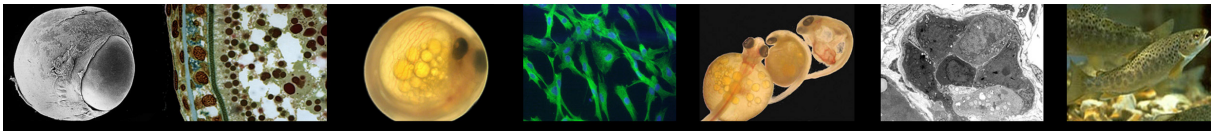
8th International Symposium on Reproductive Physiology of Fish ; June 3-8, 2007 - Saint-Malo (France). Abstract Book

Guylène Roudaut, Catherine Labbé, Julien Bobe



8th International Symposium on

Reproductive Physiology of Fish

June 3-8, 2007

Saint Malo - France

8th INTERNATIONAL SYMPOSIUM ON REPRODUCTIVE PHYSIOLOGY OF FISH

June 3-8, 2007 - Saint-Malo, FRANCE

ABSTRACT BOOK

Edited by G. Roudaut, C. Labbé and J. Bobe

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ORAL COMMUNICATIONS

Reproductive Neuroendocrinology

O 2

Melatonin and 2-[^{125}I]Iodomelatonin binding during larval and post-larval development of sea bream (Sparus auratus): the whys and wherefores

Kalamarz Hanna (1), Fuentes Juan (2), Nietrzeba Marta (1), Martinez-Rodriguez Gonzalo (3), Mancera Juan Miguel (4), Kulczykowska Ewa (1)

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BACKGROUND: Fish larvae undergo profound and coordinated physiological, behavioral and morphologic changes as they develop into juveniles. The developmental variations are accompanied by pronounced endocrine alterations. In this study, for the first time, pineal hormone melatonin and specific binding of 2-[^{125}I]Iodomelatonin were studied during larval and post-larval development in fish.

METHODS: The larvae of sea bream (Sparus auratus) were reared under photoperiod of 12h light and 12h dark (LD). Larvae were collected daily from 1 to 10 days post-hatching (dph) and then every 10 days until 70 dph at 12.00 h. The head and trunk were separated in larvae of 30-day and above. Melatonin (Mel) and protein concentrations were measured in sonicated tissues. Mel was assayed by RIA method with preceding extraction procedure. Larvae 67 dph were divided into 3 experimental groups and allowed to develop at three different lighting regimes: i) constant light (LL), ii) constant darkness (DD) and iii) a photoperiod of 12 hours of light and 12 hours of dark (LD). Samples were collected at 12.00 and 24.00 h during 2 subsequent days. Head and trunk Mel and protein concentrations were measured as above. In addition, head and trunk specific binding of 2-[^{125}I]Iodomelatonin was assessed. Binding affinities (Kd) and binding densities (Bmax) were calculated according to a Scatchard analysis.

RESULTS: Mel concentration increased unevenly till 10 dph, but during next days was diminished. Significant head-trunk difference in Mel concentration with trunk levels as a higher was observed only in 50 dph larvae. In 67 dph larvae under LD condition, head Mel concentration showed the distinct day-night differences. However, in the trunk, this concentration was stable during the day. Under both DD and LL, head and trunk Mel decreased; under LL, the head-trunk differences completely disappeared. Under LD, Bmax was higher in head and Kd in trunk, but there were no day-night differences in these parameters.

CONCLUSION: High Mel contents in larvae till 10 dph and at 50 dph suggest a role of this indole associated with organogenesis and metamorphosis. The endogenous rhythm of Mel synthesis is not present in 67 dph larvae. During the night, the main sources of Mel are probably pineal organ and retina, but during the day the gastrointestinal tract.

O 3

Daily and annual variations of plasma melatonin in the tropical catfish "Sierra Negra" (*Oxydoras sifontesi*), related to the annual gonadal maturation

Guerrero Hilda Y (1), Cardillo Elizabeth (1), Poleo Germán (2), Marcano Daissy (1)

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BACKGROUND: Fish reproduction is known to be affected by the photoperiod. The major pathway for this action seems to be the endocrine secretion of melatonin from the pineal gland. However, in tropical freshwater fish, little is known about pathways that conveys the photic information to the gonadal axis. In the present work, we have studied the function of the pineal gland of the tropical catfish "Sierra Negra" as a putative endocrine transducer of daily and seasonal photic information.

METHODS: To determine the daily pattern of plasma melatonin concentration, adult catfish kept under natural photoperiod or under constant darkness, were blood sampled over a 24h period. To asses the annual variation, midday and midnight blood samples were obtained at four times in a year and the concentrations of melatonin were correlated to the annual gonadosomatic index (GSI) variation.

RESULTS: Daily variation. O. sifontesi exhibited a 24h rhythm of plasma melatonin concentration in both lighting conditions with significant high values during the real and the subjective night. However, melatonin concentration values measured during the subjective night (40 pg/ml) were higher than those in the real night (10-20 pg/ml).
Annual variation. Melatonin concentration shows an annual profile with the highest nocturnal values in July-October (rainy season), which correspond with the post-spawning period and the lowest values during in March-April (dry season), corresponding with the preparatory period. As expected, the GSI values showed an opposite tendency, high in the preparatory period and low in the post-spawning period.

CONCLUSION: *O. sifontesi*, as most teleosts species studied presents a daily plasma melatonin rhythm with elevated levels during the night.
The presence of a 24h melatonin rhythm under constant darkness conditions strongly indicates the existence of an endogenous circadian clock regulating the pineal gland activity.
The highest annual concentration of plasma melatonin during the rainy season and the lowest in the dry season, suggest a role for melatonin as an endocrine delivery pathway of photic information to periphery tissues.
Finally, the inverse relationship between the annual concentration of melatonin and the GSI should indicate an apparent inhibitory effect of melatonin on the gonadal maturation process, as have been described in other teleosts species.
This work was supported by grants from FONACIT-MCT (Nº 2002000292) and CDCH-UCV (Nº 09-11-5148/2003).

O 4

Effects of melatonin on the gonadotropic axis of European eel, *Anguilla anguilla*

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BACKGROUND: Although they start their oceanic reproductive migration, silver eels are sexually immature. This is due to low levels of gonadotropins (LH and FSH) resulting from a deficit in gonadotropin-releasing hormone (GnRH) and a strong dopaminergic (DA) inhibition. Environmental factors of the deep-sea oceanic migration are supposed to interact with internal factors to stimulate eel maturation. Since melatonin is known to mediate environmental signals, we have investigated its potential effects on the brain-pitutary-gonad (BPG) axis of silver female eels.

METHODS: A neuroanatomical study of melatonin binding sites was performed using [^{125}I]-melatonin on brain slices in control eels. The effect of a melatonin implantation the treatment was tested in female silver eels, after five months, on different parameters of the BPG axis. Quantitative real time PCR was used to measure the expression of GnRH (mGNRH and cGnRH-II), tyrosine hydroxylase (TH: the rate limiting enzyme of DA synthesis) in various brain regions and, on LH and FSH beta subunits in the pituitary. The plasma levels of vitellogenin (Vtg) and sex steroids (11-KT, E2) were measured by immunoenzymatic assays.

RESULTS: Melatonin treatment resulted in an increase in TH mRNA in specific brain regions. Binding of [^{125}I]-melatonin was compatible with this effect. In agreement with the enhancement of DA inhibition, mRNA levels of LHbeta and FSHbeta decreased in the pituitary. No significant effect of melatonin was shown on peripheral parameters. The mRNA levels of the various GnRH forms are currently assayed.

CONCLUSION: These results suggest that melatonin may play a negative role in eel sexual maturation, possibly by increasing dopaminergic inhibition.

O 5

The clock mechanism in Atlantic salmon (*Salmo salar*); isolation and characterisation of the molecular mechanism which entrains reproduction

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BACKGROUND: Environmental entrainment of reproduction is reliant on a self sustaining molecular mechanism or "clock". This clock is believed to provide both circadian and circannual rhythmic information to coordinate reproductive processes. To date this mechanism is poorly characterised in teleosts.

METHODS: Atlantic salmon parr were acclimated to either a long (16:8 L:D) or a short (8:16 L:D) photoperiod for 1 month. Then, brains were removed and tRNA extracted (6 individuals per treatment) over a diel cycle, starting at 2 hours (ZT2) after sunrise (ZT0) and every 4 hours thereafter (ZT6, 10, 14, 18 and 22). Sets of degenerate primers designed against vertebrate homologs of target genes were used to isolate partial sequences for Period homolog 1 & 2 (*Per 1 & 2*), Clock, Brain and Muscle Aryl hydrocarbon receptor nuclear translocator like protein 1 (*BMAL1*) and Cryptochrome 1a (*Cry1a*). 5` and 3` ends were then extended by RACE-PCR and protein sequences/structures deduced. Following which, quantitative real-time PCR assays were developed and brain expression profiles under the different photic states were characterized along with diel variations in expression in other key tissues representative of critical physiological functions e.g. gonad, liver, spleen.

RESULTS: All genes showed a high consensus (70 to 96% consensus) with their homologs in characterised vertebrates. Interestingly the genes showed differential diel expression patterns in relation to the entraining photoperiods both in terms of daily expression patterns as well as in relation to seasonal photoperiods. For example, *CLOCK* expression peaked at around ZT18 (following sunset) under a short day photoperiod (winter) however under long day photoperiods (summer) this diel rhythm no longer existed. Meanwhile, *Per 2* expression peaked just prior to sunrise (ZT22) in both seasonal conditions.

CONCLUSION: The genes targeted in this study represent the key constituents of the positive (*CLOCK* and *BMAL1*) and negative (*Per 1 & 2* and *Cry1a*) arms which form the transcription/translation-based negative feedback loop that is central to the clocks' function. The demonstration of the diel expression patterns confirms the function of the clock in Atlantic salmon. However more importantly, the seasonal differences in expression of *CLOCK* are of critical importance as it demonstrates a possible neuroendocrine link for the seasonal entrainment of reproduction in fish. While *CLOCK* had previously been linked to reproduction in salmonids through QTL mapping, the present results demonstrate, for the first time, how the environmental windows that regulate sexual maturation in salmon (transition from short to long day photoperiods) are interpreted into a neuroendocrine signal that directly entrains the reproductive axis.

O 6

Which are the functions of chicken GnRH-II and salmon GnRH in the European sea bass? a neuroanatomical, physiological and molecular approach

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BACKGROUND: The decapeptide GnRH plays a major role in controlling the release of gonadotrophin, but has also been implicated in the secretion of other hormones. However, increasing evidence suggests that GnRH serves important autocrine/paracrine actions in extra-pituitary tissues such as gonad, liver or kidney. In addition, GnRH may also act in the brain to modulate nesting and sexual behaviour, but the precise sites of GnRH actions in CNS are unclear. In perciforms, three different GnRH forms are expressed, seabream GnRH (sbGnRH), salmon GnRH (sGnRH) and chicken II GnRH (cGnRH-II). It is clearly stated that the sbGnRH form represents the main hypophysiotrophic hormone, whereas the roles of the two other GnRH forms expressed, sGnRH and cGnRH-II, remain still unknown. In this study, we present neuroanatomical, physiological and molecular evidences that suggest a role of cGnRH-II and sGnRH in the modulation of the activity of photoreceptive structures in the European sea bass.

METHODS: The central connections of the pineal organ and retina and their possible relationship with gonadotrophin-releasing hormone (GnRH) neurons were elucidated using a combination of neural tracing methods and immunohistochemical techniques. The in vitro and in vivo effects of GnRH on the secretion of melatonin were determined by RIA. The presence of GnRH receptors in the pineal organ and retina was determined by RT-PCR.

RESULTS: The dorsal synencephalon represents a target of pineal fibers and exhibits pineal-projecting cells. Combined DiI-tracing methods and immunohistochemistry revealed that retrogradely labeled cells in the dorsal synencephalon corresponded to cGnRH-II immunoreactive neurons, which represent the source of cGnRH-II fibers detected in the pineal organ. Furthermore, the in vitro and in vivo studies show a stimulatory effect of cGnRH-II on pineal melatonin secretion. These results are consistent with the expression of different GnRH receptors showed in the pineal organ of sea bass by RT-PCR. In turn, salmon GnRH cells present in the terminal nerve, at the transitional area between the olfactory bulbs and the telencephalon, send projections to the retina, which also expresses GnRH receptors.

CONCLUSION: Our results suggest a role for chicken GnRH-II and salmon GnRH in the modulation of pineal and retinal functions, respectively. In this way, these GnRH forms could be involved in the modulation of photoperiod effects on reproductive and other rhythmic physiological events in the European sea bass.

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O 7

Autocrine and paracrine factors modulating GnRH-I neuron migration : The role of GnRH-I and netrin

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BACKGROUND: Normal migration of GnRH-I neurons during early development is a crucial step in establishing a functioning reproductive system in all vertebrates. To gain a better understanding of the process of GnRH-I migration and the factors controlling it, we utilized a recently developed GnRH-I-GFP transgenic zebrafish line coupled with time-lapse confocal microscopy to track the GnRH-I developmental/migrational process in detail. In addition, we studied the involvement of GnRH-I itself and Netrin1a in the establishment of the GnRH-I system.

METHODS: GnRH-I-GFP transgenic zebrafish larvae were used to track GnRH-I migration and development using light and confocal microscopy. Knock-down (KD) of GnRH-I transcript was achieved by using two variants of GnRH-I morpholino injected into transgenic embryos at the 1-2 cell stage. Currently, we are using Netrin1a morpholino and Netrin1a over-expression to explore the involvement of Netrin1a in the migration process.

RESULTS: GnRH-I fibers began to extend from the soma at 26 hours post-fertilization in multiple tracts, including along the vomeronasal nerve. By 4-5 days post-fertilization an extensive network of GnRH-I fibers is in place innervating the optic tract, telencephalon, hypothalamus, midbrain tegmentum and hindbrain. Subsequently the GnRH-I soma migrate to the hypothalamus. The pathfinding of the GnRH-I fibers was disrupted by anti-GnRH-I morpholino, causing mistargeting, abnormal extensions and anomalous GnRH-I soma localization.

CONCLUSION: This study provides a better spatio-temporal resolution of early GnRH-I soma and fiber development including several novel findings regarding fiber behavior and localization within and outside the CNS. Our findings suggest that in zebrafish, which has two GnRH forms, GnRH-I regulates functions controled by GnRH-III in other species. Our GnRH-I KD results show that GnRH-I itself is an important autocrine factor in the establishment of the GnRH-I network. Additional Netrin1a results from ongoing studies will be reported at the conference as well.

O 8

Localization of GPR54 expression on GnRH neuronal elements and their regulation by gonadal steroids in Atlantic croaker

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BACKGROUND: Recent studies have demonstrated that the G protein-coupled receptor 54 (GPR54) and its natural ligand Kisspeptin play important roles in the control of puberty and reproduction in mammalian species. In addition, GPR54 mRNA has been localized on the neurons expressing the three forms of GnRHs in tilapia. However, a natural ligand for GPR54 has not been identified in any non-mammalian species and there is no information on changes in GPR54 mRNA or protein expression in fish brain after any experimental manipulation. Therefore, the present study was designed to localize GPR54 on GnRH neurons and to determine whether steroid hormone treatments similar to those demonstrated to influence GPR54 signaling in mammalian models could influence this receptor in a fish model, the Atlantic croaker, *Micropogonias undulatus*.

METHODS: The cDNAs of three gonadotropin releasing hormones, seabream GnRH (sbGnRH), chicken GnRH-II (cGnRH-II) and salmon GnRH (sGnRH), and a G protein-coupled receptor (GPR54), were isolated from the brain of the Atlantic croaker. The expression of three GnRH mRNAs was localized by *in situ* hybridization, and quantified in dissected brain areas by real-time quantitative RT-PCR (qRT-PCR). GPR54 protein expression was determined by Western blot analysis and GnRH peptide levels by ELISA.

RESULTS: The qRT-PCR study revealed expression of sbGnRH and sGnRH mRNAs in the olfactory bulb, telencephalon, and preoptic-anterior hypothalamic area, indicating an overlap of the two GnRH systems in these forebrain regions consistent with their localization by *in situ* hybridization. However, cGnRH-II mRNA expression was detected only in the midbrain tegmentum. The sbGnRH mRNA expression was observed also in the pituitary, thereby suggesting potential local regulation by the sbGnRH synthesized within the pituitary gland. GPR54 was also localized on GnRH neuronal elements in the brain and pituitary, providing a morphological basis for possible physiological effects. In addition, significant positive effects of 17β-estradiol (E_2) and negative effects of the progestin, 20β-S, on GnRH and GPR54 mRNAs in selected brain areas were observed in fish undergoing crudescence and those with fully mature gonads, respectively.

CONCLUSION: Significant alterations in the expressions of the GPR54 and GnRH mRNAs were observed after E_2 and 20β-S administrations consistent with their positive and negative feedback effects, respectively. The results suggest that GPR54 signaling could be an important component in the steroid feedback regulation in fish similar to estrogen effects shown previously in mammals. Moreover, this study reports a novel finding of progestin negative feedback effects on GPR54 and GnRHs in a vertebrate species.

O 9

Aromatase is expressed in progenitor cells in the brain of adult zebrafish

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BACKGROUND: Brain aromatization of androgens is particularly important for organisation of brain sexually dimorphic structures, testosterone feed-back on neuroendocrine circuits controlling the reproductive axis and regulation of sexual behaviour. The brain of teleost fish is characterized by a high aromatase activity corresponding to expression of aromatase B (cyp19b), one of two cyp19 duplicates. In teleost species, including the zebrafish, it was shown that aromatase B is expressed in radial glial cells. Such cells have a typical morphology with a small nucleus near the brain ventricles and radial cytoplasmic processes projecting towards the brain surface. Given the well-known role of radial glial cells as progenitor cells during embryogenesis, we examined the possibility that aromatase B-positive radial cells are progenitor cells in the brain of adult fish.

METHODS: Zebrafish were treated with Bromodeoxyuridine (BrdU) during 12, 24 or 48 hours and the fish were kept different times after BrdU treatment 0, 1, 2, 5, 15 and 30 days. The presence of newborn cells, their localization and their fate (migration, differentiation, apoptosis) was then analysed by a combination of BrdU immunohistochemistry and that of various cell markers.

RESULTS: Here, we further document that aromatase B is expressed in radial glial cells of the forebrain and never detected in cells expressing the markers of post-mitotic neurons, Hu and acetylated-tubulin. We demonstrate for the first time, that aromatase-positive radial cells actively divide to generate newborn cells in many forebrain regions. Such newborn cells can further divide, as shown by BrdU-PCNA double staining. We also demonstrate that, over time, newborn cells move away from the ventricles, most likely by migrating along the radial processes. Finally, using antisera to Hu and acetylated-tubulin, we document that some of the newborn cells derived from radial glia differentiate into neurons.

CONCLUSION: These data provide new evidence for the mechanisms underlying adult neurogenesis in fish. Given that estrogens are known to affect proliferation, apoptosis, migration and differentiation, expression of aromatase B in progenitor cells is likely to affect adult neurogenesis under certain physiological conditions. Such mechanisms are likely to provide the brain of adult fish with a high sexual plasticity.

O 10

Electrotonic coupling and slow synchronous oscillations in the anterior pituitary of teleost fish: a possible role for connexin 35

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BACKGROUND: In fish, hypothalamic fibers directly innervate the pituitary. When hypothalamic hormones are released from these fibers, they bind to membrane receptors on pituitary cells and lead to spike generation, a rise in cytosolic calcium and exocytosis. It is unclear whether these activities are confined to the stimulated cell or propagate to adjacent cells through gap junctions.

METHODS: We used electrophysiological and molecular techniques in a novel hypothalamo-pituitary slice preparation from tilapia to examine cell-to-cell communication. Groups of coupled pituitary cells were detected by dye injection, and single and paired recordings using whole-cell and perforated patch clamping technique were performed. To determine the molecular basis for the electrotonic coupling, we studied expression of the connexins Cx35 and Cx43 using PCR analysis and in-situ hybridization. Real-time PCR was used to analyze the endocrine regulation of connexin expression.

RESULTS: Pituitary cells at rest generated occasional spontaneous spikes and spikelets. The latter represented spikes in neighboring, electrotonically coupled cells. Dye-coupling and biophysical evidence of electrotonic coupling confirmed that clusters of pituitary cells are functionally connected, and quantitative characteristics of the coupling was determined in paired recordings. Thirty-three percent of the pairs exhibited strong reciprocal coupling. The electrical junctions were effective low-pass filters, and spikes were greatly attenuated while low-frequency waveforms readily passed from cell to cell. Transient application of GnRH to anterior pituitary cells elicited a prolonged period of voltage oscillation (~1 Hz) with bursts of action potentials, arising from the depolarizing edge of the waves. Depolarization or hyperpolarization decreased the amplitude of the oscillations, but did not modify their frequency. The oscillations were prominent in field potential recordings, indicating a high degree of regional synchrony. Cx35 and Cx43 were partially cloned from tilapia pituitary. In-situ hybridization showed that while Cx43 is mainly expressed in neural brain tissue, Cx35 is prominently expressed in the adenopituitary, where it is prevalent in the rostral pars distalis (where mainly prolactin cells are detected) and in the proximal pars distalis (where LH cells are detected). As evidence of physiological modulation of coupling, application of estradiol increased gene expression of Cx35 in vivo.

CONCLUSION: Our findings lead us to conclude that GnRH-induced oscillations are synchronized through gap junctions formed by connexin Cx35. Regulation of this coupling by estradiol and other endocrine factors may play a central role in determining complex patterns of pituitary hormone secretion.

O 11

Neuroendocrine regulation of pubertal development in grey mullet (*Mugil cephalus*)

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BACKGROUND: Molecular studies were conducted to further elucidate the neuroendocrine regulation of pubertal development in grey mullet, a relatively late maturing teleost with its reproductive process inhibited by dopamine (DA).

METHODS: The cDNAs of key genes along the reproductive axis were isolated by RT-PCR. The promoters of the mullet dopamine D_2 receptor (D_2R), ovarian aromatase (muCyp19a) and brain aromatase (muCyp19b) genes were isolated by genome walking PCR and further characterised by a reporter gene assay. The expression profile of mullet G-protein coupled receptor 54 (muGPR54), gonadotropin-releasing hormone genes (muGnRHs), muD_2R, muCyp19a and muCyp19b genes were determined by real-time quantitative PCR in female fish at different stages of pubertal development.

RESULTS: The promoter regions of muD_2R, muCyp19a and muCyp19b genes were isolated and their functionality was demonstrated *in vitro,* in COS-7, αT3 and TE671 cell lines. The basal promoter activity of the muCyp19b promoter was negatively modulated by DA via the putative cyclic AMP response element. In pubertal female mullet, significantly higher levels of expression were observed for the muGPR54, muGnRHs and muD_2R genes in the brain at the early stage of puberty relative to the advanced stage. In contrast, muGPR54 and GnRH1 expression in the ovary increased significantly at the advanced stage compared with levels observed at the early stage. In a similar manner, the expression levels of muCyp19a and muCyp19b genes in the ovary and in the brain, respectively, were significantly higher at the advanced stage of puberty than at the early stage.

CONCLUSION: Our results indicate that the stage-specific coordination of the expression of key genes along the reproductive axis contributes to the regulation of the pubertal process in female grey mullet. Insights from the promoter studies suggest the critical roles of DA and oestrogen in the overall expression of those key genes, particularly of the GnRHs, which are vital for puberty to commence. The overall findings are summarised in a conceptual model proposing the neuroendocrine regulation of the pubertal process in female grey mullet.

O 12

Insulin-like growth factor I (IGF-I) mRNA and peptide in the hypothalamic-pituitary-gonadal axis during development of the tilapia, *Oreochromis niloticus*

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BACKGROUND: In fish, steroid hormones exert important functions in gonadal sex differentiation. However, recent evidence indicates that they probably interact with other hormones in sex differentiation both at the pituitary and gonad level. IGF-I that exerts numerous essential functions in fish is a likely candidate to be involved. While some studies indicate that IGF-I occurs in fish pituitary and gonads no data exist on its potential production sites in development.

METHODS: Early developmental stages, 0-100 days post fertilization (DPF), juvenile and adult male and female monosex tilapias were investigated. The localization of IGF-I mRNA in pituitary, ovary and testis was investigated by *in situ* hybridization and that of IGF-I peptide by immunohistochemistry.

RESULTS: In the neuropituitary, IGF-I appeared in neurosecretory axons around 17 DPF while no IGF-I mRNA was detected indicating that the likely source of IGF-I are hypothalamic IGF-I immunoreactive neuronal perikarya. In the adenopituitary, IGF-I mRNA appeared at 28 DPF where it persisted throughout the fish life in ACTH and GH cells. Some developmental stages, especially around 90 DPF, expressed IGF-I in gonadotrophs. At 7 DPF, IGF-I mRNA appeared in somatic cells while only IGF-I peptide was observed in primordial germ cells (PGCs). In germ cells, IGF-I mRNA and peptide appeared at 29 DPF in the ovary and around 52 DPF in testis. In adult testis, IGF-I was expressed in spermatogonia, spermatocytes and Leydig cells and in adult ovary in small and previtellogenic oocytes as well as in numerous granulosa and some theca cells of follicles at the lipid stage and around mature oocytes.

CONCLUSION: The transient appearance of IGF-I in gonadotrophs during puberty may indicate its involvement in the proliferation of LH and FSH cells. During early development IGF-I in the PGCs may be transferred from somatic cells to the PGCs or be of maternal origin. The production of IGF-I in the germ cells likely is linked to the onset of meiosis. The expression of IGF-I in Leydig cells may indicate its role in the synthesis of male sex steroids. In general, the results suggest a crucial role of IGF-I in the formation, differentiation and function of tilapia gonads.

O 13

Is Somatolactin Related to Reproduction in Black Seabream (*Acanthopagrus schlegeli*)?

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BACKGROUND: Somatolactin (SL) has been shown to have the following physiological functions including the control of reproduction, respond to stress, background adaptation, melanosome aggregation, metabolism (Pi, Ca^{2+}, fat, energy), and acid-base balance, etc. We have recently detected high level of SL mRNA in black seabream pituitary with only one form of the SL mRNA obtained from sequencing of over 100 SL cDNA clones. To study the physiological function of SL in black seabream, we initiated a study of monthly levels of SL and gonadal somatic index (GSI) to determine whether SL is related to reproduction in black seabream; we also obtained the SL gene promoter for gene regulation studies aiming to search for possible regulatory elements related to reproduction onset.

METHODS: Black seabream were sampled monthly from a fish farm in Sam Mun Tsai, Tolo Harbour, Hong Kong from November 2004 to December 2006. Pituitaries were collected for SL mRNA detection using real-time quantitative PCR. Body weight and gonad weight were recorded and the GSI was calculated. A SL gene promoter was cloned using a DNA walking method with a *SpeedUp*™ Premix Kit (Seegene, Korea) from primers designed from SL cDNA sequence. The SL gene promoter sequence was analyzed *in silico* with TESS (Transcription element search software), dragon ERE finder (Version 2.0) and data of published fish hormone gene sequences, aiming at understanding the relationship of SL gene regulation and its possible physiological control in black seabream.

RESULTS: GSI (%) rose to peaks in Jan. 2005, Dec. 2005 and Dec. 2006 and then decreased in the following months in both male and female black seabream. The level of SL mRNA increased to the highest in Nov. 2004, Oct. 2005 and Nov. 2006, one month prior to gonad development. Moreover, a 1.6 kbp gene fragment upstream of the SL gene was cloned and sequenced. According to the *in silico* study, 8 consensus motifs $(A/T)_3$ NCAT for putative Pit-1 (pituitary specific transcription factor) binding, a putative estrogen receptor response element and a putative androgen receptor response element are proposed.

CONCLUSION: Black seabream spawn once a year, from December to March next year, and the peak of SL mRNA level appeared one month before the gonad development; indicating that SL may play a role in reproduction. From the SL gene promoter sequence obtained in this study, Pit-1, estrogen and androgen may regulate SL expression at the onset of gonad development.

O 14

Brain and gonadal aromatase as molecular and biochemical targets of endocrine disrupters in a model species, the zebrafish (*Danio rerio*)

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BACKGROUND: P450 aromatase is a crucial steroidogenic enzyme that catalyses the irreversible conversion of androgens to estrogens which are key regulators in development and reproduction. Little is known about the effect of endocrine disrupters on this enzyme in fish. This study was thus undertaken to characterise the molecular and biochemical mode of action of endocrine disrupters on ovarian (AroA encoded by *cyp19a*) and brain aromatase (AroB encoded by *cyp19b*) in a model fish species, the zebrafish.

METHODS: We first developed methods to measure brain and gonadal aromatase at mRNA, protein and enzymatic levels and characterised their baseline expression in control fish. These methods were then used to assess the *in vivo* effects of several compounds on aromatase expression/activities in brain and gonads. Zebrafish were exposed at larval or adult stage during 3 or 7 days to different compounds: a steroidal aromatase inhibitor (androstatrienedione), an imidazole fungicide (clotrimazole, known as an aromatase inhibitor *in vitro*) and (xeno)-estrogens (estradiol, ethynylestradiol, nonylphenol).

RESULTS: Exposure of adult fish to androstadrienedione inhibited aromatase activities (AA) in both brain and gonads without affecting aromatase genes expression. In contrast, the *in vivo* action of clotrimazole was more complex. In brain, a U-inverted dose-response curve was observed on aromatase B expression and activities. In ovary, *cyp19a* was induced at all the concentrations tested while AA were induced only at low concentrations.
Furthermore, we showed that (xeno)-estrogens were able to inhibit transcription of ovarian *cyp19a* gene as well as AA. In contrast, (xeno)-estrogens strongly induced *cyp19b* expression through an estrogen receptor-dependent mechanism leading to *de novo* synthesis of AroB protein in radial glial cells in the brain of zebrafish larvae. These AroB inductions by (xeno)-estrogens were also observed in adult male fish (and not in females) but the induction factors were much lower as compared to larvae.

CONCLUSION: Our results showed that brain and gonadal aromatase are molecular and biochemical targets of endocrine disrupters in fish. The tested substances elicited sex, tissue and life stage specific responses. Based on our data, it can be concluded that induction of aromatase B is a sensitive marker of estrogenicity in the central nervous system of fish. Taken together, our work provides new and relevant data concerning the effects and mechanisms of action of endocrine disrupters in fish and raised the question of the physiological and reproductive consequences of these aromatase perturbations for the organism.

O 15

Expression, regulation and endocrine regulation of gonadotropins in tilapia

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BACKGROUND: FSH is generally important for early gonadal development and vitellogenesis, whereas LH stimulates processes leading to final oocyte maturation and ovulation. Both gonadotropins (GTHs) are heterodimeric glycoproteins comprising two non-covalently associated subunits. GTHs are under a dual hypothalamic regulation when GnRH stimulates and dopamine inhibits their release.

METHODS: We produced *Oreochromis niloticus* (t) recombinant LH and FSH, as single-chain polypeptides, in the methylotrophic yeast *Pichia pastoris*. The tLHbeta, alpha or tFSHbeta mature protein-coding sequences were joined to form a fusion gene encoding a "tethered" polypeptide. A "linker" sequence was placed between the beta- and alpha- chains to assist the chimerization of the subunits, and a six-His tail was added, to enable purification of the recombinant protein. rGTHs were used to develop ELISAs, using primary antibodies against rtLHbeta or rtFSHbeta, respectively, and rtLHbetaalpha or rtFSHbetaalpha as standards.

RESULTS: rtFSHbetaalpha and tLHbetaalpha significantly enhanced both 11-KT and E2 secretion in a dose-dependent manner, similarly to mammalian GTHs, pituitary-derived FSH or LH, and tilapia pituitary extract, from tilapia testes or ovaries, respectively.
tFSH-receptor (R) and tLH-R were transiently expressed in COS-7 cells, together with the reporter gene CRE-LUC. Functional assessment of tLH-R and tFSH-R showed that each GTH induced luciferase activity of its cognate receptor.
Both LH and FSH plasma levels were increased 6 h after sGnRHa injection to tilapia females. During a reproductive cycle, both GTH levels increased during the 2nd day, probably related to the vitellogenic phase. Both GTHs levels increased toward spawning, which occurred on the 11th day. sGnRHa increased mRNA levels of GnRHR1 and GnRHR3. Both GnRH receptors activate the PKC pathway, while only tGnRHR3 activates the PKA pathway. By using a chimera between tGnRHR3 and GFP we showed that the receptor is initially localized at the plasma membrane and upon activation by sGnRHa undergoes relatively rapid endocytosis.

CONCLUSION: In this study we describe the expression of tFSH and tLH as biologically active, single-chain polypeptides using *Pichia pastoris*. The development of specific ELISAs using recombinant GTHs was used to advance the study of the distinct functions of each of these important hormones. We also showed the role of sex steroids on positive and negative feedbacks controlling the reproductive cycle.

Sex Determination and Sex Differentiation

O 16

Diversity of sex determination in fish: Positional cloning of the sex-determining gene of the platyfish *Xiphophorus maculatus*

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BACKGROUND: In contrast to the situation observed in mammals and birds, fish display an amazing diversity of sex determination systems, with very frequent switching between different mechanisms during evolution. Almost nothing is known about the molecular and evolutionary basis of this phenomenon.

METHODS: Bacterial artificial chromosome (BAC) contigs including the sex-determining region of the platyfish *Xiphophorus maculatus* and covering 2.5-3.5 megabases of the X and Y chromosomes have been constructed and sequenced. Genes have been identified and their expression has been analyzed by RT-PCR and *in situ* hybridization.

RESULTS: Thirty-nine BAC clones have been sequenced so far to completion, leading to the identification of 63 sex chromosomal genes. Interestingly, eleven of these genes are also located on the human sex chromosomes, but no synteny was found with other fish gonosomes. Through comparison between X and Y sequences, the sex-determining region has been delimited. Gene candidates have been identified for all loci involved in pigmentation, cancer formation and sexual development that are linked to the master sex-determining gene of the platyfish. Numerous genes, including the melanocortin hormone receptor gene *mc4r*, are amplified in the sex-determining region of *X. maculatus*, this being reminiscent of the situation observed on the human Y chromosome. Gonad-specific genes were identified, including two genes with so far unknown function organized in tandem and exclusively expressed in oocytes. One gene, called *swimy*, is present on the Y but not on the X chromosome and represents an excellent candidate for the master sex-determining gene. This gene encodes a ubiquitously expressed protein, with different predicted domains involved in nucleic acid binding and protein-protein interactions. Interestingly, through the use of an alternative exon in spermatogonia, a testis-specific longer isoform is produced, with different domains involved in protein degradation.

CONCLUSION: This analysis, which uncovers new types of genes involved in sexual development, suggests that the diversity of sex determination in fish is associated with the frequent formation of new sex chromosomes and possibly new master sex-determining genes. Possible reasons for this exceptional evolutionary turnover will be discussed.

O 17

Functional analysis of the medaka sex determining gene, DMY reveals its role in the regulation of germ cell proliferation in the XY gonad

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BACKGROUND: DMY (the DM-domain gene on the Y chromosome) was cloned from the sex-determining region on the Y chromosome of the teleost fish medaka and has been shown to be required for the normal development of male individuals. Recently, DMY genomic DNA was found to be inducing testis differentiation and subsequent male development in XX (genetically female) medaka by transgenic experiments. However, the function of DMY still remains to be elusive.

METHODS: For the functional analysis, DMY was knocked-down using engineered peptide nucleic acid (gripNA). Estradiol 17-beta (E2) was used to feminize the genetic males. The fry collected on the day of hatching were subjected to both histological (for germ cell counting) and whole-mount in situ hybridization (WMISH) analyses. The sex of the fry was assessed by genomic PCR of the DNA extracted from the head or tail region of each fry, using common primers for DMRT1 and DMY.

RESULTS: Some of the 0 day after hathcing (dah) DMY knock-down XY fry had germ cell numbers comparable to those of the control and normal XX fry. On the contrary, the germ cell numbers in the E2-treated XY gonads at 0 dah were found to be similar to those of the control and normal XY gonads of the same stage. However, WMISH with the meiosis specific marker gene, Synaptonemal complex protein 3 (Scp3) revealed that meiosis was induced in the gonads of the 0 dah DMY knock-down and E2-treated XY fry.

CONCLUSION: Our findings revealed that the mitotic and meiotic activities of the germ cells in the 0 dah DMY knock-down XY larvae were identical to those of the normal XX larvae, suggesting the microenvironment of these XY gonads to be similar to that of the normal XX gonad, where DMY is naturally absent. Conversely, E2 treatment failed to initiate mitosis in the XY gonad, possibly due to an active DMY, even though it could initiate meiosis. Present study has proven that the germ cells in the XY gonad can resume the mitotic activity, if DMY was knocked-down. These results demonstrate that DMY is sufficient for male development in medaka, and suggest that the functional difference between the X and Y chromosomes in medaka is a single gene.

O 18

Sex-dependent differences in DNA-methylation levels in the promoter of the gonadal aromatase gene in a fish, the European sea bass (*Dicentrarchus labrax*)

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BACKGROUND: Aromatase is the enzyme responsible for estrogen synthesis and essential for ovarian differentiation in lower vertebrates. It is well established that females have higher levels of aromatase expression and activity than males at the time of sex differentiation, and that enzymatic activity matches gene expression levels. Thus, inhibition of aromatase at this time leads to male development in genetically female fish. Recently, the promoter region of gonadal aromatase has been characterized in some fishes and several binding sites for putative regulatory factors identified. However, the molecular mechanisms by which this promoter is controlled are essentially unknown. Methylation of DNA is a powerful mechanism of gene silencing. In this study, the amount of methylation in a fragment close to the transcription start site in the sea bass gonadal aromatase promoter was determined according to phenotypic sex.

METHODS: One-year-old fish were sacrificed and their gonads removed. After DNA extraction and digestion, a ~600 bp fragment of the sea bass gonadal aromatase promoter was obtained and subjected to bisulphite treatment. The product was amplified by PCR and cloned into the pGEM-T vector (Promega). Ten clones per fish were sequenced and used to determine methylation levels in seven CpG dinucleotide positions, labeled I through VII. Average methylation per position and fish were calculated. Quantitative real-time PCR analysis was used to determine aromatase expression levels.

RESULTS: Methylation ranged from 13 to 51% in females and from 77 to 96% in males, with mean ± SEM of 39.4 ± 7.01 in the former and 83.9 ± 3.24 in the latter ($P<0.001$). In contrast, aromatase expression in testes was lower than in ovaries. In addition, differences in methylation among the seven CpG sites were observed, with position V, which corresponds to a putative binding site for transcription factor Sox, showing the highest methylation differences between males and females.

CONCLUSION: Our data clearly demonstrate differences in DNA methylation of the sea bass gonadal aromatase promoter, with males significantly more methylated than females. Thus, transcription and DNA methylation were inversely correlated. Furthermore, differences in methylation in the position V, suggest that this site is important for aromatase gene transcription and thus necessary for ovarian development. To the best of our knowledge, this is the first report of clear global sex-dependent differences in the methylation of the aromatase promoter of any vertebrate. Finally, we propose that methylation may be the mechanism by which gonadal aromatase transcription is inhibited in developing males and thus a proximate cause that prevents the development of an undifferentiated gonad into an ovary.

O 19

Foxl2 up-regulates aromatase gene transcription in a female-specific manner by binding to the promoter as well as interacting with Ad4BP/SF-1

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BACKGROUND: Foxl2 is a fork head (FH) transcription factor implicated in the early ovarian differentiation and later follicular development and maintenance in vertebrates. However, its target genes and molecular mechanisms of action are largely unknown. On the other hand, estrogens play an important role in ovarian differentiation in non-mammalian vertebrates. In fish, the ovarian form of aromatase (Cyp19a1) is responsible for ovarian production of estrogens.

