocyte sizes from 100 to 450 μm, but the increasing rate of plasma E2 was somewhat smaller than that of Vtg, and both reached a saturated level at oocyte sizes of around 450 μm. In contrast, plasma levels of T showed no marked increase until oocytes grew beyond 400 μm.

P-VI-32

APOPTOSIS DURING GAMETOGENESIS IN DIPLOID AND TRIPLOID TURBOT (*SCOPHTHALMUS MAXIMUS*)

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Completion of gametogenesis requires the progression through successive developmental stages in a strict temporal and spatial manner. Gametogenesis is monitored at selected checkpoints to detect potential problems (e.g., chromosomal aberrations or meiotic complications) halting development until these mistakes are corrected. If repair is impossible, apoptosis can be immediately triggered or an arrest of meiotic progression is eventually followed by cell death. In spite of the important function of apoptosis in the regulation of germline development and the elimination of unwanted or unneeded cells during gametogenesis, its role in the control of gonadal development in fish has yet to be elucidated. Triploidy is readily induced in fish typically resulting in chromosomal modifications that provoke abnormal gonadal development. We investigated the presence of apoptotic cells in the gonads of male and female triploid turbot as compared to diploids to elucidate the possible role of apoptosis as a regulatory mechanism during both normal and abnormal gonadal development. Using electron microscopy and immunohistochemistry with the primary antibody M30, we identified the presence of apoptotic cells in the testis of 89 males (63 diploid and 26 triploid) and 116 triploid females. Correlation between gametogenic condition and number of apoptotic cells was also evaluated. Triploidy induced deficient gonadal development in both sexes but in males meiosis was initially unaffected and spermatogenesis proceeded until spermatids. In females, meiosis disruption was evident in most fish and oogenesis was arrested at the transition to primary oocyte. Thus, apoptosis was common in the ovaries of triploids but absent in those of diploids. In contrast, apoptosis was observed in both diploid and triploid males, with a higher incidence in the former. These results suggest that apoptosis is involved in the normal regulation of spermatogenesis progression and male germ cell fate, and that the differential effects of triploidy on gonadal development include the induction of apoptosis in females.

P-VI-33

PURIFICATION OF ANAPHASE PROMOTING COMPLEX/CYCLOSOME FROM GOLDFISH OOCYTES MODIFICATIONS TO AND FACTORS INTERACTING WITH PROTEASOMES DURING THE MEIOTIC CELL CYCLE

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Destruction of cyclin B is required for exit from mitosis and meiosis. A cyclin-specific ubiquitinating system, including anaphase-promoting complex/cyclosome (APC/C) is thought to be responsible for cyclin B destruction. To learn more about the molecular mechanism of cyclin B degradation, a molecular study of the ubiquitinating system in goldfish has been undertaken. For biochemical preparation of APC/C, we first conducted the cloning, sequencing and expression analysis of goldfish, *Carassius auratus*, cdc27 that encodes a subunit of APC/C from goldfish ovary. The deduced amino acid sequence is highly homologous to cdc27 from other species. Then recombinant goldfish Cdc27C (C-terminal half of Cdc27) was expressed in *Escherichia coli*, and an antibody was raised against purified recombinant protein. Polyclonal antiserum cross-reactive with Cdc27 was obtained. By the assay using the antibody, APC/C was purified by column chromatographs.

P-VI-34

STEROID SYNTHESIS IN FOLLICULAR CELLS AND INDUCTION OF GERMINAL VESICLE BREAKDOWN IN SPOTTED WOLFFISH (*ANARHICHAS MINOR*) OOCYTES

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The small oocytes of most marine fish spcies preclude separation of the follicular cell layers (theca and granulosa) and hinder investigation of the role of the different cell layers in sex steroid synthesis. Spotted wolffish, a marine perciform species, produces large oocytes (5-6 mm in diameter) and separation of the follicular cell layers is possible. During the periovulatory period female wolffish display only low plasma concentrations of 17,20β-dihydroxy-4-pregnen-3-one (17,20β-P) but high concentrations of other sulphated 20β-hydroxylated progestins (mainly 5β-pregnane-3α,17,20β-triol-20-sulphate (3α,17,20β-P-5β-S)). The site of synthesis, and the involvement of these steroids in final oocyte maturation (FOM) in female wolffish is not known. Theca and granulosa cell layers, obtained by mechanical separation of follicles from postvitellogenic wolffish ovaries, were incubated, either alone or in combination, with radiolabeled 17-hydroxyprogesterone (17-OH) or 17,20β-P. Theca cells metabolised little of the 17-OH, and separation of the incubation products by thin layer chromatography (TLC) revealed two small peaks corresponding to testosterone (T) and androstenedione (A). In granulosa cell layers 17-OH was almost completely converted to 17,20β-P (30%) and A (50%). When 17,20β-P was used as the precursor about 60% of the radioactivity appeared as 17,20β-P-sulphate (17,20β-P-S) produced by theca cells. 17,20β-P-S was also formed from 17,20β-P in the granulosa layer, but to a lesser extent. No further conversion of 17,20β-P was observed in either of the cell layers. Eleven different steroids were tested for relative effectiveness in induction of germinal vesicle breakdown (GVBD). 17,20β,21-trihydroxy-4-pregnen-3-one (20β-S), 17,20β-dihydroxy-5β-pregnan-3-one (17,20β-P-5β) and 17,20 β-P were the most potent in that order. Sulphated steroids (i.e. 3α,17,20β-P-5β-S and 17,20β-P-S) had no GVBD inducing capacity. Since only 17,20β-P was found to be synthesised by the follicle *in vitro*, we suggest that 17,20βP may function as the maturation inducing steroid in female wolffish.

P-VI-35

REGULATION OF StAR AND STEROIDGENIC ENZYME EXPRESSION IN TROUT OVARIAN FOLLICLES

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Numerous studies have focussed on the signal transduction pathways mediating the endocrine or local hormone control of steroidogenesis in teleosts. By comparison, few studies have examined the regulation of enzymes that are responsible for cholesterol mobilization or the synthesis of steroids within the gonad. The objective of the present study was to determine the effects of gonadotropin and cAMP analogues on the expression of the steroidogenic acute regulatory protein (StAR), which is responsible for the movement of cholesterol across the inner mitochondrial membrane, and the steroidogenic enzymes, 3β-hydroxysteroid dehydrogenase (3β-HSD), P450 side chain cleavage (P450scc), and P450c17 in rainbow trout ovarian follicles. Using semi-quantitative RT-PCR, we showed that a 1 hr incubation of trout follicles (4mm) with salmon gonadotropin (SG-G100; 10 ug/ml) upregulated the expression of StAR (41%), 3β-HSD (930%), and P450c17 (58%) but had no effect on P450scc. Treatment with 8-bromo cAMP (0.15 mM) resulted in reduced expression of all the transcripts. Treatment of SG-G100 or 8-bromo-cAMP at 3 hr resulted in reduced expression of all the transcripts. Actinomycin D (10 ug/ml) attenuated basal and gondadotropin-stimulated expression of the transcripts at 1 hr and 3 hr, which suggests that the transcripts were produced *de novo*. StAR expression levels were also examined in post-ovulatory follicles incubated with 8-bromo-cAMP using northern hybridization techniques. These experiments showed significant increases (30-75%) in transcription levels at 3 hr. Collectively, these studies show that established regulators of gonadal steroidogenesis affect the expression of enzymes and proteins that are fundamental to the control of ovarian function in teleosts. This provides a framework for future studies to examine additional mechanisms by which endogenous or exogenous factors may influence ovarian function.

P-VI-36

EFFECTS OF dbcAMP ON OVARIAN STEROIDOGENESIS IN ISOLATED FOLLICLES OF ATLANTIC SALMON

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This work arose from in-vitro studies on the effects of temperature on cellular mechanisms of ovarian steroidogenesis during vitellogenesis in Atlantic salmon. Estrogen (E2) and testosterone (T) levels were measured in isolated follicles in response to treatment with 2-0-dibutyryladenosine 3'5'cyclic monophosphate (dbcAMP) and human chorionic gonadotropin (hCG). In terms of E2 production, ovarian follicles were responsive to stimulation with hCG at a level of 100 but not 10 IU ml^{-1}, but T production was not significantly elevated over basal levels by any treatment. Incubation with 0.1, 1.0 and 10 mM dbcAMP produced unexpected results. T production was dose related but E2 was not significantly elevated at any treatment concentration suggesting aromatase synthesis or activity was inhibited under these conditions. Possible cellular mechanisms may involve: post-translational phosphorylation events; interference between steroidogenic cytochrome P450 pathways non-specifically up-regulated by dbcAMP in the absence of contributions from first messenger receptor occupancy; competition between steroidogenic enzymes for reducing equivalents provided by P450 reductase. In summary, compounds that elevate intracellular cAMP in the absence of trophic hormone may be disruptive for co-ordinated steroidogenesis.

P-VI-37

APOPTOSIS IN REGRESSING FOLLICLES FROM *SOLEA SOLEA* AND *GADUS MORHUA*

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In order to measure individual egg production (realised fecundity) in wild fish we need to determine the fate of vitellogenic follicles as they regress into post ovulatory or atretic follicles. The cellular processes underlying follicular regression is thought to start with the production of apoptotic nuclei and this was investigated using the TUNEL reaction and by measuring short chain oligonucleotides (180-200 base pairs). The first part of the study focused on *Solea solea* collected from wild populations both during the spawning cycle and immediately after spawning. Although there were high levels of short chain (180-200 base pairs) oligonucleotides and apoptotic nuclei shown by the TUNEL reaction in spent ovaries of *Solea solea* there was no clear association with atretic compared to apparently normal vitellogenic follicles. Two experiments where then carried out with captive cod to produce ovary biopsy samples containing atretic and post ovulatory follicles at all stages of regression from normal vitellogenic oocytes. The production of atretic vitellogenic follicles was induced by switching the illumination from a natural cycle to continuous exposure. Fish with recently produced post ovulatory follicles were collected from a captive spawning population by only taking running females and their eggs had to show high levels (95%) of fertility. The production of apoptotic nuclei was determined in each case using the TUNEL reaction and related to traditional descriptions of each class of regressing follicle.

P-VI-38

HETEROLOGOUS GAP JUNCTIONS BETWEEN OOCYTE AND GRANULOSA CELLS IN AYU (*PLECOGLOSSUS ALTIVELIS*): FORMATION AND ROLE DURING GTH-DEPENDENT ACQUISITION OF OOCYTE MATURATIONAL COMPETENCE

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Before the gonadotropin (GTH) surge, full-grown teleost oocytes are often unresponsive to maturation-inducing hormone (MIH) and sensitivity of oocytes to MIH (oocyte maturational competence; OMC) can be stimulated by GTH. Our previous studies with Atlantic croaker suggested that heterologous (oocyte-granulosa cell) gap junctions (GJ) have a role to play during the GTH-dependent acquisition of OMC. Although an earlier study with Ayu indicated that heterologous GJ-like structures are present, the significance of GJ with respect to OMC was not explored. In this study, we cloned and characterized GJ protein (connexin; Cx) cDNAs and examined the functionality of heterologous GJ in Ayu ovarian follicles. The effects of a gap junction inhibitor on GTH-induced OMC were also determined. Four Cx cDNAs were cloned from Ayu ovary. One Cx, predicted to be 34.9 kD (Cx34.9), was expressed in both oocyte and follicle cells thus suggesting that this molecule contributes to the formation of homotypic GJ between oocyte and granulosa cells. Cx34.9 transcripts were abundant in the ovary during development of OMC and oocyte maturation. However, its level was low in pre-vitellogenic and ovulatory stage ovary. This observation suggests that Cx34.9 is involved in OMC and/or oocyte maturation. Lucifer yellow injected into oocyte diffused to follicle cells, and this diffusion was completely inhibited by a gap junction inhibitor (18-α glycyrrhetinic acid; α-GA). These results clearly indicate that GJ between oocyte and granulosa cells make functional channels capable of letting through small molecules. Further, the α-GA inhibited GTH-induced acquisition of OMC in a dose-dependent manner. In conclusion, GJ (possibly formed by Cx34.9) between oocyte and granulosa cells seems required for GTH-dependent acquisition of OMC.

P-VI-39

PROTEOME ANALYSIS: A NEW APROACH TO IDENTIFY KEY PROTEINS INVOLVED IN ACQUISITION OF MATURATIONAL COMPETENCE AND OOCYTE MATURATION

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Japanese freshwater fish, medaka (*Olyzias latipes*) spawns daily under the intrinsic hormonal control of gonadotropin and maturation-inducing hormone, 17α,20β-dihydroxy-4-pregnen- 3-one (17α,20β-DP). We maintain medaka in laboratory aquaria under the conditions of 14L:10D of lights with constant temperature (27°C) to obtain developmentally coincident oocytes. Oocytes obtained 22 hr prior to spawning were classified as maturationally competent oocytes (>950 μm in diameter; 2nd group) that already expressed 17α,20β-DP receptor at the cell surface level. At the same time, oocytes having 650-750 μm in diameter (3rd group) were collected as maturationally incompetent oocytes that would be spawning after 46 hr and expressed no receptor activities yet. These oocytes were analyzed by high performance two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) using Multiphor/Excelgel system (Amersham Bioscience) to identify key proteins involved in acquisition of maturational competence and a signal transduction pathway of 17α,20β-DP. Image analysis of stained gels showed that oocytes contained over 2,000 proteins in the range of 3.0-10.0 of isoelectric point (pI) and 5,500-2,000,000 of molecular weight (MW). The detailed comparative analyses of 2nd and 3rd group oocytes in the range of pI 4.7-6.0 and MW 5,500-66,000 revealed that 149 spots showed changes during acquisition of maturational competence. These interesting spots were composed of 59 newly appeared spots, 5 disappeared spots, 61 spots increased to three-fold, and 24 spots decreased less than 1/3rd.
The second group oocytes were also analyzed for changes at phosphorylation and dephosphorylation levels of proteins by using anti-phosphoamino acid antibodies (anti-serine, threonine and tyrosine) at 1, 5, and 15 min after the hormonal treatment during an early phase of the signal transduction. In the range of pI 4.7-6.0 and MW 5,500-66,000, 53 spots showed changes at varying degrees of protein phosphorylation. Among these, 22 spots were either increased or newly appeared, 4 spots were decreased, and 29 spots fluctuated in phosphorylation levels within 15 min. These specified proteins are identified by sequence analysis of composed amino acids using protein sequencer and mass spectrometer. Preliminary study revealed that L-SF precursor, vitellogenin II component, and 22KDa proteins had undergone changes during maturational competence. We will introduce this new methodology to evaluate our current results.

P-VI-40

17,20β,21-TRIHYDROXY-4-PREGNEN-3-ONE AS A OOCYTE MATURATION- INDUCING STEROIDS IN PROTANDROUS BLACK PORGY, *ACANTHOPAGRUS SCHLEGELI*

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The objectives of the present study were to investigate the maturation-inducing steroid (MIS) in marine protandrous black porgy, *Acanthopagrus schlegeli*. 17,20β,21-Trihydroxy-4-pregnen-3-one (20β-S) and 17,20β-dihydroxy-4-pregnen-3-one (DHP) were capable to induce *in vitro* oocyte maturation in black porgy. Females were injected with two successive doses of LHRH analog (10 and 50 mg/kg of fish) to induce maturation. The ovarian tissue was obtained at the MIS-sensitive stage for *in vitro* oocyte culture. Plasma luteinizing hormone and 20β-S concentrations increased significantly during the oocyte maturation. A tritiated precursor, [^{3}H]-pregnenolone, was in vitro cultured together in the maturing ovarian tissue. The tritiated metabolites were purified and identified by the methods of solvent extraction, HPLC, TLC, microchemical reaction, and recrystallization. Significant levels of tritiated 20β-S but not DHP were biosynthesized from [^{3}H]-pregnenolone on the bases of HPLC and TLC. Similar TLC profiles were found between tritiated metabolites isolated from HPLC/TLC and standard 20β-S after the reaction of acetylation. Constant specific radioactivity of tritiated 20β-S by recrystallization was further obtained in the tritiated metabolites isolated from HPLC fraction. The present data provide the first evidences that 20β-S is the physiological MIS in black porgy.

P-VI-41

HORMONAL REGULATION OF THE ZEBRAFISH (*DANIO RERIO*) OVARIAN AROMATASE GENE

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Cytochrome P450 aromatase is a steroidogenic enzyme catalyzing the conversion of androgens to estrogens (mainly testosterone to estradiol-17β). In teleosts, estradiol-17β is the main estrogen essential for hepatic vitellogenin synthesis and oocyte development. The regulation of the ovarian aromatase activity and expression has been well documented in mammals; however, little information is available on its control in teleosts.
Using a semi-quantitative RT-PCR assay, the present study examined the stage-dependent pattern of aromatase expression in the zebrafish ovarian follicles followed by investigation of its regulation in either the freshly isolated intact follicles or the cultured follicle cells. Zebrafish aromatase expression increased markedly from the pre-vitellogenic to mid-vitellogenic follicles, but dramatically decreased in full-grown follicles prior to the oocyte maturation. When incubated in vitro, the expression of aromatase steadily declined in the cultured mid-vitellogenic follicles over six days, and the level of expression in freshly isolated intact mid-vitellogenic follicles was significantly increased by hCG (10 IU/ml). The stimulation could be mimicked in the cultured zebrafish ovarian follicle cells by dibutyryl-cAMP (db-cAMP), a cAMP analog, and forskolin, an activator of adenylate cyclase, in dose- and time-dependent manners, suggesting that the intracellular cAMP pathway is involved in the regulation of aromatase expression. Furthermore, the db-cAMP-stimulated expression was effectively blocked by H89, a specific inhibitor of protein kinase A (PKA), at 10 uM, indicating that its regulation by cAMP and possibly gonadotropin(s) is mediated via the cAMP-PKA pathway. In addition, preliminary experiments showed that the effects of hCG and cAMP on the expression of ovarian aromatase were modulated by other factors such as activin and insulin-like growth factor I. The regulation of zebrafish ovarian aromatase by cAMP is consistent with a previous study by others that identified several potential cAMP-responsive elements (CREs) on the aromatase promoter based on sequence analysis; however, which CRE is functionally involved in the regulation by gonadotropin and cAMP remains to be further elucidated. [The work was substantially supported by grants (CUHK4176/99M, CUHK4150/01M and CUHK4258/02M) to W. Ge from the Research Grants Council of the Hong Kong Special Administrative Region.]

P-VI-42

PARTIAL CLONING OF 17β-HSD-I FROM THE NILE TILAPIA OVARY AND ITS EXPRESSION PATTERN DURING SPAWNING CYCLE

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In teleosts, estradiol-17β (E_2) produced by the follicle layers is well known for its function in vitellogenesis. The key enzymes presumed to be responsible for the steroidogenic pathway leading to E_2 production are cytochrome P-450 aromatase and 17β -hydroxysteroid dehydrogenase (17β -HSD). Different forms of aromatases were cloned, characterized and its importance in vitellogenesis is well documented in several teleost species. However, till this date 17β -HSD-I was only isolated using Japanese eel ovary. To understand further, we attempted to isolate 17β -HSD-I from the ovary of Nile tilapia. It is also important to understand its functional relevance, as several types of 17β -HSDs have varied functions based on their ability to catalyze different 17-keto steroids in mammals. Using degenerate primers we obtained a 548bp partial cDNA fragment homologous to 17β -HSD-I by RT-PCR. Partial sequence shows highest homology to eel 17β -HSD-I and contains the entire signature domains required to classify it with short-chain alcohol dehydrogenase family. To understand the role of 17β -HSD-I during vitellogenesis we analyzed its expression pattern by RT-PCR at different stages of oocyte development ranging from early vitellogenesis to spawning. 17β -HSD-I transcripts increase gradually during vitellogenesis and became faint or undetectable at the day of spawning. These results tend to support that E_2 production getting subdued when the fish undergo final maturation after vitellogenesis. Existing knowledge from eel 17β -HSD-I recombinant protein suggests that it converts estrone to E^2 but not androstenedione to testosterone or vice-versa. Nevertheless, our research work is in progress to obtain full length 17β -HSD-I cDNA and to understand its functional ability to convert different steroid substrates. Based on previous reports from our laboratory together with the present study, the working hypothesis of steroidogenic shift in the ovary by gonadotropins during vitellogenesis to final maturation seems to be relevant for teleosts.

Session VII

Environmental Influence on Reproduction

O-VII-1
COLD AND DARK OR WARM AND LIGHT: VARIATIONS ON THE THEME OF ENVIRONMENTAL CONTROL OF REPRODUCTION

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Environmental change synchronizes reproduction in most fishes with the environmental information being transduced though the activity of the reproductive endocrine system. In temperate habitats there is a marked climatic seasonality and this is associated with relatively short periods that are optimal for egg and larval survival, and subsequent juvenile growth. In consequence, reproduction in temperate systems tends to be truncated and is typically strongly entrained by photoperiod, with temperature acting as a secondary modifying cue. In warm temperate or tropical systems the situation is altered by the fact that there is less seasonal variability of the physical environment and reduced imperative for seasonally phased reproduction. There may still be local synchronization of reproductive activity but this tends to be more closely associated with strategies to avoid predation on eggs and hatching larvae. This review examines the endocrine machinery that underlies environmental regulation of reproduction and how it might differ in temperate and tropical environments, using salmonids and the tropical damselfish *Acanthochromis polyacanthus* as examples of the issues at work.

Salmonids offer a prime example of a temperate group where reproductive phasing is strongly photoperiod driven and have formed the basis of studies elucidating the transduction of the photoperiod signal via the pineal gland and the secretion of melatonin. It is clear that this in turn is translated into a reproductive endocrine signal but the exact mechanism remains opaque. The photolability of salmonid reproduction has lead to the use of photoperiod manipulation to advance or delay reproduction in commercial stocks. Recent experiments in Tasmania where ambient light levels are several orders of magnitude brighter than in the native range raise new questions about the process and how it responds to changes in other variables such as temperature. The developing perception is that temperature may be a much stronger modifying variable than was initially thought. In contrast to salmonids, seasonality in damselfish appears to be temperature regulated but also strongly dependent on social context and is probably also acutely regulated by proximate factors such as local food availability. We suggest a working model of environmental control whereby temperate and tropical species approach a *photoperiod-temperature-proximate variables* hierarchy of control, from different directions.

O-VII-2
THE USE OF ADDITIONAL LIGHTING AND ARTIFICIAL PHOTOPERIODS TO RECONDITION EARLY MATURING ATLANTIC SALMON (*SALMO SALAR*) IN TASMANIA

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Aquaculture of Atlantic salmon in Tasmania takes place under unique culture conditions of high light intensity; and temperatures reaching the upper thermal limit for salmonids. The traditionally low maturing Canadian stock used in Tasmania exhibits increased growth rates reaching a marketable size a whole year earlier than in the Northern Hemisphere; however, it also displays reduced times to first maturation. This presents a major financial loss to the aquaculture industry as early maturing fish must be harvested before market size is attained or the resultant poor flesh quality makes the individuals unsaleable. Ambient environmental conditions and the reproductive strategies of Tasmanian fish mean that photoperiods used to inhibit maturation in the Northern Hemisphere are of limited success, therefore this work investigates the use of artificial photoperiod to recondition previously mature Atlantic salmon to provide a larger fish with high market value. In April 120,000 Atlantic salmon displaying pronounced secondary sexual characteristics were randomly divided into 4 sea cages and fed to appetite. Three of the cages were then put under 24 hour artificial light from either May 10, June 15 or July 18. The remaining group was maintained under ambient conditions. Histological, hormonal and morphometric measurements were collected at monthly intervals until harvest in December. Fish maintained under lights attained a reversion from dark skin pigmentation typical of mature individuals to a silver sea going colouration more rapidly than the control group. In addition the ROCHEsalmofan™ score and body wall thickness was significantly greater than that of the control fish combining to produce a higher commercial quality fish. The results show that the use of artificial lights and photoperiods must be fine tuned account for Southern hemisphere conditions, however, used correctly, provides a useful tool with which to accelerate the reconditioning of previously mature Atlantic salmon under Tasmanian conditions.

O-VII-3

INFLUENCE OF PHOTOPERIOD REGIMES ON THE EURASIAN PERCH GONADOGENESIS, SPAWNING AND EGG AND LARVAE QUALITY

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The Eurasian perch, *Perca fluviatilis*, has been identified as a significant new species for freshwater aquaculture. Photoperiod and temperature, which display seasonal rhythms, are thought to be the main environmental cues in the timing of reproduction. A 10-month experiment was carried out to investigate the effects of photoperiod regimes under ambient temperature on gonadal growth and spawning quality in Eurasian perch. Fish were reared in 12 tanks (3000 L, 88 fish/tank, initial weight of 300g, age=2^{+}) in an outdoor recirculating system and subjected to 4 photoperiod regimes in triplicate: continuous light (LL), constant daylength (LD 16:8) as observed in ambient conditions, natural (NP) and simulated natural (SNP) photoperiod. Gonadal development was monitored through GSI and gonad histology on sacrificed fish. Blood samples and weight and length measures were taken monthly from 15 agged fish in each tank. Spawning, oocyte and larval qualities were determined. This study showed that LL inhibited reproduction in male and female with very low sex steroid levels being measured throughout the cycle. LD 16:8 resulted in heterogeneous gonadal development and sex steroid levels with only 54% of females and <30% of males maturing. Similar gonadal development was seen under NP and SNP, although a delay was observed under SNP, and 45% of the maturing females did not spawn. Spawning occurred at dawn and around 2 pm but was never recorded at night. The photoperiod regimes and artificial lighting affected egg quality, as fertilisation in the SNP group was very low (8%) compared to the NP treatment (58%). This study is the first record of photoperiod control of Eurasian perch reproduction. Daylight alternation appeared to be important as no fish matured under LL and seasonal daylength variations are required to control reproduction. Light characteristics (spectrum, intensity, daily changes· are not likely to be involved in the timing of gonadogenesis as fish matured under both SNP and NP, although these parameters appeared to have an effect on the spawning release and the egg quality.

O-VII-4

PHOTOPERIOD MANIPULATION OF MATURATION AND GROWTH OF ATLANTIC COD (*GADHUS MORHUA*). THE EFFECT OF TIMING, PERIOD AND SYSTEM ON SUCCESFUL APPLICATION

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Maturation 24 months post-hatch in Atlantic cod is a problem for commercial culture of this species as it leads to losses in somatic growth potential through redirection of energy to gonadal development, to alterations in flesh quality and increases in mortality. It is known that the application of continuous light from the summer solstice prior to first maturation (approximately 15 months post-hatch) can inhibit maturation in the second year when applied in enclosed light-proofed tank systems. However, the use of similar procedures has been found to have only a delaying effect when tested in uncovered cage systems. A series of experiments were designed to examine the effects of photoperiod manipulation on first maturation in cod and the lighting parameters that may influence the successful use of such procedures in cage systems. Trial one examined the effect of prolonged exposure to the previously reported successful photoperiod in a light-proofed tank system. Trial two studied the importance of timing of LL application 6-18 months post-hatch again in light-proofed tanks. Finally, light characteristics (spectral content and intensity) of photoperiod control systems used in the aquaculture industry were recorded to catalogue the influence of various environmental and physical factors. During the first trial, spawning was delayed by 12 months and somatic growth improved by 30%. However, an underlying "endogenous" maturation cycle that was not influenced by the photoperiod treatment was evident In the second trial seven experimental and one control photoperiod were tested in an enclosed tank system. All individuals were tagged and growth and maturation data were recorded for each individual. Regular samples were taken from all groups for measurement of gonadosomatic index (GSI) and hepatosomatic index (HSI). Interpretation of the response of the fish to photoperiod was assessed by measurement of plasma melatonin. This paper will discuss the effects of the different lighting environments in covered tanks and uncovered sea cages and the timing of photoperiod manipulation on maturation control in Atlantic cod and other marine species. This work was supported by NERC studentship NER/S/A/2000/03640.

O-VII-5

EFFECTS OF PHOTOPERIOD, TEMPERATURE AND GnRHa TREATMENT ON THE REPRODUCTIVE PHYSIOLOGY OF ATLANTIC SALMON (*SALMO SALAR* L.) BROODSTOCK

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Photoperiod appears to be the most important environmental cue controlling reproductive timing in salmon. However, high temperatures can delay or inhibit spawning in salmon. The present study investigates endocrine mechanisms of high temperature inhibition, and the use of GnRHa treatment to overcome this inhibition. Exposure to an accelerated photoperiod regime (AP) advanced ovulation compared with simulated natural photoperiod (SNP) However, ovulation was desynchronised in the AP group under ambient temperatures, and egg survival was low. In contrast, exposure to cooled water restored egg survival, and synchronised and advanced ovulation in both the AP and SNP groups compared with fish maintained at ambient temperature. In a follow up study, salmon broodstock reared under SNP were exposed to high (14°-16°C), ambient (decreasing from 11° to 5°C) or cold temperatures (3°-7°C) from September 28. On October 5, each temperature treatment was combined with either a GnRHa treatment, a sham treatment, or left untreated (controls). Both ovulation and spermiation were completely inhibited in the high temperature groups, while the coldwater treatment slightly advanced spawning in both sexes. Treatment with GnRHa advanced and synchronised spawning in male and female fish at all temperatures, overriding the inhibition induced by elevated temperatures. Survival of eggs to eyed stage was slightly higher in the cold water group than in ambient, while eggs of GnRHa treated fish had lower survival rates than sham and untreated controls. Results on the endocrine profiles of these studies will be presented and discussed.

O-VII-6

EFFECTS OF LOW pH ON REPRODUCTIVE BEHAVIOR OF SALMONID FISHES

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In northern Europe and America, acidification of lakes and rivers induced by acid precipitation has damaged fish populations. At present, rapidly expanding industrial activities in East Asia have led to a continuous increase in the amount of emission of acidic pollutants, and rain at acidic levels of pH 4 has precipitated throughout Japan. Therefore, the effects of acid rain on fish population has been apprehended also in Japan. In order to examine the effects of low pH on spawning behavior of salmonids which are acid-sensitive species, changes in frequency of nest-digging behavior and up-stream behavior for spawning due to pH changes were observed in mature female land-locked sockeye salmon *Oncorhunchus nerka*, brown trout *Salmo trutta* and Japanese char *Salvelinus leucomaenis*. Digging behavior and up-stream behavior were significantly inhibited in weakly acidic water made with sulfuric acid such as pH 5.8-6.4, and the land-locked sockeye salmon was the most sensitive to pH change of three species.

O-VII-7
THE MECHANISM OF TRANSCRIPTIONAL REGULATION OF VITELLOGENIN GENE IN JAPANESE FLOUNDER (*PARALICHTHYS OLIVACEUS*)

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Vitellogenin (Vg), a precursor of egg yolk protein, is synthesized in the liver in response to estrogen in teleost fishes. Recently, Vg has been used as a biomarker for the estrogenic effect of endocrine disrupting chemicals in some fishes. However, the mechanism of transcriptional regulation of Vg gene has yet to be clarified.

To elucidate how the transcription of Vg gene is regulated in the Japanese flounder (*Paralichthys olivaceus*), we first isolated the putative promoter region of the flounder Vg gene. Next, we constructed the plasmid (Vg-luc) which linked the putative promoter of the Vg gene to luciferase reporter gene and that which expressed the flounder estrogen receptor-α (CMV-fERα) or the ERβ (CMV-fERβ), and both plasmids were transiently transfected into Hepa-E1 cells (eel hepatocyte) with or without 17β-estradiol (E2). As a result, luciferase activity was significantly induced by co-transfection of the CMV-fERα or CMV-fERβ plasmid with the Vg-luc plasmid under E2 administration. The E2-dependent induction was counteracted by co-administration of tamoxifen (ER antagonist). Moreover, mutation analysis of the estrogen responsive elements (EREs) in the Vg gene promoter indicated the presence of two potential EREs. These results suggest that the expression of Vg gene is induced by E2 through both ERα and ERβ in the flounder liver.

To investigate the mechanism of transcriptional regulation of Vg gene by estrogenic chemicals, the Vg-luc, and the CMV-fERα or CMV-fERβ plasmids were transiently transfected into Hepa-E1 cells that were then incubated with *p*-nonylphenol (NP), bisphenol A (BPA), or genistein. As a result, luciferase activity was induced more intensely by co-transfection of the CMV-fERα than CMV-fERβ with the Vg-luc plasmid under NP or BPA administration. On the other hand, the activity was significantly induced at a similar level by co-transfection of the CMV-fERα or CMV-fERβ plasmid under genistein administration. These results indicate that NP and BPA induce the expression of Vg gene mainly through ERα, while genistein induces the gene expression through both ERα and ERβ. We suggest that such an *in vitro* reporter assay provides an excellent system for elucidating the action mechanism of estrogenic chemicals in fishes.

O-VII-8
REPRODUCTIVE FAILURE AND DISTURBED STEROIDOGENESIS IN FEMALE PERCH (*PERCA FLUVIATILIS*) EXPOSED TO TOXIC REFUSE DUMP LEACHATE

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Toxic leachate from many Swedish refuse dumps is passively and uncontrollably dispersed into the environment. We recently reported reproductive failures and endocrine disruption in female perch (*Perca fluviatilis*) and brook trout (*Salvelinus fontinalis*) from a leachate-contaminated water system. Our findings included low numbers of reproducing females, together with low gonadosomatic index (GSI), low brain aromatase activity and reduced circulating levels of steroids. High frequencies of skin lesions and induced EROD activity were observed in perch. To further explore the endocrine mechanism(s) behind the reproductive failures, an annual study was conducted on female perch from the leachate-contaminated Lake Molnbyggen and compared to reference perch. Steroid hormones were analysed in blood plasma with GC/HRMS, and the number of reproducing females, liver-somatic index (LSI), GSI, and brain aromatase activity were measured. Although skin lesions, such as sores, were less frequent, most of the females in Molnbyggen were still not reproducing. Those females that were reproducing had lower levels of androgens, particularly androstenedione and testosterone, during the first months of oogenesis. Lower 17β-estradiol levels as well as lower LSI were found (the latter possibly a result of a less active vitellogenesis), but no effects were observed on either GSI or the aromatase activity. The high numbers of non-reproducing females could be a direct result of a disrupted steroidogenesis. Testosterone levels may be insufficient to activate the brain-pituitary-gonadal axis, precluding the initiation of gametogenesis. The improved GC/HRMS method, provides the opportunity to analyse a wide range of steroids, including their metabolites, as well as stress hormone levels. The results show that steroid profiles are powerful and sensitive reproductive biomarkers for the early detection of endocrine disruption. This biomarker also has the potential to give information about the endocrine mechanism(s) behind the reproductive failures in leachate-exposed females.