METHODS: The present study has investigated the plausible role of Foxl2 in ovarian differentiation through transcriptional regulation of aromatase gene, using mono-sex fry of tilapia. In situ hybridization and immunocytochemistry were performed to check the co-expression of Cyp19a1, Ad4BP/SF-1 and Foxl2 in gonad. Promoter assays using mammalian cell lines were performed to test whether Ad4BP/SF-1and Foxl2 can activate the Cyp19a1 gene transcription. EMSAs were performed to confirm the binding of Foxl2 to the Cyp19a1 promoter. Pull-down assays, mammalian two-hybrid assays and double mutations were performed to confirm the interaction between Foxl2 and Ad4BP/SF-1. In vivo transgenic overexpression of Foxl2 and its dominant negative mutant were performed by injection of these GFP constructs into fertilized XY and XX eggs, respectively.

RESULTS: The expression of Foxl2 is sexually dimorphic, like Cyp19a1, co-localizing with Cyp19a1 and Ad4BP/SF-1 in the stromal cells and interstitial cells in gonads of normal XX and sex-reversed XY fish, before the occurrence of morphological sex differentiation. Under in vitro conditions, Foxl2 binds to the sequence, ACAAATA in the promoter region of the Cyp19a1 gene directly through its FH domain, and activates the transcription of Cyp19a1 with its C-terminus. Foxl2 can also interact through the FH with the ligand binding domain of Ad4BP/SF-1 to form a heterodimer and enhance the Ad4BP/SF-1 mediated Cyp19a1 transcription. Disruption of endogenous Foxl2 in XX tilapia by overexpression of its dominant negative mutant induces varying degrees of testicular development with occasional sex reversal from ovary to testis. Such fish display reduced expression of Cyp19a1 as well as a drop in the serum levels of estradiol-17β and 11-ketotestosterone. Although the XY fish with wild type Foxl2 overexpression never exhibited a complete sex reversal, there were significant structural changes, such as tissue degeneration, somatic cell proliferation and induction of aromatase, with increased serum levels of estradiol-17β and 11-ketotestosterone.

CONCLUSION: Altogether, these results suggest that Foxl2 plays a decisive role in the ovarian differentiation of the Nile tilapia by regulating aromatase expression and possibly the entire steroidogenic pathway.

O 20

The mechanism of transcriptional regulation of P450 aromatase gene by Foxl2 in Japanese flounder (*Paralichthys olivaceus*)

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BACKGROUND: Japanese flounder (*Paralichthys olivaceus*) is a teleost fish that has a XX(female)/ XY(male) sex determination system. The XX larvae can be sex-reversed to phenotypic males by rearing at high water temperature during the sex differentiation period. However, the mechanism of the sex-reversal by high water temperature remains to be clarified.
Cytochrome P450 aromatase (P450arom) is the steroidogenic enzyme responsible for the conversion of androgen to estrogen. Previously, we demonstrated that rearing genetically female flounder larvae at high water temperature caused suppression of *P450arom* gene expression in the gonad and resulted in sex-reversed males. Moreover, treatment of the XX larvae with an aromatase inhibitor (fadrozole) induced sex-reversal as well as suppression of *P450arom* gene expression. These results indicate that P450arom plays a critical role in gonadal sex differentiation in the flounder.
As a first step to analyze the regulation mechanism of P450arom in the flounder, we investigated whether the *P450arom* gene expression is regulated by Foxl2, which is a member of the winged helix/forkhead family of transcription factors and is known to be involved in ovarian development in some vertebrates.

METHODS: We first isolated *Foxl2* cDNA from the ovary and examined the expression pattern in the gonads during the sex differentiation. Next, we investigated by electrophoretic mobility shift assay whether the flounder Foxl2 binds to the forkhead binding sites in the putative promoter of the flounder P450arom gene. Moreover, we constructed the plasmid (P450arom-luc) which linked the promoter of the flounder *P450arom* gene to luciferase reporter gene and that which expressed the flounder Foxl2 (CMV-fFoxl2), and both plasmids were transiently transfected into HEK293 cells simultaneously.

RESULT: *Foxl2* mRNA was expressed highly in the female gonads but not in the male gonads during the sex differentiation. Electrophoretic mobility shift assay presented two potential Foxl2 binding sites in the *P450arom* gene promoter. Moreover, luciferase activity was significantly induced by co-transfection of the CMV-fFoxl2 plasmid with P450arom-luc plasmid.

CONCLUSION: *Foxl2* mRNA was expressed highly in the female gonads during the sex differentiation, similarly to expression pattern of *P450arom* mRNA. Foxl2 induced the P450arom transcriptional activity in HEK293 cells. These results suggest that Foxl2 regulates the expression of *P450arom* gene during the sex differentiation in the flounder.
We showed that the flounder Foxl2 mRNA especially expression in female gonads, similarly to expression pattern of P450arom mRNA during sex differentiation. In addition luciferase assay and binding assay suggested possibility that P450arom gene is induced by flounder Foxl2.These results suggest that the possibility of P450arom gene regulated by Foxl2.

O 21

Sox9a, _Sox9b_ and _Amh_ are up-regulated in the gonads during natural and temperature-induced tilapia male differentiation

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BACKGROUND: *Sox9* gene is an early sex determining gene in mammals and possibly the direct target of the major sex-determining gene *Sry*. *Sox9* gene has been identified in some teleosts due to evolutionary conservation. *Amh*, the anti-Müllerian hormone gene found in higher vertebrates has also been identified in some teleosts although its function is unclear. Expression of *Sox9* and *Amh* have not been analysed yet in the tilapia *Oreochromis niloticus,* a fish in which sex is determined by the interaction between genetic factors (XX/XY + minor factors), and high temperature (masculinising effect).

METHODS: Eight genetic all-female (XX) and eight all-male (XY) progenies were produced. XX fry were reared at both 27°C and 36°C, and XY fry were reared only at 27°C. Trunk with gonads and heads were sampled during sex differentiation from 11 to 26 days post-fertilization (DPF). Cloning of *Sox9* and *Amh* genes was performed using degenerated primers. Gene expression profiles were analysed after sequencing, by real-time PCR and *in situ* hybridization.

RESULTS: *Amh* and two *Sox9* genes have been partially cloned in *O. niloticus*. Amino acid homology was 63% between *Sox9a* and *Sox9b*. Phylogenetic analysis suggests that the two genes have been duplicated through an ancient fish-specific genome duplication. *Sox9a, Sox9b* and *Amh* are already expressed in the gonads of males at 11 DPF, whilst levels were much lower in females. Expression of *Sox9a* was always higher than that of *Sox9b*, and together with *Amh* increased in males during the sex differentiation period. *Sox9b* was strongly expressed in the temperature-treated females already at 12 DPF and increased thereafter, whereas expression of *Sox9a* only increased in masculinised females after 18 DPF. All 3 genes were expressed highly in male heads at early stages (12 DPF) of sex differentiation, with significantly elevated levels of *Amh*. Levels for *Sox9a* and *Sox9b* rose in masculinised female heads at around 26 DPF.

CONCLUSION: Our data suggests that *Sox9a*, *Sox9b* and *Amh* are important genes in tilapia testis differentiation, with early expression of *Sox9b* occurring in the temperature-dependent masculinisation process.

O 22

Role of apoptosis in temperature-dependent sex determination of pejerrey *Odontesthes bonariensis*

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BACKGROUND: The pejerrey *Odontesthes bonariensis* is a "differentiated" gonochorist that shows a striking degree of temperature-dependent sex determination (TSD). The environmental (water) temperature experienced by the fish between 1 and 7 weeks post-hatching (or 11 to 18 mm body length, depending on the temperature) modulates gonadal sex differentiation and sex ratios decrease gradually from 100% female at 19°C to 100% male at 29°C. This study investigated the possibility that apoptosis (programmed cell death) plays a role in TSD of pejerrey. Our interest derives from the fact that apoptosis has been shown to be involved in various developmental processes and tissue differentiation from insects to vertebrates.

METHODS: Two experiments were conducted in which larvae were reared at feminizing (17°C), masculinizing (29°C), and intermediate temperatures (24 and 25°C in the first and second experiments, respectively) between hatching and 10 weeks. Samples were taken weekly for histological analysis of the timing of sex differentiation and the detection of apoptosis in gonadal cells by the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) method.

RESULTS: The results of the two experiments coincided in that the number of individuals with gonadal apoptosis and the intensity of TUNEL labeling per individual increased with rearing temperature. Thus, there were only a few individuals with sparse apoptotic cells at the feminizing temperature whereas at the masculinizing temperature all individuals had intense TUNEL labeling. Individuals at the intermediate temperatures had either no or intense gonadal apoptosis. Of great importance is that the appearance of TUNEL-positive cells preceded histological differentiation of the testes by 1 and 4 weeks at the masculinizing and intermediate temperatures, respectively, and that only males showed intense apoptosis after sex differentiation. Other interesting findings were that apoptosis was largely restricted to the right gonad of each individual and that TUNEL-positive cells were found first in the anterior portion of the gonad.

CONCLUSION: These results strongly suggest that apoptosis is crucial for testicular formation during TSD in pejerrey. Combined to the information available for this species, the results also provide evidence that a) the female is the default sex in pejerrey and b) that inactivation of putative female-inducing factors (?) located in the anterior portion of the right gonad during the critical time of TSD is necessary for testicular differentiation.

O 23

17-alpha-methyltestosterone exerts its masculinizing activity by acting as an androgen rather than as an aromatase inhibitor

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BACKGROUND: For years, 17-alpha-methyltestosterone (MT) has been used as a masculinizing agent in producing all-male fish populations. The efficiency of the treatment is undoubted, but its biological mechanism remains unclear. The masculinizing capability of MT may arise from its activity as an androgen or from its inhibition of aromatase (CYP19).

METHODS: Aromatase inhibition was tested in vitro by incubating ovarian microsomes, in the tritiated water assay. Androgenic activity of MT, MT + Flutamide, and Fadrozole was tested by feeding the substances to *Schianochromis fryeri* fry and testing for the appearance of blue color. Masculinisation trials were carried out on genetically female (XX) tilapia (*Oreochromis niloticus*) fry. The fry were fed diets containing either MT; MT + Flutamide; Fadrozole or a control diet, for 28 days. Aromatase expression was determined by real-time PCR, with 18S used as a reference gene. Estradiol was extracted from homogenized whole fish, and its levels in the extract were determined by competitive ELISA. Juvenile (60-day-old) fish were sexed by examining their gonads under a microscope after prestaining with aceto-carmine.

RESULTS: When applied in vitro to ovarian microsomes, MT clearly inhibited aromatase activity, though, in the same system, inhibition by Fadrozole, a specific non-steroidal aromatase inhibitor, was 240 times stronger. Administration of MT in food resulted in development of bright blue color in *S. fryeri* fry after 14 days. Flutamide, administered concomitantly with MT, decreased the appearance of the blue color in a dose-dependant manner. Fadrozole or Flutamide alone produced no coloration; the fry retained the same grey color as the controls. In all treatments, higher aromatase expression than in the control was found during the first 2 weeks. In the third week the trend was reversed: all treatments showed less aromatase expression than the controls. After 3 weeks of feeding, the treated fry showed significantly lower levels of estradiol than the controls in whole-body homogenates. Fish in the control group were all females; both the MT and Fadrozole treatments resulted in complete masculinization (100% males), whereas fish treated with both MT and Flutamide had a sex ratio of 33% females and 67% males.

CONCLUSION: The present study showed that MT acts both as an androgen and as an aromatase inhibitor. When MT androgen activity is blocked, its masculinization activities are decreased, implying that the androgenous activity of MT is crucial for its activity as a masculinizing agent.

O 24

Ovary to testis trans-differentiation by an anti-aromatase treatment induces a male specific testicular pattern of gene expression in rainbow trout

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BACKGROUND: Changes in the gonadal steroid environment during sex differentiation is known to affect the resulting gonad phenotype in fish. However, masculinizing treatments using androgens have been shown to induce a gene expression pattern quite different from the one observed during natural testicular differentiation. In order to understand whether this is the result of this masculinization process *per se*, or only a side effect specific to the androgen treatment, we investigated the effects of an aromatase inhibitor, another effective masculinizing treatment, on gonadal gene expression in rainbow trout.

METHODS: All female rainbow trout populations were masculinized using previously known effective treatment conditions either with the anti-aromatase, 1, 4, 6-androstatriene-3, 17-dione (ATD, 50 mg/kg of food for 3 months), or the androgen, 11ß-hydroxyandrostenedione (11And, 10 mg/kg of food for 3 months). Gene expression profiles (qPCR, quantitative RT-PCR and *in situ* hybridization) of 46 genes, previously reported to be involved in gonad differentiation, were compared between control males and females and females treated with ATD or 11And during the course of the treatments.

RESULTS: Both ATD and 11And treatments resulted in a quick and complete inhibition of *cyp19a1a* (*aromatase*), along with other female differentiation genes such as *fst* (*follistatin*), *foxl2a* and *foxl2b*. Most of the testicular differentiation marker genes including *sox9a2*, *amh*, *lhx9*, *pax2, alk4, con43* and genes involved androgens synthesis like *cyp11b2.1, hsd3b1*, *cyp11a1*, *cyp17a*, *star*, *nr5a1* (*sf1*) were strongly inhibited in fish treated with 11And. In contrast, the ATD treatment was totally able to restore this male specific gene expression pattern. However, some genes including *nr0b1* (*dax1), dmrt1*, *pdgfra*, *pdgfrb* that are also known to be important for testicular differentiation, were strongly up-regulated by both 11And and ATD treatments.

CONCLUSION: Our results clearly demonstrate that in contrast with androgens, masculinization with ATD produce a gene expression pattern closely related to the one observed during natural testicular differentiation. Masculinization processes induced with either ATD or 11And share in common the quick inhibition of female differentiation, suggesting that this inhibition of the female pathway is the major required step required for an effective masculinization. In this context, the inhibition of estrogen synthesis observed in both treatments is probably the sufficient condition for inducing an ovary to testis trans-differentiation.

O 25

Do airborne contaminants cause endocrine disruption of salmonids in "pristine" Western U.S. national parks?

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BACKGROUND: Airborne contaminants have been detected in snow, lake sediment, water, and fish in alpine aquatic ecosystems. Therefore a multidisciplinary study was initiated to assess the levels and potential effects of contaminants in alpine aquatic ecosystems of Western US national parks.

METHODS: Fish, primarily salmonids, were collected from "pristine" lakes of 8 national parks ranging from north of the Artic Circle to southern California and form the Pacific Coast in the west to the rocky Mountains in the east. Other sampling matrices included, snow, lake water and sediment, lichens and willow bark, moose meat, and fish, all of which are analyzed for contaminants. We assessed endocrine disruption by quantifying circulating vitellogenin (Vg) and sex steroids and examined gonads histologically. Other physiological factors were assessed to indicate the general health of the fish.

RESULTS: Some male trout collected from parks in the Rocky Mountains exhibited significantly elevated Vg. Some trout appeared to be intersex males and others appeared to have abnormal gonadal tissue, perhaps undeveloped testes containing some oogonia or oocytes. Fish from other regions appeared normal. The frequency of gonadal anomalies in populations where this condition was extant appears higher than in old (some over 100 years) museum specimens examined for comparison. Affected specimens contained various contaminants known to be endocrine disrupting. We also found the presence of splenic and nephric pigmented macrophages in fish from several regions. The abundance of these correlated with body mercury content.

CONCLUSION: It is likely that airborne contaminants are having adverse effects in fishes in waters at locations held to be very pristine. Historic collections of salmonids reveal that the frequency of abnormal gonadal development salmonids is higher than formerly assumed.

O 26

Temporal and spatial expression of the sex-related genes during sex differentiation and sex change in the protandrous black porgy fish, *Acanthopagrus schlegeli*

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BACKGROUND: Protandrous black porgy are functional males for the first two years of life but begin to change to female during the third year. Gonadal sex differentiation occurs at the age of 4-5 months. The molecular mechanism of sex differentiation and sex change is of interesting and becomes the objectives by using black porgy as the model animal.

METHODS: Black porgy from 3-months to 3-year-old period were collected to study sex differentiation and natural sex change. Long-term E2-administration was applied for the controlled sex change. Testicular tissue was further removed in 1^{+}-year-old fish to investigate the interaction between two tissues and the effects in ovarian development. RNA interference (RNAi) technique was also applied to investigate the direct role of the gene function. Sex-related gene expression was measured by RT-PCR and real-time quantitative PCR.

RESULTS: Sf-1, Sox-9, Dmrt 1, Dax-1 and Amh transcripts in the gonad were remained low at 4 months age and significantly increased in 5-6months-old differentiated fish. In the E2-induced sex change to a female in the juvenile fish, Dmrt 1, Dax-1, Sf-1 Sox-9 and Amh transcripts were decreased but Wnt-4 and Foxl2 were increased. Dax-1 was increased after3-months E2-treatment. During the reversible sex change after E2-withdrawal, Dmrt-1 and Sf-1 in gonad had a sexually dimorphic expression with much higher in male than E-induced female; but there was no sex difference in Wnt-4, Dax-1, Sox-9 and Amh. Low Sf-1/Cyp19a with high Dax-1 transcripts in the bisexual ovary, and high Sf-1/Cyp19a with low Dax-1 transcripts in the vitellogenic oocytes were detected. Bisexual ovarian tissue developed to a vitellogenic ovary after removal of bisexual testis. Significant decrease of Dmrt 1 in testis by RNAi technique was associated with the regression of bisexual testis. Vitellogenic oocytes were developed in these Dmrt 1 RNAi-induced females.

CONCLUSION: We demonstrated the time frame in the expression of important genes during sex differentiation and gonadal development in a protandrous fish. Dmrt 1 may play critical roles in the testicular development. Bisexual testis implanted inhibitory effects on the development of ovarian tissue. High Sf-1 and low Dax-1 favored to the development in the vitellogenic ovary.

O 27

Quantification of FSH- and LH-receptor messenger RNA in gobiid fish (*Trimma okinawae*) during serial sex change

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BACKGROUND: Despite numerous endocrine studies on sex change in teleosts, no general mechanisms mediating sex change have emerged. The gobiid fish, *Trimma okinawae*, possesses ovarian and testicular tissues simultaneously in its gonad. Furthermore, *T. okinawae* is able to change sex repeatedly in both directions depending on its social surroundings. A dominant male controls a harem of several females. Removal of this dominant male results in sex change of the largest female in the harem. If placed with a larger male, the once dominant male reverts to a female. Almost all sequential sex-changing fish either protogynous or protandrous reversibly change sex in one direction. As sex change in both directions can be socially manipulated, *T. okinawae* provides an excellent animal model to elucidate the mechanisms of male to female and female to male sex change. To obtain information of pituitary-gonadal axis in sex change, we cloned cDNA of the goby GtH receptors (GtH-R: FSH-R, LH-R) together with their expressions throughout sex change.

METHODS: Experimental fishes were collected from their natural habitat. Sex was determined by genital papillae. We produced transitional fish by manipulating social status according to established methods. Transcript abundance of the two GtH receptor genes in ovary and testis of the same individual was measured by real-time PCR.

RESULTS: Real-time PCR revealed that expression of both GtH-R was mainly restricted to the active gonad of the corresponding sexual phase. Ovaries, but not testis of female phase, contained relatively high levels of FSH-R and LH-R transcripts. Interestingly, GtH-R showed remarkable changes in terms of their location with the onset of sex change. In the dominant female, the levels of both FSH-R and LH-R transcripts were high, concomitant with the onset of female to male sex change, and peaked five days after manipulation, by the time all individuals were turned into functional males.

CONCLUSION: Remarkably, the reversibility of GtH-R localization is very rapid less than one day after social manipulation. GtH-R transcripts were significant decrease in ovaries of individuals stimulated to undergo sex change from female to male. These findings suggest the ability to switch on/off GtH-R expression in gonadal tissue plays a critical role in sex change of the goby. Moreover, the spatial expression of GtH-R with sex change provide insights into how gobies prevent self-fertilization and produce only one gamete type; despite they have both ovary and testis with the same individuals.

Gametogenesis and Gamete Biology

O 28

Spermatogenesis of Atlantic cod (*Gadus morhua*)

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BACKGROUND: With decreasing wild stocks, the production of farmed cod has become increasingly relevant. However, under farming conditions in particular male cod enter puberty precociously, leading to decreased flesh quality and growth performance. As part of a program aiming to understand and eventually control pubertal testis maturation, we investigated the first (pubertal) wave of spermatogenesis in Atlantic cod.

METHODS: Testis tissue from 1.5 year-old cod was collected monthly during one year and fixed in Bouin or glutaraldehyde. Slides with 4µm paraffin or 3µm plastic resin sections were used for histological analysis or cell counting. The gonado-somatic index (GSI) was calculated; spermatogonial proliferation was assessed by immunocytochemical detection of phosphorylated histone H3 (pH3); and the TUNEL technique was used for identification of apoptotic cells. Androgen plasma levels are quantified at present.

RESULTS: The paired cod testes are composed of several, similar lobes arranged around, and drained by, one common efferent duct per testis; the two ducts fuse caudally. Testis tissue is highly dynamic as indicated by the 41-fold change (0.2 – 8.2%) in GSI during an annual cycle. Testis growth starts in August with cyst formation, spermatogonial and Sertoli cell proliferation. New cysts are formed in the apical periphery of each lobe, which continues until December and increases testis size by appositional growth. The stationary cysts proceed through meiosis and spermiogenesis. In this way, a gradient of progressively mature germ cells is established in each lobe, in which cysts with more advanced germ cells are situated closer to the efferent duct. After the stop of cyst formation in January, spermiogenesis is completed in all tubules in a wave proceeding towards the lobes' periphery. Tubules filled with sperm are characteristic of the spawning season (February to May), after which residual spermatozoa are removed via phagocytosis by Sertoli cells (June/July) from the spent testes. Since ca. 1,900 pachytene spermatocytes (counted in 6-8 cysts per animal in 5 males) were present per cyst, an early spermatogonium completes at least 11 mitotic cell cycles (*n.b.* 2^{11}=2048) before differentiating into a spermatocyte. Overall, the incidence of apoptosis was low and occurred mainly during the last stages of spermatogonial proliferation.

CONCLUSION: During cod spermatogenesis, a marked gradient of development is established with undifferentiated spermatogonia in the periphery of testis lobes and the most advanced germ cells near the efferent duct, reflecting a tight spatio-temporal regulation of a testis lobe's histo-architechture. With 11 mitotic cell cycles and a low incidence of apoptosis, cod spermatogenesis is considerably more effective than in many mammalian species, but typical for other teleosts (e.g. guppy, tilapia, zebrafish). From a regulatory point of view, it will be interesting to study entrance in, as well as exit from, the period of spermatogenic cyst formation in the apical "germinative zone" of the cod testis lobes.

O 29

Transcriptional analysis of spermatogenesis regulation in trout

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BACKGROUND: Understanding molecular and cellular mechanisms involved in hormonal control of gamete proliferation and maturation is essential for the development of new methods of induced breeding or of managed sterility of broodstocks. Our initial data support the view that the transition from prepubertal to maturing testis results from regulated changes in the expression of large numbers of genes (Mazurais et al, 2005). Here we summarize different global analyses of gene expression patterns obtained during spermatogenetic development, and under the influence of different sex steroid exogenous treatments.

METHODS: We used a 9K trout cDNA microarray from the AGENAE project to analyze gene expression in gonads selected by histological examination in stages I/immature to VIII/spermiation, and also in isolated germ cell populations enriched in spermatogonia, spematocytes or haploid cells. In other experiments, males in stage I/II were submitted to sex steroid supplementation (androgens, estradiol, 20betaDHP) by hormone implants(5 to 15 samples per treatment). The expression values were submitted to statistical tests to detect differential expression and/or hierarchical classification in order to group genes or samples on the basis of correlations in gene expression changes.

RESULTS: The classification of 8000 expression values, in 50 samples, nicely separated testis samples in relation to their stage of differentiation. Genes could be classified in at least 5 main clusters that reflect cellular processes implicated in specific steps of testis development, for example: extra-cellular matrix genes changing at the time of tubular organisation; cell cycle and mitotic spindle regulation genes increasing in relation to rapid mitosis stage; transcripts for meïotic and post-meïotic proteins concurring with intensive appearance of spermatocytes. *In vivo* supplementation with sex steroids induced changes in numerous gonadal transcripts (1% to 10 % of the studied genes) including up or down regulation of genes known to be involved in the regulation of steroidogenesis, cell proliferation and germ cell differentiation. Many of the transcripts whose relative abundance changed after sex-steroid treatment displayed a striking pattern of expression over the gonadal cycle. For example, in testosterone regulated genes, 3 groups could be distinguished that were respectively up regulated -in prepubertal stage I, -at the beginning of rapid spermatogonial proliferation, -in meiotique/post meiotique stages. Many of these genes also displayed a relative high expression in fully mature testis (Somatic cells and spermatozoa only).

CONCLUSION: Associated with the in situ localisation of some of the genes and with the search for steroid responsive elements in gene promoters, these studies progressively allow for a comprehensive description of gene regulations involved in the key steps of spermatogenesis.

O 30

Cytokine and cell adhesion molecule expression pattern in the gilthead seabream (Sparus aurata L.) testis

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BACKGROUND: The reproductive cycle of the gilthead seabream, a protandric hermaphroditic seasonal breeding teleost, can be divided to four stages: spermatogenesis, spawning, post-spawning and resting or testicular involution during the first and the second reproductive cycle, respectively. Leukocytes, including acidophilic granulocytes (professional phagocytic granulocytes), macrophages and lymphocytes are present in the gonad of the gilthead seabream. Acidophilic granulocytes are recruited to the testis in response to a physiological need at post-spawning and during testicular involution before sex change and display functional adaptation to the testicular microenvironment. Thus, they show impaired respiratory burst and phagocytic activities, but produce IL-1beta, a pro-inflammatory cytokine.

METHODS: Total RNA was extracted from gonad fragments (n=4-5 gonads/month) during the first two reproductive cycles and treated with DNase I. The SuperScript III RNase H- Reverse Transcriptase (Invitrogen) was used to synthesize first strand cDNA with oligo-dT18 primer from 1µg of total RNA, at 50ºC for 60 min. Real-time PCR was performed with an ABI PRISM 7700 instrument (Applied Biosystems) using SYBR Green PCR Core Reagents (Applied Biosystems). For each mRNA, gene expression was corrected by the S18 ribosomal RNA content in each sample, and in all cases, each PCR was performed with triplicate samples.

RESULTS: We first analysed a group of molecules related with leukocyte infiltration: E-selectin, a cell adhesion molecule expressed by endothelial cells, and the CCL4-like chemokine. E-selectin and CCL4-like transcript levels increased from spermatogenesis to post-spawning in both reproductive cycles. Moreover, CCL4-like mRNA also increased at resting stage. The pro-inflammatory cytokines IL-1beta and TNF-alpha were also expressed in the gonad. While the highest levels of TNF-alpha mRNA levels were observed at spawning and the beginning of post-spawning, the IL-1beta mRNA levels mainly peaked at the end of the first reproductive cycle, during resting, and in the second reproductive cycle at the end of spermatogenesis. Finally, the mRNA levels of the anti-inflammatory cytokine TGF-beta1 reached their highest levels during post-spawning in the second reproductive cycle.

CONCLUSION: Our data demonstrated a particular expression pattern of anti- and pro-inflammatory cytokines throughout the two first reproductive cycles of the gilthead seabream, suggesting a role for these molecules in spermatogenesis. We hypothesize that the tightly regulated expression of chemokines and adhesion molecules might determine the infiltration of different types of leukocytes during the different stages of the reproductive cycles.

O 31

Roles of extracellular Ca^{2+} and pH on motility and flagellar waveform parameters in sturgeon spermatozoa

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BACKGROUND: Studies of sturgeon sperm have suggested a key role for Ca^{2+} in activation of motility. External concentrations of Na^{+} or K^{+} also regulate the activity of axoneme through their internal level in sturgeon spermatozoa. How these factors eventually affect the flagellar waveforms is not known. The present work provides new information showing that pH controls the Ca^{2+} induced asymmetry of flagella in sturgeon sperm.

METHODS: The experiments were carried out on sperm of sterlet sturgeon, *Acipenser ruthenus***.** The roles of Ca^{2+} ($CaCl_2$ from 0.1 to 20 mM) and pH (7.0, 8.0, 9.5 and 10.4 in 30 mM Tris-HCl) upon sperm swimming pattern were tested. Distilled water with 0.1 % of BSA was used as control. For testing the sperm swimming pattern, 1.0 µl of sperm was directly mixed within a 49 µl of distilled water or swimming medium. Sperm motility was recorded right after activation using a 3 CCD video camera mounted on a dark-field microscope under stroboscopic light. Sperm motility (velocity and percentage of motile cells) and flagellar waveform (number of waves, wave length and wave amplitude) parameters were analyzed on successive video frames by a micro image analyzer.

RESULTS: In distilled water, the percentage of motile cells was 80-100 % with velocity of 170 µl s^{-1} at 15 sec post sperm activation. All sperm flagella produced symmetric bending waves with 2 to 3 waves of large amplitude traveling the whole way along the flagellum. In absence of Ca^{2+}, no change was observed in terms of symmetric pattern of waves when pH was increased from 7.0 to 10.4, but differences were significantly observed in terms of sperm velocity, wave length and amplitude. Interestingly, Ca^{2+} in the 0.35 to 20 mM range at high pH (9.5 and 10.4) induced the assymetry of the flagellar waveforms. Increase of Ca^{2+} higher than 2.5 to 5.0 mM decreased significantly sperm velocity and percentage of motile sperm after 30 sec post activation. Significant differences were observed in terms of sperm velocity, wave length and amplitude at different Ca^{2+} concentrations and pH. In pH and Ca^{2+} close to that of seminal plasma, sperm cells had a lower proportion of damage such as blebs and curling of the flagellum.

CONCLUSION: The present study suggests Ca^{2+} regulatory effects on swimming patterns of sturgeon spermatoza, may be throughout interaction of Ca^{2+} with an axonemal Ca^{2+}- dependent regulatory protein. Further study of Ca^{2+} effects on the axoneme are required for better understanding of the mechanism by which Ca^{2+} induces swimming pattern changes in sturgeon sperm flagella.

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O 32

Application of fish sperm cryopreservation to selective breeding in France: from laboratory to commercial fish farms

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BACKGROUND: Technical feasibility of sperm cryopreservation is published for most farmed species today. Nevertheless, only a very limited number of applications by the industry are observed. As French fish farmers have initiated selective breeding programs at the beginning of the nineties with the counselling of SYSAAF for genetics, an important project was set up on cryopreservation in collaboration with INRA and IMV Technologies. The objective was to develop extenders, protocols, adapted devices, know-how and procedures to make this technology affordable to the fish breeding companies. This presentation illustrates the successive steps followed since 1994.

METHODS: Based on INRA expertise in cryobiology and gamete biology, multispecies extenders were developed and tested in fish farms before they were launched for commercialisation by IMV Technologies. These extenders include StorFish™ to collect and conserve sperm, OvaFish™ to wash eggs from coelomic fluid, faeces and blood, CryoFish™ to cryopreserve sperm, and ActiFish™ to maximise sperm motility and increase egg fertilisation rate. Then, a complete on-farm procedure was set up and standardized to print straws with a unique reference, to collect sperm from ejaculates or from testicular extracts, to fill and close 0.25 or 0.5 ml TBS™ straws with the automated machine MRS1™ (4000 straws/h), to freeze sperm in a specific freezer that allow a fast and repeatable freezing process, to manipulate straws in an adapted container before straws storage in LN_2.

RESULTS: Maintenance of extender performances from laboratory to farm scale was observed provided that the standardized processes were respected. Appealed by the reliable fertilization rates obtained, fish breeders have invested in sperm cryopreservation for 3 mains reasons: to secure their breeding programs from potential technical, genetic or pathological problems, to optimise mating schemes, and to keep genetic materials for a future evaluation of the genetic progress. In practise, assistance for on-farm cryopreservation of the broodstocks was provided by SYSAAF to fish breeders, together with parentage assignment with fingerprints to get genealogical pedigree. Today, 6 private cryobanks owned by fish breeders or INRA are running for rainbow trout, brown trout, sea bass, sea bream and turbot. At the end of 2006, 50 080 straws from 1554 males from 26 lines or generations from the lines of these 5 species were produced. Transfer of the procedure to high security 0.3 to 0.5 ml CBS™ was also successfully operated.

CONCLUSION: The procedure described here allowed the transfer of sperm cryopreservation from the research domain to fish farm application. The high cost of maintenance of individual cryobanks and the high risk of accidental losses prompted the setting up of a cooperative cryobank whose development will associate aquaculture breeding companies, research organisation and the French National Cryobank. For sanitary reasons, the use of high security CBS™ straws was approved for the forthcoming patrimonial depository of aquatic species in the French National Cryobank.

O 33

Characteristics of membrane progestin receptors on fish gametes and evidence for their involvement in oocyte maturation and sperm motility

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BACKGROUND: Recently a novel cDNA was discovered in spotted seatrout ovaries encoding a protein that has the characteristics of the membrane progestin receptor (mPR) mediating maturation-inducing steroid (MIS) induction of oocyte maturation in this species. Preliminary in vitro studies showing the MIS rapidly stimulates the motility of Atlantic croaker sperm suggests an mPR may also be involved in the maturation of male gametes. Additional characterization of mPR on fish gametes is required, however, to confirm their involvement in oocyte maturation and sperm motility

METHODS: Plasma membrane localization, steroid binding characteristics, G protein coupling and activation of second messenger pathways were investigated with wildtype mPRs in oocytes and sperm and recombinant mPR in transfected breast cancer cells using established immunodetection, radioreceptor and signal transduction protocols, respectively. The effects of pharmacological modulation of these signal transduction pathways on MIS induction of oocyte maturation in seatrout and sperm motility in croaker were investigated using in vitro assays.

RESULTS: Immunoblotting with mPR antibodies showed bands of the appropriate sizes on Western blots for mPR dimers (80kDa) and monomers (40kDa) in plasma membrane preparations from goldfish and croaker oocytes, seatrout, croaker and Southern flounder sperm, and cells transfected with seatrout mPR. The mPR was localized to the midpiece on whole croaker and seatrout sperm preparations, consistent with it having a role in sperm motility in these species. Membrane preparations from all these tissues and species demonstrated specific progestin binding typical of steroid membrane receptors. Treatment with progestins resulted in G protein activation and alterations of adenylyl cyclase activity in seatrout oocytes, sperm and cells transfected with the receptor. Moreover, these changes in intracellular signaling were associated with oocyte maturation and increased sperm motility. Finally, pharmacological blockage of these signal transduction pathways attenuated the stimulatory effects of progestins on oocyte maturation and sperm motility.

CONCLUSION: The results are consistent with physiological roles of mPR in both oocyte maturation and increased sperm motility in several teleost species. Oocyte, sperm and recombinant mPR membrane proteins all have the characteristics of function progestin membrane receptors. Our proposed model of progestin action on gametes involves activation of mPRs which are coupled to G proteins. Activation of inhibitory G proteins in oocytes causes in inhibition of adenylyl cyclase activity and G protein activation in sperm stimulates adenylyl cyclase activity, resulting in oocyte maturation and sperm motility, respectively.

O 34

Progestin is an essential factor for the initiation of the meiosis in spermatogenesis and early oogenesis in fish

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BACKGROUND: Meiosis is an indispensable process of sexual reproduction. However, detailed information on the regulatory mechanisms that initiate meiosis is not available. Progestins are important steroids regulating final maturation in male and female vertebrates. In male teleosts, it is known that progestin induces spermiation and sperm maturation. However, a role for progestin in early spermatogenesis or meiosis has not yet been described.

METHODS: In this study, we examined the functions of progestin on the initiation of meiosis in male Japanese eel, female Japanese huchen and common carp. Using several *in vitro* gonadal culture systems in male and female, we have analyzed the role of 17a, 20b-dihydroxy-4- pregnen-3- one (DHP), a natural progestin in teleost fish, in controlling spermatogenesis and early oogenesis.

RESULTS: DHP and its receptors were present in the testis at an early stage of spermatogenesis. Using eel testicular culture systems, DHP was shown to induce DNA replication in spermatogonia. Although 11-ketotestosterone, a known initiator of spermatogenesis, also stimulated DNA synthesis in spermatogonia, antibodies against DHP prevented DNA replication when added during the period in which meiosis was initiated. DHP treatment also induced the expression of meiosis specific markers, such as DmcI and Spo11. Furthermore, Spo11 expression and synaptonemal complexes (SC), specific features of the meiotic prophase, were detected in testicular fragments cultured with DHP in some germ cells that showed morphological characteristics of undifferentiated spermatogonia.
In huchen and carp ovarian organ culture, DHP induced DNA synthesis of oogonia. In addition, the expression of Spo11 was observed histochemically and SC were ultra-microscopically detected in some germ cells. Therefore, DHP is also implicated in the regulation of early oogenesis from oogonial proliferation to initiation of the first meiotic division.

CONCLUSION: These data suggest that DHP, a progestin, is an essential factor for the initiation of meiosis in both spermatogenesis and oogenesis.

O 35

Functional specificity of gonadotropin receptors in rainbow trout (Oncorhynchus mykiss) assayed *in vitro* using heterologous mammalian cells

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BACKGROUND: The hormonal specificity of fish gonadotropin receptors has been studied in different species but marked differences have been reported, depending on the fish order or the gonadotropins used. To better understand the distinct role and the specificity of gonadotropin receptors in salmonids, functional characterization of trout gonadotropin receptors (rtFSHR and rtLHR) and their gonadal gene expression pattern have been studied.

METHODS: Trout gonadotropin receptors were transiently expressed in COS cells and exposed to increasing amounts of highly purified homologous and heterologous (chinook salmon) gonadotropins. Receptor activation was detected using a cAMP responsive luciferase reporter gene. Gene expression of gonadotropin receptors was determined by quantitative real-time PCR during the first male and female reproductive cycle.

RESULTS: The molecular cloning of rtFSHR showed similar but also different structural determinants in the ligand binding domain, compared to other vertebrate FSHR, including fish and mammals. Regarding rtFSHR, a significant ($p<0.05$) maximal 2.5 fold induction of the reporter gene expression was seen in presence of 100 ng/ml onwards. No reporter gene induction was observed in presence of rtLH. Similar results were obtained using chinook gonadotropins (cFSH and cLH). Regarding rtLHR, a significant ($p<0.05$) 8.9 fold induction was obtained at 1000 ng/ml, and the EC_{50} was 117 ng/ml. A high dose of rtFSH (1000 ng/ml) was also able to induce a significant ($p<0.05$) and reproducible two fold induction of the reporter gene expression. cLH induction resulted in a similar dose-dependent responsiveness to that obtained in presence of rtLH (EC_{50} = 98 ng/ml). Unexpectedly, contrary to rtFSH, cFSH had no significant effect on rtLHR transactivation at the tested doses. In female, rtFSHR gene expression decreased at the end of vitellogenesis but peaked at maturation and ovulation. rtLHR transcript relative abundance progressively increased over the reproductive cycle to reach its maximum at ovulation. In male, rtFSHR and rtLHR transcripts relative abundance was similar with a high increase in spermiating animals.

CONCLUSION: Our data demonstrate that trout gonadotropin receptors have different ligand selectivity *in vitro*: rtFSHR is only activated by FSH whereas rtLHR is activated by both gonadotropins. However, the high rtFSH doses required to activate rtLHR questions the physiological relevance. The high rtFSHR gene expression in spawning animal suggests that FSH may play an important role in regulating gonadal functions, not only at early stages but also at final stages of male and female reproductive cycles, in addition to the LH pathway.

O 36

Characterization of sea bass gonadotropin receptors: seasonal expression and specific activation

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BACKGROUNG: The follicle-stimulating hormone (FSH) and the luteinizing hormone (LH) play central roles in vertebrate reproduction. These gonadotropins act through their cognate receptors to stimulate testicular and ovarian functions. Two cDNAs coding for the sea bass FSH and LH receptors were previously cloned (sbsFSHR and sbsLHR). Their characterization can help to understand the combined roles of the gonadotropins in the regulation of sea bass reproductive cycle.

METHODS: The genomic structure of the receptors was studied by PCR amplification. Receptor expression in ovary and testis during the reproductive cycle was analyzed by real-time PCR. Cellular localization of *sbsFSHR* mRNA was done by in situ hybridization. Recombinant sea bass single-chain gonadotropins were obtained from the culture medium of specific CHO stable clones. FSH and LH receptor activation were measured in HEK293 stable clones through a CRE-luciferase transactivation system.

RESULTS: Comparison of sea bass FSH and LH receptors was performed at different levels. *sbsLHR* is more conserved than *sbsFSHR* when comparing their mRNA and genomic primary structures with other gonadotropin receptors, particularly in the extracellular domain. Seasonal expression of these receptors in the gonads was analyzed together with other physiological parameters. In females, both receptors follow the same expression profile, although *sbsFSHR* expression level is on average three-fold higher than that of *sbsLHR*. In males, both receptors follow a bimodal profile and the maximum expression peak for *sbsFSHR* is advanced respect to the highest expression of *sbsLHR*. In situ hybridization showed that the *sbsFSHR* is expressed in previtellogenic and early vitelogenic oocytes. Ligand activation studies showed that each receptor responds exclusively to its specific sea bass hormone in a dose-response manner. However, sbsLHR could be activated by bovine LH and bovine FSH, while sbsFSHR was only activated by bovine FSH.

CONCLUSION: The described results aimed to understand the role of the gonadotropin receptors in male and female sea bass, under the light of the reproductive strategy of these species. Contrary to what has been observed in other fish gonadotropin receptors, sea bass FSH and LH receptors display specificity for their homologous hormones. The promiscuous activation of sbsLHR by mammalian hormones recommends caution in the biotechnological application of heterologous hormones, if these have not been previously evaluated.

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O 37

Photoperiod-induced accellearation or inhibition of ovarian maturation in Atlantic salmon: localisation and quantification of gonadotropin receptor expression

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BACKGROUND: Early sexual maturation is a major problem in salmon farming, and methods to delay puberty including photoperiod control have been developed. However, these protocols are not always fully effective, and there is limited knowledge on the activation of the brain-pituitary-gonad axis during puberty in salmon that are essential for further improvement of methods to fully control puberty. Thus, the current study investigates expression of some key genes during puberty under different photoperiod regimes in female Atlantic salmon.

METHODS: Previously immature, 3 years old salmon were maintained in sea cages under ambient light conditions (NL). An initial control sample was collected on January 8. Starting February 1, half of the animals were exposed to additional constant light (LL); the other half remained under NL conditions. Samples were collected February 18, March 19, April 25, and June 11, when the experiment was terminated as maturation was clearly visible in all females of the NL group, while the LL treatment group contained females with small and large ovaries. Real-time, quantitative (rtq)PCR systems were developed to analyse expression of the ovarian gonadotropin receptor (FSHR and LHR) mRNA levels. In situ hybridization of receptor expression was performed with digoxigenin-incorporated labelled cRNA probes.

RESULTS: The cellular site of expression of the both FSHR and LHR were the follicular granulosa cells, while the FSHR was also expressed in by the androgen steroid-producing cells in the theca layer. The expression of LHR was low in ovarian samples collected in January and in the animals sampled in June, in which LL-treatment resulted in an inhibition of ovarian development. A strong up-regulation of LHR expression was recorded in animals with oocytes in full vitellogenesis of both the NL and the LL treatment groups, however the latter showing the highest values recorded. Also with regard to FSHR expression, females in full vitellogenesis showed high levels but the differences were less pronounced between developmental stages than when considering LHR expression; the lowest levels of FSHR expression were recorded in the oil droplet stage ovaries from the start control group. In contrast to LHR, peak levels for FSHR expression were recorded in the vitellogenic females of the NL-treated group that were less far progressed through vitellogenesis than the LL-treated group. Analysis of pituitary gonadotropin subunit expression, and of samples collected from females on February 18, March 19, and April 25 is ongoing.

CONCLUSIONS: The data available now suggests that FSHR expression peaks first, followed by a peak of LHR expression in larger follicles and is well correlated with changes in ovarian weight, histology and circulating 17beta-oestradiol levels.

O 38

Arachidonic acid affects fecundity and spawning performance in female Atlantic cod (Gadus morhua l.)

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BACKGROUND: The capacity for Atlantic cod juvenile production has increased from a few 100 000 to about 100 mill. in Norway over the past 3-4 years. Broodstock management and nutrition is vital in order to ensure a stable production of viable offspring, and good practises here will promote general fish health, production efficiency, and the quality of the final product. Arachidonic acid (ARA) concentrations are higher in eggs of wild fish than in farmed fish. Further, supplemental ARA improved spawning performance and egg quality in Japanese flounder and Atlantic halibut. The present study was designed to investigate potential effects of ARA on the spawning performance of Atlantic cod.

METHODS: Two-year old Atlantic cod of both sexes (n=3200) were equally distributed into eight experimental sea cages after completion of their first spawning in May 2005. Four experimental groups were established and held in duplicate cages. The groups were fed diets with different levels of ARA corresponding to 0.5, 1, 2 and 4% of total fatty acid content. Monthly samples were taken for analysis of plasma hormones, ovarian morphology, ovarian ARA content and gene expression of key genes. In January 2006, 30 females and 18 males from each experimental group were transferred to covered, circular tanks (Ø 3m; volume 8m3) supplied with egg collectors connected both at the surface and bottom. Total and buoyant egg volumes, fertilisation rate and egg diameter were registered in all batches. Hatching rate and larval viability at d3 posthatch were registered weekly. Mortality in spawning females was also monitored.

RESULTS: Ovarian ARA levels were highly correlated to dietary ARA levels. The total as well as relative fecundity were highest in the group receiving 1% dietary ARA, and lowest at 4%. No significant differences were found in egg diameter, fertilisation rates or hatching rates. Female mortality during and after spawning showed marked differences between the experimental groups. Between 30% and 60% of the females in each group died during or shortly after spawning. In the groups given intermediate ARA levels, a high proportion of females died during spawning, apparently due to a failure to ovulate or release their eggs (irregular spawners). The groups given either low or high ARA had few irregular spawners, but a higher proportion of females that died after spawning were completed.

CONCLUSION: ARA affected spawning performance in Atlantic cod in the present study. The highest and lowest dietary ARA concentrations both appeared to give lower fecundity. However, mortality due to irregular spawning was higher in the groups given intermediate ARA levels, indicating that ARA may have had a negative impact. Plasma steroid analyses and ovarian histology are in progress.

O 39

Swimming increases gonadotropin sensitivity of European female silver eels as shown by q-PCR of the estradiol receptor and vitellogenin in the liver

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BACKGROUND: When European silver eels (*Anguilla anguilla*) disappear in the ocean for their 5,500-km semelparous spawning run to the Sargasso, they are still in a prepubertal stage. Further sexual development appears to be blocked by dopaminergic inhibition of hypothalamus and pituitary activity. We hypothesize that swim exercise is the crucial trigger for releasing this inhibition. Recently, we found that swimming stimulated the incorporation of lipid droplets in the oocytes. In this study, we investigated the effect of long-term group-wise swimming on vitellogenesis and sensitivity for gonadotropin stimulation. For this purpose we developed new molecular probes for the expression of 17β-estradiol receptor α (E2-Rα), vitellogenin 1 (VTG1) and 2 (VTG2) in the liver.

METHODS: Migratory female silver eels (n= 54; body-length= 75 ± 1 cm, body-weight= 749 ± 25 g, eye index= 14.3 ± 0.2) were anaesthetized, PIT-tagged and sampled for blood after which they were randomly divided over 9 groups of n= 6. One group was dissected at the start. An oval shaped swim-gutter (6.0*4.0*0.8 m) was constructed to allow simulated migration for 1.5 (907 km) and 3 months (1,420 km) in 6,000-l natural seawater at 18 ºC. Other eels rested during the experimental period. After swimming and resting for 1.5 and 3 months four groups were dissected. Four other groups were sampled and injected with 20-mg carp pituitary extract (CPE) for 3 weeks after which they were dissected. Oligos were designed from the reported sequences of *A. japonica* for VTG1, VTG2, 'house-keeping gene' β-actin and from *Salmo salar* and *Oncorhynchus mykiss* for E2-Rα. RNA was isolated from liver samples of CPE-matured eels for RT-PCR and for cloning of the genes in the pCRII-TOPO vector. This protocol was then applied to quantify gene expression in the livers of the experimental animals. Plasma estradiol (E2) was measured by ELISA and VTG was determined indirectly through blood calcium (paired samples).

RESULTS: Swimming eels showed a diminished expression in the liver of E2-Rα, VTG1 and VTG2. They also had lower E2 and VTG levels in the blood. However, when eels were subsequently stimulated by 3 weekly CPE injections, the expression of the same genes and plasma levels of E2 and VTG strongly increased. This increase appeared stronger in swimming eels in relation to the swim period.

CONCLUSION:
1. Continuous swimming inhibits hepatic vitellogenesis.
2. Sensitivity appears to increase in relation to the swim period.

O 40

Use of continuous light and triploidy to control sexual maturation in farmed Atlantic cod (*Gadus morhua*)

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BACKGROUND: Early maturation is considered one of the greatest problems in cod mariculture. Fish stop growing during spawning and food fed for somatic growth is lost through spawning resulting in elevated feed costs (low food conversion efficiency). We examined two approaches to impede sexual maturation: (i) continuous light and (ii) induction of sterility by triploidy. Induced triploidy as a means of producing sterile populations for aquaculture has been investigated extensively for Atlantic salmon and other salmonids, but not for any gadoids.

METHODS: Atlantic cod (*Gadus morhua*) stocked to sea cages in the Bay of Fundy were maintained either under continuous light or natural ambient photoperiod. Six 400 W submerged lights were established on a 70 m polar circle cage containing 1 yr-old cultivated cod. Lights were kept on year round from June 2005 to spring 2007. Gametes were stripped from several parents in 2004 and 2005 for the purpose of generating triploids. Zygotes were pressure shocked (5 min at 8500 psi) at specific intervals post-fertilization. Larvae from each cross/shocking were reared separately. Blood samples of young juveniles were analysed for red blood cell DNA content to ascertain ploidy. Diploids and triploids were reared in isolation and then subsequently pooled.

RESULTS: Continuous light delayed onset of sexual maturity by ~ 4-5 months. Females exposed to 24 h light retained the state of ripeness for two months rather than releasing eggs shortly after GSI values peaked as was observed in the control cage. Cod triploids, perhaps the first-ever generated worldwide, were produced using pressure shock. Preliminary trials in 2004 and 2005 showed that treatments beginning at 180 °C-min post-fertilization led to 100% triploid offspring. Triploid cod are performing well compared to diploids though greater numbers incorporating sea cage trials will be required to fully evaluate their potential for cultivation.

CONCLUSION: Continuous light delayed maturation but did not stop gametogenesis. Triploidy was successfully induced with treatments identical to those previously used for producing triploid halibut.

O 41

Induction of spermiation, ovulation and spawning in Atlantic bluefin tuna (*Thunnus thynnus*) using GnRHa delivery systems

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BACKGROUND: Fueled by the increasing demand for bluefin tuna for the sashimi-sushi market in Japan, Europe and the United States, in the last decade there has been a booming fattening industry developing around the Mediterranean coast. Tuna fattening is a "capture-based aquaculture", and involves the capture of wild fish and their culture in floating cages. The recent expansion of this industry is considered threatening to the wild population, and there is a great interest in developing broodstock management, spawning and larval rearing methods for bluefin tuna.

METHODS: Mature migrating bluefin tuna were obtained in June 2003 around the Balearic Islands, Spain using a purse seine and transported (n = 40) to two 25-m diameter cages near shore. In June 2004 and 2005, fish were implanted by a diver with gonadotropin releasing-hormone agonist (GnRHa)-implants and an individual visual tag. Some fish were used as non-treated controls. After 2, 3, 5, 6 or 8 days, all fish were sacrificed and their gonads removed for maturity evaluation.

RESULTS: Underwater GnRHa-implantation is a very time-consuming operation and was 84% successful. Plasma GnRHa was a significantly higher (ANOVA, $P < 0.05$) in GnRHa-implanted fish, demonstrating the ability of the implants to deliver the necessary amounts of GnRHa. Males implanted with GnRHa were in spermiating condition and all but one GnRHa implanted females had undergone ovulation, as indicated by the presence of post-ovulatory follicles. Fertilized eggs were collected from one of the cages 3 days after GnRHa treatment, and the hatched larvae were identified as bluefin tuna. Three days after treatment, ovulated eggs were also collected from two GnRHa-treated females and were fertilized *in vitro*, producing viable larvae.

CONCLUSION: This is the first report in any tuna species, on the hormonal induction of oocyte maturation, spermiation and spawning, and underlines a real possibility for controlling reproduction in captive bluefin tuna, as well as other tunas of interest. The results could spark worldwide efforts at inducing spawning in wild-caught, captive-reared bluefin tuna which are widely maintained in many parts of the world by the fattening industry.

O 42

Syngenic and xenogenic germ cells transplantation in the Nile tilapia (*Oreochromis niloticus*)

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BACKGROUND: Spermatogonial transplantation, developed in 1994 by Brinster and colleagues, is a fascinating and powerful technique utilized to investigate reproductive biology, mainly the aspects related to spermatogenesis and the stem cell biology. This technique also offers great potential for studies involving biotechnology, transgenic animals, and the preservation of the genetic stock of valuable animals or endangered species. Although germ cell transplantation is relatively well characterized for mammals, there is no study utilizing this approach for fish or other lower vertebrate. Due to its fast growth, relative small size when it reaches sexual maturity, good adaptability to different environmental conditions and economical importance, the tilapia (*Oreochromis niloticus*) is an excellent experimental model. So, we have been investigating the viability of adult tilapias as a recipient model for syngenic (tilapia to tilapia) or xenogenic (frog to tilapia) germ cells transplantation.

METHODS: All approaches necessary for spermatogonial transplantation, such as endogenous spermatogenesis depletion, obtention, selection and labeling of spermatogonia, and the transplantation through the urogenital papilla, were standardized recently in our laboratory. The transplanted testes were evaluated by light, confocal, and fluorescence microscopies.

RESULTS: The syngenic transplants showed the presence of PKH26 labeled germ cells forming spermatogenic cysts with varied sizes and different stages of development, in tilapia seminiferous epithelium. At approximately 8 weeks post transplant, round and small PKH26 labeled cells, resembling released spermatozoa that were probably originated from transplanted spermatogonia stem cells, were observed in the tilapia seminiferous tubule lumen. Regarding xenogenic transplantation, ongoing studies in our laboratory are also showing that frog (*Rana catesbeiana*) germ cells are able to colonize and form spermatogenic cysts in the tilapia testes.

CONCLUSIONS: The results for syngenic transplants indicated that successfully transplanted spermatogonial stem cells were able to colonize, proliferate and differentiate in the tilapia testes. Therefore, this teleost might represent a good experimental model to investigate the germ cell biology and the testis function in fish. Moreover, this technique could be also utilized as a potential approach for fish bioengineering, preservation of genetic stock of endangered fish species or fish strains carrying commercially valuable traits. Regarding the xenogenic transplants, the preliminary results found suggest that tilapias might also be an appropriate potential model to investigate spermatogonial stem cell biology and spermatogenesis in lower vertebrates.

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O 43

Production of all donor-derived offspring from surrogate parents by interspecies transplantation of spermatogonia using sterile triploid recipient

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BACKGROUND: Recently, we reported the successful transplantation of germ cells between two different species in salmonid. Such technique of xenotransplantation of germ cells can be used to facilitate the seed production of commercially valuable species or endangered species. However, in the previous study, the xenogeneic recipients produced not only donor-derived but also recipient-derived gametes, leading a low production rate of donor-derived gametes and offspring. In this study, we examined the feasibility of using infertile triploid masu salmon as a recipient to increase the contribution efficiency of donor-derived gametes.

METHODS: Donor germ cells were prepared from transgenic male rainbow trout (*Oncorhynchus mykiss*) carrying a green fluorescent protein (*Gfp*) gene driven by the *vasa* gene regulatory regions. Approximately 15,000 testicular cells containing 6,000−10,000 spermatogonia were transplanted into the peritoneal cavity of newly hatched triploid masu salmon (*O. masou*).

RESULTS: Two years after the transplantation, milts were obtained from 10 out of 29 (34.5%) triploid male masu salmon recipients. In females, 5 out of 50 (10.0%) recipients ovulated. Whereas, control triploids without receiving germ cells of rainbow trout did not show any sign of spermiation or ovulation, suggesting the gametes obtained from the recipients were all donor-derived. To test this hypothesis, first, the milts from those 10 triploid masu salmon recipients were artificially inseminated with eggs from wild-type rainbow trout. As a result, the all-10 males yielded embryos all of which inherited the donor-derived haplotype, i.e., *Gfp*-labeled germ cells. Next, the eggs from the female recipients were artificially inseminated with the spermatozoa produced by the male recipient. In all experiments, hatchlings with donor-derived haplotype were successfully obtained. Randomly amplified polymorphic DNA analysis demonstrated that the DNA fingerprint of the all hatchlings (18 offspring examined) derived from the triploid masu salmon parents was identical to that of rainbow trout. In addition, restriction fragment length polymorphism analysis of mitochondrial DNA revealed that the mitochondria retained by the all hatchlings were derived from rainbow trout-eggs.

CONCLUSION: We successfully developed a technique to produce surrogate parent that produce only donor-derived gametes, which enables it to obtain all donor-derived offspring from xenogeneic recipient parents. Moreover, to our best knowledge, this is the first report of successful production of donor-derived functional eggs from xenogeneic recipients by germ cell transplantation in all vertebrates.

O 44

Transplantation and cryopreservation of blastoderm in salmonids

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BACKGROUND: Transplantation of dispersed blastomeres is a method of genetic transfer. Donor contribution to the resulting chimera is random and germ-line transmission is low, if any. Donor transmission rate can be dramatically improved when a blastoderm fragment is transplanted instead of separate blastomeres. We have developed a non-destructive technology for entire blastoderm manipulations in salmonids enabling blastoderm transplantation and cryopreservation, and production of high donor-rate chimeras.

METHODS: Entire blastoderms of rainbow, cutthroat and brown trout embryos at developmental stages from early to late blastula were accessed *in vivo* through cutting a large, closable window in chorion above the blastoderm of egg fixed to a Petri dish side with cyanoacrylate glue. A series of blastoderm transplantation experiments was performed in order to establish a technology for high donor-rate germ-line transmission. We performed also interspecific transplantations. We studied location-specific transplantation effects by transplanting specific parts of donor blastoderm into specific locations in recipients and we tracked migration and distribution of donor cells. We tested various protocols for cryopreservation of entire blastoderms. Some of produced chimeras were reared until their sexual maturity and then examined for the efficiency of germ-line transmission of donor genome.

RESULTS: Survival of chimeras depended on donor and recipient developmental stage (the highest around midblastula stage), blastoderm replacement or insertion rate (the lower rate, the higher survival) and the location of replacement or insertion in the host. Generally, survival of chimeras was low (0 – 25%, varying in different experiments). Cells from donor blastoderm fragment remained static throughout blastulation stage in upper and middle parts of chimeric blastoderm, whereas cells from the lower part of transplant intensively migrated along blastoderm margins. High donor-rate germ-line transmission was found in chimeras which reached sexual maturity. Interspecific transplantation of the entire blastoderm or blastoderm fragments resulted in incorporation and development of chimeras, however no normally developed embryos was obtained. The best protocol for cryopreservation of blastoderms resulted in over 80% survival of blastomeres.

CONCLUSION: The developed technology for *in vivo* manipulations of entire blastoderm in salmonid species, also including slowing down the development, attaching an egg to Petri dish and individual tracking of its development, has been tested in multiple experiments throughout several years in three laboratories. The technology enables production of high donor-rate germline chimeras, location-specific transplantation, cryopreservation of entire blastoderms, as well as other experimental approaches including elimination of chorion during incubation, injections, labeling, or blastomere sampling.

O 45

Intrafollicular EGF signaling network in the zebrafish ovary and its control

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BACKGROUND: In vertebrates, the pituitary gonadotropins - follicle-stimulating hormone (FSH) and luteinizing hormone (LH) - play vital roles in controlling ovarian development and function. Their activities in the ovary are mediated or modulated by a variety of locally produced peptide or protein factors that form an intimate regulatory network within and between the ovarian follicles. In the past few years, a variety of such factors have been identified and characterized in the zebrafish ovary including activin and epidermal growth factor (EGF), and lines of evidence from our laboratory and others point to the existence of an ovarian network of communication involving these factors.

METHODS: RT-PCR was performed to analyze the expression profiles of EGF family members and their receptor EGFR during zebrafish follicle development. To examine spatial patterns of gene expression within the follicle, the somatic follicle layers and oocytes were mechanically separated for RT-PCR analysis. Pharmacological approaches and Western blotting were used to analyze signal transduction pathways in the zebrafish follicle cells.

RESULTS: Activin is predominantly expressed in the somatic follicle cells surrounding the oocyte, whereas its receptors and intracellular signaling SMAD proteins are abundantly expressed in the oocyte. In contrast, our recent experiments demonstrated that EGF and EGF-like peptides such as transforming growth factor alpha (TGF alpha), heparin-binding EGF (HB-EGF) and betacellulin (BTC) were exclusively or predominantly expressed in the oocyte, whereas their common receptor EGFR was exclusively expressed in the follicle cells. One of the effects of EGFR activation by EGF in the follicle cells was to increase the expression of activin subunits but suppress that of activin-binding protein follistatin. Interestingly, EGFR expression increased significantly during zebrafish folliculogenesis, in parallel to that of gonadotropin receptors (FSHR and LHR), whereas its ligands (EGF, HB-EGF, TGF alpha, and BTC) all exhibited rather constant expression. Further evidence showed that EGFR expression in cultured zebrafish follicle cells was stimulated by a novel cAMP-dependent but PKA-independent pathway that involved p38 MAPK.

CONCLUSION: Our previous and present studies suggest that activin may act as a paracrine signal from the follicle cells to oocyte, whereas EGF and its related peptides may serve as paracrine signaling molecules from the oocyte to control the function of somatic follicle cells. EGFR may represent a controlling point of the EGF-EGFR network in the zebrafish ovary that is subject to endocrine regulation, particularly by the pituitary gonadotropins.

O 46

Identification of differentially expressed ovarian genes across previtellogenic stages of oogenesis in salmon

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BACKGROUND: Little is known about the regulation of previtellogenic oocyte growth in fish, therefore the goal of this study was to identify differentially expressed ovarian genes during primary and early secondary oocyte growth in coho salmon, *Oncorhynchus kisutch*. This semelparous teleost exhibits synchronous development of a single clutch of ovarian follicles, thus allowing for stage-specific gene expression analyses.

METHODS: Reciprocal suppression subtractive hybridization (SSH) cDNA libraries were generated from ovaries with perinucleolus (PN) or cortical alveolus (CA) stage oocytes. A total of 288 clones were sequenced per library and their identity determined by comparing sequences to the GenBank and trout EST databases. Differential expression of selected genes was further confirmed across stages with quantitative RT-PCR (QPCR).

RESULTS: The library containing genes upregulated in the PN stage consisted of 160 nonredundant genes; 80% were identified as similar to annotated genes, 15% were homologous to unannotated ESTs, and 5% were novel. Representative genes included: 1) ferritin, a storage protein; 2) cathepsin B, a yolk protein-associated protease; 3) alveolin, which is involved in chorion hardening; and 4) several cyclins important to cell cycle control. The library containing genes upregulated in the CA stage consisted of 153 nonredundant genes; 71% were similar to annotated genes, 16% were homologous to unannotated ESTs, and 13% were novel. Representative genes included: 1) serum lectin, a C-type lectin likely involved in the fertilization response; 2) somatic lipoprotein receptor, a possible very low-density lipoprotein receptor; 3) cathepsin D, an enzyme involved in yolk processing; and 4) retinol dehydrogenase, an enzyme involved in retinoic acid synthesis. QPCR results confirmed that a number of genes identified by SSH were differentially expressed across the PN and CA stages. Zona pellucida-related genes and yolk processing enzymes were highly expressed in PN stage follicles, while serum lectin and retinol dehydrogenase were upregulated during the CA stage. Other putatively regulated genes revealed by SSH are currently being validated by QPCR.

CONCLUSION: A number of differentially expressed genes were identified that are known to play important roles in oogenesis, such as zona pellucida formation, sequestration and processing of yolk proteins, apoptosis, and the fertilization response. Interestingly, a number of genes involved in vitellogenesis were expressed in PN stage follicles, more than one year prior to the initiation of yolk incorporation. The SSH libraries were dominated by genes expressed in the oocyte, therefore cDNA libraries using theca/granulosa cell enriched tissue are being constructed.

O 47

Targeted cod (CodArray) gene chip: A genomic tool for evaluating the role of androgens on the growth of previtellogenic oocytes in Atlantic cod (*Gadus morhua*)

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BACKGROUND: Despite the concept of life-history strategies, the internal signals that regulate fecundity are not known. Prior to vitellogenesis (previtellogenesis), androgens appear to play a pivotal role in stimulating oocyte growth. In this study, we have used the analytical power of suppression subtractive hybridisation (SSH) with subsequent array development to open a possible revealing window in understanding the functional aspects of oocyte development.

METHODS: We constructed a targeted cod cDNA array containing 200 differentially-expressed genes using suppression subtractive hybridisation (SSH) after in vitro exposure of previtellogenic gonad tissue from Atlantic cod (*Gadus morhua*) to a complex mixture of androgens. The targeted CodArray gene chip was hybridized using total RNA from cod previtellogenic oocyte cultures exposed to 11-ketotestosterone (11-KT) and testosterone at physiological relevant concentrations (0, 10 and 100 μM) for 5 and 10 days. Array analyses demonstrated a number of differentially expressed genes, and specific genes were selected and their expression patterns verified using real-time PCR.

RESULTS: Transcripts isolated by SSH were from a diverse range of genes involved in hormonal regulation, signal transduction, cell growth control, intra/extracellular structure, general stress, apoptosis/protein degradation, biometabolism, transcription, translation, transport/binding and DNA structure, as well as transcripts with no presently known function in fish. Subsequent printing of cDNA clones to the CodArray gene chip and hybridisation of sample RNA from androgen-exposed tissue showed 0.5-3.5 -fold significantly altered transcript levels for a wide range of genes. Interesting, testosterone treatment showed increased levels of two different genes associated with the zona pellucida, whereas these genes were not altered after exposure to 11-ketotestosterone. Moreover, 11-KT treatment showed an apparent induction of cyclin-B, which is suggested to control the timing of early embryonic cell cycle.

CONCLUSION: Despite its high economic significance, the Atlantic cod is not a well-studied species from an endocrinological standpoint. In this study, we present the differential gene expression profile of more than 200 genes after androgen exposure, most of them being poorly studied or even unknown in fish. Thus, the results of the present study show that SSH and the array technology is a useful method to discover novel androgen-responsive genes, indicating androgen control of early follicular and oocyte growth.

O 48

Reproductive consequences of an immune challenge during the periovulatory period in trout

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BACKGROUND: An immune challenge with bacterial lipopolysaccharide (LPS) in trout results in the activation of the apoptotic program in the ovary and causes the death of follicular cells surrounding the oocyte. However, little is known about the consequences of follicular apoptosis on gamete viability or survival, or about the cellular and molecular mechanisms that are involved in mediating this effect. Given that TNFα is highly induced in immune cells in response to LPS, we hypothesize that TNFα could be an important mediator of the effects of LPS in the ovary. In this study, we have investigated the effects of LPS administration on egg survival and also the direct effects of recombinant trout (rt) TNFα on trout ovarian follicles.

METHODS: Preovulatory female trout received a single i.p. injection of LPS, their timing of ovulation was recorded and survival at eyed stage was determined. To determine the effects of rtTNFα on steroid production and gene expression, intact preovulatory trout follicles or isolated granulosa and theca layers were incubated with various concentrations of rtTNFα in the absence or presence of a partially purified gonadotropin preparation. Steroid production will be measured by RIA and gene expression by microarray analysis as well as by real-time PCR. The effects of rtTNFα on granulosa cell viability were assessed by incubating isolated granulosa layers in the presence or absence of rtTNFα for aproximately 24 hours, labelling cells with propidium iodide and visualizing fluorescence under a fluorescent microscope. Apoptosis in granulosa cells was measured by flow cytometry.

RESULTS: Preliminary results from the in vivo treatment with LPS indicate that LPS-injected females ovulated earlier than saline-injected females and that ovulation was partial. Furthermore, fertilization of ovulated eggs from LPS-injected females resulted in a marked decrease in survival rate at eyeing (2.5%). We have also obtained preliminary evidence that exposure of trout follicles to rtTNFα in vitro causes a decrease in granulosa cell viability and an increase in the number of granulosa cells undergoing apoptosis. Currently, the effects of rtTNFα on follicular steroid production and on the global effects on gene expression by microarray are being analyzed.

CONCLUSION: We have preliminary evidence indicating that LPS could cause the premature ovulation of non-competent eggs, resulting in a marked decrease in embryonic survival. Furthermore, preliminary evidence indicates that rtTNFα could directly decrease the viability of granulosa cells by inducing apoptosis, supporting our hypothesis that the previously reported increase in follicular apoptosis induced by LPS could be mediated by rtTNFα in the ovary. Overall, these results would suggest that a pathogen-induced activation of the immune system prior to ovulation could increase TNFα–mediated apoptosis in the trout ovary and result in a potentially important decrease in egg quality.

O 49

Identification and characterization of a novel subfamily of aquaporin-1-related water channels neofunctionalized in oocytes of marine and catadromous teleosts

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BACKGROUND: In marine teleosts, oocyte hydration is an essential mechanism that occurs during meiotic maturation that renders the egg buoyant in seawater, contributing to their survival and dispersal in the ocean. In sea bream (Sparus aurata), oocyte hydration is regulated by the interplay between yolk protein hydrolysis, that creates the osmotic gradient, and a novel aquaporin (AQP), SaAQP1o (now named Aqp1b), that facilitates water uptake into the oocyte. Aqp1b is highly expressed in the ovary, unlike SaAQP1 (now named Aqp1a), which is the ortholog of mammalian AQP1 and is ubiquitously expressed. Here, we aimed at investigating the origin of Aqp1b and explore its posttraslational regulation in the oocyte.

METHODS: AQP1-like sequences from teleosts and other vertebrates were obtained from database searching and cDNA cloning using degenerate oligonucleotide primers. Phylogenetic analyses were performed by using the neighbor-joining (NJ) method and the robustness of the phylogenetic results were tested by bootstrap analysis with 1,000 repetitions and by the standard interior-branch test. Gene expression analysis was done by RT-PCR on different adult tissues including maturing (hydrated) ovaries. The Xenopus laevis oocyte swelling assay, combined with immunofluorescence and Western blot, was employed to test the functionality of Aqp1a and Aqp1b. Specific regulatory sites in the C-terminus of Aqp1b were investigated by chimera construction and site-directed mutagenesis.

RESULTS: The reconstruction of the molecular phylogeny of AQP1 suggest that vertebrate AQP1 evolved from a common ancestor gene. However, teleosts suffered an early duplication event of this gene giving raise to aqp1a and aqp1b In extant marine and migrating (catadromous) teleosts that spawn highly hydrated eggs in seawater, such as sea bream, sole (Solea senegalensis) and eel (Anguilla anguilla), aqp1b mRNA was predominantly expressed in the ovary, where it encoded a functional water channel. In contrast, in freshwater species that spawn non-hydrated eggs, such as zebrafish (Danio rerio) or medaka (Oryzias latipes), aqp1b transcripts were not detected in any tissue. Heterologous expression of sea bream Aqp1a and Aqp1b in X. laevis oocytes revealed that Aqp1a was more efficiently translocated into the plasma membrane than Aqp1b. Because the C terminus of Aqp1b is the region more divergent with respect to Aqp1a, a chimera protein was constructed replacing the C terminus of Aqp1b by that from Aqp1a. Oocytes expressing the chimera protein exhibited higher permeability than those expressing wild-type Aqp1b, suggesting the presence of regulatory sites at the C terminus of Aqp1b. Mutation of a diLeu motif located in the Aqp1b C terminus into diAla completely inhibited oocyte water permeability by retention of the protein in the endoplasmic reticulum.

CONCLUSION: We conclude that aqp1b is encoded by a gene unique to teleosts that represents a neofunctionalized water channel adapted to oocytes in marine and catadromous species, thereby contributing to a water reservoir in eggs and early embryos. In freshwater species that spawn non-hydrated eggs, aqp1b becomes a pseudogene or it is lost in the genome. Furthermore, we demonstrate that the C terminus of Aqp1b contains sites for Aqp1b trafficking regulation where a diLeu motif might play a key role.

O 50

Sub-functionalization of a rainbow trout divergent paralog of the Sex Hormone-Binding Globulin (SHBG) results in an ovarian-specific expression of a protein that could participate in the estrogenic control of ovarian functions

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BACKGROUND: Sex hormone-binding globulin (SHBG) is classically known to bind and transport sex steroids in the blood, thus regulating their bioavailable fraction and access to target cells. However, SHBG is also able to bind to steroid target cell membranes and to directly modulate steroid action. Using microarrays, we identified a novel gene that displayed an extremely good correlation with the ovarian gene expression profile of aromatase (*cyp19a1*) both during ovarian sex differentiation, late-vitellogenesis and oocyte maturation. We report here the phylogenic and functional characterization of this gene that encodes for a previously uncharacterized protein exhibiting sequence similarity with fish and other vertebrates SHBG.

METHODS: The cDNA of this novel SHBG, named OSHBG (ovarian sex hormone binding globulin) was obtained from a pooled tissue library and fully sequenced. After screening EST libraries and fully sequenced fish genomes, a phylogenetic analysis of SHBG and OSHBG sequences was carried out using the Figenix platform (http://www.up.univ-mrs.fr/evol/figenix/index.html). Spatio-temporal gene expression was analyzed by real time RT-PCR, *in situ* hybridization and *in situ* immuno-cytochemistry using a mouse anti-peptide antibody. The steroid binding characterization was carried out on recombinant trout SHBG and OSHBG proteins expressed in COS7 cells.

RESULTS: No ortholog sequence of the trout *oshbg* gene was found in non-salmonid fish or other vertebrates. Phylogenetic analysis performed using the full OSHBG amino acid sequence demonstrates that this gene is a highly divergent paralog of the trout SHBG. Gene expression studies showed that, in contrast to the classical trout *shbg* that is predominantly expressed in the liver, *oshbg* is specifically co-expressed with *cyp19a1* in the granulosa cells of the ovarian follicle. *oshbg* is also expressed at much lower levels in muscle and stomach but not in any of the other tissues studied, including liver and testis. Similarly to SHBG, recombinant trout OSHBG binds to estradiol-17beta (E2) and testosterone but not cortisol.

CONCLUSION: In the present study we identified and characterized a functional rainbow trout divergent paralog of the classical SHBG. This paralog probably arises from salmonid-specific genome duplication. Together, our observations show that *oshbg* has rapidly evolved and results from a gene duplication followed by sub-functionalization characterized at least by new tissue expression specificity. Finally, the high correlation between *oshbg* and *cyp19a1* ovarian expression profiles strongly suggests that *oshbg* participates in the estrogenic control of ovarian functions.

O 51

Changes in steroidogenic enzyme, steroidogenic acute regulatory protein, steroid receptor, gonadotropin receptor and transcriptional factor mRNAs in ovarian follicles during oocyte growth and maturation of medaka (*Oryzias latipes*)

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BACKGROUND: The medaka usually spawns daily within 1 hr of the onset of light when acclimated to a 14-hr light/10-hr dark photoperiod at 27^{o}C. Under these condistions, largest follicles in ovary undergo germinal vesicle breakdown at 6 hr before lighting and are ovulated at 1 hr before lighting. We have previously reported that in medaka, estradiol-17β and 17α,20β-dihydroxy-4-pregen-3-one (17α,20β-DP) are the two major steroid hormones controlling oocyte growth and maturation. In the present study, we used real time-PCR to determine the levels of mRNAs encoding steroidogenic enzymes (P450scc, 3β-HSD, P450c17, 17β-HSD, P450arom, and 20β-HSD), steroidogenic acute regulatory protein (StAR), steroid nuclear receptors (PR, ERs, AR, and GR), steroid membrane receptors (mPRs), gonadotropin receptors (FSHR and LHR), and a transcriptional factor (Ad4BP/SF-1) in ovarian follicles of medaka during oocyte growth, maturation and ovulation.

METHODS: Mature medaka (orange-red type) were purchased from fish farm (Yatomi, Aichi Prefecture, Japan) and maintained under reproductive condition (14 hr light/10 hr dark) for at least 2 weeks before use. Every three hours from 47 to 2 hr before ovulation (16 different stages of development), 30 follicles were collected from 4 medaka fish and divided them into 3 microtubes, each one with 10 follicles. Immediately, follicles were homogenized in RNA extraction buffer and total RNA was isolated by RNeasy spin column. mRNA expression level of each gene was quantified by real time-PCR using specific primers.

RESULTS: Each gene showed characteristic expression patterns and according to these patterns the gene were classified into 5 groups. Genes, whose expressions were (i) maintained during late vitellogenic stage and then down regulated: FSHR, P450arom, 17β-HSD type1 and 3β-HSD; (ii) maintained until final oocyte maturation stage: LHR, P450scc, StAR, ERs and ARα; (iii) transiently increased just before final oocyte maturation stage: P450c17; (iv) transiently increased during final oocyte maturation stage: PR; (v) constantly expressed: 20β-HSD, mPRγ and GR.

CONCLUSION: These results indicate that the expression of genes examined in the present study changes dynamically, depending on the developmental stages of medaka ovarian follicles. It has been suggested that a distinct shift from the secretion of estradiol-17β to 17α,20β-DP in response to gonadotropin occurs immediately prior to oocyte maturation stage. Coincident with this event, expression of FSHR and some other genes dropped dramatically at -32 hr, indicating the shift point of steroidogenesis. And then, granulosa cells gradually acquire the ability to produce 17α,20β-DP by 21 hr before ovulation. It has been proposed that 17α,20β-DP is secreted around 12 hr before ovulation to induce oocyte maturation. A major finding in the present investigation was that the expression pattern of PR nuclear receptor was synchronized with deduced 17α,20β-DP secretion pattern. This finding brings the possibility that PR nuclear receptor has a key role in final oocyte maturation or ovulation.

O 52

Estradiol alters the microRNA expression profile in the zebrafish (*Danio rerio*)

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BACKGROUND: MicroRNAs (miRNAs) are non-coding RNAs that negatively regulate gene expression at the post-transcriptional level. Each miRNA may target the expression of several genes. To date, little is known about signals that induce miRNAs expression in general and about hormonal regulation of miRNAs in particular.

METHODS: Microarrays displaying ~ 240 zebrafish mature miRNAs specific probes were hybridized using RNA samples originating from whole body extracts of male zebrafish exposed to Estradiol-17 beta (E2) for 1, 4, 12 and 24 hr. Eight specific miRNAs were selected from the differentially expressed identified miRNAs and their expression was analyzed by real-time RT-PCR in total body extracts and four different tissues (liver, intestine, skin and gills). In addition, the expression pattern of the three estrogen receptor genes was also elucidated by real-time PCR in total body extracts and examined tissues.