P-VII-1

PHOTOPERIOD AND TEMPERATURE AFFECTS SEASONAL OVARIAN GENE EXPRESSION OF P450 AROMATASE AND GONADOTROPIN RECEPTORS IN ATLANTIC SALMON (*Salmo salar* L.) BROODSTOCK

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Photoperiod and temperature modulate the seasonal timing of ovulation and affect egg quality in Atlantic salmon broodstock. In the current study, exposure to an accelerated photoperiod regime (AP) advanced ovulation compared with simulated natural photoperiod (SNP), both under ambient temperature and in cooled water. However, ovulation was desynchronised in the AP group under ambient temperatures, while exposure to cooled water synchronised and advanced ovulation in both the AP and SNP groups. The abundance of transcripts for Atlantic salmon aromatase (P450arom), a putative Follicle-Stimulating Hormone receptor (FSH-R) and a putative Luteinizing Hormone receptor (LH-R) were determined by rtq-PCR throughout the annual reproductive cycle, and correlated to ovarian development and plasma levels of estradiol and testosterone. In the SNP group, the P450arom expression increased 25-fold, with a maximum at the late vitellogenic stage, and declined prior to spawning. FSH-R expression increased 4-fold during vitellogenesis, declined at the late vitellogenic stage, but increased 12-fold post ovulation. LH-R expression increased 20-fold during vitellogenesis, and declined to basal levels after spawning. In the AP group, the increase in P450arom at vitellogenesis was advanced by one month, while the decline before spawning was advanced further to two months. The decline of FSH-R expression at the late vitellogenic stage was also advanced by one month, while there was no increase in FSH-R expression at spawning in the AP group. In the AP group, the increase in LH-R expression at vitellogenesis was not advanced, but was doubled to a 40-fold increase. The cold water treatment (given just before spawning) was found to further decrease the P450arom expression prior to spawning in the SNP group and markedly increase the LH-R expression. The cold-water treatment showed no effect on the expression of any of the genes in the AP group, presumably due to the advanced reproductive stage (postovulatory) of this group at the time of sampling and few sampling points.

P-VII-2

EFFECT OF *IN VITRO* XENOESTROGENS ON STEROIDOGENESIS IN MATURE FEMALE FISH, *CHASMICHTHYS DOLICHOGNATHUS*

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The purpose of this study was to investigate the *in vitro* effects of xenoestrogens on ovarian steroidogenesis of the longchin goby, *Chasmichthys dolichognathus*. Fully vitellogenic oocytes were incubated in the presence of xenoestrogens, diethylstilbestrol (DES), bisphenol A (BPA), and nonylphenol (NP) at concentrations of 100 ng/ml, using radiolabeled 17α-hydroxyprogesterone as precursor. Steroids were extracted from media and isolated oocytes, and the extracts were separated and identified by TLC and GC-MS. The identities of the major metabolites were progestogens [17α,20α-dihydroxy-4-pregnen-3-one (17α20αOHP) and 17α,20β-dihydroxy-4-pregnen-3-one (17α20βOHP)], androgens [androstenedione (A4) and testosterone (T)] and estrogens [estrone (E1) and estradiol-17β (E2)]. Followings are the summary of the results obtained. DES inhibited both androgen and estrogen synthesis, while it had a stimulatory effect on the production of progestogens. 2) BPA inhibited estrogens synthesis, but stimulated the androgens synthesis. 3) NP inhibited androgens, but stimulated the estrogens synthesis. Both BPA and NP did not affect on the progestogens synthesis. These results suggest that NP demonstrated estrogenic activity on the three chemicals. DES and BPA appear to act as an anti-estrogen.

P-VII-3

THE EFFECTS OF DIFFERENT DAYTIME LIGHT INTENSITIES ON THE DIEL PATTERNS OF PLASMA MELATONIN IN FINGERLING AND BROODSTOCK RAINBOW TROUT

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The effect of different light intensity on diel melatonin release by two sizes of rainbow trout was investigated. Two sets of fish: fingerlings (25 ± 2.5 g mean weight) and broodstock (1672 ± 1.5 g mean weight) rainbow trout were stocked in six 1.8 m^3 cylindrical fibreglass tanks at densities of 16 kg/m^3. Three replicates for each fish size were set up with either 500 W or 300 W lamps to create light intensities of 1250 lux or 700 lux. After two weeks acclimation, samples of blood were taken from the fish at 7 time points over a 24-h period and the plasma melatonin levels determined by radioimmunoassay. The results showed that light intensity affected the night-time melatonin release in both groups of fish. Under 700 lux the difference was not significant between the two size-groups. However, the concentrations of night-time-plasma melatonin under 1250 lux were significantly higher ($P<0.05$) in the fingerlings (602 ± 75 pg/ml to 492 ± 29 pg/ml) than in the broodstock (314 ± 83 pg/ml to 265 ± 55 pg/ml). Although varying metabolic rates and differential sensitivities to light in the different sizes of fish may explain these results, it is also possible that amplitudinal differences in melatonin are more important in younger fish.

P-VII-4

PHYSIOLOGICAL FUNCTIONS OF THE SPAWNING ENVIRONMNET ON FINAL MATURATION AND SPAWNING IN JAPANESE DACE, *TRIBOLODON HAKONENSIS*.

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Japanese dace congregates at the spawning ground that is formed at rapids of the middle reaches of rivers and lays eggs on fine gravel. It is considered that the suitable environment for spawning has not only ecological significance but also physiological roles on reproduction. In the present study, spawning induction tests were carried out under various experimental conditions to elucidate indispensable environmental conditions for spawning. Moreover, we investigated the possible endocrine responses of dace to the environmental stimuli. In the spawning test, mature fish of both sexes were placed to ponds that were provided with or without gravel and/ or water current. Spawning was induced only when ponds were provided with both gravel as a spawning substrate and sufficient water current. In contrast, spawning was not observed in ponds that had none or only one of these two factors. In the endocrine response examination, both or single sexes of mature dace were placed in tanks with the spawning environment. The serum concentration of steroids in both sexes and the amount of expressible milt of males were measured. Females and males transferred to spawning environment immediately spawned. At that time, there were significant increase in serum 17α, 20β-dihydroxy-4-pregnen-3-one (DHP) levels and were significant decrease of the level of other steroids (estradiol-17β: E2, testosterone: T, 11-ketotestostrone: 11-KT), in addition, the amount of expressible milt considerably increased. When only females were transferred to spawning environment, DHP and E2 levels showed the similar change and most fish ovulated. When only males were transferred to spawning environment, there were significant increase in the amount of expressible milt and significant decrease in T and 11-KT levels. Meanwhile, there was no change in DHP. These results indicate that spawning environment (specific combination of spawning substrate and water current) is a necessary condition for spawning of dace and the environmental factor affects as a trigger of endocrine change of final maturation.

P-VII-5

MOLECULAR CLONING OF vitellogenin cDNA IN Rockfish (*Sebastes schlegeli*) and effects of estrogenic substances on its gene expression

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Environmental xenoestrogens have been found to affect reproductive system of teleost, although most studies have focused on freshwater species. Vitellogenin (VTG) is the major yolk protein, synthesized in the liver under the control of the pituitary–gonadal axis, whereas this protein is induced by some xenoestrogenic substances such as nonylphenol (NP) in juvenile female and male fishes. In the present study, we have isolated a partial cDNA which encodes a VTG of rockfish, *Sebastes schlegeli*, in order to assess the use of VTG mRNA as a tool to monitor environmental xenoestrogens in marine eco-system. Rapid amplification of cDNA ends (3'-RACE) polymerase chain reaction (PCR) was used to amplify VTG cDNA prepared from estrogen-treated male liver, and the obtained PCR products were subcloned and sequenced. DNA sequence analysis of the cloned PCR products confirmed the presence of the predicted 3'-coding region as well as the 3'-untranslated region. The deduced amino acid sequences of the rockfish VTG (rfVTG) showed high homology to rainbow trout VTG (81%) and Atlantic salmon VTG (82%), respectively. Northern blot analysis showed the transcript of rfVTG to be 3.5 kilo bases. Next, we examined *in vivo* effects of NP on VTG mRNA expression in juvenile male rockfish using Northern blot and semi-quantitative RT-PCR method. In this experiment, the mRNA levels of rfVTG were increased by NP (10–25 ppm) treatment (24–72 hours). We also observed increased VTG protein levels in the rockfish liver by NP after 48 hours. Taken together, these results suggest that the obtained rfVTG cDNA could be used for monitoring purposes in marine system.

P-VII-6

GONADAL DEGENERATION IN SUB-ADULT PEJERREY (*ODONTESTHES BONARIENSIS*) MALES DURING EXPOSURE TO WARM WATER

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High water temperature has ben shown to induce germ cell death and sterility in juvenile pejerrey (*Odontesthes bonariensis*) and other teleost fishes but the mechanism of heat-induced cell loss has not been clarified. This study examined the dynamics of gonadal degeneration in pejerrey sub-adults subjected to different levels of thermal stress and the possibility that apoptosis (programmed cell death) is implicated in this process. One year-old males with a mean body length of 15 cm were exposed to three temperature regimes: 24°C (control), 29°C, and 31°C. The latter group was transferred back to 24°C at 36 hours after the fish started displaying sings of the thermal stress. Fish from all groups were sampled after 0, 12, 24, 36 and 48 hours, 1, 2, and 4 weeks, and 4 months. The gonads were excised and fixed in 10% formalin, immersed in 20% sucrose, embedded in OCT compound, and frozen at -80°C. Portions of the gonads were also frozen at -80°C for biochemical analysis. Cryostat-sections were stained with Acridine Orange (AO, 0.05 μg/ml for 5 min), which shows differential staining of normal (green color) and apoptotic (orange - red color) cells and analyzed by fluorescence microscopy. Caspase-3 activity, an early biochemical step in apoptosis, was analyzed by fluorometry using the synthetic caspase-3 substrate Ac-DEVD-AMC (N-acetyl-7-amino-4-methylcoumarin). The number of AO-stained cells per unit area of the cross section of the gonad increased in heat-stressed gropus between 24 and 36 h at a rate approximately proprotional to the rearing temperature. AO-stained cells in fish reared at 29°C continued to increase during the first week and then reached a plateau approximately equivalent to 6 times the level observed in the control group. The group exposed to 36°C for 36 h showed a slow but steady decrease in stained cells after transfer to 24°C. Caspase-3 showed transient peaks of activity at 12 and 36 h in fish maintained at 31 and 29°C, respectively, and the magnitude of the peaks was proportional to rearing temperature. Histological examination of the gonads after 4 months showed the presence of complete and partial sterility in fish kept at 29°C but not in the controls. The results of AO staining and caspase-3 activity are consistent with apoptosis and suggest that this form of degenerative process might be implicated in heat-induced gonadal degeneraiton and sterelity in sub-adult pejerrey.

P-VII-7

MOLECULAR CLONING OF TWO TYPES OF SPIGGIN cDNA IN THE THREE-SPINED STICKLEBACK, *GASTEROSTEUS ACULEATUS*

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Spiggin is the glue protein produced by the kidney of breeding male sticklebacks (*Gasterosteidae*) and used for nest building. Its synthesis is strongly up-regulated by androgens. Currently this protein is used as a biomarker for androgenic chemical substances. However, details of spiggin protein molecule have not been well described. In the present study, we isolated and characterized two different types of cDNAs encoding spiggin (SPG-I and SPG-II) in three-spined stickleback, *Gasterosteus aculeatus*. The deduced open reading frame of SPG-I and SPG-II cDNAs encoded 616 and 639 amino acid residues, respectively. Although there was a marked structural difference (continuous 63 nucleic acids insertion (or deletion) in N-terminal side) between the two cDNAs, the deduced amino acid sequence of SPG-I showed high homology (80%) with that of SPG-II. In addition to the structural similarity, Northern blot hybridization using specific probes for each revealed that both types of spiggin mRNA were clearly detected in breeding male kidney. These results suggest that both types of cDNAs encode protein molecules that are functional and essential for the final product, the glue protein spiggin. Northern blot hybridization also detected multiple length of SPG-I mRNA, although only one signal was detected in SPG-II mRNA. Quite recently, from the re-screening of mature male kidney cDNA library, we isolated two SPG-I cDNAs that showed higher total length than the SPG-I cDNA described above. Currently we are analyzing the nucleic acid sequence of these cDNAs. The present results suggest that the stickleback glue protein for nest building is composed of multiple spiggin molecules.

P-VII-8

PHOTOPERIOD-INDUCED PRECOCIOUS MALE SEXUAL MATURATION IN ATLANTIC SALMON (*SALMO SALAR*)

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Current dietary androgen treatments for sex inversion of Atlantic salmon are inefficient. Many animals are sterilized while others typically exhibit testicular malformations and lack patent sperm ducts which preclude normal stripping and recovery for use in a subsequent season. Against this background, the question of treatment optimization is complicated by the fact that fish must be retained as prospective broodstock for 3 years before treatment efficacy can be properly evaluated. The present study was conducted to assess the potential for photoperiod induction of precocious male sexual maturation to facilitate treatment efficacy to be evaluated within one year of application. Treatment fish were maintained on a short day photoperiod (8L:16D) from first feeding (mid Aug) until the summer solstice (late Dec) at which time they were returned to a natural photoperiod (42°S). Controls were maintained on natural photoperiod throughout. The following autumn (May) fish were euthanased and examined on dissection for sexual maturation. In photo-manipulated populations, the incidence of precocious male sexual maturation was 60-90% whereas in controls the highest incidence was 25%. This probably reflects the fact that maintenance on a short day photoperiod allows greater numbers of fish to accumulate the energy reserves required for sexual maturation prior to experiencing the increase in photoperiod which is the recognized Zeitgeber for its commencement. Accordingly, the time required for completion of sexual development in the majority of animals was reduced from 3 years to 8-9 months. This protocol is now being used routinely for assessment of sex inversion treatments for commercial use in the Tasmanian salmon farming industry.

P-VII-9

EVALUATION OF A 40-DAY JUVENILE ZEBRAFISH (*DANIO RERIO*) ASSAY FOR DETECTION OF ENDOCRINE DISRUPTING CHEMICALS

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The purpose of the present study was to evaluate a test system using juvenile zebrafish (*Danio rerio*) for detection of endocrine disrupting chemicals. The zebrafish were exposed to 17β-estradiol (estrogenic), 17α-methyltestosterone (androgenic) or flutamide (anti-androgenic) during the sex labile period from day 20 to 60 post hatch. Following exposure, the endocrine disrupting effects were evaluated by sex ratio determinations and vitellogenin measurements. In the 17β-estradiol experiment, a significant feminization and a significant dose-dependent induction of vitellogenin were revealed after exposure to actual concentrations of 54, 96 or 250 ng/l. In the 17α-methyltestosterone experiment, a significant change in sex ratio towards males was recorded in all exposure groups (26-1000 ng/l). A significant reduction in vitellogenin concentration was recorded in groups exposed to 100, 260 or 500 ng/l 17α-methyltestosterone. In the flutamide experiment, a significant change in sex ratio towards females was recorded at 100 µg/l, whereas no significant effects were observed on the vitellogenin concentrations. The present study shows, that the developmental stage 20 to 60 days post hatch in the zebrafish is a sensitive period for estrogenic, androgenic or anti-androgenic chemicals. Consequently, the juvenile zebrafish is a suitable test organism in screening bioassays for detection of endocrine disrupting chemicals.

P-VII-10

THE EFFECT OF WATER FLOW ON SPAWNING IN MEDAKA, *ORYZIAS LATIPES*

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The effect of water flow on spawning was investigated in medaka, *Oryzias latipes*, in order to study environmental influence on fish reproduction and to obtain basic information for environmental conservation. Pairs of sexually mature medaka were kept in glass aquaria with standing water at 25°C and 16L/8D photoperiod. Medaka spawned almost every day under this condition. Pairs spawned for more than four days in five consecutive days were selected as continuously spawning pairs. When such continuously spawning pairs were subjected to a flow of water (approx. 11 cm/s) (experimental group), spawning rate significantly decreased. The average days of spawning in this period was 1.57 ± 0.43 (mean ± SEM; N=7) compared to 3.29 ± 0.42 (N=7) in controls kept in standing water for five days. After water flow was stopped, spawning was resumed in the experimental group and the spawning rate in the following five days increased to 3.14 ± 0.67. Spawning rate of the control group during the same period was 2.71 ± 0.68. These results indicate that water flow inhibited spawning but gonadal maturity of fish was maintained. Swimming ability of medaka was also estimated by measuring the water velocity to which fish could not maintain their position. The water velocity in which adult medaka could not keep their position was 46.0 ± 2.1 cm/s. These results indicate that water velocity that inhibits spawning was much lower than the critical water velocity for swimming of the fish. The results of present study suggest that there are environmental conditions in which fish can maintain its gonadal maturity but will not spawn. Gonadal maturity is commonly used as a parameter to examine the reproductive success of fish, but it does not seem to reflect whether spawning is performed properly. Therefore, for adequate environmental conservation, it is proposed to employ estimation of actual spawning as one of the key parameters for assessing whether life cycle of a fish species can be completed under given environmental conditions.

P-VII-11

METABOLIC EFFECTS OF ESTROGENIC OR ANDROGENIC EXPOSURE IN *ZOARCES VIVIPARUS*

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Sexually mature males or females of the eelpout *Zoarces viviparus* were exposed in the ambient seawater to the synthetic estrogen ethinylestradiol (EE2) in different doses during late spermatogenesis and early pregnancy, respectively, or females were exposed to methyltestosterone (MT) during vitellogenesis and early pregnancy, and metabolic effects were investigated. A significant dose-related increase in the level of vitellogenin (VTG) and calcium was observed in plasma of the fish exposed to EE2 and E2. The increased synthesis of VTG was also observed in the liver of the estrogen-exposed fish by PCR. In females during vitellogenesis exposure to MT resulted in a significant decrease in the circulating yolk-precursor. During early pregnancy, however, no effect could be observed by the MT-exposure on the level of circulating VTG. A significant increase was observed in the erythrocyte nucleoside triphosphate (NTP) concentration in the blood of female fish exposed to the high dose of MT (500 ng/L) during early pregnancy. Hepatic activity of different enzymes related to protein and glucose metabolism or indicative of oxidative stress response was investigated and dose-related changes were observed.

P-VII-12

EXPOSURE TO INSECTICIDES INHIBITS EMBRYO DEVELOPMENT AND EMERGENCE IN ATLANTIC SALMON (*Salmo salar* L.)

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There is increasing concern over the continual decline in wild stocks of Atlantic salmon throughout the NE Atlantic. This has been attributed to a variety of factors operating in both the freshwater and marine environments. For instance in freshwater, salmonids may be exposed to a suite of contaminants during sensitive periods in their life cycle. Exposure to environmental levels of pesticides have previously been shown to deleteriously effect Atlantic salmon reproduction, by disrupting pheromone-mediated spawning. The aim of this present study was to investigate the impact of two insecticides on the early life-cycle stages of the salmon. These insecticides (the synthetic pyrethroid cypermethrin and the organophosphate diazinon) are the principal active components in sheep dip treatments used in many areas of Europe. Sheep-dipping normally occurs at the time of salmon spawning and both insecticides have been routinely measured in a number of tributaries supporting spawning salmonids. During the study, eggs and milt from salmon were briefly exposed to pesticide-dosed water during fertilisation, mimicking conditions that would occur in the spawning tributaries. There were 6 groups, each with 600 fertilised eggs: Control; 0.05μgl-1 and 0.10μgl-1 diazinon; 0.05μgl-1 and 0.10μgl-1 cypermethrin; and a mixture of 0.05μgl-1 diazinon and cypermethrin. After exposure embryos were placed in separate artificial redds supplied with clean water and allowed to develop. Temperature, emergence and mortality were monitored daily. There was a significant difference in the pattern of emergence between the groups. The fry from the two cypermethrin-dosed groups and the lowest dose of diazinon emerged earlier and later compared to the other treatments. These groups also had fewer fry emerging compared to the control. Exposure to cypermethrin also reduced the subsequent survival of the fry. There was only a 30% survival from fertilisation to 1month post-emergence in the 0.05μgl-1 group, compared to 75% survival in the control group. The results suggest that exposure to environmentally relevant levels of pesticides for a brief period during fertilisation may effect both the timing of emergence, and the subsequent survival of the fry. This may have implications for juvenile survival and recruitment to the population

P-VII-13

DISPOSITION OF 4-TERT-OCTYLPHENOL IN FLOUNDER PLATICHTHYS FLESUS FOLLOWING A SINGLE ORAL DOSE

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Alkylphenol polyethoxylates is a large group of non-ionic surfactants used both as detergents and in many industrial formulations. One of the biodegradation products of the alkylphenol polyethoxylates, octylphenol, have in several papers been reported to exert estrogenic effects on male fish in the form of vitellogenin induction, e.g. after dietary exposure in rainbow trout *Oncorhynchus mykiss* and flounder *Platichthys flesus*, after water exposure in rainbow trout *O. mykiss*, roach *Rutilus rutilus* and Japanese medaka *Oryzias latipes* and after intraperitoneal injections in rainbow trout *O. mykiss*.
In a previous experiment oral exposure to 4-*tert*-octylphenol resulted in an induction of vitellogenin and a significant accumulation of 4-*tert*-octylphenol in liver, muscle and testis tissue of male flounders. The aim of the present study was to establish the time course of 4-*tert*-octylphenol accumulation and vitellogenin induction in male flounders fed a single dose of 4-*tert*-octylphenol (50 mg OP kg^{-1}). Fish were sampled 3, 6, 12, 18, 24, 48, 72, 144 and 216 hours after the feeding. The results on measurements of plasma vitellogenin and 4-*tert*-octylphenol concentrations in plasma and tissues will be presented and discussed.

P-VII-14

THE EXISTENCE OF EXTRA-RETINAL AND EXTRA-PINEAL PHOTORECEPTIVE ORGAN REGULATING GONADAL DEVELOPMENT OF AYU (*PLECOGLOSSUS ALTIVELIS*)

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Gonadal development of ayu *Plecoglossus altivelis* is induced by shortening of the photoperiod and inhibited under long photoperiod. The gonadal development is inhibited under long photoperiod even without both the eyes and pineal organ, which are well known photoreceptive organs in fish, suggesting the existence of unknown photoreceptive organ in ayu. However, there is no report whether shortening of the photoperiod induces the gonadal development without both the eyes and pineal organ or not. We removed both the eyes and pineal organ of ayu (Ex+Pinx ayu) and kept them under long photoperiod (LD20:4, 19 °C) for 1 week. Thereafter the fish were divided into two groups and kept under short photoperiod (LD4:20, 19 °C) or long photoperiod (same as above) for the next 3 weeks. The same experiment was also conducted in intact ayu. The gonads were dissected out on the day when the photoperiod was changed (Day 0) and after 3 weeks (Day 21) and calculated gonadosomatic index (GSI). In intact ayu, there was significant increase in GSI under short photoperiod both in male and female on Day 21 compared with that on Day 0, whereas no significant difference was observed under long photoperiod both in male and female. GSI on Day 21 was significantly higher under short photoperiod than that under long photoperiod. Thus, the gonadal development is clearly regulated by photoperiod in intact ayu as expected. In Ex+Pinx ayu, significant increase in GSI was also induced under short photoperiod both in male and female after 3 weeks. GSI unchanged under long photoperiod in female, but slightly increased in male. GSI on Day 21 was significantly higher under short photoperiod than that under long photoperiod. Thus, the gonadal development was also induced under short photoperiod even without both the eyes and pineal organ, suggesting the existence of extra-retinal and extra-pineal photoreceptive organ in ayu.

P-VII-15

EXPRESSION OF UBIQUITIN C-TERMINAL HYDROLASE IS REGULATED BY ESTRADIOL-17β IN TESTIS AND BRAIN OF THE JAPANESE COMMON GOBY

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We previously cloned the cDNA for the *Mr*=27000 testicular protein (27kD protein) of a teleost, the Nile tilapia, *Oreochromis niloticus*. The predicted amino acid sequence of the 27kD protein was homologous to those of the ubiquitin carboxy–terminal hydrolases (UCH) reported in mammals. In addition, the recombinant 27kD protein showed the ubiqutin-releasing activity. According to these results, the 27kD testicular protein was identified as the tilapia UCH. Northern blot analysis showed that the UCH mRNA was expressed in ovary and brain in addition to testis. Immunohistochemical study with the antibody to the tilapia recombinant UCH revealed that UCH was present in A and early B type spermatogonia, spermatids and spermatozoa in testis. In brain, UCH was localized especially on the olfactory bulb and olfactory epithelium in olfactory rosetta, while in ovary UCH was present especially in pre-vitellogenic oocytes. These results suggest that the tilapia UCH is involved in protein degradation in these tissues as one of enzymes which belongs to so-called ubiquitin-dependent protein degradation known as one of the cellular regulatory mechanisms. Considering the tissue specificity, UCH could be a convenient biomarker for studying the brain-gonadal axis and the effect of the endocrine disruptors on the axis. In order to elucidate the effect of the endocrine disrupting chemicals on the brain-gonadal axis, the level of UCH mRNA both in brain and in testis were quantified with the competitive PCR assay using the Japanese common goby (*Acanthogobius flavimanus*), which is widely distributed in coastal area of Japan. After exposure the male goby to bisphenol A, nonylphenol and estradiol-17β(E2) for 3 weeks, the level of UCH mRNA both in brain and in testis were measured. Although the exposure to bisphenol A and nonylphenol could not induce significant change in the mRNA expression, the exposure to E2 at a nominal concentration 100ng/L induced significant increase of mRNA level in both of these tissues. This result suggests that the transcription of UCH gene is E2-inducible.

P-VII-16

EFFECTS OF REARING WATER QUALITY ON EXPRESSION OF BRAIN CORTICOTROPIN-RELEADING HORMONE AND GONADAL DEVELOPMMENT IN GOLDFISH (*CARASSIUS AURATUS*)

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Stress has been shown to interfere with physiological functions, such as growth, immune system or reproduction. Corticotropin-releasing hormone (CRH) is the first hormone in the response of the hypothalamic-pituitary-interrenal axis. It stimulates the release of adrenocorticotropin, which in turn stimulates the production of cortisol in the adrenal gland. Thus, CRH plays a role in the regulation of the peripheral response to stress. In a previous study, we investigated the effects of changes in water quality in a closed recirculating system on physiological function of goldfish. This approach resulted in reduced reproductive performance given reduced gonado-somatic index (GSI) and number of LHβ immuno-reactive cells, and delayed spermatogenesis and oogenesis. Serum cortisol levels were also elevated. These results suggest that stress affects physiological function in this species. However, the physiological mechanisms that mediate the effects of stress on reproductive function are still unknown. The aim of this study was to analyze the relationship between stress and reproduction; as a first step, we repeated the above water quality experiment and investigated the effects of stress on brain CRH gene expression. Fish were reared in a density of about 20 g/L at 20°C and under natural photoperiod in a closed recirculating system for 13 weeks. In the experimental group, fish were reared without water change, while in the control group, the 50 % of water was changed every day. The pH was held constant in both groups. During the experimental period, total ammonia nitrogen, nitrate, phosphate and pH were measured. The histological observation of gonad was curried out. At the end of the experiment, mRNA levels of goldfish CRH in the brain were measured by RT-PCR. In the experimental group, total ammonia nitrogen, nitrate and phosphate were increased over the experimental period. mRNA encoding CRH in the brain seemed to be expressed stronger compared to that in the control group, although, no differences were histologically observed in gonadal development between control and experimental group. It is suggested that deterioration of rearing water quality may stimulate brain CRH gene expression in this species.

P-VII-17

EFFECT OF PERSISTANT ORGANIC POLLUTANTS ON FECUNDITY OF HIGH ARCTIC SVALBARD CHARR (SALVELINUS ALPINUS) KEPT IN CAPTIVITY

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Persistant organic pollutants, radioactive compounds, and long-range transport of heavy metals have been documented in the high Arctic region. The organic compounds break down slowly, are fat-soluble and accumulate in the stored fat of Arctic animals. Thus, bio-magnification and hazardous effects on the living organisms, populations and ecosystems may occur. At the Norwegian University of Science and Technology (NTNU - Trondheim the insidious potency of Aroclor 1254 (PCB) was tested on the high-Arctic Svalbard charr (Salvelinus alpinus L.). It was demonstrated that 50 mg PCB/kg body weight (given per os) caused a significant reduction in egg batch volume (and thereby also the number of eggs) spawned by the Dieset charr. The PCB load also reduced survival of fertilized eggs during incubation. Furthermore, the parental PCB exposure reduced the offsprings body length growth (after hatching), while death rate and number of fry with visible body deformations increased significantly. Aroclor 1254 injected into parr (2 x 25 mg/kg BW) did not affect the parr-smolt transformation if tested as changes in seawater tolerance.

P-VII-18

EFFECT OF HIGH WATER TEMPERATURE ON BRAIN-PITUITARY-GONAD AXIS OF THE RED SEABREAM DURING ITS SPAWNING SEASON

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Many aspects in fish reproduction are strongly influenced by water temperature. Effects of temperature on the commencement and termination of sexual maturation and spawning have been intensively studied in freshwater species, such as cyprinids, however, our knowledge is still scarce for marine teleosts. In the present study, we examined the effect of high water temperature on brain-pituitary-gonad (B-P-G) axis of a perciform fish red seabream, *Pagrus (Chrysophrys) major* during its spawning season (April-May). Males and females (6 fish each) were kept in a tank and subjected to constant temperature of either high (H-group; 24 °C) or low (L-group; 17 °C, same temperature as ambient temperature at the start of the experiment), and reared for 19 days. Females were sampled at the beginning of the experiment, and after 10 or 19 days. Histological observation of ovary was performed, and mRNA levels of seabream (sb) GnRH in the brain, the mRNA levels of LHβ, FSHβ and GnRH receptor (GnRH-R) in the pituitary were measured by real-time quantitative PCR. H-group had quit spawning in 9 days, while L-group had continued spawning till the end of the experiment. The ovaries of H-group were regressed and gonadosomatic index (GSI) decreased on Day 10 and remained low on Day 19, while L-group remained matured and GSI was maintained high levels till the end of the experiment. All mRNA levels, except FSHβ that showed no significant changes during the experiment, were lower in H-group than L-group both on Day 10 and 19. These results suggest that the increase of water temperature is the termination factor of the spawning season of red seabream, and that the high water temperature suppressed the gene expressions of several hormones and the receptor in B-P-G axis of this species.

P-VII-19

COMPARISON OF IN VITRO VITELLOGENIN SYNTHESIS BY ALKYLPHENOLS TREATMENT IN PRIMARY CULTURE OF CHINESES MINNOW, *RHNCHOCYPRIS OXYCEPHALUS* HEPATOCYTES

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Three alkylphenols with nonylphenol (NP), bisphenol-A (BPA) and 4-tert-octylphenol (OP), were compared the estrogenic potential using *in vitro* vitellogenin (VTG) synthesis in primary cultures of chinese minnow, *Rhynchocypris oxycephalus* hepatocytes. The liver was carefully removed from 20-30 fishes and hepatocytes were isolated using collagenase. The isolated hepatocytes were cultured using Leibovitz-15 medium at 18". Two days after culture, 10^{-6} to 10^{-3} M concentration of each NP, BPA and OP were added to the culture media. The VTG secreted into the culture media was measured by enzyme-linked immunosorbent assay (ELISA), which developed using antibody prepared from egg homogenates of chinese minnow. The treatment of each NP, OP and BPA at a concentration of 10^{-3} M to the medium caused death of hepatocytes. The VTG synthesis was induced by NP, BPA and OP treatments, however, these influences on VTG synthesis were different between each alkylphenols. The VTG synthesis was the highest at 10^{-4} M of NP and OP, and 10^{-5} M of BPA in the test concentrations, respectively. These results suggest that estrogenic potential is highly sensitive in the BPA than NP and OP in chinese minnow hepatocytes.

P-VII-20

LUNAR CYCLE IN THE REPRODUCTIVE ACTIVITY IN RABBITFISHES

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The lunar-related spawning cycle is one of pronounced characteristics in reef fishes. Using four rabbitfish species that exhibit a definite lunar-related spawning cycle, we have examined relationship between lunar cycle and reproductive activity. It was found that each species has a specific lunar phase for synchronous spawning. Histological observations of gonads revealed that gametes develop toward and were released at the expected lunar phase. Changes in the plasma steroid hormones were correlated well with gonadal development. In the female fish, plasma levels of estradiol-17β (E2) and 17 α20β-dihydroxy-4-pregnen-3-one (DHP) increased at the phases of vitellogenesis and final oocyte maturation, respectively. In the male fish, on the other hand, 11-ketotestosterone (11-KT) and DHP peaked at the phases of spermatogenesis and spermiation, respectively.*In vitro* experiments showed that addition of human chorionic gonadotropin (hCG) was required for production of those steroid hormones. These results suggest that the gonadal development is repeated at an interval of approximately one month according to the lunar cycle and regulated by rhythmic secretion of gonadotropin from the pituitary. When the reproductive activity was compared with fluctuations of environmental factors, it was considered that initiation and termination of reproductive season were related to fluctuations of water temperature and/or photoperiod. There are the observatory facts that the larvae of the rabbitfish appear in the shore with the expected lunar phase. Therefore, it is considered that the cues from the moon are percepted not only by mature fish but also by immature fish. It is hypothesized that when environmental factors became suitable for reproductive activity, synchronous gonadal development starts with lunar cycle.

P-VII-21

THE REPRODUCTIVE SYSTEM OF THE MALE EELPOUT (*ZOARCES VIVIPARUS*): EFFECTS OF XENO-ESTROGENS ON TESTIS STRUCTURE, SERTOLI CELLS AND BIOCHEMICAL COMPOSITION OF SEMEN

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The eelpout (*Zoarces viviparus*) being a marine, viviparous fish with internal fertilization shows characteristics different from external fertilizing fish. In the male this is reflected in its testicular structure by pronounced Sertoli cells, the composition of seminal fluid and the sperm cells. Our studies have shown that xeno-estrogens such as alkylphenols severely affect the reproductive system of male eelpouts. Exposure to octylphenol (OP) or 17β-estradiol (E2) during different times of the spermatogenetic process caused induction of circulating vitellogenin and negatively affected the testicular structure and/or the biochemical composition of the seminal fluid (e.g., milt volume, spermatocrit, calcium- and magnesium-concentration). Some of these effects were inhibited by the anti-estrogen ZM189,154 indicating that the effects are partly mediated via the estrogen receptor. Sertoli cells were one of the sites affected shown by a changed histology and by reduced testicular gamma-glutamyl transpeptidase (gamma-GTP) activity. We have localised gamma-GTP to the Sertoli cells in eelpout and propose that this enzyme is a potential marker of Sertoli cells in fish. As the Sertoli cells secrete the seminal fluid, the observed effects on these cells and the semen indicate a possible disruption of the synthezising/secretory function of Sertoli cells by estrogenic exposure.

P-VII-22

EFFECTS OF TEMPERATURE ON GONADAL DEVELOPMENT OF MUDSKIPPER (*PERIOPHTHALMUS MODESTUS*)

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Periophthalmus modestus, an amphibious mudskipper fish inhabiting tidal flats in East Asia, spawns and breeds from late spring to summer. The gonadal development starts with increasing ambient temperature in early spring. There have been many studies on the effect of temperature on gonadal development and maturation in teleosts. However, it has not been investigated in mudskipper. In the present study, we exposed the fish to various temperature regimes to examine effects of temperature on gonadal development. Immature males and females were collected in the Ariake Sound in early February and maintained at either 18°C or 30°C. The daylength was kept at 14 hours light – 10 hours dark. Fish of each group were sampled every 26 days from early February to late April, and their gonadal development and plasma vitellogenin concentration were examined. In addition, gonads and blood samples were obtained from wild fish. In high temperature group (30°C), gonadosomatic index (GSI) of both males and females significantly increased in late March (day 52), and reached maximum levels in late April (day 78). In this group, spermatogenesis started in early March (day 26). Yolk globules accumulated into the oocyte, and serum vitellogenin concentration increased in females in late March. In contrast, sperm production and vitellogenesis were not observed in the low temperature group (18°C) during the experimental period. In the wild fish, an increase of GSI in males and accumulation of yolk globules in females started in late April, when the environmental temperature became higher than 20°C. These results suggest that temperature plays an important role in gonadal development and maturation in the spring- summer- breeding mudskipper.