RESULTS: The miRNA microarray results show several tens of miRNAs to be differentially expressed in whole fish samples at different time points after exposure to E2. The up-or down-regulation of eight miRNAs was verified by real-time PCR for samples taken after 12 and 24 hr of treatment and in four different tissues. The increased expression of estrogen receptor1 [*esr1* (ER alpha)] and the expression of vitellogenin in the treated fish, confirmed the efficacy of the E2 treatment. While *esr1* is predominantly expressed in the liver, the results also show a significant up-regulation of *esr2a* (ER beta2) but not of *esr2b* (ER beta1) in the other tested tissues. A search of zebrafish ortholog genes with conserved 3' UTRs by acceptable computational target prediction programs, revealed a list of predicted gene targets for the selected eight miRNAs such as *myc* and *hox* genes that serve as important transcription factors.

CONCLUSION: We investigated the regulation of the zebrafish miRNAs under stimulation of estradiol and found tens of miRNAs that were up- or down-regulated at different time points after exposure. Of these miRNAs, eight were also found to be regulated in different tissues. Our results are the first example for estrogen regulation of miRNAs and of the possible involvement of miRNAs in estrogen regulated processes such as vitellogenesis and reproduction. These results are also relevant to the effect of endocrine disrupting chemicals and cancer research, where a role for estrogen or functionally related compounds, has been demonstrated. The results suggest novel pathways for the pleiotropic and multi-gene function of estradiol.

O 53

Revealing the genes associated with vitellogenesis in the liver of the zebrafish (Danio rerio) using gene expression microarray profiling

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BACKGROUND: Vitellogenesis is associated with the expression of several genes regulating synthesis of proteins in the liver. Among them, most notably are the vitellogenins (Vg's) that are secreted to the plasma as phospholipoglycoproteins and form major components of the egg yolk. Vg's are also associated with retinal as the main retinoid deposited in zebrafish oocytes. The concurrent synthesis of the Vg and its associated prosthetic groups including lipids, retinal, carbohydrate and phosphorous, raises the question on the identity and regulation of genes associated with Vg synthesis and of other proteins related to vitellogenesis.

METHODS: RNA pools were prepared from livers of vitellogenic (vit) females, males and E2 treated males. mRNA profiles of these samples were established by using zebrafish gene expression chips containing 16,416 oligonucleotide probes. Differentially expressed genes were identified by ANOVA. To reveal genes of interest, clustering of the differentially expressed genes was performed according to the gene expression patterns. In order to validate the microarray results, 16 genes selected from the chip were tested by real-time PCR. In addition, the expression of other relevant genes that did not appear on the chip, were also tested by real-time PCR. In addition, RNA samples from the liver of previtellogenic (pre-vit) females were used in real-time PCR experiments.

RESULTS: Cluster analysis identified 227 up-regulated and 203 down-regulated genes in vit females compared with males. In E2 treated males, 1317 genes were up-regulated while 192 genes were down-regulated compared with untreated males. When comparing vit females with E2 treated males, 326 genes showed similar expression levels, 1231 genes were found to be up-regulated and 80 genes were down-regulated in E2 treated males. Comparison with samples from pre-vit females is now in progress. Among the 326 genes are those known to be involved in vitellogenesis, such as: Estrogen Receptor1 (esr1), genes involved in lipid metabolism (VLDL, lipoprotein lipase, PPARs etc.), genes involved in retinoids metabolism (RBP1, LRAT, RDH14), genes with putative functions in gluconeogenesis, phosphrylation and calcium binding. Surprisingly, Vg's display different expression patterns; Vg1 and Vg3 were expressed only in vit females, while Vg2, Vg4, Vg5, Vg6 and Vg7 were expressed in pre-vit females, vit females and males.

CONCLUSION: Microarray profiling of liver samples revealed expression patterns characteristic of vitellogenic females that share 326 genes with E2 treated males. The study revealed a large array of genes expressed during vitellogenesis, putative pathways for the synthesis of Vg prosthetic groups and a novel pathway for the synthesis of retinal associated with vitellogenin.

O 54

Gene expression profiles revealing the mechanisms of anti-androgen- and oestrogen-induced feminisation in fish

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BACKGROUND: Environmental anti-androgens are increasingly being recognised as potential contributing factors in the chemically-induced feminisation of wild fish because, by blocking androgen action, they can produce phenotypic effects similar to environmental oestrogens. The molecular mechanisms by which anti-androgens and oestrogens exert feminising effects, however, have not been systematically compared.

METHODS: Using a targeted real-time PCR approach, we profiled the expression responses of a suite of 22 genes involved in reproduction, growth and development (processes controlled by androgens and oestrogens) in the liver and gonad in adult male and female fathead minnow (*Pimephales promelas*) exposed for 21 days to the model anti-androgen flutamide (at 320 µg/L) and the model oestrogen 17α-ethinylestradiol (EE_2; at 10 ng/L). Gene expression analyses we conducted in conjunction with the analysis of feminising effects on phenotypic level effect measures, including gonadal growth, plasma vitellogenin (VTG) and secondary sex character development.

RESULTS: Both flutamide and EE_2 produced phenotypic effects indicative of feminisation (induction of plasma VTG, reduced gonadosomatic index, and reduced secondary sex characters), although for the chosen test concentrations EE_2 was the more potent. For the genes studied, flutamide and EE_2 produced distinct expression profiles in both liver and gonad, suggesting that they largely operate via distinct molecular mechanisms. For example, in the testis, flutamide up-regulated genes coding for enzymes involved in androgen biosynthesis (*cytochrome P450 17* [*cyp17*] and *hydroxysteroid 11-beta dehydrogenase* [*hsd11b*]) implying an inhibitory action on androgen negative feedback pathways. EE_2, in contrast, inhibited the expression of enzymes involved in androgen biosynthesis (*cyp17*, *hsd11b* and *hydroxysteroid 17-beta dehydrogenase* [*hsd17b*]). There were also some commonalities in the molecular mechanisms of flutamide and EE_2 action, including the down-regulation of gonadal sex steroid receptor expression (gonadal *androgen receptor* [*ar*] and ovarian *estrogen receptor 1* [*esr1*]), increased expression of genes coding for oestrogen-producing enzymes (*cytochrome P450 19a1* and *a2* [*cyp19a1* and *cyp19a2*]), and decreased expression of genes involved in testis differentiation (*anti-Mullerian hormone* [*amh*] and *doublesex and mab-3 related transcription factor 1* [*dmrt1*]).

CONCLUSION: The data presented show clearly that it is possible to differentiate between the effects of a model anti-androgen and model oestrogen through studies on gene expression in fish, and further demonstrate that the feminising effects exerted by these two classes of endocrine disrupting chemicals occur via both distinct and common gene pathways.

O 55

Applying transcriptomics to unravel the mechanims underpinning oestrogenic disruption of sexual development in roach (*Rutilus rutilus*)

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BACKGROUND: Wild roach (*Rutilus rutilus*) inhabiting UK rivers that contain high levels of effluents have been shown to have altered sexual development which impacts negatively on their reproductive capabilities. The effects observed, including feminisation of the male gonad, have been ascribed to oestrogenic chemicals in those effluents. However, the molecular events underlying these disruptions in gender assignment have not been established. In this work we applied a transcriptomic approach to identify the biological processes involved.

METHODS: In two separate experiments, roach were exposed from early life (encompassing the period of sexual differentiation) up to 720 days post hatch (dph) to the pharmaceutical ethinyloestradiol (EE_2; at 4ng/L) and up to 390 dph to 100% treated sewage effluent. The phenotypic sex of each fish subsequent to the exposures was determined histologically. RNA originating from control and contaminant-exposed fish was extracted and applied to a custom-built roach cDNA microarray displaying 18477 gonadal ESTs (9676 assembled sequences of which 4959 were identified) which was developed from gonadal cDNA libraries of different development life stages. Of these identified sequences, 2800 had gene ontology (GO) annotations and a further 556 encoded for enzymes. The arrays were hybridised and analysed using an ANOVA loop design. Principal Component Analysis (PCA) was used to identify the main trends in gene expression for the different treatments. Differentially expressed genes were identified and their biological significance investigated by analysis of bias of the distribution of GO terms in the differentially expressed gene lists.

RESULTS: Exposure to EE_2 for two years resulted in an all-female population of roach. Exposure to the treated sewage effluent for around one year induced duct-disruption and initial stages of germ-cell disruption in male fish. PCA of the gene expression profiles in gonads of control roach clearly separated males from females for both studies. An analysis of the gene profiles for the EE_2-exposed fish showed clear and major effects of treatment on the transcriptome. PCA on the EE_2 exposed, 'female-like' fish (that would have included genetic females and males) separated them into two groups, one showing a higher similarity to the control females, and the other (presumed feminised males) to the control males. In contrast, for effluent-exposed roach, treatment was not the main determinant on the differences on the overall transcriptome between individuals compared with controls, despite the fact that some features associated with morphological feminisation were evident in those males. 'GO-Biological Process' categories affected in both studies included those for reproduction and immune response. A more detailed analysis of the genes and biological processes affected will be presented.

CONCLUSION: Life-long exposure of roach to an environmental concentration of EE_2 induced phenotypic feminisation of males and this was associated with changes in the resulting transcriptomes of those individuals. PCA revealed that the transcriptomes of males were impacted to a greater extent compared with females in both oestrogen exposures (EE_2 and effluent).

O 56

Induction of fish oocyte maturation by DES through membrane progestin receptor

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BACKGROUND: The induction of oocyte maturation (OM) in fish by progestins is a valuable model for investigating interference of nongenomic steroid actions by endocrine-disrupting chemicals (EDCs).

METHODS: The potency of EDCs to induce or prevent the resumption of meiotic cell cycle by maturation-inducing steroid (MIS) was examined in vitro oocyte culture. Maturation inducing activity was also evaluated by the detection of synthesis of cyclin B protein. The ability of EDCs to interact with the progestin receptor mediating OM was investigated in receptor binding assays using plasma membranes from goldfish ovaries and breast cancer cells transfected with goldfish membrane progestin receptor α (mPRα).

RESULTS: An endocrine-disrupting chemical, diethylstilbestrol (DES), a nonsteroidal estrogen, triggers oocyte maturation in goldfish and zebrafish. The morphology (the time course of the change in germinal vesicle breakdown) and an intracellular molecular event (the *de novo* synthesis of cyclin B) induced by DES were indistinguishable from those induced by a natural MIS: 17α, 20β-dihydroxy-4-pregnen-3-one (17,20β-DHP). A synergistic action of DES on 17,20β-DHP-induced OM was observed. Both 17,20β-DHP- and DES-induced oocyte maturation was inhibited by an antibody against mPRα. Membranes prepared from both ovaries and mPRα–transfected cells showed high affinity, saturable, displaceable, single binding sites specific for the goldfish maturation-inducing steroid, 17,20β-DHP. DES and DES analogues (DP-DES and HEX), which induce OM in goldfish, bound to the receptor and caused concentration-dependent displacement of [^{3}H]-17,20β-DHP.

CONCLUSION: We showed that exposing fish oocytes to DES at a dose within a range similar to that used in experimental exposure to 17,20β-DHP induces oocyte maturation. This is the first report showing that EDC can potentially induce oocyte maturation like an endogenous MIS. It was demonstrated that both the ovarian plasma membrane receptor in goldfish and goldfish mPRα expressed in transfected cells displayed high affinity, limited capacity, displaceable specific binding for [^{3}H]-17,20β-DHP. The relative binding affinities of various steroids and EDCs to these two receptor preparations were similar, supporting the idea that mPRα is a MIS receptor *in vivo*. The close correspondence between binding of DES and its analogues to the mPRα protein and their OM-inducing activities suggests a mechanism of endocrine disruption mediated by binding to mPRα resulting in its activation, thereby mimicking the nongenomic action of the progestin 17,20β-DHP.

O 57

The requirement of retinoids in the reproduction of zebrafish

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BACKGROUND: The extent to which the retinoids are required in fish reproduction is largely unknown. Retinal is deposited in the egg yolk during oogenesis, as a retinoid source for the future embryo. RA signaling may be active in fish gonads, since the mediators of RA action, the RA receptors (RARs) and retinoid X receptors (RXRs), are present in rainbow trout ovaries and testes. However, no studies have examined the functional requirement of the retinoids in fish reproduction.

METHODS: RT-PCR was used to determine if the enzymes involved in the synthesis and degradation of RA [retinal dehydrogenase 2 (RalDH2) and CYP450RAI (CYP26a), respectively] and the RA receptors (RAR and RXR subtypes) are expressed in the ovaries and testes of zebrafish. Zebrafish were also exposed in vivo to an inhibitor of RalDH2, diethylaminobenzaldehyde (DEAB), for 11 d and the effects on egg production and fertilization rate were determined. In addition, real time PCR was used to determine the expression of gonadal RalDH2 and CYP26a. The final experiment examined the long-term (130 d) effects of a retinoid deficient diet on whole body and egg retinoid levels and the same reproductive endpoints were evaluated.

RESULTS: It was shown with RT-PCR that RalDH2, CYP26a, and RA receptors (RAR two subtypes) and RXR (3 subtypes) are expressed in the ovaries and testes. Exposure to DEAB decreased the number of spawned eggs by 95% and altered the expression of RalDH2 and CYP26a in the gonads. Zebrafish fed a retinoid deficient diet for 130 d, had whole body retinoids decreased in females and males by 68% and 33%, respectively. During the final 12 d of the exposure, females fed the retinoid deficient diet produced 71% fewer eggs that contained 78% less retinal than controls. Ovarian expression of RalDH2 and CYP26a was not affected by the deficient diet.

CONCLUSION: These studies have shown that the RA signaling system including enzymes that synthesize RA (RalDH2) and catabolize RA (CYP26a) and multiple receptor subtypes are expressed in the zebrafish gonads. Moreover, studies using inhibitors of retinoic acid metabolism or retinoid deficient diets demonstrate that the retinoid system is essential for the spawning of eggs. The retinoid system while often overlooked in studies of reproductive biology has an essential role in normal development and should be considered as possible target mediating the toxicity of some chemicals in the environment.

O 58

Purification and classification of egg yolk proteins derived from multiple vitellogenins in grey mullet (*Mugil cephalus*)

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BACKGROUND: Vitellogenin (Vg), an estrogen-inducible yolk precursor protein, has become an important biomarker for assessing exposure of animals to estrogenic contaminants in aquatic environments. The grey mullet is a key species for monitoring such contamination. Teleost yolk proteins (YPs) include multiple types of lipovitellin (Lv), phosvitin (Pv), and β'-component (β'-c) derived from different forms of Vg. With the exception of Pv, these YPs can be used as antigens to develop antisera for detecting specific types of Vg. The objectives of this study were to purify, characterize, and classify Vg-derived YPs in grey mullet.

METHODS: Seven yolk proteins, four large lipoproteins (YPs1-4) and three minor components (YPs5-7) including one phosphoprotein (YP7), were purified from extracts of vitellogenic ovaries by hydroxylapatite, ion exchange, and immunoadsorbent column chromatography and by gel filtration. Peptide bands of purified YPs were subjected to N-terminal amino acid sequencing and mapped against the primary structure of multiple mullet Vgs (VgA, VgB and VgC) deduced from their respective complete cDNAs, which were cloned and sequenced.

RESULTS: Purified native YP1, YP2, YP3, and YP4 exhibited relative molecular masses of 330, 325, 335, and 570 kDa, respectively. The tertiary structures of YP1, YP2 and YP3 revealed by SDS-PAGE were typical of teleost Lv, consisting of a heavy chain and a light chain, while YP4 consisted of a heavy chain and two more polypeptide bands. The YP5 and YP6 were ~34 kDa in their native state and consisted of two ~16 kDa monomers. The YP7 appeared as a phosphorylated ~25 kDa peptide in the gels. Peptide mapping revealed YP1, YP2, and YP3 to be mullet Lvs derived from VgA, VgB, and VgC, respectively, and specific antisera were raised against each form of Lv. The fourth YP (YP4) appeared to be a proteolytic variant consisting of both Lv and Pv components of VgA. The YP5 and YP6 were identified as β'-cs derived from VgA and VgB based on their structures and common, but not identical, antigenicity to anti-salmonid β'-c serum, while YP7 was identified as a Pv derived from VgB.

CONCLUSION: This is the first report on purification and molecular classification of three distinct forms of Lv from any oviparous vertebrate. The present study yielded Lv type-specific antisera, the first step toward development of type-specific Vg immunoassays for advanced assessment of environmental estrogens using Vg-based bioassays.

O 59

Yolk precursors in white perch (*Morone americana*): deduced primary structures of three types of vitellogenin (Vg) proteins and disparate binding of the different Vgs to multiple ovarian receptors

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BACKGROUND: Vitellogenins (Vgs), a family of yolk protein (YP) precursors, are produced by the piscine liver in response to estrogen (E2), released into the circulation, and taken up by oocytes *via* receptor (VgR)-mediated endocytosis. Vgs A and B are then cleaved into YPs [lipovitellin (Lv), phosvitin (Pv), beta'-component, and various Lv-Pv complexes] but VgC is not. During oocyte maturation in advanced teleosts spawning pelagic eggs, additional proteolysis of the Lv heavy chain from VgA and other Vg-derived YPs occurs, however Lvs from VgB are only partially cleaved. Prior investigations have revealed distinct functions of the different forms of Vg. However, disparate uptake by or trafficking within the oocyte of different forms of Vg or Vg-derived YPs remained to be explored.

METHODS: Three Vg transcripts were cloned from a perch liver cDNA library and sequenced. Ligand blots of ovarian membrane proteins separated by non-denaturing SDS-PAGE were conducted using digoxigenin (DIG)-labeled Vgs (A, B, and C) purified from blood plasma of E2-injected males by several steps of ion-exchange chromatography. Receptor binding assays were conducted in 96-well plates coated with solubilized ovarian membrane proteins. Binding specificity was evaluated by incubating membrane preparations with constant amounts of DIG-labeled Vg tracers (A, B, C, and AB mix) alone or in the presence of unlabeled competitive Vg ligands.

RESULTS: Two cDNAs encoded Vgs with a complete suite of YP domains which, based on comparison with Vgs from other species, were classified as VgA and VgB. The shorter cDNA encoded a Pv-less Vg classified as VgC. Three ovarian receptors were resolved in ligand blotting; one large (>200 kDa) receptor primarily bound VgA, whereas two smaller receptors (116 and 110.5 kDa) bound VgB. Binding assays revealed that individual unlabeled VgA and VgB were only able to effectively displace ~50% of membrane bound DIG-VgAB at 250 excess molar concentrations. Furthermore, VgC did not specifically bind to ovarian membranes.

CONCLUSION: This study provides the first evidence for a system of multiple ovarian VgRs with disparate specific binding to dual complete Vgs and lack of binding to Pv-less Vg. Selective processing of Lvs derived from functionally distinct parent Vgs may arise from this multiple VgR system, since receptor-ligand directed compartmentalization and processing of VgA and VgB may occur following internalization. Furthermore, since VgC does not bind to ovarian membranes, it must enter the oocyte non-specifically in the fluid phase and thus may not be subjected endosomal processing, which would explain why it is not cleaved into YPs.

O 60

The *souffle* (*suf*) Gene Controls Oocyte Maturation in Zebrafish

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BACKGROUND: At the end of oogenesis, oocyte maturation prepares the egg for fertilization. Although a plethora of genes influences oocyte maturation in vertebrates, only two genes have been confirmed genetically to be required for this process.

METHODS: To identify genes required for a biological process forward genetics has been extremely successful in the worm *C. elegans* and the fruit fly, *Drosophila melanogaster*. In a systematic mutagenesis screen in zebrafish we have isolated several mutants with an oocyte maturation defect.

RESULTS: The *souffle* (*suf)* mutant displayed in all assays the strongest phenotype. Therefore we focused on its molecular identification and characterization. To study the biological role of the *suf* gene, we investigated the cellular changes disrupted in mutant oocytes. A hallmark of oocyte maturation is the disaggregation of the nuclear envelope, also known as germinal vesicle breakdown. When eggs of wild type females were exposed to maturation inducing hormone, they undergo germinal vesicle breakdown. In contrast oocytes from *suf* mutants keep their germinal vesicle. These results indicate that the *suf* gene acts in a molecular pathway upstream of germinal vesicle breakdown before the oocyte resumes meiosis. In addition, these data imply that the *suf* gene activity is required cell-autonomously within the oocyte and not in the surrounding follicle cells to initiate oocyte maturation.
To molecularly identify the gene controlling oocyte maturation we positionally cloned the *suf* gene. *Suf* is predicted to encode a zinc finger protein of 280 kD and might be involved in vesicle transport. The identified mutation leads to a C-terminal deletion of 286 amino acids. The expression of the *suf* mRNA initiates during oogenesis right before the oocyte becomes competent to undergo oocyte maturation. We found *suf* homologs throughout the animal kingdom including invertebrates. Moreover, a homolog is also present in plant genomes suggesting that the *suf* gene family might exert a basic function conserved during the evolution of female meiosis.

CONCLUSION: Using forward genetics we isolated a key gene regulating oogenesis in zebrafish. Furthermore, the identification of the *suf* gene provides a molecular entry point to dissect the cellular and biochemical changes during oocyte maturation.

O 61

Identification of yolk proteins derived from multiple vitellogenins in Labrid teleosts that spawn benthic and pelagic eggs

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BACKGROUND: Differential proteolysis of vitellogenin (Vtg)-derived yolk proteins (Yp) during meiotic maturation has been shown to cause oocyte hydration in marine teleosts. The degree of hydration and the class of Yp that is degraded appears to depend upon whether the species spawns pelagic or benthic eggs. We hypothesise that the greater expression of one form over the other may underlie these observations. To investigate this possibility, we studied a group of closely related acanthomorph teleosts (*Labridae*, Perciformes) that spawn pelagic or benthic eggs.

METHODS: In order to understand the relationships between deposited oocyte Yps and hepatically expressed *vtg* mRNAs, we identified different classes of Yp using Western-immunoblots, N-terminal microsequencing and mass spectrometry, and mapped them to multiple forms of *vtgs* cloned from liver extracts.

RESULTS: We find that each of the investigated species expresses three forms of *vtg* (*vtgAa*, *vtgAb* and *vtgC*) in the liver, but selectively deposits the Yp products of these *vtgs* in their oocytes. In the species that spawns highly hydrated pelagic eggs (Goldsinny wrasse), the *vtgAa*-derived Yps are the only major proteins found in the oocytes. This class of Yps is extensively degraded to FAA during oocyte hydration leaving only a fragment of the lipovitellin light chain for embryonic development. In one of the investigated species that spawns benthic eggs (rock cook), limited proteolysis of the deposited Yps was associated with the appearance of a small pool of free amino acids. In this species the major Yps originate from *vtgAb* and are not degraded, while a lower amount of *vtgAa* derived Yps are partially proteolysed. In the third species that also spawns benthic eggs (cuckoo wrasse), no proteolysis occurs during oocye hydration. Our ongoing investigations suggest that the Yps of this species are almost exclusively translated from *vtgAb*.

CONCLUSION: Our results show that *vtg* expression ratios or their translational control underly the character (benthic or pelagic) of the spawned egg in this closely related family of marine teleosts.

Reproductive Strategy and Sexual Cycles

O 62

Reproductive activity of bluefin tuna (*Thunnus thynnus*) in the Mediterranean Sea: wild vs. captive broodstocks

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BACKGROUND: The bluefin tuna (BFT) has become an endangered species due to over-fishing. To resolve the contradiction between environmental and fishery needs, BFT was chosen as a prime candidate for domestication. Our study focused on comparing reproductive characteristics of captive and wild broodstocks, and assessing the effectiveness of gonadotropin releasing hormone (GnRH) spawning induction therapies.

METHODS: Wild BFT were spatially and temporally sampled across the Mediterranean Sea (MS). Morphometric parameters were recorded, and the gonadosomatic index (GSI) values were calculated. Likewise, captive BFT held in floating cages in Spain were sampled, and hormones including GnRHs, luteinizing hormone [LH], and sex steroids, were measured in control and GnRH-treated fish.

RESULTS: GSI values in wild BFT peaked during June. with fish caught at eastern MS locations exhibiting advanced sexual maturity compared to western MS BFT. Highest GSI values were recorded in June also in captive BFT, coinciding with maximal pituitary levels of sbGnRH, cGnRH-II, and LH. Moreover, no significant difference was observed in the levels of LH in the pituitaries of captive and wild BFT. The GnRH-implantation induced a rapid burst of pituitary LH at day 2-post treatment and elevated circulating LH up to day 8, at which time the experiment ended. There were significant differences in steroid hormone levels between GnRH-implanted and control. A significant peak in plasma 17,20b-P levels was observed in BFT females parallel to germinal vesicle (GV) migration.

CONCLUSION: Our results highlight June as the natural spawning season of eastern BFT stock and suggest that water temperature is a key trigger for BFT spawning. Hormone analyses show that the reproductive endocrine system in captive BFT is functioning and preparing for the natural spawning season. Endocrine manipulation with exogenous GnRH indicate a stimulatory effect on the reproductive brain-pituitary-gonadal axis of captive BFT, and draws attention to a striking resemblance in reproductive traits of BFT and the gilthead seabream. On the basis of the plasma 17,20b-P elevation in BFT at the time of GV migration we suggest a role for this steroid in inducing final oocyte maturation and ovulation in females and spermiation in males.

O 63

Gonadotropin gene expression, steroids plasma level and gonad development in Russian sturgeon (Acipenser gueldenstaedtii) grown in aquaculture

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BACKGROUND: The Russian sturgeon (Acipenser gueldenstaedtii) has been introduced to aquaculture in recent years, following the decline of natural populations and the resulting price rises. Males and females of this species mature sexually after 8–13 and 10–16 years, respectively, in nature. The factors affecting puberty are not fully recognized.

METHODS: Fertilized Russian sturgeon eggs from Russia were bred up to 7 years of age at a commercial farm. stFSHbeta, stLHbeta and glycoprotein alpha-subunit were cloned. Expression of stFSHbeta and stLHbeta in the pituitaries was determined with Real Time PCR, for 1-, 2-, 3-, 4- and 5-year-old fish. Plasma E2 and 11KT were measured by ELISA, and the gonadal developmental stage was determined by histology. Fish were sorted by gender with endoscope technology. GnRH, alone or combined with metoclopramide, was injected into 3-, 4- and 5-year-old females, and E2 levels were determined.

RESULTS: The expression levels of both stFSHbeta and stLHbeta subunits were found to be higher in 3-, 4-, and 5-year-old fish than in 1-year-olds. mRNA levels of stLHbeta were higher than those of stFSHbeta in both genders, and mRNA levels of stFSHbeta were significantly higher in females than in males. Even at 5 years, the gonads of all the female fish tested were at the pre-vitellogenic stage, with small oocytes and very low plasma estradiol levels, and they did not respond to exogenous GnRHa. Vitellogenesis started at 6 years of age, when GnRHa treatment elicited a marked increase in E2. However, among males, at 3-4 years testes already contained spermatids and spermatozoa, and showed high GSI levels, stLHbeta expression and 11-KT levels. We found that the ovarian development was highly asynchronous at the early vitellogenic stages.

CONCLUSION: The sexual development of Russian sturgeons bred in captivity was investigated according to morphological, histological and endocrinological parameters. The unusually high levels of stLHbeta gene expression in immature sturgeons indicate a still unidentified role for this hormone. The information on gonadal development in relation to age and body weight is an important management tool for sturgeon aquaculture for reproduction and caviar production.

O 64

Effects of handling on stress, ionic plasma indicator and spawning success of farmed Siberian sturgeon, *Acipenser baerii* Brandt

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BACKGROUND: In farming conditions, spawning of sturgeon is induced by hormonal treatment. There are no external indices which allow one to correctly identify spawners. Further, in order to assess the advancement of maturation it is necessary to remove some ovarian follicles for further observations. As a result, handling is necessary and this may be considered as a stressful situation which possibly worsens spawning results. The aim of the study was to test this hypothesis.

METHODS: 11-year-old females were randomly chosen among fully mature fish. Cannulated fish (N1= 16) were compared to currently managed fish (N2=23). Controlled fish were sham injected in both cases. With regard to the cannulated fish, two protocols were applied. 8 fish, referred to as non-resting females, were cannulated and injected straight away. The other 8, referred to as resting females, were cannulated, and hormonally stimulated 32h later without handling. This latter procedure corresponds to the current spawning protocol. It also allowed us to assess the effects of the injection on plasma characteristics in the absence of handling. Cannulation lasted up to about 6h post spawning. Profile stress indicators (cortisol, glucose and lactate) as well as ion contents (Ca^{++}, Na^{+}, Mg^{++}, K^{+}, Cl^{-}) were determined. Controlled fish were blood-sampled when handled. Ovarian follicles were sampled for aspect, size, germinal vesicle migration (polarization index, PI), and in vitro maturation competence (time to reach 50% of maturation (GVBD)) referred to as ET_{50}. Females and males were injected with carp pituitary extract. Spawning success was assessed by embryonic rates at mid-gastrulation. ET_{50} was calculated by maximizing the classical log likelihood function. Two or more samples were compared with U-test and Kruskall-Wallis ANOVA respectively.

RESULTS: Weight (7.4 to 9kg), ovarian follicle diameter (3.3 to 3.5 mm), PI (3 to 6%) were similar for all fish groups. ET_{50} was significantly higher in non-resting cannulated females compared with other groups (16.2 vs 11.2 and 12.1h). Embryonic survivals did not differ significantly for cannulated females (68.8 and 71.3%), but are are significantly higher than in currently spawned fish (non cannulated) (42%). Profiles are similar within the two groups of cannulated fish. Ca profiles remained at a steady state during the experiment, whatever the fish-group. In contrast, significant decreases were recorded, fairly small for K and Mg and more pronounced for Na and Cl. Lactate peaks (~400 $mg.l^{-1}$) were recorded early post handling. Glucose peaks were more delayed post handling and recovery was also delayed. Cortisol peaks (up to 200 $ng.ml^{-1}$) appeared early post handling and/or hormonal injection and disappeared rapidly.

CONCLUSION: Initial ionic values (in a narrow range) are in good agreement with the few data already published on other sturgeon species and might be considered as basic values. Surprisingly, cannulation led to better embryonic survivals, thus suggesting that additional stress impacts positively on spawning. Glucose profiles suggest that confining has no detrimental effects on spawning success. Decreases in Na and Cl might suggest that the ions are transferred to the coelomic fluid. The present cannulation protocol for spawners proved to be very efficient in exploring the changes during this key phase in sturgeon reproduction.

O 65

Sexual maturity, reproductive behaviour and fertility in the first-generation hybrids of *Blicca bjoerkna* L. × *Abramis brama* L.

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BACKGROUND: Silver bream, *Blicca bjoerkna* and common bream, *Abramis brama* are two common cyprinid fish species in European waters and have the same time and requirement for spawning activities. In rivers, anthropic activities has resulted in the destruction of suitable spawning sites what caused on artificial increase of the numbers of these hybrids. Natural hybrids from these species have been observed by several authors but few experiments have been conducted to evaluate the sexual maturity, the reproductive behaviour and the fertility of these hybrids. This study aim to further examine the effect of artificial hybridization of silver bream × common bream on these biological aspects.

METHODS: Age and size at the first sexual maturity were examined in hybrids and parental species rearing in captivity. Mature females were gravid and males produced milt. Reproductive behaviour was studied into two experimental conditions (at 20°C, in duplicate): –(i) hybrid reproduction, a female hybrid was placed to reproduce with three corresponding hybrid males and –(ii) mixed reproduction, a female hybrid was mixed with a corresponding hybrid male, a male silver bream and a male common bream. Spawning-mating relationship, numbers of participating males to the mating and aggressiveness behaviours were analysed during 1 day from 8 to 18 hours using a remote controlled video system. Mating success was evaluated 1 day after spawning from samples of 100 eggs. To test the fertility of hybrids, eggs from female hybrid were divided into four equal parts and artificially fertilized with the sperm of one of the following: a corresponding hybrid male, a hybrid male from the reciprocal crossbreeding, a male silver bream and a male common bream. Survival rate was estimated following the developmental stages (in duplicate): eyed embryos (1 day post-fertilization from samples of 100 eggs), viable hatchings (3 days post-fertilization from samples of 500 eggs), and larvae to dry food consumption (5 weeks old from samples of 50 embryos).

RESULTS: Females and males in each F1 cross have attained their first sexual maturity in the same age. Mature F1 hybrids were observed at the same moment than common bream, the later parental species. Maturity sizes of male hybrids were intermediate to parents but female hybrids were closer to the highest parental species, common bream. Parity of spawning and mating numbers was observed. Female hybrid mated simultaneously and successively with all types males except the male of common bream, resulting in fertilised eggs. Male hybrids exhibited aggressiveness and territorial activities as in parental species common bream. A successful survival rate was observed in all post-F1 (F2 and backcross) generations at each developmental stages.

CONCLUSION: Our study has demonstrated that these hybrids have the biological capacity to reproduce and to exhibit a high level of sexual activity. Post-F1 generations produced have proved that these hybrids are fertile. In phylogenetic relationships, it would be better to consider these two species in the same genus as suggested by some authors. From an ecological point of view, this situation has implications on the genetic integrity of parental species, the biodiversity preservation and the population dynamics in natural environments.

O 66

The establishment of the diploid gynogenetic hybrid clonal line of red crucian carp× common carp and its application

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BACKGROUND: What is the nature of the diploid eggs generated from the allotetraploid fish or allodiploid fish ? Why did the allodiploid fish produce the diploid eggs ? What are the biological characteristics of the diploid gynogenetic hybrid clonal fish ? What are the uses of the diploid gynogenetic hybrid clonal fis h? These questions are worth answering.

METHODS: We obtained a bisexual fertile allotetraploid hybrid population of red crucian carp (*Carassius auratus gibelio*) (♀) × common carp (*Cyprinus carpio L.*) (♂).Their tetraploidy has been maintained 14 generations from F_{13} to F_{15}.

RESULTS: The female and male allotetraploid hybrids generated the diploid eggs and diploid sperm, respectively. After activated by the UV-treated haploid sperm of scatter scale carp, but without the treatment for doubling the chromosome number which was required in the haploid eggs' gynogenesis, the diploid eggs which possessed two sets of chromosome were able to develop into the first gynogenetic generation (G_1, 2n=100). Interestingly, G_1 hybrids were all-female and were able to produced a great number of diploid eggs which developed into the second gynogenetic generation (G_2, 2n=100) in the same way as G_1. G_2 hybrids were also all-female and also produced a great number of diploid eggs which developed into the third gynogenetic generation (G_3, 2n=100) in the same way as G_1 and G_2. The G_1, G_2 and G_3 grew faster and had higher anti-disease ability than the allotetraploid hybrids. The improved tetraploid hybrids (G_1×4n) were formed by crossing the diploid eggs of G_1 with the diploid sperm of the allotetraploid hybrids. After mating themselves, 98% G_1×4n hybrids produced the tetraploid hybrid offspring with 200 chromosomes. But 2% G_1×4n hybrids produced three types of diploid offspring with 100 chromosomes: red crucian carp with single tail, goldfish with bifork tail and common carp with single tail.

CONCLUSION: We established a diploid gynogenetic hybrid clonal line (G_1, G_2 and G_3) of red crucian carp× common carp. It was conclude that the formation of the diploid eggs produced by the diploid hybrids was probably due to the pre-meiotic endoreduplication in the early oogonia. With the special reproductive way to produce the diploid eggs, the diploid gynogenetic hybrid clonal line of red crucian carp × common carp had important significances both in biological evolution and genetic breeding application. In evolution, the diploid gynogenetic hybrid clonal line will create a new diploid species with gynogenetic reproductive nature. In fish genetic breeding, the diploid eggs generated from this clonal line are the important resources of diploid gametes which can be used to produce triploid or tetraploid fish. This study also suggested that the gynogenesis had the effect to make G_1×4n hybrids diversify.

O 67

Application of Reproductive Technologies to Captive Breeding Programs for Conservation of Endangered Pacific Salmon: Trials, Tribulations, and Triumphs

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BACKGROUND: Captive breeding programs have been established to prevent extinction and aid recovery of Pacific salmon listed as endangered under the US Endangered Species Act. However, several reproductive problems limit their effectiveness, including early age of male puberty, asynchronous maturation of adults, inadequate development of secondary sex characters and behavior, and poor fertility. In contrast to commercial aquaculture, selective breeding cannot be used to mitigate these problems since preserving the genetic diversity of the wild stocks is paramount. Therefore, several studies were conducted to develop or refine methods to identify mature fish well in advance of spawning, control age and seasonal timing of reproduction, and improve gamete quality.

METHODS: Several experiments were conducted to determine effects of seasonal variations in growth rate and body composition on age of puberty in males (chinook salmon) and the rate of oocyte growth, fecundity, and egg size in females (chinook and coho salmon). Ration and fat content in the diets were varied to control growth rate and body fat levels. Gonad histology and plasma steroid levels were monitored to identify the seasonal timing of puberty onset. GnRH implants were tested as a means to advance and synchronize spawning, and improve fertility of adults spawned in captivity. Effects of GnRH implants on spawning behavior were also examined in chinook salmon maintained in an artificial stream environment. Screening for Y-chromosome specific genes in DNA extracted from fin-clips, ultrasound and plasma sex steroid levels were tested as methods to identify gender and state of gonad development.

RESULTS: Restricting body growth during the fall-winter period in yearling chinook salmon significantly reduced the percent of males maturing at age 2. Body growth had a strong positive effect on the rate of previtellogenic oocyte growth, but did not affect egg number up to the late cortical alveolus stage. GnRH implants effectively advanced spawning without negatively affecting gamete quality or spawning behavior. Maturing males could be identified as early as 7 months prior to spawning using plasma 11-ketotestosterone levels, while ultrasound was effective in both sexes up to 5 months prior to spawning. A PCR method to screen for the GH-pseudo gene was >95% accurate for identifying chinook and coho salmon males.

O 68

Environmental control of cyclical reproduction of tropical freshwater fishes: evidence from comparative experimental data

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BACKGROUND: Field data from South America, Africa, and South-East Asia clearly indicate that cyclical reproduction of tropical freshwater fishes is a common phenomenon. However, the environmental control (proximate factors) of the cyclical reproduction is poorly investigated up to now.

METHODS: Fishes were subjected to various combinations of variations of environmental factors imitating the effects of high and low water conditions. Imitation of high water condition was performed through imitation of rain (R), decrease of conductivity (C) and increase of water level (WL). Imitation of low water conditions was brought about by increase of conductivity. The reaction of the fish was measured either by using the maturity coefficient and/or by spawning or by determination of Testosteron and Estradiol titers.

RESULTS: In eight neotropical gymnotiform fishes gonad maturation and spawning could be obtained by variation of all three factors (R, C, and WL), or only two factors (WL and C) or just (C) depending on the species. The basis of the regulation is induction of gonad maturation (yolk vesicle formation and vitellogenesis) and all stages of spermatogenesis. In more than ten African mormyrid species decrease of C alone was sufficient to induce gonad maturation; spawning could be obtained in nine species. In two species of the family schilbeidae and two species of the genus *Synodontis* (mochokidae) variation of all three factors led to gonad maturation. Detailed experimental analysis in *Eutropiellus vandenveyeri* (schilbeidae) showed that imitation of R was sufficient to induce complete gonad maturation. In the South-East Asian catfish *Kryptopterus bicirrhis* variation of all three environmental factors led to complete gonad maturation. In the two notopterid knifefishes *Xenomystus nigri* (Africa) and *Notopterus notopterus* (South-East Asia) and the African *Polypterus senegalus* no clear correlation between variation of environmental factors and gonad development or sex hormone titers could be found.

CONCLUSION: We showed experimentally that cyclical reproduction is found in tropical fishes from South America, Africa, and South-East Asia. The field data indicate that many more tropical freshwater fishes are characterized by cyclical reproduction though experimental evidence is still lacking. The experimental data of the African fishes (mormyrids, schilbeid fishes, mochokid fishes, notopterid knifefishe) studied show that the proximate factors that regulate the cyclical reproduction depend on the physiology of the species and therefore represent a nice example of convergent evolution.

O 69

Density effects on reproductive performance in natural populations of the staghorn damsel *Amblyglyphidodon curacao*

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BACKGROUND: There are few studies on the effect of fish density on reproductive performance in natural populations. The present study examined this phenomenon in a tropical damselfish sampled from the wild.

METHODS: Staghorn damsels were sampled underwater by divers over 2 reproductive seasons from 4 reefs in the Cairns sector of Australia's Great Barrier Reef, chosen for differing coral cover and fish density characteristics. Plasma levels of estradiol (E_2) [females], 11-ketostestosterone (11KT) [males], and testosterone (T) [both sexes] were measured by RIA and correlated with reproductive development, and population density at each of the sites.

RESULTS: In year 1, fish from the site where density of staghorn damsels was highest were smaller than fish from other sites, and males had lowest plasma levels of 11KT (0.5 $ng.ml^{-1}$ vs highest site levels of 2.0 $ng.ml^{-1}$), but there were no differences in plasma T levels. Plasma T and E_2 levels were not different among females from different reefs. In year 2, fish densities were again highest at the same site as in year 1 and again fish tended to be smaller than at other sites. Plasma T and 11KT levels did not differ between sites among males; however, plasma E_2 and T levels in females were lowest at the high density sites (1.1 vs 2.2, and 1.4 vs 3.3 $ng.ml^{-1}$ for mean minimum and maximum sites values for E_2 and T respectively). Fecundity was also lowest at the high density site, and highest in fish from the site where fish density was lowest.

CONCLUSION: The results suggest that high density sites are characterised by fish of smaller size, lower reproductive output, and on occasion, reduced plasma levels of T and E_2 in females, and 11KT in males. This may result from increased competition for planktonic food at high density, and is consistent with a similar effect previously found by us in the brooding damsel *Acanthochromis polyacanthus*. However, these results are at variance with studies on temperate damselfishes where increased population density (and consequent intensity of behavioural interactions) results in elevated plasma levels of gonadal steroids. This suggests that population density will have modifying effects on reproductive performance in natural populations of fish but that these effects may be habitat-specific.

O 70

Comparative analysis of reproductive strategies of European freshwater fishes: applications to the domestication of new species in aquaculture

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BACKGROUND: There is currently an increasing demand for the diversification of the production of fish species in aquaculture. Yet, today, the domestication of a new species is still essentially realized by a long, costly, and usually empirical zootechnical approach, which may be optimized. Within this context, it would be useful to foresee how a new species may be domesticated. Thus, the two main objectives of the present study are: (i) to establish a numerical clustering of fish species according to their reproductive traits, and (ii) to evaluate the potential applications of the results for the domestication of new species, i.e. whether it is possible to extrapolate the knowledge acquired on one species to other ones belonging to the same group.

METHODS: Data, issued from an extensive literature search, are currently hosted within a new database called STOREFISH (acronym for STrategy Of REproduction in FISH). It comprises 80 species and 50 reproductive traits. The traits are grouped into four main categories: 7 characters for egg, 7 for larvae, 21 for breeders, and 15 for spawning conditions. Overall, 78 % of the character/species matrix cells have been successfully implemented, from around 1500 biographical references. All statistical analyses have been performed using the SPAD ® 6.0 software package on each of the four character categories either separately or all together.

RESULTS: A similar grouping was obtained in all analyses performed. Most of the species usually fell into the same four to five main groups, or guilds, except for few species which clearly displayed an odd combination of characters, e.g. the burbot *Lota lota*. These groups and their discrimination will be further discussed during the presentation as well as the identification within each functional group of a paragon or a “fish model” species.

CONCLUSION: This study presents the first attempt to establish such an extensive numerical clustering of fish species according to such a large number of reproductive traits. From a fundamental point of view, this study mainly allowed to better evaluate the part of phylogeny and ecology on the clustering of species (i.e. the convergence in reproductive strategies), and from an applied point of view, it may help in the future to fit better the rearing conditions with a new species to be domesticated by referring to some known “fish model” belonging to the same group.

O 71

Previtellogenic oocytes constitute a sizable volume of the spawn in *Pterapogon mirifica*, an apogonid with direct development and transient sexual dichromatism

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BACKGROUND: Teleosts exhibit a large variability in their reproductive strategies, including ovarian development, mating systems and spawning patterns. However, the sequence of oocyte development stages from primary growth to mature oocytes, which are the only ones ovulated, is a generalized occurrence. In contrast, up to about a third of the spawn volume in *Pterapogon mirifica* consists of previtellogenic oocytes.

METHODS: Two pairs of adult wild caught reproduced in captivity five times. Mating, spawning and egg-clutch transfer were observed and video recorded. Three egg-clutches were retrieved from the males at different times during the first 24 hrs. Ovaries from an ontogenetic series of juveniles raised in captivity and adult specimens were studied as well as ovaries from adults in different phases of their reproductive cycle.

RESULTS: The egg-clutch consisted of two markedly distinct (but joint) sections: the first in being released was composed by a mass of previtellogenic oocytes, the second part consisted of mature ova. Ovaries from adult specimens showed a marked division in oocyte composition, with a clear division between two parts. The anterior carried oocytes in different states of development, whereas the posterior part was full of previtellogenic oocytes of a similar size about 0.8-1.0 mm forming a "previtellogenic posterior mass" (PPM), which occupied between 27 and 39% of the ovaries' length, and contained between 12000 and 15000 oocytes. The younger age at which a female showed a similar division in ovary composition was nine months. Ovaries from adult females in different stages of the reproductive cycle carried between three and four discrete size classes of oocytes in addition to the PPM. A mature female had a total of 172 mature oocytes (2.8-3.3 mm), 218 vitellogenic oocytes (0.5-0.9 mm), 1160 previtellogenic oocytes, and 12548 forming the PPM. Gonadosomatic indexes of juveniles up to nine months did not show an important variation, but it increased significantly at 10 months.
A synopsis of the embryologic development and a short video of mating and egg transfer are presented.

CONCLUSION: There have been reports of apogonids cannibalizing entire or part of their incubating egg-masses. The PPM disappeared during the first day and more likely was eaten by the males. The PPM ingestion could prevent the male for eating part of the egg-clutch during the first hours of the incubation period.

O 72

Timing and determination of fecundity and skipped spawning in Atlantic cod

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BACKGROUND: Forecasting stock size is an integral part of responsible and sustainable management of marine resources, and requires intimate knowledge of stock-recruitment relationships. Models that accurately depict these relationships have therefore long been sought. These models have evolved in complexity from models using correlations between spawning stock biomass (SSB) and recruitment to models including various indices of individual condition. Life history theory further predicts that reproductive investment should depend on age and size-at-age, and incorporating age-structure also increased the explanatory power of recruitment models. Emerging evidence suggest however that there is a critical time window during early vitellogenesis, which is highly influential for potential fecundity. This time window may also be linked to the decision to skip spawning, a common occurrence in many mature fish. Knowledge of the energy reserves of the mature population during this critical time may therefore have considerable implications for stock-recruitment theory. We examined this hypothesis in North East Arctic cod (Gadus morhua)

METHODS: To examine if there is a time window that is linked to both the decision to skip spawning and final potential fecundity, wild cod caught at the spawning grounds in April were subjected to different feeding regimes in the laboratory. Fish have been sampled monthly for steroid values, length, weight and ovarian biopsies. Shortly, before the onset of spawning, all fish will be sacrificed and fecundity and the incidence of skipped spawning verified. Simultaneously, a large field sampling program to collect cod has been undertaken in the Barents Sea, the natural feeding ground of the North East Arctic cod.

RESULTS: We will use image analyses to determine fecundity of females from the laboratory. We will correlate this to fish weight and condition through the course of the experiment. Further, for potential skipped spawners, we will use steroid values and histology to access whether skipping fish never started maturation or if this occurred through massive atresia and reabsorbtion of eggs. This will again be linked to fish size and condition. The field data will be used to examine the occurrence of skipped spawning in the field and link this phenomenon to fish age and condition. Skipped spawning of male fish will also be examined in the laboratory and in the field

CONCLUSION: The results will give important information of the occurrence and mechanisms regulating fecundity and skipped spawning in cod. The studies will also shed new light on the reproductive physiology of the gadoids in general, an important group of commercial finfish, subject to overexploitation throughout their range.

O 73

Sperm competition during *in vitro* fertilization in common carp (*Cyprinus carpio* L.)

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BACKGROUND: Sperm competition occurs when sperm from different males compete for fertilization. Mating systems among fish are very diverse. Fishes show one of the widest ranges of sperm competition intensity of any animal group. The effect of sperm competition during *in vitro* fertilization has been studied. The aim of this *in vitro* fertilization study was to compare competitive success of five males using four different sperm/egg ratios.

METHODS: Sperm concentrations were evaluated for five males of common carp (M1-M5) to compose pool of sperm (heterosperm) with equal number of sperm of each of males. Four batches of eggs, each of 5g (800pcs/g), were fertilized using volume of heterosperm relevant to the ratios of 5000 (G1), 10 000 (G2), 20 000 (G3) and 100 000 (G4) spermatozoa per egg. Then, the fertilized eggs were individually incubated (in four groups G1–G4) and after hatching, progenies were genotyped. Genotyping of four microsatellite loci (MFW1, MFW6, MFW7, MFW28) with an automated sequencing device was used to determine the paternity in four groups of progenies. From 54 to 91 individuals were sampled for paternity analysis after incubation. The motility of spermatozoa was observed and recorded using a dark filed microscope at 200× magnification with stroboscopic illumination and a video camera. Velocity and percentage of motile spermatozoa were extracted from successive video frames with Micro-image software (version 4.0.1. for Windows).

RESULTS: Fertilization (23.67-94.82 %) and hatching (23.67-92.22 %) control rates of individual males were counted. Sperm velocity and percentage of motile sperm were measured in range of 85.03 to 137.56 μm s^{-1} and 1.99 to 93.45 % at 15 sec post sperm activation, respectively. Contribution of individual males whose sperm was used to compose heterosperm was very diverse in progenies. Males with very low level of sperm motility (M3 4.45%, M4 1.95%) were represented by low contribution in four groups (G1-G4) of progenies. Significant differences in contribution of males were also found among individuals with similar percentage of motile spermatozoa (M1 90.74 %, M2 93.45 %, M5 89.74%) and sperm velocity (M1 126.49 μm s^{-1}, M2 136.84 μm s^{-1}, M5 137.56 μm s^{-1}). Different level of spermatozoa per egg (G1-G4) did not change ratio of sired progenies by individual males.

CONCLUSION: Contribution of individual males whose sperm was used to compose heterosperm was very diverse in progenies. Individuals with very low level of motility were represented by low contribution in all groups of progenies. In males with similar sperm parameters, variation in number of sired offspring was high and cannot be explained only by sperm motility and velocity. Thanks are also expressed to USB RIFCH No: MSM 6007665809 and GACR no. 524/06/0817 for financial support.

POSTER PRESENTATIONS

Reproductive Neuroendocrinology

P 1

Diversity of circadian organisations and light sensitivities among teleosts species; implications for the management of reproduction in commercial culture

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BACKGROUND: The photoperiodic entrainment of reproduction in fish is highly divergent; with species entraining to signals that are most relevant to their living environment. Intensive aquaculture is increasingly reliant on artificial photoperiod manipulation to regulate reproduction to improve production performance. However, little information exists with regards to the species specific light sensitivities which will determine the appropriate systems to be used.

METHODS: Three sets of experiments were performed on three commercially important teleost species, Atlantic cod (*Gadus morhua*), Atlantic salmon (*Salmo salar*) and European seabass (*Dicentrarchus labrax*). In the first experiment, a bi-lateral ophthalmectomy was performed and plasma melatonin was analysed at mid- day and night over the subsequent 2 days. In the second experiment, pineal glands were placed in an *ex-vivo* culture system and subjected to additional nocturnal illumination of varying intensities or spectral content to define light sensitivities through melatonin analysis of the culture media. In the third experiment individuals were acclimated to a common photoperiod with groups being subjected to a range of additional nocturnal illumination from complete darkness to full strength daylight to define *in-vivo* perception of day and night.

RESULTS: Ophthalmectomy had no impact on circulating melatonin rhythms in salmon while in both cod and seabass the circulating melatonin levels were suppressed by ~50%. *Ex-vivo* pineal cultures revealed a wide difference in the pineal perception of light between the three species studied. The intensity of illumination considered comparable to complete darkness (i.e. threshold of perception), was $3.8x10^5$, $1.2x10^5$ and 3.8×10^7 $W.m^{-2}$ for salmon, seabass and cod respectively. *In-vivo* threshold levels were not reached due to technical limitations. In all species the lowest intensities tested ($8.3x10^{11}$ $W.m^{-2}$ for salmon and cod; 6.4×10^{12} $W.m^{-2}$ for seabass) resulted in elevations of melatonin to ~40-50% of night time levels. Finally, longer wavelengths were significantly less efficient at suppressing melatonin production.

CONCLUSION: These results highlight for the first time the species specificity of light perception in teleosts. The demonstration of the use of retinas for non visual light perception has implications for the definition of the neuroendocrine system used to entrain sexual maturation. The wide difference in light perception by the pineal glands between species suggests evolution and/or adaptation to the environment the species inhabit. These results have significant implications for the design of culture systems to manage reproduction during commercial ongrowing, requiring species specific lighting systems to be designed.

P 2

Melatonin receptors in the brain and the pituitary of a Venezuelan catfish, "Sierra Negra" (*Oxydoras sifontesi*): a 2-[$^{(125)}$I] iodomelatonin binding study

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BACKGROUND: Melatonin receptors are expressed in neural and peripheral tissues and mediate melatonin actions on photoperiodic control of fish reproduction and behaviour in various species. *O. sifontesi* presents an annual variation in plasma melatonin concentration inversely related to the annual gonadal maturation cycle, which suggest a putative role for melatonin as the endocrine messenger of daily and seasonal photic information to the gonadal axis. In this work we aimed to localize and to characterize the melatonin receptors in the upper division of the hypothalamus-pituitary-gonadal axis.

METHODS: The central and pituitary melatonin receptors were investigated by in vitro autoradiography, and receptor binding assay, using 2-[$^{(125)}$I] iodomelatonin (I-mel) as a radioligand. The brains were rapidly dissected after decapitation of anaesthetized fish, freeze in dry ice and processed by cryostat sectioning for autoradiography or by differential centrifugation for the membrane radioreceptor binding study.

RESULTS: We have showed that I-mel binds specifically in brain regions associated with visual areas, such as the thalamic region and the optic tectum. An intense labelling was observed in the anterior hypothalamic preoptic area. A clear, but less intense labelling was found in the pituitary. The radioreceptor assay shows that specific binding to the brain membranes was rapid, stable, saturable and reversible. The I-mel binds to a single class of receptor site with a high affinity KD of 49 +/-7.5 pM and total binding capacity (Bmax) of 4.6 +/-0.26 fmol/mg protein.

CONCLUSION: Melatonin binding sites in the *O. sifontesi* brain are highly specific. The distribution of melatonin receptors in the brain was closely similar to that reported for most teleosts species studied. However, the presence of specific binding of I-mel in the pituitary has been reported in very few species. This result, together with the labelling found in the anterior hypothalamus; support the possibility of a direct action of melatonin in regulating the activity of the reproductive neuroendocrine axis.
Further efforts are in progress to localize specific melatonin binding in the gonadal tissue and to determinate the molecular subtype of melatonin receptor expressed in this species.
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P 3

Melatonin receptors in a flatfish, *Solea senegalensis:* cloning and expression of three different receptor subtypes

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BACKGROUND: Melatonin is synthesized rhythmically in the pineal organ and retina of fish, representing a daily and a seasonal signal. Although this photoperiodic transducer has been implicated in reproduction, results are contradictory and seem to be dependent on the doses of melatonin used, the photoperiod conditions, the season and the stage of the animals. Melatonin actions are mediated through specific transmembrane G-protein coupled receptors. Three different melatonin receptor subtypes, MT1, MT2 and Mel1c, have been described in vertebrates. We report here the partial cloning and expression of MT1, MT2 and Mel1c melatonin receptors in a flatfish, the Senegal sole, *Solea senegalensis*.

METHODS: Cloning of partial sequences from three different melatonin receptor subtypes was performed using cDNAs from brain, retina and skin of Senegal sole and degenerated primers. Sequences obtained were elongated by 5' and 3' rapid amplification of cDNA ends (RACE) using sole cDNAs as template. Sequences were aligned and analyzed by using ClustalW (EMBL-EBI). RT-PCR amplification was accomplished on sole cDNAs from central and peripheral tissues using specific sole MT1, MT2, and Mel1c melatonin receptor forward and reverse primers.

RESULTS: We have cloned partial sequences of three different melatonin receptors in sole. The alignment and analysis of the percentages of identity with melatonin receptor sequences available in gene database revealed that melatonin receptors cloned in sole belong to the MT1 (Mel1a), MT2 (Mel1b) and Mel1c subtypes. RT-PCR analysis showed a high MT1 receptor (ssMT1) expression in the brain, in particular in the telencephalon, optic tectum and cerebellum, but also in the pituitary and peripheral tissues as gonad, gill, liver, kidney, spleen and skin. Sole MT2 receptor (ssMT2) expression was relevant in the retina, optic tectum, telencephalon and hypothalamus, but its expression was also evident in the medulla and pituitary. This melatonin receptor subtype is practically absent in peripheral tissues, except for the gonads and gills. Finally, sole Mel1c receptor (ssMel1c) expression was conspicuous in skin but also in the retina, different brain areas and pituitary.

CONCLUSION: The Senegal sole express three different melatonin receptors. The differential pattern of expression of these melatonin receptor subtypes in sole suggests that they might be involved in different physiological processes. Furthermore, the expression of these melatonin receptors in the retina and brain neuroendocrine areas, as well as in pituitary and gonads suggests a role of melatonin in the reproductive axis of this species.

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P 4

The pineal complex in the Senegal sole, *Solea senegalensis:* a morphofunctional and carbocyanine dye tract tracing study

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BACKGROUND: The photoperiod constitutes the main environmental factor entraining circadian and seasonal rhythms. In fish, light reception is mediated by the pineal organ and lateral eyes, which transduce night length into a rhythmic melatonin secretion. Furthermore, retinal and pineal information exits from these photosensitive structures through neural projections that reach the brain. In this study we described the anatomy and histology of the pineal complex, identified the pineal photoreceptors and melatonin-producing cells and elucidated the central connections of the pineal organ in the Senegal sole.

METHODS: The anatomy and histology of the pineal complex of sole was analyzed using conventional histological techniques. The presence of melatonin-secreting cells and pineal photoreceptor cells was revealed using immunohistochemical techniques. The central connections of the pineal organ were elucidated by means of anterograde and retrograde tracing with the fluorescent lipophilic dye DiI.

RESULTS: The pineal characteristics reflect the metamorphic changes occurring in this species. The pineal complex of sole exhibits a very long and thin stalk that exits between the cerebral hemispheres and the optic tectum, courses rostrally along the midline and arrives up to the caudal pole of the olfactory bulbs. At this level, the pineal stalk leaves its midline position and turns of 90º towards the upper-right pigmented side of the head. The pineal stalk expands into a large and elongated pineal end-vesicle, which is firmly attached to the cartilage from the cranium bone. The pineal vesicle exhibits a multilayered epithelium rich in pinealocytes and a conspicuous internal lumen. The immunohistochemical analysis revealed that most of these pinealocytes synthesize melatonin and contain photoreceptive pigments. Pinealofugal projections exit from the pineal tract and innervated the rostral preoptic area, suprachiasmatic nucleus and ventral thalamus. Further caudal, this innervation reaches the dorsal thalamus, posterior commissure, pretectal area, posterior tuberculum and dorsal tegmentum. Pinealopetal neurons were evident in the ventromedial thalamus, periventricular pretectum and posterior tuberculum.

CONCLUSION: These works have provided us an important basis to understand the integration and transduction of photoperiodic signal in the sole. The existence of overlapping terminal fields from the pineal organ and retina in several brain areas as the suprachiasmatic nucleus, ventral and dorsal thalamus and pretectal complex suggests an important role of these regions in the integration of photoperiod information in this species.

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P 5

The European sea bass expresses three different melatonin receptors: cloning, expression and daily variations

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BACKGROUND: Melatonin actions on the regulation of rhythmic processes are mediated by melatonin receptors. A family of high affinity melatonin receptors, MT1 (Mel1a), MT2 (Mellb) and Mel1c, has been cloned in different vertebrate groups. MT1 and MT2 have been detected from fish to mammals, while the expression of Mel1c has been demonstrated only in fish, amphibian and birds. Although the amino acid sequences of these receptors show a high percentage of identity, differences in their tissue distribution and expression have been shown. Furthermore, several studies have reported the existence of diurnal/circadian variations in the melatonin receptor gene expression in neural tissues in mammals and fish. The aim of the present study was to clone the different melatonin receptor cDNAs present in the European sea bass, analyze their expression pattern in neural and peripheral tissues and examine daily variations in mRNA expression in the brain and retina.

METHODS: Cloning of partial sequences from three different melatonin receptor subtypes from sea bass was performed using RT-PCR and 5' and 3' rapid amplification of cDNA ends (RACE) using sea bass cDNAs as template. The expression of MT1, MT2 and Mel1c was analyzed by RT-PCR and in situ hybridization in central and peripheral tissues using specific primers and riboprobes. Daily variations of MT1 and MT2 mRNA expression in the optic tectum and retina were examined using real-time quantitative RT-PCR.

RESULTS: We have cloned total (MT1) and partial (MT2 and Mel1c) sequences of three different melatonin receptors present in sea bass. RT-PCR analysis showed that MT1 receptor was mainly expressed in neural tissues (retina and brain) but also in gonads, while MT2 mRNA expression was mostly detected in the pituitary and Mel1c mRNA expression was more conspicuous in skin. Using in situ hybridization we have evidenced the expression of sea bass MT1 receptor in cells from the gonad, retina (photoreceptor, bipolar, amacrine and ganglion cells) and visually related areas of the brain, as well as in neuroendocrine regions, cerebellum, tegmentum and hindbrain nuclei. Real-time PCR reveals daily rhythms of MT1 and MT2 expression in the optic tectum and retina of sea bass. These rhythms are characterized by higher expression levels during the nighttime and lower expression levels during the daytime.

CONCLUSION: The expression pattern and daily variations suggests an important role of these melatonin receptors in the processing of light information, but also in the regulation of neuroendocrine systems and behavioural/motor rhythmic activity in sea bass.

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P 6

Evidence for the existence of the KISS1/KISS1R pathway in fish

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BACKGROUND: In mammals, the onset of puberty has been shown to be regulated by KISS1 and its receptor, KISS1R. The *kiss1* gene encodes a family of neuropeptides called kisspeptins, which activate KISS1R and play a role in the neuroendocrine regulation of gonadotropin-releasing hormone (GnRH) secretion. In fish, however, the mechanisms underlying the initiation of puberty are poorly understood and the role of the KISS1R/KISS1 pathway in puberty has yet to be established. In this study, we investigated the presence of this pathway in fish, using both a comparative genomics approach and empirical studies in cyprinid fish.

METHODS: A selection of bioinformatic tools were employed in order to search for the existence of *kiss1* and its receptor in the zebrafish genome. This approach involved BLAST searches and genomic comparisons across vertebrate species including human, mouse, zebrafish (*Danio rerio*), *Takifugu rubripes* and *Tetraodon nigroviridis*. The sequence information derived was used to isolate these genes from zebrafish and fathead minnow (*Pimephales promelas*). Bioinformatic approaches were used to screen upstream regions of these genes for evolutionary conserved regulatory elements. Gene expression profiles for *kiss1*, *kiss1r* in the brain, and a selection of other genes in the brain-pituitary-gonad axis known to be involved in reproduction, were measured throughout sexual development using real-time PCR.

RESULTS: Putative sequences were found for both *kiss1r* and *kiss1* in the zebrafish genome. *kiss1r* was isolated from the brains of zebrafish and fathead minnow and showed high homology with their mammalian homologs. A putative cDNA for *kiss1* was obtained from the brain of zebrafish only. The sequence for *kiss1* was less well conserved, but the region encoding kisspeptin-10 was highly conserved (80-90% homology with mammalian kisspeptin-10). *kiss1* was found in a gene cluster that is highly conserved between vertebrate species. Conserved regulatory elements were identified in the upstream region of mammalian *kiss1*, and included oestrogen and androgen responsive elements. Expression patterns of *kiss1r*, and downstream genes involved in reproduction were consistent with those found in mammals at the onset of puberty. We are now characterising the biological function(s) of kisspeptins in fish reproductive development.

CONCLUSION: In this study, we identified gene sequences for both *kiss1* and *kiss1r* in the zebrafish genome and used this information to isolate and characterise these genes from zebrafish and fathead minnow (*kiss1r* only). The conservancy of the *kiss1r* sequence and conservancy in key elements of the *kiss1* sequence, together with their expression profiles, suggests strongly that the *kiss1* pathway plays a similar role in puberty in fish as for mammals.

P 7

Kisspeptin in Nile tilapia *O. niloticus niloticus:* receptor expression during early development and effects on the Brain-Pituitary-Gonadal axis

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BACKGROUND: In recent years, the novel neuropeptide, kisspeptin (Kiss-1), has been identified in mammals as a key regulator of puberty acting through the control of gonadotropin secretion. Its receptor, GPR54, has been found to play a vital role in the signaling of GnRH production during puberty. Recently, this receptor has been cloned and sequenced in tilapia. This would suggest the Kiss mechanism to be present and play a similar role in lower vertebrates. However data in teleosts are still very limited especially regarding the ontogeny of expression in relation to puberty.

METHODS: Quantitative real-time PCR assays were optimized for GPR54 and rGnRH-I by designing primers from previously published sequences. In a first trial, diel rhythm in GPR54 expression was studied in relation to photoperiod in adult tilapia. Secondly, whole embryos or larvae and juvenile heads were sampled from fertilization to puberty (4 month post-hatch). Total RNA was extracted from all samples and cDNA synthesized. Gene expression was quantified and normalized using β-actin as a reference gene. The timing of gene expression against gonadal stage of development was assessed. Furthermore, a number of tissues were screened to localize GPR54 expression. Then, a series of short term *in vivo* and *in vitro* trials were performed to investigate the effects of mammalian kisspeptin on gonadotroph cells LH and FSH and gonadal sex steroid production as well as the Brain-Pituitary-Gonadal axis (BPG) following kisspeptin injections.

RESULTS:
No clear diel rhythmic expression of GPR54 was observed during a 24 hour period (day-night) in tilapia brains. No detectable expression of GPR54 was observed during early embryonic development until after hatching occurred whereas GnRH-I appeared to be expressed from early embryonic stages. Furthermore, tissue expression screening showed this receptor to be solely highly expressed in the brain although detected in few other tissues. Preliminary *in vitro* results showed no effects of exogenous kisspeptin on gonadal sex steroid production (T and E_2). Finally, *in vivo* kisspeptin injections appeared to have a time and dose dependant effect on the BPG axis with for example an increase in plasma sex steroids.

CONCLUSION: Taken together, these results suggest that GPR54 and overall the kisspeptin mechanism would act similarly in tilapia than in higher vertebrates, by stimulating the BPG axis. Further work is required to determine the links and interactions between the environmental signals, the endogenous circadian rhythms and the kisspeptin pathway that determine the onset of puberty and entrain reproduction. A better understanding of these mechanisms would ultimately help to develop regimes to manipulate reproduction in commercially important fish species.

P 8

Expression of a GPR54 –like but no agonistic effect of mammalian kisspeptins in trout

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BACKGROUND: Mammalian kisspeptins are natural ligands of the previously orphan G protein-coupled receptor, GPR54. In the last 3 years, observations made in human and mouse revealed a totally unexpected, fundamental role of the KISS/GPR54 system in the control of puberty and reproductive function in mammals, and was proven as an essential regulator of GnRH. *In vivo* treatment with C terminal decapeptides (Kisspeptins, $KISS_{10}$) were found to induce GnRH release, and ultimately to increase gonadotropin and steroid hormone concentrations in the blood. Therefore, if a functional the KISS/GRPR54 system does exist in fish, it could open new ways to control fertility or spawning in aquaculture species. We initiated the study of this system in trout by checking for GPR54 expression and testing the efficiency of mammalian Kisspeptins at 3 stages of the reproductive cycle.

METHODS: A trout GPR54-like cDNA obtained from a pooled-tissue cDNA library was partially sequenced and expression of this receptor was measured by Q-PCR in the anterior brain and by cDNA array in the testis. The effects of mouse and human $KISS_{10}$ on LH or testosterone plasma concentrations were assayed *in vivo* in immature and maturing males (2 intraperitoneal injections at 3 hours interval, total: 600µg/kg). $KISS_{10}$ action on ovulation/spermiation was tested in fully mature fish (2 injections of 300µg/day for 3 days). Effects were compared to those obtained after GnRH treatment (Gonazon, 40µg/kg).

RESULTS: The candidate EST obtained presented 95 % homology with the tilapia GRP54-like (Parhar, 2004), 61 % with the human GPR54, and appeared as the best potential ortholog after in silico search in fugu and zebrafish genomes. The anterior brain of the trout (containing hypophysiotropic neurons) and the testis were found to express this receptor mRNA. The expression did not vary significantly over the reproductive cycle in males and females. In the pre-pubertal male, kisspeptins had no significant stimulatory effect on circulating testosterone concentration 45 min to 3 hours post injection. Similarly, at the beginning of active spermatogenesis (LH detectable in blood) $KISS_{10}$ treatment had no positive effect on LH or testosterone. Interestingly a tendency to a decrease in circulating testosterone and LH is observed after kisspeptin treatments. At spawning time, repeated injections of kisspeptins did not stimulate the % of ovulation or the volume of sperm. KISS treatments tended rather to inhibit these parameters. As expected, GnRH treatment increased hormone plasma levels and stimulated ovulation and spermiation at spawning time.

CONCLUSION: Although a GPR54 related receptor is expressed in reproductive trout tissues, the human and mouse Kisspeptins did not have stimulatory effects either on circulating hormones or on ovulation/sperm production, indicating that these mammalian peptides have no agonistic effects on GnRH secretion in trout. Conversely, our observations evoke a slight antagonistic effect of these peptides. A GPR54 ligand potentially involved in the regulation of reproduction remains to be identified in fish.

P 9

Molecular cloning and sequence characterization of a G protein-coupled receptor (GPR54) in the Senegal sole (*Solea senegalensis*)

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BACKGROUND: *Solea senegalensis* is a promising species for aquaculture due to its high market price. However, the reproduction of this species, particularly that of males, in captivity presents some problems related to gonad maturation. Recently, it was discovered that GPR54, a G protein-coupled receptor, plays a very important role in the appearance of puberty in vertebrates since it is involved in the regulation of GnRH synthesis. However, information on this receptor in fish is very scarce. Studies on this receptor would contribute to our understanding of the appearance of puberty in fish. In this paper, we present the cloning and sequence characterization of the GPR54 receptor in *Solea senegalensis*.

METHODS: Total RNA was extracted after tissue homogenization in TRIZOL reagent from brain of adult male and female *Solea senegalensis*. GPR54 gene was cloned using degenerated primers based on conserved regions from human, rat, mouse, tilapia and zebrafish. To obtain the full genomic sequence of the gene, including intron and exon sequences and boundaries, we designed additional specific primers. Tissue expression distribution was performed using real-time PCR.

RESULTS: A ~1000 bp cDNA sequence was obtained for the Senegal sole. Further, comparison of the GPR54 genomic structure across vertebrates, from fish to mammals, showed an extremely well conserved organization of this gene, with five exons and four introns. Intron-exon boundaries were located approximately at the same position, except exon 4 which contained an additional 325 bp long fragment precisely in the Senegal sole. Exon sequence comparison resulted in a high homology with the other few fish species and mammals for which the sequence of this gene is available, indicating an extremely well conserved function and structure of the protein over evolution. A phylogenetic tree using deduced amino acid sequences confirmed the homology between these sequences.

CONCLUSION: Senegal sole GPR54 shares the main global structure of previously identified GPR54 genes in mammals and fishes. The characterization of the Senegal sole GPR54 gene sequence provides the basis for further studies on the implication of this gene in the regulation of puberty in this species and in fish in general. Currently, gene expression patterns in different experimental conditions are being investigated.

P 10

Investigations into the molecular mechanisms controlling puberty in the fathead minnow (*Pimephales promelas*)

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BACKGROUND: In mammals, the KISS1 receptor (KISS1R; also known as GPR54) and its ligand KISS1 have recently been demonstrated as key factors regulating the onset of puberty, by potently enhancing the release of gonadotropin-releasing hormone (GnRH). In fish, however, the mechanisms underlying the initiation of puberty are poorly understood and the role of the KISS1R/KISS1 pathway in puberty has yet to be established.

METHODS: In this study, full-length cDNAs for *kiss1r* and *gnrh2* were cloned and characterised from the brain of fathead minnow (*Pimephales promelas*), through reverse transcription PCR and 5'/3'-Rapid Amplification of cDNA Ends (RACE) strategies. Their patterns of expression were established in adults (in seven tissues) and in individual brains of males and females throughout sexual development (at 16 time points from 25 to 140 days post fertilisation [dpf]; n = 6 for each sex at each time point), via real-time PCR. Gonad development was determined via histology in each fish used for molecular analyses.

RESULTS: In adult fish, both *kiss1r* and *gnrh2* were expressed in brain and gonad and not in pituitary, liver, intestine, gill, or muscle tissues. In adults, there was no sexual dimorphism in expression of *kiss1r* or *gnrh2* in brain (where expression levels were approximately 16-fold higher than in gonad) but both genes were more highly expressed in testes than in ovaries (6-fold higher for *kiss1r*, 1.7-fold higher for *gnrh2*). During sexual development, neural expression profiles for *kiss1r* aligned with those for *gnrh2*. In males, expression of *kiss1r* in the brain was low until approximately 50 dpf when there was a sharp increase and maximal expression at 60 dpf. This peak in expression corresponded with the appearance of spermatogonia type B in the testis. Subsequently, expression of *kiss1r* in the brain steadily declined. In females, *kiss1r* expression in the brain was low from 25-35 dpf then, coincident with the appearance of perinuclear stage oocytes in the ovary, increased progressively to a maximal level at 70 dpf, when cortical alveoli stage oocytes were first present in the ovary. Subsequently, there was a rapid drop in expression of *kiss1r* in the brain between 70 - 80 dpf, but moderate levels of expression continued into more advanced stages of ovarian development. These temporal patterns of expression of *kiss1r* and *gnrh2* were analysed with a wider suite of genes centrally involved in sexual differentiation and development in the same fish (including *estrogen receptor 1* [*esr1*], *androgen receptor* [*ar*], *dopamine receptor d2* [*drd2*] *cytochrome P450 19a1* [*cyp19a1*] and *cytochrome P450 19a2* [*cyp19a2*]).

CONCLUSION: The data presented demonstrate a strong association between the expression of *kiss1r* and *gnrh* during puberty and further emphasise a likely role for KISS1R as a positive regulator of pubertal development in fish.

P 11

Cloning, characterization and tissue distribution expression pattern of two GnRH receptors in pejerrey. Phylogenetic relationships

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BACKGROUND: The events leading to GnRH-stimulated gonadotropin synthesis and release from the anterior pituitary gland is dependent on the presence of gonadotropin releasing hormone receptors (GnRHRs). Today it is well known that multiple GnRHRs are expressed in a single species. In this context, the main objective of the present study was to characterize the cDNA sequences encoding GnRHR in our model species, the pejerrey Odontesthes bonariensis, document their expression pattern, and also present a phylogenetic analysis including representatives of other chordate GnRHR.

METHODS: Complementary deoxyribonucleic acid from brain/pituitary tissue of adult pejerrey, was used to perform RACE PCR. Then, RT-PCR analysis was performed with specific primers in different brain areas and extra-nervous tissues and organs in order to examine the distribution of GnRHR mRNAs. Phylogenetic maximum parsimony trees were generated with the TNT program and Jackknife re-sampling with 1,000 replicates was performed.

RESULTS: Two full-length GnRH receptors were cloned, sequenced and their structure characterized.
The expression pattern profile showed that both receptors were widely expressed both in the central nervous system and also in peripheral organs. Differences in the tissue distribution expression of both receptors were determined.
The phylogenetic analysis resulted into two main lineages of GnRHRs.

CONCLUSION: The two GnRHRs molecules obtained share the structural organization of the other vertebrate GnRHRs sequences. The expression data analysis suggests that in pejerrey GnRHRs could be mediated directly or indirectly many functions besides reproduction and the existence of GnRH systems independent of hypothalamic–pituitary–gonadal axis.
The phylogenetic results allowed hypothesizing that these molecules can be grouped into two main types as already proposed.

P 12

Cloning and distribution of two distinct dopamine D2 receptors in the brain and pituitary of the European eel

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BACKGROUND: Dopamine (DA) exerts a powerful inhibitory action on LH secretion and puberty onset in the eel. However the receptor mediating this inhibition had never been isolated. The previously shown absence of D1(A,B or C) receptors on the proximal pars distalis and particularly on the gonadotropes cells, together with the fact that antagonists of mammalian D2 receptors could release the inhibition on puberty onset, prompted us to look for the presence of a D2-like receptor in the eel (brain and) pituitary.

METHODS: Degenerate oligonucleotides were designed on the basis of the conserved nucleotide sequences of D2, D3 and D4 receptors cloned in different species of vertebrates, and used in PCR experiments to isolate a eel D2-like receptor. The brain and pituitary receptor distribution pattern was analyzed, both by qPCR and ISH.

RESULTS: Two full-length cDNAs encoding two putative DA receptors were obtained from silver eel brain. They potentially encode two proteins of 455 and 451 amino acids that exhibit the typical arrangement of a seven transmembrane domain G-protein coupled receptor. Both proteins contain highly conserved amino acid residues thought to be important for the binding of DA. The deduced amino acid sequence of the cloned cDNAs show a higher sequence similarity to known D2 than D3 or D4 dopamine receptors. Phylogenetic analysis also shows that both eel sequences belong to the D2 receptor subtype. They were thus designated as eD2A- and eD2B-R. The distribution pattern of their transcripts in the brain and pituitary exhibit largely overlapping territories. The main areas of eD2-Rs expression are the olfactory bulbs, the pallial and subpallial areas of the telencephalon. More caudally, the entopeduncular nucleus, preoptic nuclei, preglomerular nuclear complex, ventral thalamus, periventricular hypothalamus, optic tectum and cerebellum, as well as nuclei associated with the cranial nerves, all contained various amounts of eD2-R transcripts. The eD2A- and eD2B-R were expressed at comparable levels, however in a few nuclei of the ventral telencephalon and diencephalon eD2B-R largely predominates or was even the only one to be expressed. In the pituitary, the strongest labeling for both transcripts was observed in the pars intermedia (MSH cells). However, in the pars distalis, stained gonadotrophs and lactotrophs cells were also clearly observed.

CONCLUSION: Thus, in contrast to mammals and birds in which only one gene coding for D2-R is present, at least two are found in the European eel, as in a few other teleosts. They likely result from the major gene duplication event that occurred specifically in the teleost lineage. The small but functionally relevant difference observed among regional distribution of the two subtypes was probably an important constraint for the conservation of the newly duplicated gene.