P-VII-23

LUNAR-RELATED SPAWNING IN HONEYCOMB GROUPER, *EPINEPHELUS MERRA*

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Honeycomb grouper *Epinephelus merra* in Okinawa coastal waters is a lunar-related spawner. However, details of spawning cycle and gonadal development in this species are not well known. In order to obtain reproductive information of honeycomb grouper related to lunar cycle, we studied the gonadal development during spawning season and observed behavior of fish in fringing reefs around Sesoko Island, which is located in the northern part of Okinawa Island. Presence of honeycomb grouper in the fringing reefs was observed everyday between April and July in 2002 and 2003. The fish used for histological study of gonadal development were collected in the fringing reefs every 3 days. Moreover, spawning at full moon phase was monitored in artificial rearing condition. Honeycomb groupers were observed everyday in the fringing reefs from April to late May (in 2003) or early June (in 2002). Gonad somatic index (GSI) was relatively low from April to early May, then increased rapidly and reached maximum level during full moon in late May or early June. Ovaries of fish with high GSI contained oocytes at tertiary yolk stage. The fish disappeared from the fringing reefs for a few days after full moon. Likewise, mature and ovulated fish were not observed in the same area. The fish reappeared in the fringing reefs few days later. This time, GSI was low and their ovaries contained oocytes at peri-nucleolus stage and yolk vesicle stage. These results strongly suggest that honeycomb grouper move outside of the fringing reefs to spawn a few days after full moon. Oocyte development started again and GSI reached maximum levels at full moon of late June or early July. Spawning behavior and spawning of fish collected from the fringing reefs were observed in artificial rearing condition during a few days after full moon. This is a first report of a direct observation of lunar-related spawning in honeycomb grouper.

P-VII-24

THE INTERACTIONS OF PHOTOPERIOD AND INSULIN-LIKE GROWTH FACTOR-I (IGF-I) IN THE CONTROL OF RAINBOW TROUT REPRODUCTION

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The use of artificial light to advance and delay spawning in rainbow trout has become an important tool in the year-round production of eggs. However, by advancing maturation fewer fish achieve the energetic status, size or stage of development required to complete maturation; as a result maturation is arrested. Therefore, hormones implicated in the control of growth (IGF-I) and energetic metabolism (leptin) are likely to play a key role in the onset and time course of puberty. This work aimed to determine potential growth and metabolic signals responsible for the initiation of puberty and further help in the understanding of reproductive control mechanisms.
In January 2000, two groups of 60 individually P.I.T. tagged virgin 2-year old female rainbow trout were exposed to either a simulated natural photoperiod (SNP) or an advancing regime (ADV), constant long-days (LD18:6) until May followed by constant short-days (LD 6:18). The aim of these photoperiod regimes being to change spawning times and modify the percentage of fish which would spawn. Monthly weight-lengths and blood samples for vitellogenin, testosterone, plasma IGF-I and leptin analysis were taken from all fish until June 2001. Under SNP, 63% of the population attained maturity and spawned successfully, while only 29% spawned in the ADV group. However, the ADV group spawned 6 months in advance of the SNP group. In both treatments, maturing fish exhibited higher plasma IGF-I levels than immature fish. Peak IGF-I levels in ADV fish occurred one month in advance of those under SNP. Under SNP, plasma IGF-I in both mature and immature fish were strongly correlated with temperature. This pattern was less obvious under the ADV regime. This work is important as it provides an insight into the mechanisms that may provide a peripheral signal to indicate growth rate and size to the fish in order that the decision to undergo puberty may be made. Furthermore, this is the first study of changes in plasma IGF-I in response to photoperiod manipulation and reproduction. I would like to acknowledge and thank NERC ROPA and the British Trout Association for funding support during these studies.

P-VII-25

REPRODUCTIVE AND TRANS-GENERATIONAL EFFECTS OF OESTRONE IN A FATHEAD MINNOW PAIR-BREEDING TEST

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The natural steroid oestrogens, oestradiol and oestrone, and the synthetic steroid ethinyloestradiol, are known to contaminate the aquatic environment throughout Europe. The oestrogenic effects of the oestradiol-17β and the ethinyloestradiol, have been well characterised in fish. These steroids are present in effluents at concentrations that induce vitellogenin synthesis and in some cases at concentrations that cause reproductive effects. Oestrone is known to be a weaker oestrogen compared with oestradiol-17β, but is often found in effluents from sewage treatment works at far higher concentrations than oestradiol-17β (up to 10-fold higher). Little is known about the effects of exposure to exogenous oestrone on reproduction in fish. In this study pair-breeding fathead minnow were exposed to oestrone at concentrations ranging between 32 and 1000ng/L. Measured exposure oestrone concentrations were greater than 70% of nominal throughout. Oestrone exposure induced a concentration-dependent induction of vitellogenin. Exposure to the highest dose of oestrone for 3 weeks resulted in a lower gonadosomatic index in both the males and females and caused a suppression of male secondary sex characteristics (tubercle number and fatpad index). Egg production was reduced at exposure concentrations of 307ng/L and 781 ng/L. There were no trans-generational effects on hatching success, sex ratio, or development in the F1 generation, but a significant reduction in growth was observed in offspring from fish exposed to oestrone at concentrations of 34 and 781 ng/L.

P-VII-26

RATES OF GROWTH AND REPRODUCTION OF FISHES AT VARIABLE TEMPERATURES

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Temperature is one of the most important abiotic factors, having a great influence on rates of growth, development and reproduction of aquatic life, in particular of fishes. Not constant but variable temperatures are the norms of existence for the majority of fishes. Many kinds of fishes produce spawn on breeding bottoms at constantly varying ambient temperatures. Spawn is exposed in this case both to daily and to short-time variations in temperature. Investigations have been made to analyze the influence of constant and variable temperatures on rates of development and survivability of embryos of loach *Misgurnus fossilis* and on a rate of reproduction of guppy *Poecilia reticulata.* A loach spawn has been kept at constant (16, 18, 20°C) and variable (18 ± 2°C, frequency of temperature sinusoidal fluctuations is two hours) temperatures. We could see that an average length of loach spawn bodies at the moment of hatching at variable temperatures is longer than that at a temperature of 18°C ($p<0.01$). At variable temperatures, a rate of embryonic and larva development turned out to be equal to that observed at a temperature of 18 or 20°C. At the moment of hatching, survivability of loach larvae accounted, at constant and variable temperatures, for 63, 71, 74 and 86% ($p<0.05$) respectively. Larva body length has decreased at variable temperatures 1.1 to 1.55 times. A higher rate of linear growth of loach larvae at variable temperatures has made their survivability to increase 1.25 to 1.43 times ($p<0.01$). At variable temperatures, a frequency, at which embryos with various abnormalities have been encountered at different phases of their development, has been averaged 15%, while at test constant temperatures, from 16 to 30%.
A rate of guppy reproduction has been investigated for five months at a constant temperature (26°C) and also in a space at variable temperatures (24-28°C). Young fishes (five males and ten females) have been placed in specially equipped aquariums to watch their breeding. It was obvious that different females have produced young guppies at variable temperatures two or three weeks earlier than at constant temperatures. The total number of young fishes at the end of experiment at constant and variable temperatures accounted for 375 and 433 species, accordingly, while each female could be able of producing 75 and 87 newly-hatched fishes. Now we can see that at variable temperatures, in the process of loach spawn development, larva body length increases, larva retains its form, embryo survivability becomes higher and embryos with different abnormalities are not encountered as frequently as before. Variable temperatures facilitate higher rates of guppy breeding. All this proves that variable temperatures are more favorable for development and reproduction of fishes other than constant temperatures.

Session VIII

Biotechnology in Aquatic Science

O-VIII-1
PRIMORDIAL GERM CELL: A NOVEL TOOL FOR FISH BIOENGINEERING

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In plants, *in vitro* cultured cells can be converted into individual plants via callus formation. Also, individual mice can be produced from embryonic stem cells. These systems provide powerful tools for the genome biology and biotechnological applications. In spite of these benefits, there are presently no systems for converting *in vitro*-cultured cells into individual fish. Since the primordial germ cells (PGCs) have the potency to be converted into individual fishes via maturation and fertilization processes, we have chosen PGCs as the initial material used for this system. As a first step, we visualized live PGCs in rainbow trout. Because the *vasa* transcript is restricted to the germ cell lineage, its regulatory regions should be activated only in PGCs. Therefore, we produced transgenic strains carrying the green fluorescent protein (GFP) gene driven by the *vasa* gene regulatory regions. The resulting transgenic embryos showed green fluorescence specifically in PGCs. As a second step, GFP-labeled PGCs were purified using a flow-cytometer. The genital ridges isolated from the transgenic embryos were dissociated by trypsin and sorted into GFP-positive and GFP-negative cells. The GFP-positive cells possessed typical morphological characteristics of PGCs. In addition, the *vasa* gene was expressed only in GFP-positive cells, confirming that they were PGCs. To obtain functional gametes derived from isolated PGCs, a technique to convert PGCs into eggs and sperm is necessary. For this purpose, we developed a method to transplant PGCs into developing embryos to incorporate them into the germ cell lineage of the recipient embryos. About 10 PGCs isolated from hatching embryos were transplanted to the peritoneal cavity of recipient hatchlings. The transplanted PGCs actively migrated towards, and were finally incorporated into, the genital ridge. These PGCs settled in the genital ridges of the recipient embryos proliferated, started meiosis, and differentiated into eggs and sperm in synchrony with the germ cells of recipient embryos. Further, the donor-derived gametes produced normal progenies through fertilization. These techniques, combined with *in vitro* culture, genetic modification, and cryopreservation of PGCs, have numerous applications in the field of fish bioengineering.

O-VIII-2
CRYOPRESERVATION AND TRANSPLANTATION OF SEXUALLY IMMATURE GONADS OF RAINBOW TROUT

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Numerous salmonid populations are declining. The genetic diversities of some of these populations are being conserved through the establishment of germplasm repositories. However, because cryopreservation of salmonid eggs and embryos has not been successful, these germplasm repositories are composed exclusively of sperm stored in liquid nitrogen. Therefore, in order to include female germplasm, the objective of this research is to determine if sexually immature ovaries transplanted following cryopreservation to a proper recipient will complete the maturational process. Since sexually immature testes of rainbow trout (RBT) have been transplanted successfully, the first experiment was to determine if RBT testes cryopreserved and stored in liquid nitrogen could undergo spermatogenesis following transplantation. The testes were removed from sexually immature males and placed in Leibovitz L-15 media at 4 degrees C to which dimethylsulfoxide (10%) was added stepwise over a two hour period. Each testis was cooled at a rate of -1.5°C/minute to -70°C and plunged into liquid nitrogen. Individual testes were thawed and surgically transplanted to isogenic male trout. Four weeks following transplantation, all fish were injected with salmon pituitary extract for 10 weeks. The transplanted testis was present in 3 of 6 animals. Fertility of sperm obtained from individual testes was evaluated by in vitro fertilization. Sperm from the intact control testes had a fertilization rate of 95 ± 3% (mean ± SEM), and sperm from transplanted testes had a fertilization rate of 78 ± 7%. The second experiment was to determine if RBT ovaries could be transplanted. In this experiment, ovaries were detached from the body wall, removed from the abdominal cavity and autografted to an ectopic site along the pyloric cecae. At five months following surgery, the animals were killed and examined; the autografted ovaries were present in 5/5 animals. Histological comparison of the size distribution of oocytes of intact and transplanted ovaries support the conclusion that the oocytes of the transplanted ovary were developing.

O-VIII-3
PRODUCTION OF DONOR-DERIVED OFFSPRING BY XENOTRANSPLANTATION OF PRIMORDIAL GERM CELLS IN SALMONIDS

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In this study, we demonstrated that the production of offspring from the xenotransplanted primordial germ cells (PGCs) for the first time in vertebrates. Germ cell transplantation is a valuable tool for germ cell research and reproductive technologies. In particular, xenotransplantation (transplanting cells from one species to a different species) of germ cells are expected to provide a new method to produce transgenic animals, and to preserve endangered wildlife. However, the production of donor-derived offspring from the xenotransplanted germ cells has never been achieved so far. The present study aimed to determine whether rainbow trout (*Oncorhynchus mykiss*) PGCs transplanted into yamame salmon (*Oncorhynchus masou*) embryos can differentiate into functional gametes that are capable of development into donor-derived offspring. Donor PGCs were prepared from transgenic rainbow trout carrying a green fluorescent protein (GFP) gene driven by a *vasa* gene promoter. Germline cells, including PGCs, of this transgenic trout were labeled by indelible green fluorescence. Approximately 20 PGCs were transplanted into the peritoneal cavity of a newly hatched salmon embryo. Fluorescence observations of recipient gonads revealed that donor PGCs were incorporated, proliferated, and differentiated into the spermatogonia in the xenogenic gonads. Further, the presence of donor-derived spermatozoa in the milt of matured salmon was confirmed by PCR analysis with GFP-specific primers. The PCR-positive milts were inseminated with trout eggs. Among the F1 offspring, embryos showing donor (trout)-derived phenotype were successfully produced. RAPD-PCR confirmed that the genotypes of such embryos were identical to that of the rainbow trout. To date, donor-derived offspring have grown up normally. Therefore, we concluded that surrogated salmon broodstock could produce live trout embryos in the offspring. Combined with *in vitro* culture, genetic modification, and cryopreservation of PGCs, the PGC transplantation technique will herald a new era in fish breeding technology.

O-VIII-4
ENHANCEMENT OF GROWTH IN CULTURED FISH USING RECOMBINANT CARP GROWTH HORMONE

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While world demand for fresh water and marine fish products is growing rapidly, there is a decline in the catches of commercially important fish species. Advances in aquatic biotechnology would be necessary in order to increase fish production. Growth rate is a particularly important parameter in the economic equation, since it would significantly reduce the time to produce market-size fish and provide a major boost to production and the profitability of the industry. To increase growth rate of fish, a number of investigators have examined two entirely different approaches: production of transgenic fish species that over express growth hormone (GH), as well as treating fish with exogenous recombinant GH. In our studies, we examined the idea that recombinant carp GH (cGH) can be used as a food supplement to improve growth rate in cultured fish. We successfully produced biologically active cGH using *E-coli*, *Bacillus subtilis* and canola plant and went on to develop a novel form of fish food containing an active form of fish GH. The specific application of this technology would be to increase growth rate and food conversion efficiency in cultured fish, using fish GH as food supplement. In case of, plant-based system, cGH was produced in *Brassica napus* as a translational fusion with the native seed oil body protein, oleosin. Natural targeting of the oleosin fusion protein to oil bodies provided for its rapid purification through a simple process of flotation-separation. The orally administered cGH was demonstrated to be functional through an activity assay measuring induction of insulin-like growth factor-I and growth rate. We tested both bacterial and plant-derived cGH on different species of fish fed diets containing the recombinant product for several weeks. Fish receiving diets supplemented with cGH exhibited significant increases in growth over controls. Examination of fish following completion of the study revealed no morphological abnormalities in animals receiving diet containing cGH. In conclusion, our findings demonstrate that recombinant cGH can be used effectively to increase growth rate in cultured fish. The production systems used in these studies are relatively inexpensive and can readily be scaled up to meet requirements for the commercial aquaculture industry.

O-VIII-5

PRODUCTION OF RECOMBINANT GOLDFISH GONADOTROPINS BY BACULOVIRUS IN SILKWORM LARVAE

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Extract of fish pituitary gland containing gonadotropins (GTH) has been used for the induction of gonadal development in aquaculture. However, it is getting difficult to collect a large amount of pituitary glands and is also difficult to estimate and control the contents of two types of GTH, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) when the extract is administered. As a substitute for the pituitary extract, we attempted to produce recombinant goldfish FSH and LH that are biological active *in vivo* by baculovirus in silkworm larvae. cDNA of goldfish GTHα, FSHβ, and LHβ subunits were engineered into baculovirus expression vector (Superworm System, Katakura Industries). The baculovirus carrying cDNA of GTHα and FSHβ or LHβ were co-infected to silkworm larvae. Five days after the inoculation, hemolymph was collected and its biological activity was examined by injecting the hemolymph into sexually mature male goldfish and female bitterling. Milt production was induced by injection of hemolymph containing the recombinant FSH or LH in male goldfish whereas ovulation in female bitterling was induced only by hemolymph containing LH. Expressions of GTHα and LHβ subunits for LH and GTHα subunit for FSH were confirmed by western blot analysis using antisera against GTHα peptide and LHβ peptide although expression of FSHβ subunit was not confirmed because of unavailability of the antiserum. This is the first demonstration of production of recombinant fish GTH that was biologically active *in vivo*. The results of present study suggest that recombinant fish GTH produced in silkworm larvae could be applied to aquaculture as a substitute for the pituitary extract if a method of a large scale production is established. Also, since the recombinant FSH and LH are not contaminated from each other unlike pituitary extract, differential use of FSH and LH might be able to establish a more efficient method for the induction of gonadal maturation of fish.