P 13

Gonadal and steroid feedback on the hypothalamus-pituitary axis in striped bass (Morone saxatilis)

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BACKGROUND: The objective of the present study was to expand our understanding of the mechanisms of gonadal steroid feedback regulation of the hypothalamus-pituitary (HP) axis during several reproductive stages (juvenile, pubertal, adult) throughout the life cycle of the striped bass (*Morone saxatilis*).

METHODS: We investigated the effects of bilateral gonadectomy and steroid replacement on the endocrine correlates of the HP axis, such as GnRH, GnRH-receptor and GtH gene expression levels, as well as GnRH peptide and LH protein levels, *in vivo*. We also developed a brain-slice culture method and utilized pituitary cell cultures to investigate the direct effects of estrogen on these correlates at the level of the brain and the pituitary *in vitro*.

RESULTS: Our findings indicate that: 1) During their development, the gonads play an important role in providing feedback to the HP axis in all stages investigated. These feedback patterns change during the transformation from the juvenile to the adult and throughout the adult reproductive cycle. The pathways involved use both steroidal and non-steroidal feedback as regulatory mechanisms. 2) Gonads, through their steroids, become more involved in regulating the HP axis during reproductive development and their primary feedback target appears to be gene transcription in the pituitary. 3) The observed changes in gonadal feedback throughout the adult reproductive cycle probably reflect the physiological requirements of gametogenesis. 4) The responsiveness of the HP axis towards steroids initially appears during puberty and further increases in adult females. Steroids, in the adult, solely affect the pituitary in the early stages of gametogenesis, while in the later stages GnRH expression in the brain is also regulated by steroids. However, the nature of the feedback is dependent on estrogenic and/or androgenic pathways. 5) Our *in vitro* studies showed that estrogens act directly at both the levels of the brain and the pituitary in adult female fish. Based on these findings, it appears that the activity along the endocrine reproductive web of female striped bass intensifies with age, and that prior cycles of oocyte development may prime the HP axis to respond faster and more vigorously in subsequent years.

CONCLUSION: This study provides an increased resolution and a broader perspective on the mechanisms involved in gonadal and steroid feedback regulation of GnRH neural activity and its targets at the level of the pituitary in striped bass.

P 14

Levels of Estradiol 17-beta(E_2) and immunolocalization of Estrogen Receptor alpha(ER-α) in the different regions of the brain of Indian major Carp *Labeo rohita*

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BACKGROUND: Estrogen plays an important role in the regulation of gonadotropin production, sexual differentiation and activation of sexual behavior in vertebrates. Three estrogen receptor [ER] subtypes have been described in teleost fish, namely ER-alpha, two ER-beta subtypes, called ER-beta1 and ERbeta2 and ER-gamma. Among vertebrates, teleost fish have the greatest capacity for estrogen production in the brain. Numerous researches have been conducted on maturation inducing steroids (MIS) in the Indian major carps. In contrast there has been very little focus on the localization and distribution of the steroid receptors.

METHODS: The sexually matured brains of male and female *Labeo rohita* were collected for a complete reproductive cycle. Sampling was done at regular interval of every month. The brain was divided into seven regions, each region homogenized with saline and extracted for E2. The E2 levels in the different regions were measured with HPLC and confirmed with RIA. The brain regions were processed and taken for estrogen alpha (ERα) immunolocalization studies. The immunoreactivity was observed and microscopic photographs were taken.

RESULT: The levels of E2 were varied in different brain regions and it reached peak value in the breeding season. The female showed four times higher level of E_2 than the male. The highest level was found in the pituitary, 1200 pg/g in the female and 312 pg/g in the male. The second highest level was recorded in the hypothalamus, 1029 pg/g in female and 301 pg/g in male. The lowest levels were found in cerebellum and medulla, male did not showed any result in medulla. The distribution of the ERα has been found higher in the pituitary,olfactory, hypothalamus, optic and cerebrum

CONCLUSION: The results were clearly indicating that the level of sex steroid E2 reaches peak in the month of July, which is breeding season for *L .rohita.* The brain regions that showed high levels of E2 are pituitary, hypothalamus, preoptic and optic, olfactory and cerebrum. To support to this evidence the estrogen receptors also highly concentrated in these regions of the brain. The ER alpha positive neurons were distributed in different areas of the brain, particularly in pituitary, optic, hypothalamus and olfactory regions. The expression of ERα in the following brain regions was already demonstrated in gold fish and zebra fish, olfactory bulb, cerebral cortex, septum, preoptic area, bed nucleus of the stria terminalis, amygdala, paraventricular hypothalamic nucleus, thalamus, ventral tegmental area, substantia nigra, dorsal raphe, locus coeruleus, and cerebellum. The presence of ERα in the brain of different animals showed very high amount in the optic region and the same evidence were made in this species also. We conclude that the abundance of ERα and the level of E2 were recorded high in the fore and mid brain, both showed its higher presence in breeding season.

P 15

Emergence of estrogen signalling in the brain of zebrafish embryos and larvae

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BACKGROUND: It is now well admitted that estrogens have important actions in the development and functioning of the central nervous system through modulation of neural differentiation and cell survival *in vitro* and *in vivo*. The classical pathways of estrogenic effects involve nuclear receptors acting as ligand-dependant transcription factors that regulate the expression of target genes either directly, by binding to an estrogen responsive element (ERE), or indirectly through interaction with others transcription factors. While only two estrogen receptors (ER), namely ERα and ERβ have been clearly characterized in mammals, birds and amphibians, two ERβ exist in teleost fish, ERβ1 and ERβ2, resulting from a duplication of the ERβ gene. We have recently shown that aromatase B (AroB, the product of the *cyp19b* gene) in adult zebrafish is expressed in radial glial cells and is strongly up-regulated by estradiol, an effect that involves ER and an ERE located in the proximal *cyp19b* promoter.

METHODS: In this study, we studied the emergence of estrogen signalling by looking at the spatiotemporal expression of three estradiol receptors and aromatase B using in situ hybridization and quantitative real-time PCR and determining the onset of AroB up-regulation by estradiol.

RESULTS: Our results show a tissue-specific distribution and a differential spatiotemporal expression of ER mRNA with a significant induction of the expression of the three receptors between 24 and 48hpf. In situ hybridization shows that ERβ1 and ERβ2 are expressed early during development in the neuroendocrine regions, while ERα was not detected in the brain until three weeks hpf. Between 24 and 48hpf, the expression of AroB mRNA also strongly increases, but this increase is partly blocked by the pure anti-estrogen ICI 182-780. This pattern suggests a concordance between expression of AroB and ER mRNAs. In accordance with these data, from 48hpf to 130hpf, AroB mRNA up-regulation induced by estradiol is abolished by ICI 182-780.

CONCLUSION: ERβs are expressed early in the brain of zebrafish as shown by both in situ hybridization and quantitative PCR. Basal aromatase B expression increases between 24 and 48 hpf an effect that probably involves ER as it is blocked by ICI. Aromatase B is strongly induced by E2 staring at 24 hours an effect that involves classical ERs. Altogether, the data show that functional ERs are expressed in the brain of zebrafish between 24 and 48hpfs.

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P 16

Comparative distribution of three estrogen receptors in the brain-pituitary complex of the rainbow trout

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BACKGROUND: The classical pathways of estrogenic effects involve nuclear receptors acting as ligand-dependant transcription factors and regulating expression of target genes either directly, by binding to cis-regulatory elements (ERE), or indirectly through interaction with others transcription factors. While only two estrogen receptors (ER), namely ERα and ERβ have been clearly characterized in mammals, birds and amphibians, two ERβ exist in teleost fish, ERβ1 and ERβ2, resulting from a duplication of the ERβ gene. Although the first fish ER, ERα, was cloned in the rainbow trout, information on ERβ was lacking in this important model species.

METHODS: In this study, we obtained sequences corresponding to two ERβ forms rainbow trout, rtERβ1 and rtERβ2. The distribution of these receptors was studied by means of in situ hybridization in the brain, pituitary and gonads of male and female rainbow trout in comparison with that of the previously identified ERα. Comparisons were always made on adjacent sections.

RESULTS: The overall distribution of rtERs mRNA in the brain was similar in males and females whereas it was clearly different in the pituitary between male and female trout. A clear signal was detected with the three receptors in the telencephalon and in the preoptic area, although abundance of the messengers was not identical. In contrast, the three receptors showed distinct expression patterns in the mediobasal hypothalamus, in which ERα and ERβ2 were found more ventrally than ERβ1 in both males and females. In the pituitary of males, ERα was more abundant in the pars intermedia whereas, ERβ1 and ERβ2 were mostly observed in the proximal pars distalis. In the female pituitary, all three receptors were found in the proximal pars distalis, ERβ1 being the most abundant.

CONCLUSION: The three ER are expressed in the brain and the pituitary of the rainbow trout. However, within one sex they show a clearly different pattern of expression in the brain and the pituitary. Furthermore, clear differences exist between males and females in the pituitary. These data suggest that the three ERs are likely to mediate differential functions in the brain and the pituitary.

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P 17

AURATUS.CA: a cyprinid cDNA microarray system to profile neurohormone action and endocrine disruption in the brain

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BACKGROUND: Neuropetides, neurotransmitters, steroids and thyroid hormones interact to control hypothalamo-pituitary hormone secretion and feedback regulation of reproduction, growth and metabolism. While it has been known for many years that sex hormones regulate gene expression, very little is known about the roles of neurotransmitters to affect transcription in the brain. We have addressed this issue by developing a mixed goldfish-common carp cDNA array to profile neuroendocrine control under normal conditions and following exposure to endocrine disrupting chemicals.

METHODS: We have produced a 10,000 cDNA (in duplicate) microarray on glass slides. Targeted homology cloning of goldfish neuropeptides, neurotransmitter synthesis enzymes, ionotropic and G-protein coupled receptors and random sequencing of a suppressive-subtractive cDNA library is continuing. Carp cDNA were sequenced from multiple tissue libraries (see carpBASE). We employ exogenous control cDNAs and extensive normalization procedures followed by Statistical Analysis of Microarrays (SAM) methods to obtain candidate regulated genes. Real-time PCR is used to validate microaaray results.

RESULTS: The majority of cDNA sequences obtained to date have homologs in zebrafish or other model vertebrates. We are determining the effects of short (24h) and long-term (days to weeks) exposure to estradiol, aromatase inhibitors and thyroid hormone (T3) on gene expression in the telencephalon and hypothalamus. I.P. injection of dopamine or GABA receptor agonists rapidly (5-6 h) affects global gene expression. We are also profiling the effects of estrogenic EDCs and neurotoxins. Overall, 1-5% of cDNAs represented on the array are affected by the treatments. Statistically significant ($q<5$) changes for most genes are in the range of 1.3-2.0 fold. PCR-verified and a small number of genes are more highly regulated are the focus of our attention. These include aromatatse B, estrogen receptor alpha, isotocin, secretogranin-III, ubiquitin-conjugating enzyme 2, adenylate cyclase and others.

CONCLUSION: As goldfish ESTs become available they are deposited in GenBank. The AURATUS.CA microarray is a useful tool to profile neurohormone action, however, there is a significant challenge to study gene expression in brain. Transcript levels are tightly regulated and rarely change more than 2-fold. Extensive use of controls and rigorous statistical and bioinformatic analysis are required to obtain verifiable candidate genes regulated in the telencephalon and hypothalamus of the goldfish. Many regulated genes have not previously been associated with neurohormone action or are unclassified ESTs with functions that remain to be discovered. Funded by NSERC.

P 18

Sexual changes in the neurosteroids of Indian major carp, *Labeo rohita (Ham.)*

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BACKGROUND: The brain is a steroidogenic organ that expresses steroidogenic enzymes to produce neurosteroids. Steroids in the brain arise both from local synthesis and from peripheral sources and have a variety of effects on neuronal function. However, evidence for the range of steroids present in brain or of the pathways for their synthesis and sexual differentiation or regulation of reproductive cycle of the steroids are not yet known. The synthesis of these sex steroids, testosterone (T) and estradiol-17β(E_2) by secretary cells like glial and astrocytes and conversion of androgens into estrogen with a help of complex of aromatase enzymes were studied.

METHODS: The synthesis of T and E_2 in the different regions of the male and female brain was studied by incubating the brain regions with the sex steroid precursor 17α– hydroxy progesterone (17α-OHP). After incubation the medium was extracted with ethyl acetate for the sex steroids. The synthesized T and E_2 were identified by TLC and quantified by HPLC. Aromatase activity was studied using aromatase assay with tritiated androstenedione incubation.

RESULTS: The synthesis of sex steroids are sex dependant and the male and female showed higher amount of T and E2 respectively. Pituitary, olfactory, optic and hypothalamic areas showed higher levels sex steroids, the highest recorded was 789pg/g T in the male and 867pg/g E2 in the female. The aromatization study confirms that the distribution and concentration of the aromatase enzymes are regions specific and the female showed higher level of aromatization than male.

CONCLUSION: These results confirm that there is *insitu* synthesis of sex steroids in the *L.rohita* brain. The pituitary, olfactory, optic and hypothalamus areas are mostly involves in synthesis of T and E2. The synthesis of T and E_2 from 17α-OHP indicates the expression of enzymes, P-$450_{17,20}$lyase, 17β-HSD and P-450aromatase during the final reproduction. The aromatase activity is significantly high in female than the male brain. Further, this study leads to understand the control mechanism for the expression of the neurosteroid synthesis and the sexual dimorphism.

P 19

Biological Activity of Recombinant Orange-spotted Grouper (*Epinephelus coioides*) Gonadotropin Hormone IIβ Produced in Insect Cells by the Baculovirus Expression System

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BACKGROUND: The orange-spotted grouper, *Epinephelus coioides*, is a marine fish widely cultured in Southern China. It is a protogynous hermaphroditic fish. Females reach sexual maturity at about 5 years of age, and the transition of the sex gland from female to male begins at about 7 years of age. To study the biological activities and physiological relevance of the two gonadotropins, the availability of homologous hormones was essential.

METHODS: As a first step in unraveling the mechanism involved in the sex change and gonadotropin function of the grouper, gonadotropin hormone IIβ (GtH IIβ) subunit was expressed in insect cells by the baculovirus expression system, and the biological effects of the recombinant protein were determined with in vitro and in vivo approaches. The cDNA sequence encoding orange-spotted grouper GtH IIβ subunit was cloned into baculovirus transfer vector and expressed in insect Sf9 cells.

RESULTS: The results showed that a band about 16.8 kDa could be detected by SDS-PAGE and Western blot. The recombinant grouper GtH IIβ (rgGtH IIβ) could stimulate the secretion of testosterone (T) and estradiol-17β (E_2) from the gonad in a static incubation system in a time-dependent, but not a dose-dependent, manner. Using in vivo bioassay, the mRNA levels of two aromatases (P450aromA and P450aromB), GnRH, GtH IIβ, GtH Iβ and GtHα in the pituitary, gonad, and hypothalamus were determined in different groups of orange-spotted groupers treated respectively with rgGtH IIβ, human chorionic gonadotropin (hCG), and a culture medium of insect cells transformed with an expression vector without GtH IIβ subunit. The mRNA levels of P450aromA and P450aromB rose dramatically after injecting rgGtH IIβ intraperitoneally, which was consistent with the secretion of sex steroid hormones. The mRNA levels of GnRH dropped in the pituitary, hypothalamus, and gonad, and the mRNA levels of GtH IIβ, GtH Iβ and GtHα in the pituitary of the experimental group expressed at a higher level than that of the hCG group.

CONCLUSION: These results were in accord with the long positive feedback loop of gonadotropin on gonad sex steroid hormone and the short negative feedback loop of gonadotropin on GnRH mRNA level. These results indicate that the rgGtH IIβ was successfully expressed by the baculovirus-insect expression system and that the rgGtH IIβ has biological activity. (This work was supported by the grants from the Major State Basic Research Development Program of China (973 Program) (No. 2004CB117402), and the Guangdong Province National Science Foundation (No. 20023002) to Dr. Wen-Sheng Li.).

P 20

Identification and characterization of a novel splice variant of gonadotropin alpha subunit in the common carp (*Cyprinus Carpio*)

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BACKGROUND: Gonadotropin (GtH), containing a common alpha subunit and a hormone-specific beta subunit that confers its biological specificity, is a pituitary glycoprotein hormone that regulates gonadal development in vertebrates. Alternatively-spliced mRNA is found from the gene related to the reproduction process such as gonadotropin-releasing hormone (GnRH). However, to date, no alternatively-spliced variant of gonadotropin (GtH) mRNA has been identified.

METHODS: Pituitaries and ovaries of the common carp were collected prior to reproduction. Total RNA was extracted using the SV Total RNA Isolation System Kit. By RT-PCR, a novel splice variant of GtH alpha subunit has been identified. The amino acid sequences of the alpha subunit protein were analyzed by protein-protein blast. In addition, the characteristics of the novel splice variant were studied with Western blot and Co-immunoprecipitaion.

RESULTS: In the study on the common carp, we found that the alpha subunit transcript GtH-alpha 357 is 357 nucleotides (nt) consistent with that of being reported. In addition, an alternative splicing transcript GtH-alpha 291 was identified here by RT-PCR which is 291nt and exists not only in the pituitary but also in the ovary. The analysis of GtH-alpha 291 amino acid sequence by the SignalP server predicted that the GtH-alpha 291 residues subunit might characterize as a signal peptides. In the secretion experiment, GtH-alpha 357 subunit could be secreted out of Hela cells while GtH-alpha 291 could not, which confirmed the prediction. Co-immunoprecipitation assay proved that GtH-alpha 291 subunit is able to interact with both FSHβ and LHβ as GtH-alpha 357 does.

CONCLUSION: This study finds an alternative splicing transcript of a GtH alpha subunit. Further studies are necessary to elucidate the specific role of this variant in the regulation of gonadal development and sexual maturation.

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P 21

Effect of immunoneutralization of FSH and LH on GnRH-stimulated tilapia

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BACKGROUND: According to the model based on the salmon, it is accepted that in fish, FSH is involved in spermatogenesis and vitellogenesis in early reproductive stages, whereas LH appears to be involved in later stages, and to regulate spermiation and ovulation. The specific separate roles of these gonadotropins have never been monitored in fish with asynchronous ovaries that contain, at the same time, oocytes at various stages of development.

METHODS: We produced Oreochromis niloticus (t) recombinant (r) LH and FSH, as single-chain polypeptides, in the methylotrophic yeast Pichia pastoris. Specific antisera were raised against rtFSHbeta and rtLHbeta used in immunoneutralization studies. Male tilapia were allocated into five groups. The control group was injected i.p. with normal goat serum. At the designated time 0, the second, third and fourth groups were injected with sGnRHa at 15 microg/kg. The second group received sGnRHa only. The third was immunized with 0.2 ml rtFSHbeta or rtLHbeta antiserum together with the sGnRHa injection. The fourth group was immunized i.p. with rtFSHbeta or rtLHbeta antiserum 12 h before and together with the sGnRHa injection. A fifth group was injected only with anti-rtFSHbeta or rtLHbeta at time -12 h. Blood sampled taken at 4-h intervals until 24 h after treatment were stored at -20deg C pending assay for steroids.

RESULTS: We tested the effects of immunoneutralization of endogenous FSH and LH on the ability of GnRH peptide to stimulate 11-KT. 11-KT levels increased following sGnRHa injection, but a concomitant injection of sGnRHa and anti-rtFSHbeta postponed 11-KT peak by 4 h. Also fish injected twice with anti-tFSHbeta, showed a 4-h delayed 11-KT peak; however, the level of 11-KT was dramatically suppressed for 24 h in fish injected with anti-rtFSHbeta alone Generally the same pattern was observed when fish were immunoneutralized with anti- rtLHbeta, except that the reduction in the steroid level was less pronounced.

CONCLUSION: One of the most effective ways to determine the physiological role of a biological factor is to eliminate it from the system in question. One means of achieving such elimination is immunoneutralization, i.e., the administration of specific antisera produced against the factor. In the present study, injection of antibodies against recombinant tFSHbeta and tLHbeta into male tilapia reduced and delayed the 11-KT response to sGnRHa. Moreover, in the presence of anti-FSH and anti-LH, not only the GnRH-stimulated 11-KT level, but also the basal steroid levels were reduced. This strongly implies involvement of both FSH and LH in 11-KT secretion in tilapia.

P 22

The effect of di (2-ethylheksyl) phthalate (DEHP) on LH secretion in Prussian carp (Carassius auratus gibelio B.) and common carp (Cyprinus carpio L.)

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BACKGROUND: Di(2-ethylhexyl) phthalate (DEHP) is a widely used plasticiser, especially of polyvinyl chloride (PVC) products. It enters environment during production, use or after disposal of products containing phthalates. DEHP is known for its endocrine-disrupting properties, but there is few data on its influence on fish reproduction.

METHODS: In the in vitro experiment dispersed common carp pituitary cells were cultured in media containing DEHP at the concentrations of 10^{-5} or 10^{-4} M and salmon GnRH analogue (sGnRHa) at the concentrations of 10^{-9} M. In the in vivo experiment Prussian carps were kept for two weeks in water containing DEHP (0.6, 3 or 15 $\mu g \cdot dm^{-3}$) and then they were injected with sGnRHa (20 $\mu g \cdot kg^{-1}$). Blood samples were collected at the beginning of the experiment and after sGnRHa injection. The determination of LH levels in culture media and in blood plasma was determined with ELISA method. The influence of DEHP on LH secretion was evaluated.

RESULTS: In vitro experiment: after 6 hours of incubation, LH concentration in the group incubated with 10^{-5} M of DEHP was significantly higher when compared to the control group. In contrast, LH concentration in the group incubated with sGnRHa + 10^{-5} M of DEHP was significantly lower in comparison with the group incubated with sGnRHa alone. DEHP at the concentration of 10^{-4} M didn't affect the spontaneous LH secretion, however after 26 hours of incubation LH level in the group treated with this concentration of DEHP in combination with sGnRHa was significantly lower when compared to the group incubated with sGnRHa alone. In vivo experiment: after two weeks of exposition, LH levels in all DEHP-treated groups were visibly (but insignificantly) higher when compared to the control group. LH levels in these groups didn't increase significantly after sGnRHa injection, in contrary to the control group, in which LH level increased significantly at 6 hours after injection.

CONCLUSION: We showed that DEHP can modify the secretion of LH in common carp and Prussian carp. In general, obtained results suggest that DEHP stimulates the spontaneous LH release, but inhibites GnRH-stimulated secretion. This effect was more clear in our in vitro experiment, however, in the case of in vivo experiment, the same tendency was observed. We suppose that DEHP can influence the reproduction of fish in the wild and disturb the response of cultured fish to hormonal stimulation of spawning.

P 23

Seasonal-dependent effect of endosulfan exposure on gonadotropins of Cichlasoma dimerus (Perciformes, Cichlidae)

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BACKGROUND: There is an increasing awareness that chemicals dumped into the aquatic environment are capable of acting as endocrine disruptors in wildlife by interfering with the normal functions of hormones that regulate reproduction. The organochlorine insecticide endosulfan (ES) is still used in several countries despite showing high toxicity to non-target animals. In fish, it causes histopathological alterations in pituitary and testes. Luteinizing hormone (LH) and follicle-stimulating hormone (FSH) are synthesized in the vertebrate pituitary controling gonadal steroidogenesis and gametogenesis. The aim of this study was to determine the effect of ES exposure on LH and FSH expression in the Southamerican freshwater cichlid C. dimerus, comparing between high and low reproductive seasons.

METHODS: Laboratory-reared adult fish were exposed for two months to environmentally relevant concentrations of ES (0.03, 0.1, 0.3 and 1 ug/l) with water renewal every 48 hours. Two separate experiments were performed, one during the summertime (January-March), when reproduction is at its peak for this species, and one during winter (July-September). At the end of each experiment fish were sacrificed and pituitaries homogenized for Western blot (WB) or processed for immunohistochemistry (IHC), in order to detect LH and FSH. Gonads and liver were processed for histology.

RESULTS: In summer animals, WB showed a decrease in the pituitary ir-FSH content and an increase in that of LH in fish exposed to high ES concentrations. These changes in gonadotropins content correlate with the morphometric analysis of ir-cells: LH ir-cells of summer ES treated animals show a marked increase in cell area and a small increase in cell diameter when compared to those of control animals; FSH ir-cells present a smaller area and diameter in treated fish than in control fish. In the experiment carried out during the winter no clear difference between treated and untreated animals was found. No major histological alterations were observed in liver or testes at either season; as expected, testes of summer animals had a greater amount of sperm than their winter counterparts, however treated males appeared to possess more sperm when compared to control animals at both seasons.

CONCLUSION: This preliminary work supports the role of endosulfan (ES) as an endocrine disruptor in C. dimerus. Results indicate the existence of seasonal variations in gonadotropin responsiveness to ES, probably due to a different regulation of GtHs at both seasons. These results provide a novel approach to understanding the impact of an ecologically relevant endocrine disruptor on fish reproductive function.

P 24

Involvement of high hydrostatic pressure in the stimulation of eel sexual maturation

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BACKGROUND: European silver eels are blocked at a pre-pubertal stage resulting from low levels of pituitary gonadotropins (LH, FSH). This is due to a double central control: a deficit in gonadoliberin (GnRH) and a strong dopaminergic (DA) inhibition. Since eels are thought to undergo sexual maturation during their oceanic reproductive migration, we hypothesise that some environmental factors would be determinant in this phenomenon. We investigated the specific effects of high hydrostatic pressure (HP), linked to a migration at depth, on the brain-pituitary-gonad (BPG) axis of silver eels.

METHODS: Female and male silver eels were subjected to 101 ATA (=1000m depth) for several weeks, using a hyperbaric chamber. Control eels were kept at 1 ATA. The effect of HP was tested on different parameters of the BPG axis. Quantitative real time PCR was used to measure the expression of GnRH (mGNRH and cGnRH-II), tyrosine hydroxylase (TH: the rate limiting enzyme of DA synthesis) in various brain regions and, on LH and FSH beta subunits in the pituitary. The effects of HP on gonadal activity were estimated by measuring gonadosomatic index (GSI), oocyte diameter and plasma levels of vitellogenin (Vtg) and sex steroids (E_2, 11-KT).

RESULTS: At brain level, TH mRNA levels tended to decrease in olfactory bulbs, telencephalon including the preoptic area and in medulla oblongata. At the pituitary level, LHbeta expression tended to increase while FSHbeta expression decreased in both sex, leading to an increase in the LHbeta/FSHbeta ratio, as observed during experimental maturation. In females submitted to HP, we observed a significant increase in oocyte diameter and plasma levels of 11-KT and E_2. A similar trend was observed for 11-KT plasma levels in males. In females, Vtg plasma levels also significantly increased. The mRNA levels of the various GnRH forms are currently assayed.

CONCLUSION: These results suggest that HP could play a specific positive role in the activation of eel BPG axis although additional internal and external factors are necessary to induce eel sexual maturation.

P 25

Molecular characterization of GnRH and GnRH receptors in Atlantic cod (Gadus morhua)

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BACKGROUND: Atlantic cod (Gadiiformes) is an economically and culturally important species in the north Atlantic region, but early sexual maturation and variable gamete quality in brood-stock hampers development of effective cod farming. Whereas the pituitary-gonad axis has been studied for some time, information is scarce regarding the neuroendocrine regulation of puberty in cod. We therefore have initiated a research project investigating the physiology and regulation of brain GnRH systems and their impact on pituitary gonadotropes, combining molecular and electrophysiological approaches. Here, we present a molecular characterization of the different forms of GnRH and GnRH receptors (GnRH-R) in cod.

METHODS: Total RNA was extracted from brains and pituitaries sampled from maturing cod. A combination of RT-PCR and RACE-PCR were applied to isolate GnRH and GnRH-R cDNAs, using degenerate primers based on conserved regions from available teleost sequences. Phylogenetic analyses based on deduced amino acid sequences of the isolated cDNAs were performed using ProTest 1.3 and PHYML. mRNA expression analyses during puberty were performed using newly developed SYBR-green based qRT-PCR assays.

RESULTS: The isolated GnRH ligand cDNAs each encodes a signal peptide, the GnRH decapeptide, and a GnRH-associated peptide (GAP), which is connected to GnRH by a Gly-Lys-Arg sequence. The GnRH-R cDNAs each encodes a 300-400 aa protein containing seven hydrophobic transmembrane domains and N- and C-terminal regions. Sequence and phylogenetic analyses have confirmed the identity of the isolated cDNAs. We are currently investigating the mRNA expression patterns in brain and pituitary through pubertal development.

CONCLUSION: The isolated cDNAs provide us with important tools for further investigations on the physiology and regulation of GnRH systems in cod.

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Electrophysiological properties of pituitary cells in primary culture from Atlantic Cod (Gadus morhua)

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BACKGROUND: The purpose of this study was to characterize the basic electrophysiological properties of cod pituitary cells, to assess whether they may control hormone secretion through similar pathways as in mammals.

METHODS: Primary cultures of pituitary cells from maturing Atlantic cod were prepared by trypsin treatment and mechanical dispersion. Electrophysiological recordings were performed using the perforated patch clamp method. Large cells, assumed to be gonadotropes, were selected for recordings.

RESULTS: Spontaneous action potentials were observed in about 30 % of the cells. The action potentials displayed a fast initial spike followed by a prolonged plateau. Correspondingly, the inward current elicited by depolarizing steps consisted of both a transient, tetrodotoxin-sensitive Na+ component and a nifedipine-sensitive Ca2+ component that was sustained when Ba2+ replaced Ca2+ as current carrier. The outward current was partially blocked both by tetraethylammonium and 4-aminopyridine.

CONCLUSION: Large pituitary cells from Atlantic cod display electrophysiological properties necessary for controlling hormone secretion via action potentials and Ca2+ influx. The voltage-activated ion channels present in these cells largely correspond to the ion channels of pituitary cells in other teleosts (goldfish, Carassius auratus, and tilapia, Oreochromis mossambicus) and mammals, although differences exist regarding the shape and duration of action potentials.

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P 27

Synergistic action of androgens and cortisol on gonadotropin regulation in European eel, *Anguilla anguilla*

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BACKGROUND: The sexual maturation controlled by the gonadotropic axis is until now poorly understood is eel species. Indeed, eel remain at a prepubertal stage due to a deficient production of pituitary gonadotropins (Luteinizing hormone, LH and Follicle-stimulating hormone, FSH), until the oceanic reproductive migration. Since eels are fasting along the migration and maturation, it is important to decipher the possible interactions between cortisol and eel reproduction. Previous studies have underlined the role of sexual steroid feedbacks and cortisol on LH regulation. Concerning FSH, no informations are available due to the lack of molecular tools.

METHODS: To investigate the potential role of Estrogens (E2), androgens (Testosterone, T and the non-aromatisable androgen Dihydrotestosterone, DHT), cortisol (F) or their combination, on LHβ and FSHβ regulation, we performed *in vitro* experiments using primary culture of pituitary cells. Expression of gonadotropin subunits (LHβ and FSHβ) was analyzed using quantitative real time PCR. In addition, LH content in cell cultures was measured using RIA. The effects of sex steroids, F and their combination on pituitary LH content were also investigated in *in vivo* experiments.

RESULTS:
In vitro, T, DHT or F treatment, administered alone, induced an increase in LHß mRNA levels as well as in LH content, but no effect was seen after E2 treatment. Furthermore, a synergistic stimulatory action was observed after cell treatment by combinations of F and androgen (T or DHT) on LHß mRNA, as well as on LH content. Concerning the FSHß expression, no variations were observed after treatment with androgens, F or their combination. In contrast, FSHß mRNA was stimulated by E2 treatment.
In vivo, no or moderate effects were observed on LH content after androgens or F treatments, but the *in vivo* treatment by the combination F and T induced a large increase in pituitary LH content.

CONCLUSION: F, which is implicated in the mobilization of energy and metabolite stores during the reproductive migration, can also play a positive role in the control of the eel gonadotropic axis. F acts in synergy with androgen to specifically stimulate LHß expression. These results observed *in vivo* as well as *in vitro* indicate that the synergy is exerted directly at the pituitary cell level.

P 28

In vitro* regulation by activinB and IGF-I of gonadotropin expression in European eel, *Anguilla anguilla

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BACKGROUND: Eels remain blocked at a prepubertal stage if their reproductive migration is prevented. This is due to a deficit of pituitary gonadotropins (Luteinizing hormone, LH and Follicle-stimulating hormone, FSH) and understanding their regulation remains essential for future management of eel reproduction. A previous study of our group has revealed the stimulatory effect of the insulin-like growth factor (IGF)-I on LH production, but effects on FSH regulation remained unknown due to the lack of molecular tools.

METHODS: In the present work, we investigated potential regulators of the two gonadotropins, LH and FSH using primary culture of eel pituitary cells. Cells were treated by various doses (10^{-11} to 10^{-7}M) of IGF-I (human recombinant), or of a gonadal peptide known as a main stimulator of FSH in mammals, the activin (human recombinant activin B). The expression of the gonadotropin subunits, LHß, FSHß and the glycoprotein alpha (GPα) were analyzed using quantitative real-time PCR.

RESULTS: IGF-I showed a dose-dependent stimulatory effect on LHß transcript levels in agreement with our previous study using LH RIA. In contrast, IGF-I had no effect on FSHß mRNA levels. Concerning the effect of activin B, no stimulatory effect was observed on LHß mRNA levels, while a strong and dose-dependent increase was observed on FSHß expression. No variations were observed for GPα mRNA levels.

CONCLUSION: These data obtained in pituitary cell culture showed direct and specific regulations of the expression of eel gonadotropins by two peptide hormones: IGF-I specifically stimulates LHß expression whereas activin B exerts its positive action on FSHß.

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Sequence analysis of Somatolactin from Cichlasoma dimerus SL and its relation with MCH and GnRH systems

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BACKGROUND: There is increasing evidence that somatolactin (SL) is a multifunctional hormone. In Cichlasoma dimerus, a Southamerican cichlid fish that shows dramatic changes in body coloration during sexual activation, we found clear evidence of a possible involvement of SL together with melanocyte stimulating hormone (MSH) and melanin concentrating hormone (MCH), in background colour adaptation. However, there is scarce information on the regulation of SL release. This study is focused on the relation between SL and the neuropeptides MCH and GnRH. In addition we report the characterization of partial C. dimerus SL (cdSL) cDNA.

METHODS: Adult fish were processed for double-labelled immunohistochemistry (IHC) in order to analyze a possible close association between SL, MCH and GnRH. In addition, pituitary glands were removed from C. dimerus and cultured during 4 days in 96 wells plate with L15. On day 1: Medium was removed and fresh medium was added with or without GnRH (0.01 to 10 μM) or MCH (0.01 to 10 μM); after 24 hr the medium was removed. In pituitaries treated with MCH fresh medium was added for recovery of hormonal release; on day 3, medium with or without GnRH (1 μM) was added and finally, after 24 hr mediums were remove. Each culture medium removed was stored frozen for further analysis by western blot. Furthermore, degenerate primers were used to characterize, by PCR and further sequence analysis, the internal region of cdSL cDNA from pituitary gland.

RESULTS: We found a clear morphological association between MCH fibers, originating from the nucleus lateralis tuberis (NLT), and SL cells. Besides, we found GnRH fibers in association with SL cells. O the other hand, we observed in the NLT, GnRH fibers in close association with MCH soma and fibers. The analysis by immunoblots showed that after 24hs with MCH or GnRH, SL release increases significantly in a dose-dependent manner ($p<0.05$). With GnRH (1 μM) addition after the stimulus was withdrawn and after reaching the basal level of secretion was reached again.
Additionally, the partial cdSL cDNA contains 879-bp and encode a partial signal peptide and the mature protein of 207 aa. The cdSL shares 59-86% nucleotide sequence identity with other teleost SLs, has a N-Glycosylation site at position 121 and includes the seven characteristic cysteine residues of the mature peptide.

CONCLUSION: Results of double-labelled IHC and in vitro cultures of pituitary glands taken together propose that MCH and GnRH play a role in the cdSL release. On the other hand the characterization of cdSL indicates that it is highly related to Perciform fish. The cdSL characterization will allow us to develop the necessary tools for future physiological studies on SL function.

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Prolactin of the rice field eel (*Monopterus albus*) : cDNA sequence, genomic structure, and mRNA expression during ovarian development

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BACKGROUND: Prolactin (PRL) is a multifunctional pituitary hormone, which affects a variety of physiological processes in mammals, including reproduction. In fish, most studies suggest that prolactin is a fresh water-adapting hormone, but the information on its roles in reproduction is still limited.

METHODS: PRL cDNA was cloned with 3' and 5' RACE from the rice field eel pituitary. The genomic DNA sequence was obtained with PCR using genomic DNA as template. The tissue distribution of PRL mRNA was determined using the semi-quantitative RT-PCR analysis, and the expression during ovarian development was investigated by real-time PCR analysis.

RESULTS: The full-length cDNA of rice field eel PRL consists of 1577 bp, and the open reading frame encodes a protein of 212 amino acids, which consists of a putative signal peptide of 24 amino acids and a mature protein of 188 amino acids. Sequence analysis revealed that PRL of rice field eel shares highest degree of sequence identity with Perciformes. Like most other vertebrates, PRL gene structure of rice field eel is conserved and contains 5 exons and 4 introns. RT-PCR analysis showed that in addition to the pituitary gland, high levels of PRL mRNA were also expressed in the discrete brain areas. Real-time PCR showed PRL mRNA expression in the pituitary was not altered significatly during ovarian development. However, in the ovary, the PRL mRNA was also detected with real-time PCR analysis, and the expression level was significantly increased at mature stage.

CONCLUSION: This is the first report on PRL in Synbranchiforme fish. High expression levels of PRL in the discrete brain areas suggests that PRL may be improtant for the function of brain in the rice field eel. The significantly elevated PRL mRNA level in the mature ovary implies that PRL may also have an improtant paracrine role in the maturation of ovary and/or in the early embryogenesis.

P 31

Somatolactin endocrinology during sexual maturation of female Atlantic salmon (Salmo salar): expression of the somatolactin alpha, somatolactin beta and somatolactin receptor genes

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BACKGROUND: Somatolactin (SL) is a pituitary hormone of the growth hormone (GH) and prolactin (PRL) family, and is only found in fish. There are two distinct forms, SL alpha (SLa) in all fish and SL beta (SLb) exclusively in freshwater fish. SL is thought to have multiple functions in body coloration, energy mobilization, acid-base balance, Ca-balance, osmoregulation, stress and an undetermined role in sexual maturation.

METHODS: Three-year old female Atlantic salmon were sampled on 15 occasions during a 17-month period spanning from August 2004 to December 2005, a month after they spawned. The fish were held in seawater netpens until May 2005, when they were moved to freshwater tanks after sampling. Ovaries and pituitaries were sampled for RNA extraction, and relative gene expression was measured using real time quantitative PCR (RTqPCR). For this purpose Atlantic salmon SLa, SLb and SL receptor (SLR) cDNA was sequenced.

RESULTS: Ovarian SLR relative expression showed no up- or down-regulation during the maturation process. Pituitary expression of both SLa and SLb increased before and during spawning with subsequent down-regulation to baseline levels during ovarian atretion. There was also a transient increase in SLa and SLb expression prior transfer from seawater to fresh water in May. SLa had a 30-fold higher expression than SLb, but SLb showed stronger up- and down-regulation. SLa and SLb expression is strongly correlated. SLb is also strongly correlated to gonadosomatic index and plasma testosterone levels when May is omitted.

CONCLUSION: Previous studies indicate a role of SL in the sexual maturation in fish. We have confirmed the increased mRNA expression of SLa during gonadal maturation, and shown for the first time that SLb expression also changes before and during spawning. This suggests a role of both SL forms in final female ovulation of Atlantic salmon. The constant expression level of ovarian SLR indicates no regulation at the level of receptor expression in this organ. The transient increase in pituitary expression of both SL forms prior to transfer from seawater to freshwater may indicate a freshwater-adapting function in salmonids or a stress response in fish able to migrate to seawater but retained in fresh water.