O-VIII-6

PRODUCTION OF BIOLOGICALLY-ACTIVE RECOMBINANT GOLDFISH GONADOTROPINS IN TRANSGENIC RAINBOW TROUT

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This research was conducted to develop a novel method for producing recombinant proteins. We investigated the feasibility of using transgenic rainbow trout eggs as a bioreactor for producing large amounts of recombinant proteins with accurate post-translational modifications. Utilizing this system, we attempted to produce two recombinant goldfish gonadotropins (gfGTHs), follicle-stimulating hormone (gfFSH) and luteinizing hormone (gfLH).
In order to enhance the assembly of α- and β-subunits, cDNAs of these subunits were fused tandem to produce single-chain gfGTHs (sc-gfGTHs), sc-gfFSH and sc-gfLH. Two expression vectors, which contained scGTHs-cDNA driven by the medaka β-actin promoter, were constructed and introduced into fertilized eggs of rainbow trout. Transcripts of the foreign gfGTH genes were detected in the blastoderms of gastrula embryos by RT-PCR and the proteins were detected by western blotting. A deglycosylation experiment using N-glycosidase F revealed that sc-gfGTHs were N-glycosylated. Further, to examine the biological activities of sc-gfGTHs, testicular and ovarian tissue fragments excised from goldfish were cultured in media containing blastoderm homogenates inclusive of sc-gfFSH or sc-gfLH. As a result, both sc-gfGTHs enhanced the production of testosterone and estradiol-17β dose-dependently in ovarian tissue fragments. Testosterone production was also enhanced in testicular tissue fragments by addition of either sc-gfGTH.
In this study, we successfully produced recombinant gfGTHs which were N-glycosylated and biologically active. Further, the trout eggs can produce recombinant proteins at a low cost since they do not require expensive serum and medium that are essential for cultured cell systems. These results suggest that rainbow trout eggs can be a useful bioreactor for producing recombinant proteins with complex structures including GTHs. Further studies are needed to develop a method to produce recombinant proteins in larger quantities.

O-VIII-7

PRODUCTION OF CHANNEL CATFISH (*ICTALURUS PUNCTATUS*) RECOMBINANT GONADOTROPINS USING THE S2 DROSOPHILA CELL LINE

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The three subunits composing luteinizing hormone (LH) and follicle stimulating hormone (FSH) of the channel catfish (the common α-subunit,β -LH and β-FSH) were produced separately in the S2 cell system as C-terminus, His-tagged proteins. In addition, the α-subunit without the tag was co-expressed with each of the β subunits using a bi-cistronic vector. S2 cells secreted all three subunits and the LH and FSH heterodimers into the medium however the α-subunit had the highest accumulation and the β -FSH the lowest. The proteins were partially purified from the medium by IMAC. The LH αβ -heterodimer induced estradiol production in late vitellogenic oocytes of a gilthead sea bream indicating that the S2 expression system is suitable for gonadotropin expression. LH increased estradiol production in a dose dependent manner (putative concentrations of 2 to 30 ng/ml) with maximum secretion of 10.5 pg/hour/100 mg tissue (36 fold greater than control). This rate of estradiol production level is equivalent to that obtained with 10 units/ml of hCG. The FSH αβ heterodimer and the three different monomers did not induce estradiol secretion in this stage of oocyte. A homologous bioassay using early recrudescent ovaries and testes of catfish will be presented. These recombinant proteins will be used to study the differential action of LH versus FSH as the gonad develops. Purified recombinant β-subunits will be used to raise specific antibodies.

O-VIII-8

REVERSE GENETICS IN ZEBRAFISH

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To determine the function of a gene in vivo requires analysis of loss-of-function effects. However, fish system has lacked effective methods that inactivate a specific gene function till recently. We are developing/evaluating two techniques to generate mutations in known genes in the zebrafish: 1) screening for mutations that remove specific genes following gamma-ray induced mutagenesis, and 2) screening for point mutations in specific genes following chemically induced mutagenesis. We have also tested effectiveness of morpholino-modified antisense oligonucleotides method and double-stranded RNA interference method on transient inactivation of specific genes expression. Taking engla and englb genes that may function in muscle patterning as examples, the evaluation of these methods will be discussed.

P-VIII-1

CRYOPRESERVATION OF TROUT PRIMORDIAL GERM CELLS: A NOVEL TECHNIQUE FOR PRESERVATION OF FISH GENETIC RESOURCES

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The aim of this study was to develop a method for preserving the genetic resources of endangered fish species and of domesticated fish strains carrying commercially valuable traits. The conditions for cryopreservation of primordial germ cells (PGCs) were optimized using rainbow trout. Genital ridge, an embryonic tissue containing PGCs, was excised from 30-day-post-fertilization (dpf) transgenic trout embryos, in which PGCs were visualized with the GFP gene driven by *vasa*-gene regulatory sequences. The genital ridges were cryopreserved with the medium containing 0.5% bovine serum albumin, 1% glucose, and various dosages of the respective cryoprotectants (dimethyl sulfoxide (DMSO), glycerol, 1, 2-propanediol (PROH), or ethyleneglycol (EG)). After overnight preservation in liquid nitrogen, the genital ridges were rapidly thawed and dissociated by trypsin (0.5% in PBS containing 1 mM Ca^{2+} and 10% fetal bovine serum). The survival rates of the frozen/thawed PGCs were assessed by their green-fluorescence intensity and ability to exclude trypan blue dye. As a result, the highest survival (72.3%) was obtained when the cryomedium contained 1.8 M EG. In the next step, the ability of the PGCs, cryopreserved with 1.8 M EG, to resume gametogenesis was examined by transplanting them into peritoneal cavity of wild-type hatchlings. The recipients were dissected at 5, 15, and 30 days post transplantation to observe the colonization and proliferation of the donor PGCs in the recipients' gonads. On the fifteenth day post transplantation, colonization of the GFP-exhibiting donor PGCs was observed in the gonads of the recipients, though the donor PGCs were located only on the peritoneal wall at 5 days. Further, at 30 days, the proliferation of donor PGCs was observed. Therefore, the ability of the frozen/thawed PGCs to migrate, colonize, and proliferate in the recipients was confirmed. In short, we succeeded in establishing the basic technology to regenerate individual fish from cryopreserved PGCs. These techniques could also make it possible to establish a fish-PGC bank, which is particularly useful to conserve genetic diversity of wild fish populations.

Session IX

Aquaculture

O-IX-1

INDUCED MATURATION AND SPAWNING: OPPORTUNITIES AND APPLICATIONS FOR RESEARCH ON OOGENESIS

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Oogenesis begins with formation of primordial germ cells that transform into oogonia and initiate meiosis, becoming oocytes that are encapsulated within ovarian follicles. These early processes in oogenesis are under intense investigation; however, routine methods for their control have yet to be developed. Oocytes then undergo meiotic arrest and grow by orders of magnitude, incorporating lipids, yolk proteins (YPs), and other materials. We know much about derivation of YPs from circulating precursors (vitellogenins, Vgs) and proteolysis of YPs to generate specific products that play different roles in oocyte hydration (egg buoyancy) and in embryonic and larval nutrition. However, little is known about mechanisms of oocyte lipidation. Fully-grown follicles acquire maturational competence, enabling oocytes to resume meiosis (mature) in response to maturation-inducing steroid hormone, after which they are expelled from the follicle (ovulated) for spawning. An alternative developmental fate of follicles is atresia, a complex process involving oocyte destruction and the transformation of granulosa cells into phagocytes that consume YPs and lipids for recycling. Spawning must be induced shortly after completion of oocyte growth or females of many species will initiate atresia and become unspawnable. Broodstock management requires that we be able to stimulate oocyte growth and detect completion of oocyte growth, acquisition of maturational competence, and onset of atresia. Our research on temperate basses, genus *Morone*, revealed that oocyte lipidation and deposition of YPs are independent processes that can be separately controlled by manipulating daylength and water temperature. In these fishes, termination of oocyte growth is marked by disappearance of intact Vg from ovarian biopsy samples. Competence of females for induced spawning is predicted by the ability of their biopsied follicles to initiate oocyte meiosis *in vitro* in response to insulin-like growth factor I. Atresia can be delayed for months by holding gravid females at abnormally low temperature, a procedure known as "cold banking." The onset of atresia can be detected by checking biopsied follicles for edema in their granulosa cell layer. These novel findings hold strong promise for application to other farmed fishes.

O-IX-2

EFFECTS OF SPAWNING CONDITIONS ON MULTIPLICATION OF THE CAUSATIVE VIRUS OF VIRAL NERVOUS NECROSIS (VNN) IN BROODSTOCK OF STRIPED JACK (*PSEUDOCARANX DENTEX*)

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Viral nervous necrosis (VNN) is a serious viral disease in seed production process of marine fishes all over the world since 1990. The present study was tried to investigate the effects of multiple spawnings and rearing conditions for spawning (hormone injection and rearing density) on multiplication of the causative virus (striped jack nervous necrosis virus: SJNNV) of VNN in broodstocks of striped jack *Pseudocaranx dentex*. Plasma cortisol was measured as an indicator of primary stress response caused by the rearing conditions. The level of plasma cortisol in spawners was gradually elevated with the increased number of spawnings of broodstocks. Both SJNNV and plasma antibodies against SJNNV became detectable when the number of spawnings reached more than 10 times in each season. Plasma cortisol levels also increased by HCG injection and with a higher rearing density of broodstocks. In the higher rearing density, plasma glucose, which is an indicator of secondary stress response, also increased. These results suggested that conditions such as more-than-10-time multiple spawnings, HCG injection and higher rearing density would allow SJNNV to multiply in the spawners by reducing their resistance or immunity, which causes serious mortality of offsprings by the vertical transmission of SJNNV from spawners.

O-IX-3
DOMESTICATION OF GREATER AMBERJACK (*SERIOLA DUMERILI*) AT THE OCEANIC INSTITUTE IN HAWAII

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The greater ambjerjack (*Seriola dumerili*) is one of several seriolid species having outstanding potential for warm-water aquaculture. Substantial commercial production already exists for amberjack in Asia, where it is recognized for its adaptability to conditions of intensive culture, extremely fast growth, and high market value. However, the majority of current production is based on collection of fingerlings from the wild. Limited supplies of wild fingerlings have correspondingly restricted commercial production, which is further exasperated by dwindling supplies of natural stocks. OI began working on the greater amberjack in the mid-90s, achieving the first natural spawns with wild-caught adults in 1999. Although maintaining actively spawning stocks of wild-collected amberjack has proven difficult, the successful closing of the life-cycle has allowed for the use of captive reared progeny as broodstock. Growth rates under high-density using commercially available pelleted diets have been exceptional, attaining weights of over 2 kg in the first year and 5 kg in two years. In captivity, male amberjack began maturing within the first year, and actively spermiating at 21 to 22 months of age. Female amberjack mature slightly slower than males, with a rapid increase in gonadosomatic index and onset of spawning at 24 months of age. Under ambient photoperiod and temperature in Hawaii, F1 broodstock have now spawned for a period of almost two years without interruption. They exhibit some seasonality in reproductive output, but remain in reproductive condition throughout the year. Since the initiation of spawning, four tanks with 20 fish/tank, have yielded an average of 13 spawns/tank/month with mean fecundity of 154,000 eggs/spawn and mean fertility rate of 43%. Ongoing research at OI is directed at optimizing broodstock, hatchery, and nursery technologies toward securing a reliable year-round supply of amberjack fingerlings for both on-shore and off-shore grow out. Positive prospects for the off-shore sector were recently demonstrated through successful back-to-back grow out runs of Pacific threadfin (*Polydactylus sexfilis*) in a large (2600m^3) submersible cage. This has led to the establishment of the first commercial offshore cage operation in the United States.

O-IX-4
COMPARISON OF EGG AND LARVAL QUALITY BETWEEN NATURAL AND INDUCED SPAWNS OF RED SNAPPER *LUTJANUS CAMPECHANUS*

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Red snapper, *Lutjanus campechanus* is an important commercial and recreational species in the Gulf of Mexico that can be hormone induced to spawn by one injection of Human Chorionic Gonadotropin (HCG) at 1,100 and 550 IU/Kg for females and males respectively. Natural spawning of red snapper under controlled conditions is also possible. How egg and larval quality was affected by spawning method was evaluated during the 2002 spawning season at the facilities of Claude Peteet Mariculture Center, Gulf Shores, AL, USA. Wild mature red snapper were captured with hook and line during five fishing trips and were induced to spawn. Hormone induced spawns with at least a 50% fertilization rate were included in the study. Domesticated brooders were maintained in six 13.2 m^3 tanks under controlled environmental conditions. They were fed diets based on squid, herring and shrimp to satiation, 3 times a week. Natural spawns were collected from five out of six tanks but only fertilized spawns with at least 100,000 floating eggs were included in the study. Parameters such as fertilization rate, hatch rate, larval survival, egg and oil globule diameter and volume and size of spawn were used to assess egg and larval quality.
The fertilization rate, hatch rate and larval survival at 36 hours post hatching (hph) were significantly higher for the natural spawns (91.6%, 83.6%, 54.2%) relative to hormone induced spawns (79.3%, 53.2%, 33.9% respectively). The numbers of floating and fertilized eggs per spawn were not statistically different between the two treatments but were higher in natural spawns. Eggs from natural spawns had significantly greater egg and oil globule diameter (804.1μm, 139.2 μm) relative to induced spawns (793.2μm, 125 μm). Eggs from natural spawns had a 38% larger oil globule volume. The number of eggs per ml averaged 2,135 for natural spawns and 2,217 for induced spawns. At the onset of exogenous feeding red snapper larvae are known to have almost no yolk reserves and only a small amount of oil globule. The results of this study show that natural spawns of red snapper may not only contribute to higher egg quality and larval survival up to 36 hph, but also larvae from natural spawns may have a better chance of survival through first feeding due to greater energy reserves.

O-IX-5

EFFECT OF NUTRITIONAL QUALITY OF BROODSTOCK DIETS ON REPRODUCTIVE PERFORMANCE AND ARACHIDONIC ACID LEVEL OF MANGROVE RED SNAPPER, *LUTJANUS ARGENTIMACULATUS*

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Mangrove red snapper broodstock (7-year old) were kept in 3 units of 6-m diameter by 3m deep floating net cage and fed every other day at 5% of their total body weights pelleted diets for a year to determine its influence on reproductive performance. Three diets were used: a basal diet (diet 1), fortified diets pprepared by additionally supplementing the basal diet with either a mixture of vitamin C + vitamin E + glutathione + soybean lecithin (diet 2) or the mixture + squid meal + squid oil (diet 3). Broodstock fed diet 3 had higher mean hatching rates, percent of normal larvae and cumulative survival rate of eggs to normal larvae and larval survival index than those of broodstock fed diets 1 or 2. However, the distribution of arachidonic acid (ARA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in neutral and polar lipids of eggs remained relatively similar irrespective of diet. In the present study, EPA level was consistently higher than arachidonic acid level in eggs of captive stock in all diets. In comparison, ovaries of wild mangrove red snapper and the whole body tissue of wild fry showed high ARA and DHA but low EPA levels, resulting in a higher ARA/EPA and DHA/EPA ratios. This may suggest that essential fatty acids, specifically ARA and EPA, of the broodstock diet supplied to mangrove red snapper are not in appropriate proportion for this species and will be used as guideline for the improvement of the broodstock diet.

O-IX-6

THE FIRST PRODUCTION OF GLASS EEL IN CAPTIVITY: FISH REPRODUCTIVE PHYSIOLOGY FACILITATES GREAT PROGRESS IN AQUACULTURE

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It has been more than a quarter of a century since Yamamoto and Yamauchi first obtained fertilized eggs and larvae of the Japanese eel (*Anguilla japonica*) by hormonal treatment. However, to date no one has yet succeeded in the production of glass eel. Therefore, studies to develop techniques to consistently obtain good quality gametes and to successfully rear hatched larvae are currently being conducted. Weekly injections of salmon pituitary extracts (SPE) were administered to feminized, cultivated Japanese eels at a dose of 20 mg/fish. This induced vitellogenesis and caused oocytes to reach the migratory nucleus stage. Later, a majority of the females that received an injection of SPE at a priming dose, followed 24 h later by 17,20β-dihydroxy-4-pregnen-3-one (DHP), ovulated 15 to 18 h after the final injection. In cultivated males, repeated injections of human chorionic gonadotropin (hCG) at a dose of 1 IU/g BW/week induced spermatogenesis and spermiation. A majority of the males spermiated after the 5th or 6th injection of hCG, and sperm motility peaked 24 h after each injection. Since potassium ions were revealed to be an essential constituent for the maintenance of motility in the eel spermatozoa, artificial seminal plasma containing KCl was designed as a diluent of milt, and enabled the preservation of milt for several weeks in refrigeration. As a result, artificial fertilization performed immediately after ovulation with pre-diluted and stocked milt consistently resulted in the production of high-quality gametes. Recently, a slurry-type diet made from shark egg yolk has been found to be a suitable feed for captive-bred eel larvae. Although preleptocephalus larvae can be reared with this diet beyond the depletion of their yolk and oil droplet stores, it remains inadequate because larvae reared this way cannot be raised to the glass eel stage. Therefore, the diet was improved by supplements of krill hydrolysate, soybean peptide, vitamins and minerals. Larvae fed on this new diet have grown to 50 to 60 mm in total length, and have begun to metamorphose into glass eels approximately 250 days after hatching.

P-IX-1

SPAWNING INDUCTION AND REPRODUCTIVE PERFORMANCE MODULATION BY HORMONAL TREATMENT IN CHILEAN FLOUNDER (*PARALICHTHYS ADPERSUS*)

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Paralichthys adpersus is a new potential flatfish specie for culture in Chile. So far, there are few reports about its reproductive behavior and performance. In this study we carry on exogenous hormonal reproductive control in order to modulate the egg production. For this proposal we employed gonadotropin releasing hormone analogue (GnRHa), bovine growth hormone (bGH) and 17β estradiol (E2), in oil injections. Three year old fish were separated in the following four groups: a) control, b) E2 (20 µg/Kg b.w.), c) GnRHa (20 µg/Kg b.w.) and d) GnRHa (20 µg/Kg b.w.) + bGH (20 µg/Kg b.w). The first injection was administrated June 15th during the egg growth phase (vitellogenesis) and a second injection one week later. Then, weight increment, gonadosomic index (GSI) and plasmatic total protein, E2 and glucose concentrations were evaluated monthly. In the spawning season peak (November), ovulation induction was done under GnRHa (20 µg/Kg b.w.) injection in all groups. The eggs were obtained by handling striping after two days post injection, and fertilized with a semen pool, and the reproductive performance evaluated. The final weight increment percentage did not reflect statistical difference, however GnRHa+bGH treatment showed higher growth rate with 133.81 +/- 10.36% respect with its initial weight. The control group showed a lower value of 119.07 +/- 7.48 %. The final GSI did not showed statistical difference between groups, mainly because each mean presented a high standard error value. However, the E2 group had the highest average of 8.29 +/- 4.14 %, meanwhile the control showed a lower one of 6.17+/- 3.08 %. Significant differences were observed in egg mortality after fertilization between groups. The GnRHa+bGH group presented the highest mortality (90.0 +/- 5.19 %). The GnRHa group presented a lower value of 61.11 +/- 11.48 %. Both treatments showed significance differences ($p < 0.05$) versus the control group (22.64 +/- 3.8%). These preliminary results showed that the hormonal treatments during the vitellogenesis, modulate the reproductive performance. Although the control group presented the best egg viability performance; nevertheless the other treatments could produce an advance in oocyte maturation time. Then the eggs could be over matured at spawning moment, reflected in its survival during incubation.