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Physiological Mechanisms of Imprinting and Homing Abilities in Salmon: From Behavioral to Molecular Approaches

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BACKGROUND: Salmon have an amazing ability to migrate thousands kilometers from the open ocean to their natal stream for reproduction after several years of oceanic feeding migration. It is now widely accepted that some specific factors of the natal stream are imprinted to particular nervous systems of juvenile salmon during downstream migration, and that adult salmon evoke these factors to recognize the natal stream during homing migration. For a better understanding on the mechanisms of imprinting and homing abilities in salmon, three different analyses from behavioral to molecular approaches have recently been applied using anadromous chum salmon (*Oncorhynchus keta*) as well as lacustrine sockeye salmon (*O. nerka*).

METHODS: The first is behavioral analysis on swimming speeds of Japanese chum salmon migrating from the Bering Sea to Japan by means of a micro-data logger with a propeller that can measure swimming speed of fish. We tagged a maturing chum salmon in the central Bering Sea that was retrieved by a set net along the eastern Hokkaido coast 67 days after the release. The average swimming speed was 60–70 cm per sec, and vertical rate was 42.3–47.7 km per day. The estimated horizontal rate indicates that chum salmon traveled 2,763 km in 67 days, which is almost equivalent to the distance between points of release and retrieve.

RESULTS: The second is molecular biological analysis on identification of an olfactory imprinting-related genes of lacustrine sockeye salmon by a subtractive hybridization technique of representational difference analysis (cDNA-RDA) using olfactory system of 1- (smolt stage) and 3-year-old fish (feeding migration stage). We have obtained a partial clone from a subtractive cDNA library of 1-year-old fish (SOIG; Sockeye salmon Olfactory system Imprinting related Gene). The SOIG contains a 756 bp open reading frame that codes for a protein with 252 amino acid residues. By northern hybridization, the SOIG mRNA was only expressed in the olfactory epithelium. *In situ* hybridization showed that the expression of SOIG mRNA was observed in the olfactory receptor cells and basal cells of the olfactory epithelium.

CONCLUSION: The third is olfactory analysis on discriminating ability odorants of the natal river. To test whether amino acid mixtures of the home stream have attractive effects on chum and sockeye salmon upstream movement, behavior experiments were conduced in the two-choice test tank using mature chum salmon in the Osaru River and sockeye salmon in the Lake Shikotsu. The artificial home stream water was prepared by the amino acid and related substance composition of the Osaru River water or the Lake Shikotsu hatchery water. A total of 44 male chum salmon and 151 sockeye salmon of both sexes was tested, and 85.7% chum salmon and 75.8% sockeye salmon were found in the arm running the artificial home stream water. These results from behavioral to molecular approaches will discuss with the physiological mechanisms of imprinting and homing abilities in salmon.

P 33

Effects of amino acid mixtures on upstream selective movement of four salmonids

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BACKGROUND: The upstream homing migration of salmon has been thought to be controlled mainly by their olfactory functions. From our recent electrophysiological and behavior experiments, we proposed that amino acids dissolved in the home stream water might be home stream odors for chum salmon. The purpose of this study is to test effects of amino acid mixtures on upstream selective movement of four salmonids in the two-choice test tank.

METHODS: Behavior experiments were conducted to test amino acid mixtures have attractive effects on upstream selective movement of mature male chum salmon (*Oncorhynchus keta*), masu salmon (*O. masou*), sockeye salmon (*O. nerka*), and pink salmon (*O. gorbuscha*). These experiments were carried out in the two-choice test tank consisted of two water inlet arms and one pool, which had one outlet at the end of pool. Either the artificial home stream water containing amino acid mixtures or natural lake water was added to the water inlet of left or right arms. The fish movement was monitored and the number of fish moved to each arm was counted. The two test pairs of natural and artificial solution were used: (1) natural lake water, (2) artificial home stream water and natural lake water.

RESULTS: In pair (1), all species showed no selectivity for either arm. In pair (2), percentage of upstream movement of 4 salmonids was 82.3, 63.6, 53.3, and 46.7 in pink, chum, masu, and sockeye salmon, respectively. In contrast, percentage of upstream selective movement in the arm running their artificial home stream water containing amino acid mixtures was 85.7, 75.0, 71.4, and 57.1 in chum, masu, sockeye, and pink salmon, respectively.

CONCLUSION: These results indicated that the artificial home stream water containing amino acid mixture had different effects on upstream selective movement among four salmonids. Pink salmon showed the highest upstream movement and the lowest accurate selectively to the artificial home stream water. Although the homing accuracy of four salmonids has not been examined in detail, it is believed that pink salmon stray off into the non-home stream most frequently. It is quite interesting to note that the relationship between the olfactory functions and the homing accuracy among four salmonids.

P 34

A novel assay based on the reproductive behaviour of the three-spined stickleback for the detection of anti-androgens

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BACKGROUND: Endocrine disrupting chemicals released into the environment have been reported to cause adverse effects in many aquatic species, particularly fish. Accordingly, physiological endpoints, most notably vitellogenin synthesis in response to oestrogenic exposure, have been developed. In addition to the synthesis of vitellogenin, the unique presence of an androgen regulated glue protein, spiggin, in the kidney of the three-spined stickleback, Gasterosteus aculeatus, during the breeding season makes it the only recognised teleost species with an unambiguous biomarker for both (anti-)oestrogens and (anti-)androgens. In comparison to the physiological effects there remains a paucity of studies on the effects of chemicals on fish behaviour despite the fact that subtle alterations in hormonal balance may affect behavioural patterns. The reproductive behaviour of the stickleback has been extensively studied and well characterised. Here we report the development of an assay based on physiological and behavioural endpoints of the male three-spined stickleback in response to anti-androgen compounds.

METHODS: The natural reproductive behaviour of the male three-spined stickleback can been divided in three phases: nest building, courtship and parental care. The current behavioural assay involves the initial two phases. An experiment protocol detailing the period and type of exposure, the tank design, the number of replicates, the number of treatments and the environment conditions is described. Various relevant behavioural parameters have been identified and a specific recording method developed. The ability of test chemicals to antagonise the normal production and action of androgens was investigated using as endpoints spiggin production and reproductive behaviour of male sticklebacks.

RESULTS: Unexposed sticklebacks exhibited normal reproductive behaviour and had high spiggin content in their kidneys, while the spiggin content and the number of nests built by males exposed to anti-androgens were reduced and even inhibited in the high concentrations. Furthermore, the zigzag dance, normally displayed by males during courtship, was affected by means of a reduction in number.

CONCLUSION: The disruption of the normal reproductive behaviour appears to be parallel with the already established biomarker spiggin. The behavioural assay developed using the three-spined stickleback provides a sensitive, relatively inexpensive and non-invasive method for the detection of anti-androgenic activity.

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Hormonal induction of tench (*Tinca tinca* L.) with the same treatments at two sequential reproductive seasons

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BACKROUND: The reproduction of a majority of cyprinid fishes which is important in industrialized aquaculture is based on hormonally spawning induction. The main objectives in the optimization of techniques capable of inducing spawning with some degree of confidence are as follows; (1) induction of final gamete maturation, (2) synchronization of final gamete maturation, (3) the ability to induce spawning in fish that cannot reproduce in captivity and (4) the ability to survival and well prosper of broodstock after hormonal treatment. The aim of the present study was to study ovulation rate by injection of different hormones in tench (*Tinca tinca*) as a function of suquential spawning season.

METHODS: Hormonally induced artificial propagation of individually marked broodstock was performed identically at two sequential reproductive seasons. In both years, four separate groups of females significantly not different at weight were intramuscularly injected by CPE (dose 2 mg per kg body weight, b.w.), Supergestran–containing GnRHa Lecirelin (D-Tle^6) GnRH*m* ProNHEt (20 µg kg b.w.), Ovopel and Dagin at 20.9 °C. Ovopel and Dagin treatments containing GnRHa and dopamine inhibitor was used at recommended doses from producer of treatments. In control group, no injection was carried out in broodstock.

RESULTS: At the first year of spawning season, a high ovulation rate was observed in groups following injection of CPE (86 %), Supergestran and Dagin (78 %) and Ovopel (72 %). The highest ratio of stripped eggs to broodstock weight was observed following injection of Dagin (8.0±3.2) compared to other groups: 7.3±3.0 (Ovopel) and 7.4±3.6 in CPE. At the second year of spawning season, the ovulation rate was slightly changed and reached to 75% in Dagin, 68% in Supergestran, 58% in Ovopel and 55% in CPE. But, the highest ratio of stripped eggs to broodstock weight was again observed in Dagin 7.4±6.1 compared to other groups: 6.4±3.0 (CPE) and 4.5±2.6 in Supergestran. In both first and second reproductive season, ovulation rate and ratio of stripped eggs to broodstock weight were significantly lower ($P<0.05$). Broodstock which has been injected by hormones showed lower survival rate at the second reproductive season e.g. Ovopel (79%) and Dagin (77%), compared to control (88%). At the second spawning season, some differences were observed between groups which have been injected at the first year of the reproductive season.

CONCLUSION: The present study suggested using of Dagin (containing GnRHa and dopamin inhibitor) for inducing ovulation. In addition, ovulation ratio and ratio of striped eggs to broodfish weight could be changed according to hormonal treatment. Changes of the survival of broodfish may suggest possible negative effects of injected hormones may be due to physio- endocrinological mechanisms.

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Nutritional status of Atlantic salmon (Salmo salar) broodstock
- Maternal effects on early expression of leptin, IGF-I, IGF-IR, GH and GHR

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BACKGROUND: Growth and energy homeostasis is influenced by a variety of hormones. In fish and other vertebrates the IGF-I/GH axis plays an integral role in regulating differentiation, growth and reproduction. Leptin is a peptide produced by adipocytes signalling nutritional status. Recent studies point out several roles of leptin, including the regulation of sexual maturation and reproduction. The effect of nutritional status on reproduction is postulated to reflect the action of unknown metabolic signal(s) that are recognized by the brain and serve as indicators of metabolic state. Leptin receptors have been reported in the brain as well as in the ovary and testis in rats; hence leptin may act as a metabolic signal to the reproductive system acting through the brain-pituitary-gonad axis and directly on the gonads.
Leptin has just recently been identified in teleosts, therefore it is still not known whether the physiological roles of the hormone in an ectotherm fish might differ from those described in mammals. In order to start the characterisation of the roles of leptin in fish reproduction and during early development, we selected Atlantic salmon as a model because of their large eggs and larvae and tools available for expression studies.

METHODS: At spawning five Atlantic salmon females (Salmobreed), fed a commercial brood-stock diet were characterised for nutritional status and hormonal expression. The fertilized eggs were sampled every 50 - 100 day degrees until first feeding and expression of leptin, IGF-I, IGF-IR, GH and GHR was assessed by real-time PCR, in *situ hybridization* and Alcian blue staining (colouring technique prior to embedding).

RESULTS: The females used showed very good egg quality with good fertilization percentage (96%) and survival rates around 90-92%. Quantification and localisation of gene expression are underway and the results will be presented and the maternal effects of nutritional status on hormonal expression of the offspring will be discussed.

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P 37

Thyroid hormone regulation of gene expression in the neuroendocrine brain of adult goldfish (Carassius auratus)

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BACKGROUND: Thyroid hormones (THs) are important for adult brain function, although their specific roles are not well defined. Thyroid and reproductive systems in fish are linked. One possible route for TH regulation of reproduction is through modulation of expression of neuroendocrine genes. Our objective was to determine the effects of increased TH levels on gene expression in the neuroendocrine brain of adult goldfish, with focus on genes related to THs and the gamma-aminobutyric acid (GABA) system.

METHODS: Sexually regressed adult male goldfish were exposed to waterborne triiodothyronine (T3; 0.02 and 0.1 micromolar) for 2 and 6 days (d). Plasma levels of total T3 were determined by enzyme immunoassay. For both 2 d and 6 d low and high exposures, real-time PCR (RT-PCR) was used to measure telencephalon gene expression of TH receptors and deiodinases (DIs), and GABA synthetic and degradative enzymes. The AURATUS.CA carp-goldfish microarray (10,000 cDNAs in duplicate) was used to obtain an expression profile for T3 action in the telencephalon for 6 d high T3 exposure. Data were assessed using Statistical Analysis of Microarray procedures.

RESULTS: Two day low and high, and 6 d low and high T3 exposure raised total plasma T3 by approximately 10, 42, 3 and 30 fold, respectively. The low T3 dose had no significant effect on expression at either time point (significance: $p<0.05$). In the 6 d high T3 group, DI3, which is responsible for deactivating TH, was 1.9 fold higher than control, with an accompanying 1.3 fold decrease in DI2, which is responsible for synthesizing active T3 from T4, indicating a homeostatic response to the T3 challenge. GAD67 expression was 1.5 fold lower in the 2 d high T3 group than control. GABA-T expression was 1.3 fold lower in the 6 day high T3 group than control. Approximately 5% of genes on the array were affected by T3 (1.5 fold or greater; $q<5$), with 530 down-regulated, and 5 up-regulated. Among those down-regulated were known thyroid-responsive genes (e.g. myelin-basic protein), GABA system genes (e.g. GABA-A receptor subunit gamma2), and other important neuroendocrine factors (e.g. NPY, GnRH receptor type A). Microarray results require RT-PCR confirmation.

CONCLUSION: These results indicate that THs affect expression of GABA system genes. We have identified many other TH-regulated genes in goldfish brain. Some are known to be TH-regulated in mammals, while others have not previously been associated with TH action or are unclassified expressed sequence tags with functions that remain to be discovered. Whether the candiate genes identified in the telencephalon are differentially regulated in the hypothlalmus of males versus females over the course of the reproductive cycle is the target of future work. Funded by NSERC.

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Thyroid hormone function in the European sea bass, *Dicentrarchus labrax*: Cloning and expression of deiodinase enzymes and thyroid hormone receptors

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BACKGROUND: In teleosts, thyroid hormones (THs) have been implicated in a variety of physiological processes, including reproduction. Thyroid hormones (TH) homeostasis is regulated by the hypothalamic-pituitary-thyroid axis and deiodinase enzymes. Three different deiodinases have been cloned in vertebrates including fish; deiodinase type 1 (Dio1), type 2 (Dio2) and type 3 (Dio3). Recently, Dio2 and Dio3 have been involved in the photoperiodic response of gonads in several mammalian and avian species. THs act via nuclear hormone receptors (TRalpha and TRbeta), which seem to change significantly their expression along the reproductive cycle in some fish species. In this work, we have cloned Dio2, Dio3, TRalpha and TRbeta cDNA sequences from the brain of the European sea bass and studied their tissue distribution in this species.

METHODS: Cloning of specific sequences of the four genes of interest was performed using brain cDNA from sea bass and degenerated primers. 3´UTR was obtained for sea bass Dio2, TRalpha and TRbeta, and the complete sequence (3´ and 5´UTR) was obtained for the sea bass Dio3. cDNA of the different tissues from adult animals was prepared and specific primers were used for the analysis of the expression using Reverse-Transcriptase PCR.

RESULTS: Dio2 and Dio3 were expressed in all central areas including neuroendocrine centers (telencephalon-preoptic, hypothalamus) and photoreceptor organs (retina and pineal gland) as well as in the pituitary. At the peripheral level, Dio2 was expressed in gonad, gill, liver, muscle, kidney, anterior and medium digestive tract, while Dio3 was also expressed in the heart but not in muscle. TRalpha and TRbeta were mainly present in the telencephalon-preoptic area, optic tectum, cerebellum and retina, but also in the olfactory bulb, hypothalamus, pituitary and pineal. At the peripheral level, TRalpha was highly expressed in the gonad and the kidney, while TRbeta was abundant in gonad, kidney and liver.

CONCLUSION: Dio2, Dio3 and TRs were widely distributed in different sea bass tissues. Nevertheless, their presence along the reproductive brain-pituitary-gonad axis strongly suggests a role of THs in the regulation of the reproduction in sea bass. Furthermore, the expression of Dio2, Dio3 and TRs in the retina and the pineal organ might be related with a role of THs in the modulation of the activity in the two main photoreceptor structures that synchronize reproductive events.

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Photoperiod modulates deiodinase expression and activity in the European sea bass, *Dicentrarchus labrax*

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BACKGROUND: The reproduction in fish is a seasonal process controlled by environmental factors as photoperiod. Thyroid hormones in vertebrates have also been involved in reproduction and appear modulated by the photoperiod but the mechanism underlying their daily and seasonal rhythms remains unclear. Thyroidal status is finely controlled by a complex system which includes a negative feedback regulation of the hypothalamic-pituitary-thyroid axis and a peripheral regulation by iodothyronine deiodinases (Dio1, Dio2 and Dio3). Recently, a photic regulation of deiodinase expression has been reported in several avian and mammalian species, showing that type 2 iodothyronine deiodinase is involved in the photoperiodic response of gonads in Japanese quail and Syrian hamster. The aim of this work is to study if photoperiod modulates deiodinase enzymes in fish.

METHODS: 30 days-old sea bass were maintained under constant light (LL), constant dark (DD) or 12 hours light-12 hours dark (LD; control group) regimes. After 10 days, a representative number of fish was weighted and measured. Several pools of each experimental group were frozen in liquid nitrogen at mid-light (ML) and mid-dark (MD). The mRNA expression of type 2 (Dio2) and type 3 (Dio3) deiodinases, beta subunit of thyrotrophin (beta-TSH) and proliferating cell nuclear antigen (PCNA) were analyzed by Real Time PCR. Besides, Dio2 activity was measured by using 125I-T4 as substrate.

RESULTS: Somatic growth (weight and lenght) was higher in control and LL animals that in sea bass maintained at constant darkness (DD). DD condition also determines a reduction in PCNA expression in relation to LD and LL regimes. Dio3 mRNA expression increased in both LL and DD conditions in relation to control animals while beta-TSH mRNA expression was higher in DD condition. No statistically significant differences in Dio2 mRNA expression were observed among the three experimental conditions. However, there was a statistically significant difference in Dio2 expression between ML and MD periods (ML>MD). Dio2 activity was lower in LL than in DD and control animals (LD>DD>LL). Differences between ML and MD Dio2 activity were also present in the three experimental groups.

CONCLUSION: Our results demonstrate a light modulation of growth, cell proliferation and thyroid hormone metabolism in fish. The increase of beta-TSH in DD condition might reflect a feedback mechanism at the hypothalamus-pituitary-thyroid axis. Furthermore, our data also indicate for first time in fish, the existence of daily variations in Dio 2 mRNA expression and activity.

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Annual changes of serum 11-KT compared with GTH and E2 in cultured Amur sturgeon (*Acipenser Schrenckii*)

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BACKGROUND: In order to know the gonad development of cultured Amur sturgeon and make the artificial propagation perfect, annual changes of serum GTH, 11-KT and E2 were measured, which were related to the gonad development.

METHODS: The Amur sturgeons were divided into two groups with 3-4 and 5-6 years old separately. Serum sample was collected from the caudal vein and separated by centrifugation during one year.11-KT was measured through enzyme immunoassay kit while E2 and GTH were determined with radioimmunoassay. Fragments of gonad were collected at the end of experiment by biopsy.

RESULTS: The serum concentration of 11-KT of male sturgeon changed significantly and seasonally. The highest concentration appeared in August (240ng/ml). The trend was similar between age 3-4 and 5-6. It was higher from June to October than the other months. Simultaneously, the secretion peak of 11-KT of the female was from June to October. The concentration was 0.5~10.5ng/ml in age 3-4 while 0.1~200ng/ml in age 5-6 among the female individuals. The serum 11-KT was in the same levels with female and male sturgeon age 5-6. And the secretion rhythm was also same. The serum concentration of E2 was similar among the individuals age 3-4. The highest concentration was found in March both male and female. However, among the individuals age 5-6, the serum concentration of E2 was different significantly from January to April. The annual changes of E2 were not distinct in ovary stage II females. The trend of GTH annual change was similar between male and female. The highest concentration of GTH was mostly found in March. The annual change of GTH was consistent with that of E2.

CONCLUSION: It was suggesting that spermatocyte meiosis and spermatogenesis of cultured Amur sturgeon occurred during June to October. It was obvious that the secretion of 11-KT had the positive relationship with vitellogenesis. If the sex of cultured Amur sturgeon were distinguished according to the levels of 11-KT, it would be better to choose the fish younger than 3 years old and the vigorous secretion period from June to October.

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Structure, gene expression and production of recombinant follistatin from the marine fish *Sparus aurata*

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BACKGROUND: Follistatin (FS) is an activin-binding protein that prevents activin from binding to its receptor and neutralizes its activity. FS also binds other members of the transforming growth factor-β (TGF-β) superfamily. The aim of this study was to clone and characterize FS from fish, study its expression and produce recombinant FS in order to raise anti-fish FS antibodies for future studies on reproduction and growth.

METHODS: *Sparus aurata* FS (saFS) was cloned from 7-day larval RNA using RT-PCR, 3'RACE and 5'RACE. Gene expression of FS was determined by RT-PCR using total RNA from embryos, larvae and tissues. Recombinant saFS was produced in CHO-K1 cells using a construct of saFS precursor cloned in pcDNA-*myc*-His vector. The same construct was injected to muscles of *S. aurata* fry. Recombinant saFS production was assessed by Western blot and immunohistochemistry using anti-*myc* antibodies.

RESULTS: A full-length cDNA coding for saFS was cloned from 7-day larval RNA. It contains the N-terminal domain and three FS domains. Amino acid sequence of mature saFS shows 81-91% identity with FSs from other fish species, amphibians, avians and mammalians. The mature protein is 290 amino acids long, similar to other fish FSs and the short isoform of *Xenopus* FS but longer by 2 residues than mammalian FS288. Ontogeny of FS expression revealed the presence of FS transcripts throughout larval development and also in embryos 12 h post-fertilization. FS is expressed in several adult tissues including gonads, with variable levels of expression. Western blots analysis of cell lysate and conditioned media from CHO-K1 cells, transfected with pFS-*myc*-His, demonstrated production and secretion of FS. FS was also detected by immunohistochemistry in *S. aurata* muscle fibres injected with pFS-*myc*-His DNA.

CONCLUSION: The structure of saFS is highly conserved compared to FSs from other vertebrates and the mature saFS is highly homologous to that of fish, amphibian, avian and mammalian FSs. Presence of FS in *S. aurata* embryos confirms its role during vertebrate embryonic development. The recombinant fish FS is produced both in transfected mammalian cells and *in situ* by muscle fibers of fish injected intra-muscularly with the DNA construct.

Sex Determination and Sex Differentiation

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Androgen synthesis in the silverside *Odontesthes bonariensis*

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BACKGROUND: Early testis differentiation in fish with genetic sex determination seems not to be mediated by androgens. However, the role of androgens as early mediators of sex determination and/or differentiation in testes of fish with temperature sex determination (TSD) could not be excluded. We choose the silverside *Odontesthes bonariensis* as a model with a strong TSD and investigated the androgen production in several organs including testes, ovaries, kidneys and liver of juvenile and adult fish. We observed that 11-oxygenated androgens are produced almost exclusively in testes. Next studies will be directed to determine if 11-oxygenated androgens are able to be produced by male gonads during the determining and differentiating time of the species.

METHODS: Explants of immature, in spermatogenesis and spermiant testis and of immature ovaries of *O. bonariensis* were incubated in the presence of the following precursors: [1,2,6,7-^{3}H]-17-hydroxy-4-pregnene-3, 20-dione (^{3}H-17P), [1β-^{3}H] androstenedione (^{3}H-A4) and [1,2,6,7-^{3}H] testosterone (^{3}H-T). Brain, liver and kidney were incubated with ^{3}H-A4. The metabolites were analyzed by TLC, revealed by autoradiography, and further analyzed by HPLC and co-crystallization with authentic standards. Gonads were fixed for classic histology in order to determine their stage.

RESULTS: The main metabolites produced by juvenile and adult testis were adrenosterone, 11-ketotestosterone (11KT) and 11β-hydroxy androstenedione (11OHA4). In presence of 17P, immature testis produced 11KT as major androgen whereas testes from sexually mature fish produced mainly 11OHA4. Spermiating testis incubated with 17P produced 11OHA4 as major steroid whereas the same testis incubated with T presented 11KT as the most prominent steroid. None of these 11-oxygenated androgens were detected in immature females neither when incubated with 17P4 nor A4. Liver did not produced any of the 11-oxygenated androgens found in testis but kidney produced minor amount of 11OHA4.

CONCLUSION: As in other teleost fish, testes of differentiated silverside produced 11-oxygenated androgens as major androgens while ovaries did not produced these kind of steroids. 11KT and 11OHA4 seems to be involved in the regulation of spermatogenesis as it was found in other fish, and are prominent during spermiating time opening the question about their role during this period. A part from masculine gonads only the kidney had the possibility to produce 11OHA4, but in little amount. The fact that 11-oxygenated androgens are produced mainly by testes will allow us to study the androgen synthesis capacity during the very early stages of testes development in all-male silverside induced by temperature.

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The role of estrogen in gonadal sex differentiation in protandrous anemone fish *Amphiprion clakii*

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BACKGROUND: Previously we demonstrated the histological process of gonadal sex differentiation in the protandrous anemonefish *Amphiprion calrkii*. The undifferentiated gonads of all specimens first differentiated into ovaries at 60 days post hatching (dph), and the oocytes developed gradually and increased in number as the ovaries grew between 90 and 180 dph. Some cysts of spermatogenic germ cells appeared scattered throughout the ovaries at 210 dph, and the gonads formed the ambisexual gonads with both ovarian and testicular tissues at 270 dph. Immunohistochemically, we showed that endogenous steroid hormones play an important role in anemonefish sex differentiation. To clarify the role that estrogen plays in sex differentiation in the protandrous anemonefish, we examined the effects of an aromatase inhibitor (fadrozole, AI), estrogen antagonist (tamoxifen, TF), and estradiol-17ß (E2) on sex differentiation.

METHODS: The AI and TF were added to food at respective doses of 20 or 200 µg/g and 20 or 2000 µg/g. In addition, food containing both AI 200 µg/g and E2 2.0 µg/g was also prepared. We began administering AI, TF or both AI and E2 to the different groups at 30 dph and continued for 60 days, including before and during ovarian differentiation. To observe the effects of AI, TF, and E2, we examined the gonads of 4-6 fish from each group. Gonads were fixed with Bouin's solution at 4ºC and sectioned at 7µm for hematoxylin and eosin (HE) staining.

RESULTS: All the fish in the control group had the ovaries with an ovarian cavity and developed oocytes. However, there was no testicular tissue in the ovaries. All the fish treated with AI 20µg/g or TF 20 or 2000 µg/g had ovaries with developed oocytes. In these gonads, ovarian cavity formation was delayed, but no testicular tissue was found. Two of six fish treated with AI 200µg/g had gonads with few oocytes and testicular tissue including spermatogenic germ cells. Significantly fewer oocytes and delayed ovarian cavity formation were observed in the gonads of the remaining fish. By contrast, all the fish that received AI in combination with E2 had developed ovaries with a complete ovarian cavity, developed oocytes, and ovigerous lamella.

CONCLUSION: In this study, ovarian cavity formation was suppressed in fish treated with low-dose AI or TF. High-dose AI (200 µg/g) induced precociously testicular tissue including spermatogenic germ cells together with fewer oocytes in the ovaries. In contrast, all the fish that received AI in combination with E2 had developed ovaries. These results suggest that endogenous esrogen is involved in ovarian differentiation in the anemonefish.

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Seasonal changes of serum sex steroids concentration and aromatase activity of gonad and brain in red-spotted grouper (Epinephelus akaara)

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BACKGROUND: In contrast to the numerous studies on seasonal changes of sex hormones in gonochoristic species, there have been relatively fewer studies on the relationship among gonadal development, sex steroids levels and aromatase activity in natural sex-reversing species.

METHODS: Levels of serum sex steroids (estradiol-17β, E_2; testosterone, T; 11-ketotestosterone, 11-KT) in male, female and natural sex-reversing (implanted with electronic labels) red-spotted grouper (*Epinephelus akaara*), and aromatase activity of gonad and brain in both male and female were investigated throughout an annually reproductive cycle. E_2 and T were determined by radioimmunoassay (RIA), while 11-KT by enzyme-linked immunosorbent assay (ELISA). Aromatase activity in the gonad and brain (forebrain, midbrain and hindbrain) was measured by a radiometric method.

RESULTS: In females, serum E_2 and T peaked during vitellogenesis, but in males and natural sex-reversing fish, 11-KT, T and E_2 reached peak during spermatogenesis. In addition, in females, serum 11-KT levels (monthly means: 0.32±0.03 ng/ml) which were very low did not significantly fluctuate during the annual reproductive cycle. In breeding season, females displayed higher E_2 levels than males and sex-reversing fish, while males and sex-reversing fish showed higher 11-KT levels and, to a lesser extent, higher T levels than females. Furthermore, the changing pattern of sex steroids in males was similar to that in natural sex-reversing fish, and a second peak of serum androgens 11-KT and T appeared in December both in male and natural sex-reversing fish; significantly higher serum 11-KT levels were observed in natural sex-reversing fish than that in females from December to April. In females, but not in males, aromatase activity of brain and gonad demonstrated significantly seasonal changes (exhibiting a peak in breeding season); moreover, aromatase activity in females was higher than that in males. Furthermore, significantly lower aromatase activity in testis was observed in breeding season, in contrast to that in ovary.

CONCLUSION: The present findings indicated that changes of serum sex steroids levels and aromatase activity in red-spotted grouper were closely associated with sex inversion. In addition, the present results also suggested that sex inversion in red-spotted grouper peaked mainly from December through March.

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Distinct Cytochrome P450 Aromatase Isoforms in Common Carp (Cyprinus carpio): gene specific tissue distribution and sex differences in adults

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BACKGROUND: The hormonal balance between estrogens and androgens appears crucial in the process of sex differentiation in developing teleost fish. This balance relies on the availability and activity of steroid synthesizing enzymes, and in particular cytochrome P450 aromatase (CYP19), the terminal enzyme in the steroidogenic pathway catalyzing the conversion of androgen (e.g. testosterone) into estrogens (e.g. estradiol). In many fish species, two forms of cytochrome P450 aromatase, expressed in both the brain and the ovary, have been implicated in controlling ovarian development. The current project will build on base-line research on the early gonadal development by exploring the sex-specific expression of aromatase in adults and also through ontogeny and gonad differentiation and development.

METHODS: The current work involved molecular cloning using RACE PCR to isolate cDNA encoding the two distinct isoforms, brain and ovary (termed cyp19b and cyp19a, respectively) in adult carp (*Cyprinus carpio*). Expression analysis of aromatase genes in adult and embryonic/juvenile carp will involve real-time quantitative PCR using SYBR green chemistry. This will examine tissue and sex-specific expression in adults. A larval rearing experiment will determine onset and changes in aromatase expression through development and with temperature as a modulator.

RESULTS: The cloned cDNA for cyp19b was a 2853bp fragment with an open reading frame (ORF) of 511AA, the cyp19a cDNA was 1782bp in length with an ORF of 518AA.Sequence and phylogenic analysis showed that these P450 isoforms were orthologous with previously described cyp19b and cyp19a in many other teleost fishes (e.g. zebrafish, goldfish, medaka, nile tilapia). Real-time analyses and larval rearing are currently underway.

CONCLUSION: The underlying mechanisms of sex differentiation in carp are unknown. Aromatase is known to control the conversion of testosterone to estrogen, and therefore may have an influence on gonadal sex differentiation. In order to investigate aromatase expression, it was shown that two isoforms (brain and gonadal) exist in common carp. Aromatase expression will be examined in adults and during ontology to determine timing and onset of aromatase gene expression as a proxy for molecular sex differentiation. The influence of temperature on biological processes involved in gonadal differentiation such as aromatase activity will also examined to determine an influence on sex ratio.

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Molecular cloning and expression of three estrogen receptors in the protogynous wrasse, *Halichoeres trimaculatus*

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BACKGROUND: In the previous studies, we have found that removal of estrogen is a key step to initiate sex change of protogynous wrasses. The effects of estrogen may be mediated through hormone receptors to maintain the functions of ovary in female while downstream regulation of those receptors may initiate sex change. Nevertheless, the molecular mechanisms of estrogen action in protogynous sex change remain unsolved.

METHODS: Threespot wrasses, *Halichoeres trimaculatus*, were collected from coral reef in Okinawa, Japan. The wrasse estrogen receptor alpha (ERα) cDNA was amplified by PCR using gene specific primers based on database information. To obtain ERβ1 and ERβ2 cDNAs, degenerate primers were designed from conserved regions of ERβ cDNA available in the database and subsequent RT-PCR was performed. The abundance of all ER transcripts in various tissues of initial-phase (IP) males (primary males), females (primary females) and terminal-phase (TP) males were determined by real-time PCR.

RESULTS: Two major fragments of ERβ were amplified from wrasse ovary by RT-PCR using degenerate primers. One fragment made-up partial cDNA sequence of 863 bp was called wrasse ERβ1, which displayed high nucleotide identity to Atlantic croaker ERβ, zebrafish ERβ1 (esr2b) and Japanese eel ERβ. Another fragment made-up partial cDNA sequence of 944 bp was called wrasse ERβ2, which displayed high nucleotide identity to Atlantic croaker ERγ, zebrafish ERβ2 (esr2a) and Nile tilapia ERβ. In order to quantify the wrasse ERα, ERβ1 and ERβ2 by real-time PCR, specific primer sets were designed and specificity was confirmed by the observation of single amplification products and by dissociation protocols in detection ranges of at least six orders of magnitude. Expression of wrasse ERα, ERβ1 and ERβ2 was determined in various tissues of IP males, females and TP males. The higher level of expression of ERα was observed in the ovary and liver of females but lower level was found in the testis and liver of males. On the other hand, expression of ERβ1 and ERβ2 was higher in testis than in ovary. However, the constant expression of ERβ1 occurred in intestine and liver with the exception of ERβ1 in the liver of TP males. The low levels of expression of all those ERs were observed in the brain and heart of all populations of wrasses.

CONCLUSION: Here, we cloned ERβ1 and ERβ2 and determined the expression level of three ERs of protogynous wrasses to investigate the role of ERs in mediating the action of estrogens. The high level of expression of ERα in the ovary suggests that it may act as a main mediator of estrogen action in the ovary. On the other hand, high level of ERβ1 and ERβ2 in the testis gives the evidence that ERβs may play common testicular functions such as spermatogenesis in testis. Therefore, present findings suggest that three ERs have different and sex-specific functions in protogynous sex change of wrasse.

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Identification of evolutionary conserved oestrogen responsive elements (EREs) in teleosts

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BACKGROUND: The biological effects of oestrogens are mainly mediated through binding to specific receptors, activation of oestrogen responsive elements (EREs) and modulation of gene transcription. Environmental oestrogens are able to modify the oestrogen homeostasis resulting in disruption of extensive gene networks. We aimed at generating a comprehensive (genome-wide) understanding of the biological functions of oestrogens using a comparative genomics approach. This information could be used to predict putative molecular pathways susceptible to modulation by exposure to environmental oestrogens.

METHODS: We developed a bioinformatic approach to identify the presence of functional EREs in the promoter regions of all available genes in the zebrafish genome (*Danio rerio*).
A java program (NNICE) was constructed to identify conserved DNA elements (or protein motifs) and their functional probability using trained neural networks. NNICE was implemented using a neural network, trained on 93 experimentally proven ERE sites, to identify the presence of EREs in the promoter region (up to 10 kb) of all available genes for zebrafish. NNICE further evaluated the likely functionality of the EREs identified, by determining their evolutionary conservancy in 2 other fish species (*Takifugu rubripes* and *Tetraodon nigroviridis*). Genes were only categorized as having a functional ERE in their promoter region when it was present in all 3 fish species.
The bioinformatic predictions were validated by comparison of the results obtained with a list of genes differentially expressed in zebrafish following exposure to a model oestrogen (17alpha-ethinyloestradiol). The biological context of the oestrogen responsive gene lists identified was investigated by analysing the bias in the distribution of Gene Ontology (GO) terms in this list compared with all genes tested.

RESULTS: We identified 1,398 genes containing conserved EREs in their promoter region and therefore, potentially, under the direct control of oestrogens. GO analysis identified a number of biological processes over-represented in this gene list, including *cell growth and-or maintenance, metabolism,* and *cell cycle*. There was a significant overlap between the list of genes predicted to be responsive to oestrogens using our bioinformatic approach and an experimentally derived gene list (microarray) of differentially expressed genes following exposure to oestrogen ($P<0.00001$).

CONCLUSION: This study provides novel insights into the potential biological processes regulated by oestrogens and how they may be impacted by exposure to environmental oestrogens at the whole genome scale. Furthermore, the bioinformatic tools developed here could be used to investigate other regulatory elements using a comparative genomics approach.

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The mechanism of transcriptional regulation of Mullerian inhibiting substance in Japanese flounder (*Paralichthys olivaceus*) and medaka (*Oryzias latipes*)

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BACKGROUND: Mullerian inhibiting substance (MIS), also known as anti-Mullerian hormone (AMH), is a glycoprotein belonging to the transforming growth factor superfamily. In mammals, MIS is responsible for regression of Mullerian ducts, anlagen of the female reproductive ducts, in the male fetus. In Japanese flounder (*Paralichthys olivaceus*) and medaka (*Oryzias latipes*), teleost fishes which do not possess the Mullerian ducts, *MIS* mRNA is expressed in the supporting cells of the gonads during gonadal sex differentiation. However, the mechanism of transcriptional regulation of *MIS* gene in teleost fish has yet to be clarified.
As a first step to analyze the mechanism of transcriptional regulation of MIS in Japanese flounder and medaka, we investigated whether the *MIS* gene expression is regulated by the steroidogenic factor 1 (SF-1))/Ad4 binding protein (Ad4BP) and the liver receptor homologue 1 (LRH-1), which are orphan receptors that regulates the expression of *MIS* gene in mammals.

METHODS: We first isolated the 5'-flanking regions of the flounder and medaka *MIS* genes, and investigated using electrophoretic mobility shift assay whether SF-1/Ad4BP and LRH-1 bind to the Ad4 binding sites in the putative promoters of *MIS* genes in the flounder and medaka. Moreover, we constructed the plasmid (MIS-luc) which linked the promoter of medaka *MIS* gene to luciferase reporter gene and those which expressed the SF-1/Ad4BP (CMV-SF1) or LRH-1 (CMV-LRH1), and both plasmids were transiently transfected into Hepa-E1 cells simultaneously.

RESULTS: Japanese flounder SF-1/Ad4BP and LRH-1 bound with a potential Ad4 binding site in the 5'-flanking region of the flounder *MIS* gene, while medaka SF-1/Ad4BP and LRH-1 bound with at least two potential Ad4 binding sites in the 5'-flanking region of medaka *MIS* gene. Moreover, luciferase activity was significantly induced by co-transfection of the CMV-SF1 or CMV-LRH1 plasmid with MIS-luc plasmid.

CONCLUSION: SF-1/Ad4BP and LRH-1 bound with potential Ad4 binding sites in the 5'-flanking regions of *MIS* genes in Japanese flounder and medaka. Moreover, their receptors induced the MIS transcriptional activity in Hepa-E1 cells. These results suggest that SF-1/Ad4BP and LRH-1 are directly involved in transcriptional regulation of *MIS* genes in Japanese flounder and medaka.

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Anti-Müllerian-hormone (amh) and its receptor (amhrII) homologous genes of medaka (Oryzias latipes) are involved in the process of sex differentiation

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BACKGROUND: The long-term goal of our studies is to analyse the molecular control mechanisms of spermatogenesis and the communication between germ line and soma in vivo and in an in vitro model (primary cell cultures). Our model organism, the medaka, possesses a male heterogametic XX/XY sex determination system and in certain strains the early identification of males and females is possible already 2 days after fertilisation. This facilitates the molecular analysis of sex determination, sex differentiation and spermatogenesis. A similar set of homologous genes controls these processes in mammals and fish but the genes may play different functional roles.