P-IX-2

MEDIA AND METHODS FOR THE CRYOPRESERVATION OF EUROPEAN EEL (*ANGUILLA ANGUILLA*) SPERM

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Methods for the hormonal induction of gonad maturation in the European eel have been developed. However, these treatments use to take several weeks and out-dated maturations can succeed avoiding egg fertilization. The main objective of the present study was to evaluate different media and methods for the European eel sperm cryopreservation. Four different solutions has been used as basis for freezing media: a) Tanaka medium (decribed by Tanaka et al., 2002): (in mM) NaCl 137, $NaHCO_3$ 76.2, TAPS 20, pH 8.2; b) K30 (described by Ohta et al., 2001): NaCl 134.5, $NaHCO_3$ 20, KCl 30, $MgCl_2$ 1.6, $CaCl_2$ 1.3, pH 8.1; and two media isoionic with the European eel seminal plasma (see Perez et al., in this Congress): c) P1: NaCl 125, $NaHCO_3$ 20, KCl 30, $MgCl_2$ 2.5, $CaCl_2$ 1, pH 8.5; d) P2: NaCl 70, $NaHCO_3$ 75, KCl 30, $MgCl_2$ 2.5, $CaCl_2$ 1, pH 8.5. All media were supplemented with 10% v/v DMSO as cryoprotectant. The addition of L-a-phosphatidylcholine (1.4 g/100 ml, Tanaka et al., 2002) to freezing media has been assayed. Sperm motility was evaluated before cryopreservation by activation with sea water, and samples showing >50% motile cells were selected. Different dilution factors (1:5, 1:20, 1:100) of fresh sperm in the freezing media were evaluated as well. Freezing was carried out in vapour of liquid nitrogen during 10 min, followed by the immersion of 0.25 ml straws in liquid nitrogen for final freezing. Samples were thawed by immersion in a water bath at 20°C during 45 s. Sperm motility was evaluated after cryopreservation, at final dilution of 1:1000 in sea water (1000 mOsm/kg). Best results were obtained with Tanaka and P1 solutions (plus DMSO and phosphatidylcholine) with sperm diluted 1:5 and 1:100 respectively. This study was carried out in collaboration with Valenciana de Acuicultura, S.A. and financiated by a Project from Presidencia Generalitat Valenciana (CTIDIA/2002/117). J.F.A. have a research contract of the Ramon y Cajal Programme, cofinanciated by the Spanish Ministery of Science and Technology and the Univ. Politecnica de Valencia.

P-IX-3

THE EFFECT OF PHOTOPERIOD ON THE REPRODUCTIVE PERFORMANCE OF THE NILE TILAPIA, *Oreochromis niloticus*

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Tilapias are now a major aquaculture species with production levels of over a million tonnes annually. The hatchery production of fry is still very inefficient due to of the relatively low fecundity of these mouth brooding species combined with an inability to synchronise spawning to generate discrete production batches. This requires the industry to maintain large numbers of broodstock in order to obtain their fry requirements. Any methodology that enables farmers to synchronise the reproductive cycles of their broodstock would have immense practical advantages. Light is already known to play an important role in the initiation of gonad maturation in other fish species. In order to investigate the effect of photoperiod on tilapia reproduction four different photoperiods were evaluated including short (6L:18D), intermediate (12L:12D), long (18L:6D) and continuous illumination (24LL). Over a period of six months 291 spawns were registered, in which, 65 spawns were observed in 6L:18D, 61 in 12L:12D, 90 in 18L:6D and 75 in 24LL respectively. The Inter-Spawning-Interval (ISI) was evaluated, the shortest ISI was found in 18L:6D with 14.7 ± 0.5 (mean ± SEM) days, although, the longest ISI was recorded in 12L:12D with 19.7 ± 0.8 days. Nevertheless, regarding eggs size, the biggest eggs were produced under 12L:12D with 2.47 ± 0.02 mm in diameter and 7.0 ± 0.17 mm^3 in volume, although, their spawning synchrony and fecundity were inconsistent. These results suggest that long days were the most suitable for egg production as well as spawning synchrony.
This work has been supported by The National Council of Science and Technology (CONACYT-Mexico).

P-IX-4

REPRODUCTIVE BIOLOGY OF THE BULLSEYE PUFFER (*SPHOEROIDES ANNULATUS*)

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The bullseye puffer (*Sphoeroides annulatus*) is a commercially important species that has good aquaculture potential. Maturing and mature bullseye puffers migrate to estuarine lagoons from February to June forming an artisanal fishery. These fish appear to spawn in the area of the fishery, close to the mouth or in the estuarine lagoons. Spawning also appears to be in relation to the phases of the moon. Histological studies of bullseye puffer ovaries from fish that were held in captivity showed that vitellogenesis starts in March / April, 1-2 months before the start of the spawning season. Both captive fish and fish brought from the wild during the spawning season matured to stage 5 or 6 of vitellogenesis and did not exhibit any further maturation development. At the end of the season in June / July the vitellogenic oocytes became atretic and the gonads changed to the resting phase for the period from August through to December. Controlled experiments showed that both wild fish and fish held in captivity for one year can be induced to spawn good quality eggs using luteinizing hormone releasing hormone analogue (LHRHa). The percentage of fish (groups of n=11) spawning in captivity was increased from 18% in the control group (saline injection) to 82% using either multiple LHRHa injections (Day 0: 20µg/kg bw; day 2: 40 µg/kg; day 4: 80 µg/kg) or slow release implants (75 or 150 µg) of LHRHa. Eggs were 0.70±0.02 mm in diameter, transparent, demersal, adhesive; contained a small cluster of oil globules, a large yolk cell and a smaller blastodisc. No significant differences in fertilisation rates were found between eggs spawned from groups of fish (n=7) that were not treated with LHRHa (97.3 ± 1%) and groups induced to spawn with LHRHa injections (97.3 ± 0.8%) or implants (90.2 ± 2.8%). High hatching rates (87 ± 3.5%) were obtained using a closed (no water exchange) aerated incubation system filled with filtered and UV treated water. No significant differences in hatching rate were found between groups (n=5) of wild fish (82.6±17.0%) and fish held in captivity for one year (83.1±10.1%) that were induced to spawn with LHRHa injections. Collectively, this work suggests that maturation dysfunction stops final oocyte maturation from being completed in captivity. The present study showed that this dysfunction can be controlled with LHRHa treatments, which produced good quality eggs with high fertilization and hatching rates from fish maintained in captivity. This is an important advance in the development of this species for aquaculture.

P-IX-5

PUBERTY IN ATLANTIC COD AGE AT FIRST MATURATION IN RELATION TO SEASON, GROWTH AND ENERGY ACQUISITION DURING THE FIRST YEAR OF LIFE

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The interest in cod farming has increased tremendously during the past few years. A major bottleneck is that cod of both sexes enter puberty before the fish has reached market size. Spawning leads to reduced appetite, weight loss and increased mortality. Recent experiments have indicated that growth rate, body size or energy stores during the first year after hatch may affect age of puberty. However, the exact timing of the determination of age at puberty is unknown. Consequently, the aim in this project was to analyse puberty in groups of cod offered different rations (40, 70 and 100 % of satiation) at varying times of year (from October until May) and different duration (2, 3 or 4 months). Endocrine and histological tools were used to monitor the timing of reproductive events. The treatment effects will be presented.

P-IX-6

ENZYME TREATMENT FOR ELIMINATION OF EGG STICKINESS IN TENCH (*TINCA TINCA* L.), EUROPEAN CATFISH (*SILURUS GLANIS* L.) AND COMMON CARP (*CYPRINUS CARPIO* L.)

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Enzyme treatment to eliminate egg stickiness in tench, European catfish and common carp was compared with standard methodology in an attempt to decrease time consuming under hatchery conditions. Eggs of tench and European catfish were exposed to an alcalase enzyme MERCK EC 3.4.21.14 solution 3 minutes after egg activation for 2 minutes. In tench the highest hatching rate (87.1 %) was found with 10.0 $ml.l^{-1}$ enzyme treatment. The traditional desticking procedure in tench involving milk/clay treatment gave a hatching rate of 74.1 % and required 1 hour. The best enzyme concentration in European catfish was 20 ml of enzyme per litre. The traditional desticking procedure in European catfish clay treatment gave a similar hatching rate but procedure required 30 minutes. The eggs of common carp were successfully prevented from stickiness with ALCALASE DX (PLN 04715) using two applications of enzyme from 8 to 20 minutes after fertilization at two level of enzyme concentration (2 $ml.l^{-1}$ and 20 $ml.l^{-1}$) with fertilization and hatching between 80 to 87 %. Traditional technique of elimination of eggs stickiness in common carp can be shortened from 70-80 minutes to 21 minutes when enzyme treatment is used instead of milk or urea.

P-IX-7

THE INDUCED BREEDING AND LARVICULTURE IN THE ORANGE-SPOTTED GROUPER, *EPINEPHELUS COIOIDES*

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Groupers are important and highly valued maricultured fish along the coasts of South China Sea, especially in Guangdong Province where the cultured production of groupers is the largest. Currently, more than ten species of groupers are cultured in China, among which, the most common cultured species are orange-spotted grouper (*Epinephelus coioides*), banded grouper (*E. awoara*), black saddled grouper (*E. fario*) and red-spotted grouper (*E. akaara*).
In collaboration with Guangdong Daya Bay Fishery Research Center, studies on induced breeding and larviculture of the orange-spotted grouper (*Epinephelus coioides*) were carried out. The major research progresses were: (1) Mass healthy broodstock population with reasonable sex ratio was selected, intensive culture of broodstock was carried on by nutritional enrichment, hormonal induction and environmental control, and more than 80% female and male brooder reached sexual maturation synchronously and achieved spontaneous spawning in indoor spawning tank. The optimal temperature for spawning ranged from 24≧ to 27≧. Large amount of fertilized eggs were obtained, the rate of fertilization and hatching reached 80%. (2) Essential techniques for food preparation (e.g. live food culture, food enrichment, microcapsule formulated feed, etc) and suitable culture conditions (e.g. temperature, water quality, stocking density, diseases control, etc) during larviculture were developed, mass large-size (body length (3~4cm) fingerlings were produced. (3) The effects of gonadotropin-releasing hormone (GnRH), gonadotropin (GtH) and sex steroids on gonadal development、sexual maturation and sex reversal in adult fish were determined; females over 2 or 3 years old could be induced sex reversal by oral administration of 17-methyltestosterone at the dosage of 50mg/Kg diet within 2~3 months and became low-age functional male brooder.

P-IX-8

COMPARISON OF EGG QUALITY BETWEEN WILD AND CAPTIVE BULLSEYE PUFFER (*SPHOEROIDES ANNULATUS*)

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Egg quality was compared between eggs from bullseye puffer (*Sphoeroides annulatus*) that had been maintained in captivity for one year and wild puffer caught during the spawning season. The fish in captivity were maintained in 7000 lt fiberglass tanks with 24 hour aeration and water exchange (>150 % daily). Prior to vitellogenesis (4 months before spawning season) the fish were fed fresh squid at 2% body weight (bw), puffer fish diet at 1% bw (42% protein, 14% lipids) and trout diet (Silvercup) at 1% bw. Wild brood fish were caught by hook and line and transported to the research center and subsequently maintained under the same conditions. Pairs of fish, a fish from each group were selected by oocyte size (>0.5mm) and induced to spawn with two injections, day 0: 20 µg/kg bw and day 2: 40 µg/kg bw of luteinizing hormone releasing hormone analogue (LHRHa). The fish were stripped on the morning of day 3 and the following parameters recorded: length and weight of fish, relative fecundity, egg size, percentage fertilization and symmetry of the first divisions. A total of 10 batches of 200 eggs were taken from each fish and incubated separately, and percentage hatch recorded for each incubator. The experiment was repeated 5 times. Mean hatch rates per fish were all greater than 60.5±15.4% and a 2 way ANOVA (between groups and individuals) showed there was no significant difference between mean hatch rate of captive (83.1±10.1%) and wild (82.6±17.0%) brood fish. However, there were significant differences ($P<0.05$) between individuals indicating that there was variation in egg quality between individuals, this variation was normally distributed for each group with no difference between groups. Student t test (normal distributions) and Mann Whitney test (non-normal distributions) showed no significant differences between captive and wild fish respectively for the following parameters: length of fish (31.8±2.6 v 32.3±0.8 cm), relative fecundity (851,557±466,885 v 773,050±283,692 eggs/kg bw), egg size (0.72±0.04 v 0.72±0.05 mm) and percentage fertilization (93.9±5.1 v 95.2±5.6 %). There was a significant difference ($P<0.05$) between symmetry of cell divisions between the captive (94.3±3.4%) and wild (96.7±4.5%) fish. None of the parameters recorded exhibited a correlation with percentage hatch. The present study clearly demonstrates that good quality eggs can be produced from captive bullseye puffer, this is an important step for the management of this commercially important species that has good aquaculture potential.

P-IX-9

SPAWNING INDUCTION IN MALE RAINBOW TROUT *ONCORHYNCHUS MYKISS*, USING GNRH ANALOG

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In this experiment the advantage of using GnRHa (D-Ala6 desGly10) mGnRH ethylamid on the induction of spawning in male Rainbow trout, *Oncorhynchus mykiss*, and its effects on synchronization and acceleration of spermiation, the quality and quantity of milt and the fluctuation of testosterone levels was examined.

For this purpose, 40 male rainbow trout in early spawning season were treated by GnRHa dissolved in propylene glycol in 0.30, 40, 80 μg/kg B.W. The fish was checked 3 days apart and spermiated fish was stripped. The milt volume was measured by mesor 10 ± 0.01 ml and 0.2 ml of milt was used for fertilizing of 2000 eggs. The fertilized eggs were under care until eyed stage.

Results demonstrated that, spawning was very synchronous and also accelerated in treated groups in contrast to control. After 6 days, the cumulative spermiation reached to 40, 49 and 79% in groups 2 to 4 respectively, but until then none of the male in control group was spawned. Three days later, on day 9, when the spawning rate reached 57, 86, 100% in groups 2 to 4, only 38% of male fish could spermiate in control. Hormone injection also reduce the mean time needed for spawning from 16 ± 3.67 days in control to 14.3 ± 2.2, 9.2 ± 0.75 and 6.6 ± 0.29 days in groups 2 to 4 respectively ($p>0.05$). The total volume of milt stripped from each brood fish, increased from 10.3 ± 2.29 ml in control to 15.9 ± 2.3, 18.24 ± 2.3 and 21.99 ± 2.3 ml in others ($p>0.05$). Testosterone levels significantly increased in 12 to 24 hours after first GnRHa injection (but not In control). This experiment demonstrated that GnRHa could cause acceleration in spermiation and also shorten the period of spawning duration.

P-IX-10

EFFECT OF ESTRADIOL-17ß ON GROWTH AND INSULIN-LIKE GROWTH FACTOR-I mRNA EXPRESSION IN THE LIVER OF JAPANESE EEL, *ANGUILLA JAPONICA*

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It has been shown that sex-steroids accelerate growth of juveniles in some teleosts. The present study examined the effect of estradiol-17β (E2) on growth and liver expression of two types of insulin-like growth factor-Is (IGF-I-Ea1 and Ea2) in the Japanese eel, *Anguilla japonica*. Gonadally-undifferentiated juvenile eels were fed an E2-containing diet (20 μg E2/g) at an amount of 10% body weight once a day. Once a month, body length was measured, and then, mRNA levels of IGF-I-Ea1and Ea2 in the liver were measured by RT-PCR. E2 fed-eels showed 1.4 fold increases in body length compared to the control after 3 months. In the control group, IGF-I mRNA levels in the liver were higher in large size fish than in small size fish at all sampling times. Unexpectedly, in the E2-fed group, IGF-I mRNA levels in the liver were lower in large size fish than in small size fish. Although incubation of liver slices with recombinant eel growth hormone at concentration of 100 ng/ml for 12 to 24 hr resulted in an increase in IGF-I-Ea1 mRNA levels, inhibiting effect, rather than additive effect, of E2 on IGF-I mRNA expression was seen at concentrations of 10^{-5} M to 10^{-7} M. These results suggest that E2 stimulates growth of juvenile eel through IGF-I mRNA expression in non-hepatic tissues.

P-IX-11

RESULTS OF SPONTANEOUS SPAWNING OF YELLOW SNAPPER (*LUTJANUS ARGENTIVENTRIS PETERS*, 1869) REARED IN INLAND PONDS IN LA PAZ, BAJA CALIFORNIA SUR, MEXICO

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The yellow snapper Lutjanus argentiventris has been a target species for commercial farming in northwest Mexico. Since1996, this species has shown to be amenable to handling and captivity and adapted to stress at the culture facilities of the Northwest Biological Center (CIBNOR) Mexico (25-m long x 8-m wide x 1.8-m deep earth-ponds covered with plastic liners). This species has not shown sexual dimorphism after 7 years in captivity. Age at first maturity in both sexes was reached after 4 years (weight: 1025 ± 114 g; length 480 ± 3 cm). Snappers held under natural conditions showed natural courtship followed by a voluntary multispawning season. Daily spawned pelagic eggs were drained through a central pipe, collected in a mesh filter and were volumetrically evaluated (number of eggs/mL). The average egg diameter was estimated as 0.750 ± 0.012 mm. The aim of this work was to evaluate fecundity in relationship with the natural photoperiod and water temperature in ponds. Results showed that spawning started with the increase of summer temperature and daylength photoperiod. A few spontaneous spawning were obtained from April to June 2001 (.less than 1000 eggs/ mL). The main spawning season was from July to September, with a peak in September (7,730 eggs/mL). Control of reproduction of this species by photoperiod manipulation will be our future investigation at CIBNOR.

P-IX-12

STUDY ON THE LEVELS OF SEX STEROIDS AND ITS RELATION WITH OOCYTE DEVELOPMENT AND PROPAGATION QUALITY IN PERSIAN STURGEON (*ACIPENSER PERSICUS*)

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For study on the levels of sex steroids testosterone (T), 17β-estradiol (E2), progesterone (P4) and its relation with stage of oocyte maturity in Persian sturgeon, 86 specimens were sampled and investigated. Results showed that before vitellogenesis T, E2 and P4 levels were relatively low (0.25, 0.55 and 0.32 ng/ml respectively). But during the vitellogenesis (IIIth stage of oocyte maturity), the levels of T, E2 and P4 increased considerably and reached up to 8.55, 4.53 and o.52 ng/ml respectively ($P< 0.05$). In IVth stage, when nucleus had migrated toward animal pole, level of sex steroids decreased (T=7.44, E2 = 2.65 and P4= 0.36 ng/ml) and in the case of E2 and P4 level it was Significant ($P<0.05$). In order to determine the effect of physiological state of breeders on the propagation quality in Persian sturgeon, blood samples were obtained from 41 ripped females, before pituitary gland suspension injection (range of germinal vesicle(GV) "Polarization Index" were 5-8 %). The level of the sex steroids (T; E2 and P4) were determined and propagation quality (fertilization rate, survival rate of incubation and larval stages) were recorded during development of eggs and larval stages.Females based on the fertilization rate were divided in two groups and higher than 50% fertilization rate named as first and lower than 50% as second group. The T and E2 were quite high in first group and there were significant differences in T and E2 levels of two groups of females ($P<0.01$). The means of T and E2 in first group were 4.26 and 1.44 ng/ml and in second group were 1.7 and 0.93 ng/ml respectively. Changes in T and E2 levels were correlated with the fertilization rate but there was no significant difference and correlation between P4 levels and fertilization rate. Survival rate of incubation stage and first larval stage (yolk absorption stage) in the first group were 73.7% and 89.25% and in second group were 13.9% and 79.78% respectively, and the difference were significant ($p<0.001$, $p<0.05$). But there were no differences in the second larval stage (exogenous feeding stage $P>0.05$).

P-IX-13

SEASONALITY OF PIGMENTATION AND MATURATION IN ATLANTIC SALMON *SALMO SALAR*

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Links between sexual maturation and flesh pigmentation of Atlantic salmon were investigated to try to better understand the phenomenon referred to by the industry as the 'spring drop' which involves is a reduction in flesh pigment uptake during the spring months before final sexual maturation. In January 1999, post-smolts (100g ± 1.2g) were stocked into 9 cages (12x12m) and grown in triplicate under the following conditions: unlit cages with hand feeding, additional artificial light (1x400W submersible light) and hand-feeding, and a combination of artificial light and ad libitum feeding through an Aquasmart feed system, aimed at producing groups of fish at different stages of maturation. Each of the 3 replicates from each treatment was fed a diet containing either pure astaxanthin, pure canthaxanthin, or a mixed diet containing both astaxanthin and canthaxanthin. Significant decreases in flesh pigment concentration were observed during the April sample point regardless of culture conditions or the type of dietary pigment, although reductions were more apparent for canthaxanthin than astaxanthin. A reduction in flesh pigment concentration also occurred in some treatments in the February sample, all be it to a lesser extent. In the fish fed pure pigmented diets there was more efficient incorporation of the canthaxanthin with higher flesh concentrations compared to the groups fed pure astaxanthin. However, when presented with equal quantities of both pigments in the mixed diets, there was slightly improved incorporation of astaxanthin. There were no significant differences for overall numbers of maturing fish per treatment at the end of the trial although there was a significant reduction of lipid content in maturing fish ($p<0.05$). Overall, fish weight rather than the length of exposure to the pigment appeared to be the most significant factor in determining the level of flesh pigment concentration. This study provided clear evidence of reduced pigmentation uptake during the spring months and also demonstrated the potential of husbandry practices such as artificial lighting and feed management strategies to reduce the impact of seasonality on the quality of market-sized fish.

P-IX-14

INFLUENCE OF FACTORS OF AMBIENCE ON PARTICULARITIES OF SALMON REPRODUCTION CYCLE

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In modern aquaculture the biotechnology of management of fish growth and development have a big value which increases from year to year. It concerns Russian salmon farming also, where the most significant objects in european part are atlantic salmon and rainbow trout, and in the region of Russian Far East: pink salmon and chum salmon.
The manipulating by such powerful factors as, for instance, temperature and salinity (apart or in the combination) gives many possibilities in optimization of traditional technologies and constructing the new technologies, which based on the stimulation of processes of growth and development with the aim to get in short time the high-quality fry for further using this one in aquaculture.
The use of recirculating systems opens possibilities as well in reducing of negative affect of ambient factors which could be met for instance at pink salmon cultivation (high and low temperature, low oxygen in water etc.) as for increasing of growth rate and elevation of smoltification in cultivation of Pacific salmons with long freshwater period.
For Russia using of recirculating systems is limited their high cost, high power consumption, difficulties of service. One of the alternative decision, suitable rather for Russian conditions is using module type of the fish-culture farm. This equipment gives possibility of ambient conditions regulation, is easy in service and operation. Modules could be produced and equipped at work and delivered then by any type of transport, adjustment and start in the usage is realised during several days. Using a principle of modularity make it possible fast establishing of small hatcheries and large fish farming enterprises for different freshwater and marine fishes.

P-IX-15

IMPROVED FERTILIZATION RATES BY USING A LARGE VOLUME SPAWNING TANK IN RED SPOTTED GROUPER (*EPINEPHELUS AKAARA*)

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In the spawning seasons of 1995 and 1996, about 60 *E. akaara* were reared in a 50 m^3 octagonal tank (6 m wide and 1.7 m deep) at the Tamano Station of JASFA. They spawned spontaneously in the tank however the fertilization rates were as low as 17% and 22% in each season. In the 1996 season, an artificial fertilization was carried out to confirm whether they matured normally in the tank. Six pairs were selected from the spawning group and artificially fertilized. The fertilization rates were high (55 - 100%) and the fish were concluded to fully mature in the tank. Further, the spawning behavior was also observed, fish dashed as a pair vertically from the bottom to the water surface and spawned at the surface. In most cases, the pair jumped from the surface and swam horizontally on the surface before or after spawning. These phenomena suggested that the insufficient tank volume may have obstructed the spawning behavior and affected the fertilization rate. In the 1997 and 1998 seasons, 40 fish were reared in a 350 m^3 square tank (10 m square and 3.5 m deep) at the Hakatajima Station of JASFA. The fertilization rates increased to 43% in 1997 and 46% in 1998. Although pairs frequently jumped from the surface, they spawned under water seven times in the 1998 season. In these cases, the fertilization rates of the eggs were high (80 - 95%). These results indicate the low fertilization rate was derived from the insufficient tank volume. Rearing in a larger tank was effective to improve the fertilization rate of *E. akaara* because pairs require a large space to complete the spawning behavior.

P-IX-16

OCCURRENCE OF SPONTANEOUS POLYPLOID FROM THE EGGS OBTAINED BY ARTIFICIAL INDUCTION OF MATURATION IN THE JAPANESE EEL (*ANGUILLA JAPONICA*)

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Both male and female Japanese eel, *Anguilla japonica*, have immature gonads under ordinary culture conditions; oocytes at the pre-vitellogenic stage and the early stage of proliferation of spermtogonia. Thus we developed the methods for artificial induction of maturation in male and female Japanese eel. Females receive weekly injections of salmon pituitary extracts for induction of vitellogenesis followed by an injection of 17, 20 β-dihydroxy-4-pregnen-3-one (DHP) for induction of ovulation. Males receive weekly injections of hCG for induction of spermatogenesis and spermiation. Since the fertility and hatchability of the eggs vary even if eggs were fertilized just after ovulation, we measured the relative DNA contents of the embryos and the parents by flow cytometry to examine the genetic soundness of the eggs obtained by these hormonal treatment. Eggs hatched about 38-40 hours after the artificial fertilization, and at 72 hours after the fertilization we sampled the live embryos to examine the DNA contents of the cells. All of the blood cells from 45 females and sperm cells from 24 males showed the steady ploidy level (2C and 1C, respectively). There were 12.4 % abnormal polyploid embryos in the total of 1127 embryos from 50 females. Most of them were triploid (77.9% of abnormal embryos), and the others exhibited haploid, tetraploid, pentaploid, and aneuploid. Percentages of diploid embryos from each female varied from 100 % to 36 % (89.5±2.0%, n=50). There was no significant correlation between individual fertility or hatchability and percentage of diploid in the embryos from one female. In a preliminary experiment, percentage of triploid increased rapidly with delay for 3 or 6 hours of artificial fertilization. One of the candidates of the cause for occurrence of polyploid is difference of ovulation time among the eggs in the ovary after the injection of DHP.

P-IX-17

MATURATIONAL FACTORS AS INDICATORS OF EGG QUALITY IN JAPANESE EEL (*ANGUILLA JAPONICA*)

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In teleosts, oocyte maturation is controlled by three mediators. Gonadotropin, the first mediator, stimulates ovarian follicle cells to synthesize maturation-inducing hormone (MIH). MIH acts on fully-grown oocytes, inducing oocyte maturation by activation of maturation-promoting factor (MPF; consists of cdc2 and cyclin B) in the ooplasm. Mos and the mitogen-activated protein kinase (MAPK) pathway are also activated by MIH stimulation and regulate cdc2 activity. Following MPF activation, oocytes arrested at prophase I resume meiosis and matured eggs become fertilizable upon reaching metaphase II. Female Japanese eel does not mature in captivity unless treated with hormones such as salmon pituitary homogenate (SPH). 17α, 20β-dihydroxy-4-pregnen-3-one (DHP), which is the MIH in Japanese eel, is also often injected to induce oocyte maturation and ovulation. Fertilizable eggs can be obtained by this treatment; however, the fertilization rate is highly variable, possibly due to aberrations of meiosis during DHP-induced oocyte maturation. We aimed to identify maturational factors that can serve as indicators of egg quality in eggs from artificially matured Japanese eel. Female Japanese eels received weekly injections of SPH. Oocyte maturation and ovulation were induced by DHP. Ovulated eggs were analyzed by SDS-PAGE and by Western blotting using anti-phospho MAPK, anti-MAPK, anti-goldfish or -mouse cdc2, anti-Bufo or -Xenopus cyclin B and anti-goldfish Mos antibodies. In addition, ovulated eggs were fixed for histological observation. Anti-phospho MAPK and anti-MAPK detected a MAPK band of 44kDa. Both the 35kDa inactive and 34kDa active band of cdc2 were detected by anti-goldfish cdc2. Cyclin B and Mos were not detected at this time. Several chromosomes were observed histologically. Those chromosomes aligned on a vertical spindle appeared normal; however, chromosomes on a horizontal spindle or dispersed chromosomes seemed unusual. Correlation of the fertilization rate with biochemical or histological features of the eggs is currently under analysis.

P-IX-18

Induced ovulation using LHRH and artificial fertilization in devil stinger, *Inimicus japonicus*

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Luteinizing hormone-releasing hormone (LHRH) analog has been used successfully to induced ovulation and spawning in some commercially important fishes. Additionally, artificial fertilization is a commonly used technique to obtain maximal fertilization of eggs to produce embryos and larvae. In order to obtain a large quantity of fertilized eggs in devil stinger, LHRHa treatment followed by artificial fertilization were conducted. Before LHRHa injection, ovarian cannulation was carried out to determine the oocytes diameter of all experimental fish. Fish were injected with LHRHa (100μg/kg BW) and were checked for ovulation by stroking the abdomen from the anterior to posterior direction every 6 hour after injection. Ovulation was induced between 24h and 48h after LHRHa treatment. The latency period after hormone injection was related to the initial oocyte diameter. Females with initial oocyte diameter >700μm ovulated faster than with <700μm oocyte. Minimum size of oocyte required for ovulation with LHRHa injection was 574μm. Changes in fertilization and hatching rates at various times after ovulation were examined. Small portion of eggs were gently stripped from an ovulated female and subsequent fertilization and hatching trials necessitate repetitive partial stripping at 6h interval. Stripped eggs were inseminated with pre-diluted semen and mixed well. Seawater was added after few minutes to initiate sperm motility. Fertilized eggs were incubated at 20°C until hatching. Percent fertilization and hatching indicated 100% and 70%, respectively, when eggs were fertilized after the first detection of ovulation (0h post-ovulation). Fertilization rate significantly decreased when eggs were obtained 12h post-ovulation, while hatching rate showed a marked decrease at 6h post-ovulation. These results indicate that the injection of LHRHa was effective for induction of ovulation, and that artificial fertilization should be carried out immediately after ovulation to obtain good results.

P-IX-19

MORTALITY OF EURASIAN PERCH, *PERCA FLUVIATILIS*, DURING THE SPAWNING SEASON. DO REPRODUCTION AND ENVIRONMENTAL CONDITIONS HAVE A STRESS-MEDIATED IMMUNOSUPRESSIVE EFFECT?

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During the spawning season, Eurasian perch broodstock, *Perca fluviatilis*, show high mortality rates. Different photoperiod regimes applied throughout an entire cycle of reproduction all led to low mortality rates (10-15 %) during gonadogenesis (August-March). Mortality rates were much higher during the spawning season (April-May), increasing between 225 and 450 % with natural and artificial photoperiod regimes, respectively. These results suggest effects of reproduction and environmental conditions on the broodstock health. Thus, a second trial investigated the physiological status (immunology and stress) of a population of maturing perch during a spawning season (from 21 March to May 2002). Broodstock (251 fish, mean weight of 151g) were collected from a lake in the East of France and reared in a 3000-L tank using a water-recirculating system. Six males and six females were blood sampled every two weeks to assess sera cortisol levels and lysozyme, complement and phagocytic activities. Pathological examinations were performed on five fish at days 16 and 34. Mortality was recorded daily and reached almost 100 % by the end of the trial. This study was the first to investigate stress and immune response of a percid fish, Eurasian perch, during the spawning season. Fish displayed high cortisol levels (44 to 92 $ng.ml^{-1}$) indicating they were stressed during the spawning season. Moreover, perch displayed a low lysozyme activity (10-80 $u.ml^{-1}$), a very low complement activity (10-15 $u.ml^{-1}$) and a high phagocytic activity (600-800 $u.ml^{-1}$) compared to data from other fish species. The weakness of the non-specific immune response may be stress-related and may have allowed parasites and bacteria detected (*Trichodina sp.*, *Flavobacter sp.*, *Aeromonas hydrophila)* to infect the fish. In conclusion, perch mortality during the spawning season seems to be related to a non-adapted physiological status that would make them more sensitive to their environment.

P-IX-20

AQUACULTURE STATUS IN IRAN: PAST, PRESENT AND FUTURE

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Aquaculture is called the art of cultivating the natural produce of water; the raising or fattening of fish in an enclosed pond. During the last decades, the importance of aquacultural sciences have grown dramatically in Iran. More than 600 species of finfish and shellfish in the Iranian fisheries sectors, Northern, Southern, and Inland fisheries, provide the good and sometimes unique fish resources of the country. The aquatic production increased from 354189t to 399000t in 1991 to 2001, respectively. The main divisions of aquaculture activity are included cold-water fish, warm-water fish, sturgeon production, and shrimp culture. The value of salmonid culture (as main species of cold-water fish) and carp production (as main species of warm-water fish) have increased from 775t, 214,62t in 1991 to 121,70t and 280,60t in 2001. In addition, the shrimp culture rises from 136t in 1994 to 7630t in 2001. But the catch of sturgeon in the Caspian Sea has decreased from 1583t in 1991 to 680t in 2001. The main reason in the later is human activity in the Caspian Sea that affect on ecological condition such as feeding, migration, and reproduction of sturgeon in the Sea.
The aim of the present paper is to compare the aquaculture production (including catch from the Seas and production of fish farms and shrimp farms) between 1991-2001 in Iran and to describe the problems of aquaculture and to suggest some solutions to improve the aquacultural techniques for aquaculturists.
Several problems affect on the aquaculture production in Iran that the important problems are as follows:
1. The native and traditional culture techniques.
2. No attention is paid to modern biotechnology in aquaculture, including genetic study and chromosome manipulation on the broodstocks, investigation on artificial reproduction from hormone to gene, and etc.
3. The low human consumption of aquatic animals, especially shrimp consumption

As a conclusion, (1) the commercial breeding and cultivation of sturgeon is necessary to produce artificial Caviar and to conserve the natural population in the Caspian Sea, (2) the role of modern biotechnology in artificial breeding and cultivation of fish in fish farm is very important for management of the activity, such as: broodstock management, artificial fertilization, larvae culture, production of fingerlings and fattening, (3) the cultivation of shrimps and other aquatic organisms such as algae culture, marine fish culture can increase the value of aquaculture in Iran.

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