METHODS: The cellular differentiation can be followed genetically by studying the expression of stage-specific or cell type-specific marker genes like vasa (typically expressed in stem cells) or dmc1 and scp3 for meiotic- and protamine for postmeiotic gene expression. Further marker genes are available that allow to distinguish between somatic and germ line cells. The expression analysis was performed by RT-PCR and ISH.

RESULTS: We have cloned and characterized the anti-Müllerian-hormone gene (amh) and its corresponding type II receptor gene (amhrII) from medaka. The cloning of the medaka AMH type II receptor gene is the first example for a non-mammalian species. Recently, amh homologous genes have been identified in a few teleost fishes although teleosts do not develop a Müllerian duct. We present the sequence and expression data of medaka amh and amhrII from fertilisation up to adult fish. Special focus of our work was the difference between the male and female expression patterns. No sexually dimorphic expression of amh and amhrII during gonad development was observed while in adult females the expression of amhrII is weaker than in males. Medaka amh and amhrII are coexpressed in somatic cells of both gender.

CONCLUSION: The presented data shows the gonad specific expression of medaka amh and amhrII in somatic cells and no sexually dimorphic expression pattern of amh was occurred like described for the amh gene from other teleosts. Also the aromatase (cyp19a1) gene expression, a potential target of AMH signalling in mammals, was not observed to correlate with amh or amhrII expression in medaka. A function of AMH and its type II receptor during gonad formation and maintenance in fish is likely.

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Role of Mullerian inhibiting substance on gonadal sex differentiation in medaka (*Oryzias latipes*)

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BACKGROUND: First appearance of morphological sex differentiation in medaka is that the genetic female (XX) acquires more number of germ cells than the genetic male (XY) by the hatching stage. Mullerian inhibiting substance (MIS), also known as anti-Mullerian hormone, is a glycoprotein belonging to transforming growth factor β superfamily. In mammals, MIS is responsible for regression of Mullerian ducts, anlagen of the female reproductive ducts, in the male fetus. However, the role of MIS in gonadal sex differentiation of teleost fishes, which do not have the Mullerian ducts, has yet to be clarified.

To elucidate the role of MIS on gonadal sex differentiation in medaka (*Oryzias latipes*), which is a small laboratory fish with several advantages such as a short generation time, small genome size, and also several useful strains, we performed expression and functional analyses of MIS during the sex differentiation in medaka.

METHODS: We first isolated a *MIS* cDNA from medaka testes and examined the expression pattern of *MIS* mRNA in gonads of both sexes during sex differentiation period by *in situ* hybridization. Next, we performed loss-of-function experiment of MIS by injecting *MIS* antisense oligo (*MIS-AS*) into fertilized eggs of the *olvas-GFP* transgenic medaka line, which allows us to monitor germ cells by GFP fluorescence.

RESULTS: *MIS* mRNA was expressed specifically in the somatic cells surrounding the germ cells of both sexes during the sex differentiation. In loss-of-function experiment of MIS, before primordial germ cells reach the gonadal region, there were no significant differences in the number of GFP-positive germ cells of both sexes in *MIS-AS*-injected embryos compared to the controls. After the sex differentiation, there were a small numbers of the germ cells of both sexes in *MIS-AS*-injected embryos as compared to controls.

CONCLUSION: Loss-of-function of MIS resulted in suppression of germ cell proliferation in both sexes during the sex differentiation, indicating that MIS is required for germ cell proliferation during early gonadal sex differentiation in medaka.

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The expression pattern of AMH in Atlantic salmon (*Salmo salar*)

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BACKGROUND: Anti-Müllerian hormone (AMH), also called Müllerian inhibiting substance (MIS), is a member of the TGF-β superfamily. It was first discovered in mammals, as a crucial signal for the regression of the Müllerian ducts in the male embryo. Probable homologues have been found in all tetrapods and in fish. In mammals AMH is expressed in the Sertoli cells of the testis. A similar expression pattern has been found in Japanese eel. The function of AMH in fish is not well known, but it has been suggested to have a repressing effect on spermatogenesis in Japanese eel. The current study aims to investigate the expression and function of AMH in salmon (*Salmo salar*), and thus increase the knowledge about this protein in fish.

METHODS: Sequences of cDNA of salmon *AMH, Vasa, SCP3, Sox8, Sox9, Cyp11A* and *Cyp19A1* were obtained by RACE cloning. Samples of salmon from before hatching and for approximately six months onwards, in addition to samples from larger, juvenile fish were analyzed by rtPCR or fixed in paraformaldehyde to make sections for in situ hybridisations and immuno-histochemistry. Protein extracts were analyzed using a rabbit polyclonal antibody.

RESULTS: In situ hybridisations of juvenile testis showed expression of AMH mRNA in Sertoli cells. When studying RNA samples from before hatching to the alevins/fry stages, we found that AMH was undetectable until just before hatching and onwards. In males beginning puberty, AMH mRNA levels seemed to drop slightly. We further investigated the expression of other factors known to play a role in sex determination/differentiation and maturation process, such as Vasa (germline marker), SCP3 (marking the onset of meiosis), Sox9 (a regulator of AMH in mammals), Cyp11A (p450scc) (testosterone production), Cyp19A1 (estrogen production).

CONCLUSION: AMH mRNA seems to be expressed in juvenile fish approximately from hatching and onwards. In the zebrafish, AMH expression starts 17 days post fertilization, when the gonad is still undifferentiated. This might also be the case for salmon, in situ studies of embryos and alevins should be able to answer this.
Further, we found a decrease in the AMH levels as the fish matures. It has been shown earlier that the expression of AMH in the Sertoli cells decreases when the level of testosterone increases at the onset of puberty.

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Rainbow trout anti-Müllerian gene (*amh*) and its expression during sex differentiation and gametogenesis

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BACKGROUND: Anti-Müllerian hormone (AMH) also called Müllerian-inhibiting substance (MIS) is a member of the transforming growth factor superfamily which plays a major role during reproductive development in eutherians. In fish, AMH was initially described in the Japanese eel and called « spermatogenesis-related substance ».

METHODS: BAC library was screened with a rainbow trout cDNA probe. The positive BAC clones were sequenced. The sequences were aligned using CLUSTAL and homologies were searched by Blast. The promoter region was checked for the presence of transcription factor binding sites. *In situ* hybridization was performed on male and female gonads at different stages during the sex differentiation period (from 32 to 90 days post-fertilization) and in adults at different stages of gonad maturity.

RESULTS: We isolated and characterized a genomic sequence encoding a member of the transforming growth factor beta superfamily highly similar to AMH. In rainbow trout this genomic *amh* sequence spans a region of 3,400 bp. Based on the alignment with EST clones we determined that the *amh* gene is encoded by 6 exons (and 5 introns). Characterization of 1000 bp of the 5' flanking genomic region, revealed that this part of the promoter only contains a highly conserved SF1 binding site. But, surprisingly there is no SOX binding site, despite the essential role of Sox9 in the regulation of AMH in mammals. Expression of *amh* is localized by *in situ* hybridization both in male and female gonads during sex differentiation in some somatic cells surrounding germ cells with a stronger quantitative expression in the differentiating testis. Later this expression is restricted in the Sertoli cells of the testis and in the granulosa cells of the ovary.

CONCLUSION: Our data first suggest that SF1 might be a major regulator of *amh* gene in trout since, like in other species, a consensus SF1 binding site could be found. The absence of SOX binding site is a noticeable difference with the mice where Sox9 was shown as a major activating factor. *amh* expression pattern is conserved between the rainbow trout and eutherians in adult with a localization in Sertoli cells and granulosa cells, but clearly different during gonad differentiation as it is expressed both in males and females in rainbow trout in contrast with a testicular specific expression in mammals.

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The *gSOX11b* gene is predominantly expressed in the ovary of the orange-spotted grouper *Epinephelus coioides*

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BACKGROUND: *Sox*, a family of genes related to the mammalian *sex-determining region Y* (*SRY*) gene, are found throughout the animal kingdom, and are involved in the regulation of diverse developmental processes including sex determination. The orange-spotted grouper, *Epinephelus coioides*, is a protogynous hermaphroditic teleost, and a favorite marine food fish in Southeast Asia. Our previous studies have shown that *CYP19a* gene of the orange-spotted grouper was down regulated during sex change from female to male, and its promoter region contains SRY binding sites, suggesting that *SRY*-related genes may be involved in the regulation of *CYP19a* gene, and thus sex change. In this report, we cloned a *Sox* gene from the ovary cDNA library and analyzed its expression.

METHODS: The *gSox11b* gene was cloned from the ovary cDNA library with PCR method. The mRNA expression was analyzed with Northern blot and (semi-)RT-PCR.

RESULTS: The full-length *gSox11b* cDNA spans 2253bp, which encods a protein of 389 amino acids. Southern blot analysis revealed that there might be only one copy of *gSox11b* gene in the orange-spotted grouper genome. One-Step RT-PCR coupled with Southern blot analysis showed that *gSox11b* mRNA is wide-expressed at all tissues except the blood cells of the two-year-old orange-spotted grouper, and at all stages during the embryonic development. Northern blot analysis showed that *gSox11b* is expressed at a very high level in the ovary, but at very low levels in the brain and spleen, and undetectable in the adipose tissue of the two-year-old orange-spotted grouper and the testis of the natural sex-reversed male. Semi-quantitative RT-PCR analysis showed that the mRNA expression of *gSox11b* was significantly decreased during 17α methyltestosterone-induced sex change.

CONCLUSION: The *gSox11b* gene is predominantly expressed in the ovary of the orange-spotted grouper. The decreased mRNA expression of *gSox11b* gene during induced-sex change is parallel with the decreased expression of *CYP19a* gene as demonstrated previously, which suggests that *gSox11b* may be involved in the regulation of *CYP19a* gene and worth further study.

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Ol4E-T, a eukaryotic initial factor 4E binding protein of medaka fish (*Oryzias latipes*), interacts with Nanos and Vasa

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BACKGROUND: During early development, the germ cells are set aside from the somatic cells and they migrate to the germinal ridge to form the gonads along with the somatic cells. In *Drosophila*, *Chlamynomodas elegans*, anuran amphibians, and zebrafish, the germ line is predetermined by localization of the germ plasm. Recently, it was found that the medaka embryos also have the granule of germ plasm similar to that of zebrafish embryos. In *Drosophila*, a number of genes are required for specific steps of pole cell formation and further germ line development, such as *oskar*, *vasa, nanos* and *tudor*. Vasa and Nanos are well conserved throughout the animal kingdom. These two genes have been identified in medaka also as germ cell markers. Further, in *Drosophila* the homolog of eukaryotic initial factor 4E-transporter (4E-T), Cup was found to be engaged in localization and repression of *oskar*. It also interacts with Nanos to promote the normal development of the ovarian germline. However, the mechanism of germ plasm formation remains elusive. In this study, a homolog of Cup was isolated from medaka and named as Ol4E-T.

METHODS: Ol4E-T was obtained by RACE using primers based on the conserved sequences. The expression pattern was checked by Northern blot, RT-PCR, real-time RT-PCR, and in situ hybridization on gonad sections and whole embryos with DIG-labeled probes. The interactions of Ol4E-T with eIF4E, Nanos, and Olvas were studied by yeast two hybridization assays.

RESULTS: Ol4E-T cDNA is 3723 bp long and the ORF spans 2727 bp. It encodes a protein of 908 amino acids, with a molecular weight of 100.5 kD. In the genome, it spans 10.7 kb with 19 exons. Sequence analysis showed that Ol4E-T has a putative bipartite nuclear targeting sequence (NLS) and three putative nuclear export signals (NES). Ol4E-T is predominantly expressed in the gonads as shown by the Northern blot and RT-PCR. In situ hybridization revealed that this gene is expressed exclusively and strongly in the oocytes in the ovary and spermatogonia in the testis. Ol4E-T is deposited maternally in the zygotes. Eeven though its expression is ubiquitous in the embryos, the head and gonadal regions shows much more strong expressions. Yeast two hybridization showed that Ol4E-T interacts with eIF4E with the conserved motif, YTKEELL. The interaction was impaired when the mutant of this motif was used. Yeast two hybridization also found that Ol4E-T can interact with Nanos and Olvas.

CONCLUSION: Ol4E-T is the medaka homolog of Cup and 4E-T. It is a putative shuttle protein between the nucleus and cytoplasm. Ol4E-T binds to the eIF4E with the motif YTKEELL. Its expression is confined to the oocytes in the ovary and spermatogonia in the testis. Ol4E-T aids the embryonic development of medaka as a maternal factor. Like *Drosophila* Cup, Ol4E-T also may have functions in the assembly of germ plasm and specification of germ cells.

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Deceased Dax-1 and increased Sf-1 expression for the development of vitellogenic ovary in the protandrous black porgy, *Acanthopagrus schlegeli*

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BACKGROUND: Black porgy fish, a marine protandrous hermaphrodite, are functional males for the first two years of life but begin to sexually change to female during the third year. Therefore, the objectives were to study the possible roles of Sf-1 and Dax-1 in the male sex differentiation and gonadal development, especially related to the development of vitellogenic oocytes.

METHODS: Juvenile black porgies were collected from 3-months to 1-year-old period to study male sex differentiation. Estradiol (E2, 6 mg/kg feed)-administration was given to juvenile and 1^+-year-old fish to induce sex change to study the controlled female sex differentiation. Fish from 2^+- to 3-year-old period were collected to investigate the natural sex change. Testicular tissue was further removed in 1^+-year-old fish to investigate ovarian development. Transcripts of Sf-1, Dax-1 and genes related to steoidogenic enzymes were measured by RT-PCR and real-time quantitative PCR.

RESULTS: In control fish, Sf-1 transcripts were significantly increased right after sex differentiation (4-5 months age) and reached to highest levels after the age of 8 months. Dax-1 transcripts remained no change during the period of sex differentiation and then increased after the age of 8 months. E2-administration induced an early sex change in 1-year-old fish with low expression of Sf-1 and Dax-1 transcripts, but ovarian Dax-1 transcripts were increased to high levels after 8 months age. Ovarian Cyp19a was increased after one month of E2-administration but then decreased later. High Sf-1 and Dax-1 transcripts were detected in the testicular tissue before sex change. In contrast, low Sf-1 and high Dax-1 transcripts were detected in the bisexual ovarian tissue. Ovarian tissue was induced by E2-administration with the profiles of low Sf-1, high Dax-1 and low steroidogenic genes in ovarian tissue. In the testis-excised fish (1^+-year-old) and natural sex changing fish (2^+-yeasr-old), high Sf-1, low Dax-1 and high Cyp19a were detected in the ovarian tissue (vitellogenic ovary).

CONCLUSION: The data suggest that testicular development requires cooperative function of Sf-1 and Dax-1. In contrast, Sf-1 and Dax-1 have the antagonistic interaction for the ovarian development. Low Dax-1 and low Sf-1 expression may regulate the development of vitellogenic ovary in the protandrous black porgy.

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Roles and transcriptional regulation of Dmrt1 during gonadal sex differentiation in tilapia (*Oreochromis niloticus*)

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BACKGROUND: Dmrt1 has been implicated in male sex determination and gonad differentiation in vertebrates. In tilapia, Dmrt1 is expressed higher in male gonad than female from 5 days after hatching; while the morphological differentiation of gonad occurs around 25 days after hatching. Treatment of XX fry with androgen resulted in up-regulation of Dmrt1 and sex reversal to male; while treatment of XY fry with estrogen resulted in down-regulation of Dmrt1 and sex reversal to female. However, the target genes and transcriptional regulation of Dmrt1 gene remains elusive.

METHODS: Promoter assays using mammalian cell lines were performed to test whether Dmrt1 can regulate the transcription of steroidogenic genes, such as Cyp19a1, StAR, P450c17, Cyp11a1 and Cyp11b. Pull-down assays and mammalian two-hybrid assays were performed to confirm the interaction between Dmrt1 and other transcription factors. In vivo transgenic overexpression of Dmrt1 and its dominant negative mutant were performed by injection of these GFP constructs into fertilized XX and XY eggs, respectively. Furthermore, the 2.5kb promoter region of tilapia Dmrt1 was isolated, inserted into pGL3-basic promoter-reporter vector, and promoter assays were performed using several transcription factors, such as Ad4BP/SF-1, LRH-1, Creb, Gata4 and Dax1.

RESULTS: Under in vitro conditions, Dmrt1 alone can suppress the basal as well as Ad4BP/SF-1 and Foxl2 mediated Cyp19a1 transcription. Dmrt1 can also suppress Ad4BP/SF-1 mediated StAR and P450c17 but activate the Cyp11a1 and Cyp11b promoter activity. Dmrt1 can form heterodimers with Ad4BP/SF-1 and Foxl2 by direct physical interaction. Under in vivo conditions, Disruption of endogenous Dmrt1 in XY tilapia by overexpression of its dominant negative mutant induces varying degrees of testicular degeneration, increased expression of Cyp19a1 and increased serum levels of estradiol-17β, while XX fish with wild type Dmrt1 overexpression exhibited significant follicular degeneration, abnormal ovarian cavity formation, down-regulated aromatase expression and a drop in the serum levels of estradiol-17β. Sequence analysis showed that there are putative binding sites of ER, Ad4BP/SF1, Creb and Gata4 on the promoter of Dmrt1; in luciferase assay, LRH-1 and Ad4BP/SF1 can activate, while Dax1 and Creb can repress the basic or LRH-1 and Ad4BP/SF1 activated Dmrt1 promoter activity in a dosage dependent manner.

CONCLUSION: Altogether, these results suggest that Dmrt1 plays a decisive role in the testicular differentiation of the Nile tilapia by down-regulating aromatase expression and possibly by shifting the entire steroidogenic pathway towards androgen production. LRH-1, Ad4BP/SF1, Creb, Dax1 and Gata4 may be condidate factors in controling the expression of Dmrt1.

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Differential expression of dmrt1 and ovarian aromatase during sex differentiation period in pejerrey, Odontesthes bonariensis

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BACKGROUND: The process of sex determination in teleosts can be controlled by genetic and/or environmental factors. Our experimental model, the pejerrey, is a gonochoristic inland water fish with a very strong temperature sex determination (TSD) system. The proportion of females decreases gradually from 100% at 19°C to 50% around 23-25°C and 0% at 29°C. Dmrt1 is a transcription factor playing an important role in male sex differentiation in vertebrates. On the other hand, the ovarian form of cytochrome P450 aromatase (cyp19A1), the enzyme that catalyzes the conversion of androgens to estrogens, has been implicated in female sex differentiation in TSD reptiles, amphibians, and fish. However, the simultaneous expression of both genes during the period of sex determination/differentiation has been poorly studied in fish.

METHODS: Larvae were stocked in three 200-l tanks and kept at 17°C (feminizing), 25°C (mixed-sex producing) and 29°C ± 0.5°C (masculinizing temperature). Larvae (n=5 at 17 and 29°C, n=10 at 25°C) were sampled weekly from hatching to week 8. The expression profiles of dmrt1 and cyp19A1 were determined by Real Time PCR during the sex determination/differentiation period.

RESULTS: The abundance of dmrt1 mRNA showed significant differences among larvae maintained at 17°C and 29°C. Its expression significantly increased after week 4 at 29°C, remaining low throughout the study at 17°C. On the other hand, cyp19A1 expression increased from week 6 at 17°C but remained at low levels at 29°C. Both differences were observed before histological sex differentiation of the gonads (week 7 and 6 for females at 17°C and 25°C, respectively, and week 7 and 6 for males at 25°C and 29°C, respectively). The expression levels of both genes at the mixed-sex producing temperature were bimodal, showing similar values to those at either feminizing or masculinizing temperatures.

CONCLUSION: Dmrt1 and cyp19A1 genes showed differential expression during the sex differentiation period at feminizing, mixed-sex producing and masculinizing, temperatures. The expression patterns of the two genes support the idea that both are involved in the process of sex differentiation in this species.

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Characterization and expression profiles of DMRT1, AMH, SF1 and P450aro genes during gonadal sex differentiation in Patagonian pejerrey *Odontesthes hatcheri*

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BACKGROUND: Temperature sex determination (TSD) has been documented in many fish species. Our understanding of this mechanism is greatly limited by the difficulty in discerning the genotypic sex of embryos/juveniles prior to morphological sex differentiation. In order to redress this problem, our group recently developed a strain of *Odontesthes hatcheri*, a species with genotypic sex determination (GSD) and TSD at intermediate and extreme temperatures, respectively, that possesses a SNP sex-linked marker. In this study, we used this strain (BBF1) to analyze how some sex differentiation genes are expressed in this species before and during gonadal sex differentiation at a sexually neutral, intermediate temperature.

METHODS: The genes SF1, DMRT1, P450aro and AMH of *O. hatcheri* were cloned, sequenced and their structure analyzed. Their expression levels during gonadal differentiation and early development (from 0 to 7 weeks after hatching, or wah) at 21ºC were then analyzed by Real-Time RT PCR. In all samples, RNA was isolated only from the trunk whereas the caudal portion was used for DNA extraction and sex genotyping using a PCR-based SNP marker specific to the strain.

RESULTS: *O. hatcheri* had one single copy each of SF1, P450aro and AMH but three DMRT1 isoforms originated by differential splicing: DMRT1a, DMRT1b and DMRT1c. The expression profiles of the three DMRT1s were not sexually dimorphic during the period analyzed. AMH, in contrast, showed upregulation in males from 1wah while P450aro showed upregulation in females from 2wah. SF1 was equally regulated in both sexes, but the expression pattern from 2wah to 4wah (with an apparent peak at 3wah) mimicked those of AMH and P450aro.

CONCLUSION: Three isoforms of DMRT1 were identified in *O. hatcheri* but the function of each variant during gonadogenesis remains to be elucidated. The differential expression of P450aro and AMH genes soon after hatching indicates that sex in this species is probably determined soon after or even before hatching and that these genes may play an import role during gonadal differentiation since ovaries and testes become histologically distinguishable at the fourth week. The similar expression of SF1 with AMH and P450aro genes is consistent with a regulatory mechanism of SF1 over AMH and P450aro genes as described for some fishes and mammals.

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Molecular cloning of Dmrt1, Foxl2 and Cyp19 in Southern catfish and their possible roles in sex differentiation

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BACKGROUND: Southern catfish is a good model for studying gonadal sex differentiation in fish. Under laboratory conditions, the fry develop into all-female individuals without any treatment. Our previous work has investigated the histology of the gonad during early sex differentiation of this all-female population. However the mechanism of this process is still unknown.

METHODS: Full length cDNAs of Dmrt1 (a, and b isoform), Foxl2, Cyp19a and Cyp19b were isolated from the southern catfish by RT-PCR and RACE. Tissue distribution of these genes were investigated by RT-PCR. All-female Southern catfish fry were treated with fadrozole (F), tamoxifen (T) and 17β-estradiol (E2) respectively, from 5 to 25 days after hatching (dah). The influence of these treatment on the expression levels of these genes were studied by semi-quantitative RT-PCR at 65 dah. Influence of the treatment on gonadal sex differentiation and sex ratio were checked by histological analysis.

RESULTS: Dmrt1a and Dmrt1b were found to be expressed in the gonads, being higher in the testis. A low expression level of Dmrt1b was also detected in the intestine and kidney of the male. Foxl2 was found to be expressed extensively in the brain, pituitary, gill and gonads, with the highest level in the ovary. Cyp19b was found to be expressed in the brain, spleen and gonads, while Cyp19a was only expressed in the gonads and spleen. In the F, T and F+T treated groups, Dmrt1a and Dmrt1b were up-regulated in the gonad whereas Foxl2 and Cyp19a were down-regulated, while Cyp19b in the gonad remained unchanged. Furthermore, down-regulation of Foxl2 and Cyp19b was also detected in the brain. In the E2 treated group, Dmrt1a and Dmrt1b were down-regulated to an undetectable level in the gonad whereas Foxl2 and Cyp19b were up-regulated in the brain. Consistent with the observed changes in the expressions of these genes, 56%, 70% and 80% sex reversed male individuals were obtained in the F, T and F+T treated groups, respectively.

CONCLUSION: Foxl2, Cyp19a (gonad only) and Cyp19b are expressed higher in the female brain and gonad, thus favoring ovarian differentiation. On the other hand, Dmrt1 are expressed higher in the male gonad, thus favoring male sex differentiation. Treatment with F, T and F+T of the all-female fry reversed the expression patterns of these genes and caused sex reversal of the treated fish. Taken together, our results indicate that Dmrt1, Foxl2, Cyp19a and Cyp19b are important genes implicated in sex differentiation in Southern catfish.

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Gene expression during gonad transformation in zebrafish: The analysis of three candidate genes and identification of new testis markers

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BACKGROUND: Zebrafish (*Danio rerio*) is an extensively studied model organism for vertebrate biology. In contrast to the vast amount of data accumulated about most aspects of its biology, our knowledge about the sex determination and gonad differentiation process in zebrafish is rather poor. Although the morphology of gonad differentiation - through a peculiar "juvenile ovary-to-testis" transformation process - has been described, its molecular mechanism has not been studied so far.

METHODS: In our study, *vas:egfp* transgenic zebrafish were used to select individuals undergoing gonad transformation, during which the gonadal EGFP signal decreases and eventually disappears in males. The expression of a selected set of candidate genes has been analyzed in these individuals by real-time PCR and *in situ* hybridization. The global expression profile of the transforming gonads was also analyzed and compared to that of non-transforming female gonads using a 6.5K "Gonad UniClone" zebrafish cDNA microarray developed in our laboratory.

RESULTS: Ovarian aromatase (*cyp19a1a*) was found to be down-regulated during gonad transformation. Anti-mullerian hormone (*amh*) showed reciprocal expression level to *cyp19a1a*; it became up-regulated in those regions where *cyp19a1a* had been expressed before transformation, i.e. in the somatic cells immediately surrounding oocytes. The expression of 11β-hydroxylase (*P45011β*), coding for an enzyme involved with the generation of 11-keto testosterone (11-KT), was also found to be up-regulated during this process. However, it was expressed later than *amh* and was not localized in the somatic cells around the oocytes. Comparative analysis of transcriptomes between transforming and non-transforming gonads (i.e. differentiating testes and ovaries) also identified novel genes (>100) showing differential expression (at least 3 folds). The characterization of these genes is in process.

CONCLUSION: Our results support the hypothesis that AMH may inhibit the expression of *cyp19a1a*, creating a suboptimal environment for the survival of oocytes and eventually leading to the transformation of juvenile ovary to testis. 11-KT was proposed earlier as the inducer of testis differentiation in protogynous marine sex changers, but it apparently does not fulfill the same role in zebrafish owing to the timing and localization of its expression. Our gonad-derived cDNA microarray has revealed a set of novel genes with potential roles in vertebrate gonad differentiation.

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Gene expression profile in XX and XY gonads during sex differentiation in the Nile tilapia, *Oreochromis niloticus*

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BACKGROUND: In the Nile tilapia, expression of aromatase (cyp19) is characteristically detected in indifferent XX gonads from a very early developmental stage. In addition, XX gonads differentiate to testis, if the aromatase expression is inhibited. Together, these facts indicate that estrogen production in the early larval stage is critical for determining gonadal sex differentiation. The aim of the present study was to demonstrate expression profiles for genes involved in steroid physiology such as steroidogenic enzymes, steroid receptors and related transcription factors, during gonadal sex determination and differentiation in the Nile tilapia.

METHODS: Using all-female and all-male tilapia fry, gonadal ridges were isolated throughout the sex differentiation period. Total RNA was extracted from each sample, then used for one-step real time RT-PCR analyses. At the same time, sex-reversal experiments were conducted, and changes in gene expression patterns were investigated in the same manner.

RESULTS: In the XX gonads, the expression of foxl2 and cyp19 mRNA was increased 5 days after hatching (daf). Transcript levels of other steroidogenic enzymes, such as cholesterol side-chain cleavage, 3b-hydroxysteroid dehydrogenase, and 17a-hydroxylase/c17-20 lyase, were significantly increased after 10 dah. In the XY gonads, expression of foxl2 and cyp19 was not detected throughout the sampling period. The mRNA levels of other steroidogenic enzymes were equal to those in the XX gonads before 10 dah, but unlike XX gonads, abundance of these transcripts in XY gonads was maintained at low levels thereafter. Changes in levels of steroidogenic enzymes in both sexes seemed to be reflected in a change in ad4bp/sf1 expression. Steroid hormone receptors, such as estrogen receptor a, b1 and b2 and androgen receptor a and b, were all detected at relatively high levels from the first sampling day in both sexes, and there was no sexual dimorphisms. Masculinization of XX gonads with androgen or aromatase inhibitor suppressed expression of almost all steroidogenic enzymes, and ad4bp and foxl2, whereas it upregulated dmrt1 expression. In contrast, feminization of XY gonads with estrogen induced expression of steroidogenic enzymes, ad4bp and foxl2, and meanwhile, resulted in suppression of dmrt1 expression.

CONCLUSION: The present study indicates that steroidogenesis is activated much earlier in the indifferent XX than XY gonad. The characteristic cyp19 expression in the indifferent XX gonads was appeared to be regulated by ad4bp and foxl2, while other steroidogenic enzymes (not all) seemed to be regulated mainly by ad4bp. This study also suggests that estrogen production in early XX gonads possibly suppresses dmrt1 expression, the inducer of testicular differentiation.

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Characterization and expression of genes involved in sexual differentiation in Atlantic halibut (*Hippoglossus hippoglossus*)

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BACKGROUND: Elevated ovarian aromatase (cyp19a1) expression has been shown to be a critical factor for ovarian differentiation in many animals, including fish. We have previously observed that cyp19a1 is expressed exclusively in the ovary in sexually differentiated Atlantic halibut. In juveniles, cyp19a1 expression increases prior to histological ovarian differentiation. On the other hand, 11β-hydroxylase (cyp11b1) is expressed in testis but not in ovary. This study further characterizes genes related to sexual differentiation and examines their expression to understand sex differentiation at the molecular level.

METHODS: Partial sequences of over 20 genes were obtained either from ESTs generated in the Pleurogene project (http://pleurogene.ca/index.php) or by RT-PCR using degenerate primers based on conserved amino acid sequences. The PCR products were cloned and sequenced and primers designed for real-time PCR. Gene expression was examined by real-time PCR in 14 halibut tissues from sexually differentiated fish.

RESULTS: In addition to the three genes previously examined, ovary- and brain-aromatase (cyp19a2) and 11β-hydroxylase, we obtained partial cDNA sequences of (1) steroidogenic enzyme genes including cholesterol side chain cleavage enzyme (P450scc), 17α-hydroxylase/C17, 21 lyase (P450c17), 3β-hydroxysteroid dehydrogenase (3βHSD), 11βHSD, 17βHSD1, 17βHSD8 and 20βHSD; (2) sex steroid hormone receptor genes including estrogen receptor (ER) α and β, and androgen receptor (AR), and (3) genes possibly related to gonadal differentiation including three SRY-like box genes, SOX3, SOX5 and SOX9, anti-Mullerian hormone (AMH), steroidogenic factor 1 (SF1), liver receptor homologue-1 (LRH1), Wilms' tumor 1 (WT1), fibroblast growth factor 8 (FGF8), GATA binding protein 6 (GATA6), forkhead box protein L2 (FOXL2), seminal plasma glycoprotein (SPG120), sex steroid binding globulin (SHBG), and glucocorticoid receptor (GCR). We also included doublesex and mab-3-related transcription factor 1 (DMRT1) gene in the expression study. Except SPG120, these sequences showed 78 – 94% identity to published sequences from other fish species. Real-time PCR analysis of RNA from sexually differentiated halibut showed differences in expression level in the gonads but not in brain. 11βHSD, 17βHSD1, WT1, and SOX3 exhibited higher expression in the ovary than testis, while P450c17, AMH, SF1, SOX9 and DMRT1 exhibited higher expression in the testis.

CONCLUSION: We characterized over 20 genes related to sex differentiation in Atlantic halibut and examined their expression in tissues from sexually differentiated fish. Several genes showed differential expression between the sexes and their expression will be examined in developing juveniles to investigate their role in sexual differentiation.

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The T-box transcription factor, T-box 1 (*tbx1*), is a potential new player involved in rainbow trout (*Oncorhynchus mykiss*) testicular differentiation

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BACKGROUND: The T-box transcription factor gene family is characterized by the presence of a conserved T-domain and is widely involved in essential cascades required for normal development, i.e., early embryonic cell fate decision, regulation of extra embryonic structures development and organogenesis. Among this family, the Tbx1 gene has been thoroughly investigated in many aspects of embryonic development. However, no information was available on its putative role during gonad sex differentiation in any vertebrate species. A genomic screen using rainbow trout cDNA microarrays that has been developed in our laboratory highlighted several genes showing specific ovarian or testicular expression profiles. This work reports on a first characterization of one of these genes encoding a putative T-box transcription factor.

METHODS: The clone corresponding to the differentially expressed signal found following microarrays analysis was sequenced entirely. Protein and cDNA homologies were searched using blast strategies against various databases including a rainbow trout Expressed Sequence Tag (EST) database (http://sigenae.org/). Phylogeny of the deduced proteins was carried out using the Figenix software platform (http://www.up.univ-mrs.fr/evol/figenix/index.html). Gene expression profiles were analyzed by real time RT-PCR on differentiating gonads sampled on all male and all female genetic populations (from 32 to 90 days post fertilization, dpf).

RESULTS: The temporal expression profile of this gene was analyzed by real-time RT-PCR. This analysis confirms that this gene was over-expressed during testicular differentiation with a clear differential expression between testis and ovarian differentiation detected as early as 40 dpf. Blastx searches reveal that this gene encodes a T-box transcription factor exhibiting a good homology with vertebrate T-Box 1 genes. Search against rainbow trout ESTs database identifies another highly related trout gene. Phylogeny reconstruction shows that these two genes are duplicated paralogs (*tbx1a* and *tbx1b*) of the *Tbx1* gene.

CONCLUSION: We reported here for the first time in any vertebrate that T-box 1 could be involved in testicular differentiation. At present, this hypothesis is only supported by the occurrence of a testis-specific expression profile during gonad differentiation in rainbow trout. Some additional experiments are currently under way in our laboratory to better characterize the implication of these two Tbx1 paralogs in trout sex differentiation. They will be presented and discussed.

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Sex Dimorphic Expression of Gene Markers in Zebrafish Gonad Development

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BACKGROUND: No sex determining gene has been identified in zebrafish and very limited knowledge about the sex differentiation process is available to date. Zebrafish become sexually mature at about 3 months of age, but its gender is not easily distinguishable by appearance before 2 months. Histology study by Japanese pioneers has reported that oocytes can be observed in the population at 3 weeks postfertilzation while testis-ova can be seen from 4 to 8 weeks followed by normal testis structure.

METHODS: We manipulate monosex population by steroids. Masculinized population is achieved by testosterone treatment while feminized population can be obtained by mating this sex reversed male with wildtype female. Among the offspring, super female can be obtained, which can give birth to feminized population. The gender of the fish is confirmed by histology (paraffin sectioning followed by HE staining) of the gonad at 6 months of age. Gene expression patterns are analyzed by whole mount RNA in situ hybridization at 2.5 and 3 weeks..

RESULTS: Sex dimorphic expression is observed in genes including *cyp11a1*, *cyp19a* and *sox9a* by 3 weeks of age. *Cyp11a1* is expressed in cytoplasm of the oocytes in female but somatic cells in male gonad. *Sox9a* is expressed in both male and female gonads but down regulated in female by. In our histology study, primordial germ cells are observed to have differentiated into gonocytes by 10 days postfertilization (dpf). We observed the onset of expression of the early somatic steroidogenic gene, *cyp19a*, also at 10 dpf corresponding to such gonocytes differentiation. Its expression persists until 3 weeks of age when down regulation is found in male gonads, but remain high in female gonad.

CONCLUSION: This study demonstrates the sex dimorphic expression of *cyp11a1*, *cyp19a* and *sox9a* by 3 weeks of age in zebrafish. This reveals an earlier time point on the onset of sex differentiation by 3 weeks of age before any histological changes is reveal in the male gonad. The on set time of *cyp19a* expression in gonad in correspondence with the gonocytes differentiation at 10 dpf also suggested a possible role of *cyp19a* in germ cell differentiation.

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Masculinization by 11beta-hydroxyandrostenedione of female rainbow trout induces a marked dysregulation of gonadal gene expression profiles

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BACKGROUND: Fish gonadal sex differentiation is very sensitive to sex steroids treatments providing an efficient strategy to control the sexual phenotype of fish for aquaculture purpose. However the mechanisms of action of such treatments are poorly understood. This study was conceived to investigate what are the main effects of an androgen masculinizing treatment on gonadal gene expression profiles in the rainbow trout during sex differentiation.

METHODS: Gonads were sampled from both in control populations (genetic all-male and all-female fish), and in all-female fed with 11beta-hydroxyandrostenedione (10mg / Kg of food for 3 months). In each group, 20 to 100 gonads were sampled and pooled in duplicates at various stages of larvae development. These samples were analyzed according to a large-scale expression profiling approach using rainbow trout microarrays combined with detailed gene ontology (GO) analysis to characterize the most important molecular features occurring during gonad masculinization by the androgen treatment.

RESULTS: 2,474 genes were characterized as upregulated or downregulated in the gonads of female rainbow trout masculinized with androgen in comparison with control male and female populations. These genes can be classified in 13 k-means clusters of temporally correlated expression profiles. GO data mining reveals that this treatment triggers a marked down-regulation of genes potentially involved in early oogenesis processes (GO mitotic cell cycle, nucleolus, protein biosynthesis), and an up-regulation of the translation machinery (GO ribosome) along with a down-regulation of proteolysis (GO proteolysis, peptidase and metallopeptidase activity). Genes considered as muscle markers (GO muscle contraction) and genes annotated as structural constituent of the extracellular matrix (GO extracellular matrix) or related to meiosis (GO chromosome and GO meiosis) were found significantly enriched in the two clusters of genes specifically up-regulated in androgen treated females. Finally GO annotations related to sex differentiation and steroid biosynthesis were particularly found in a cluster of genes showing a clear over-expression only in control males ; and interestingly none of these genes was stimulated by the androgen treatment.

CONCLUSION: This study clearly shows that androgen treatment results in a marked dysregulation of gene expression profiles when compared to natural testicular or ovarian differentiation. The complete inhibition of the female gene expression patterns along with the absence of similarity with the male specific gene expression patterns suggest that androgens acts mainly through an inhibition of female development rather than through a direct stimulatory effect on testicular differentiation.

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Left-Right gene expression asymmetry in gonads of rainbow trout, *Oncorhynchus mykiss*, following masculinization treatments with androgens

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BACKGROUND: In vertebrates, despite a bilateral symmetrical appearance of the body plan, some internal organs are organized in an asymmetrical left-right (L/R) manner. Gonads are generally regarded as symmetrical paired organs with the well-known exception of birds. However, a few reports do exist on L/R asymmetry of gonads in other vertebrates. When reported in fish, these asymmetries are often associated with genetic or hormonal perturbations. We therefore investigated in rainbow trout whether some genes exhibit or not a L/R asymmetry in gonads following masculinization induced by an androgen treatment.

METHODS: An all female genetic population of rainbow trout larvae, was treated by repetitive immersions with 17α-methyltestosterone (17αMT, 3 immersions of 2 hours at 10, 15 and 21 days after 50% hatching in 10 mg 17αMT / l of water). The right and the left gonads were sampled separately 3 months after fertilization (1 month after the last treatment) on 33 treated animals and 5 control females. Histological examination of gonads was performed 9 months after fertilization. Gene expression profiles were determined by real-time PCR analysis of 18 genes with a characteristic expression pattern during either natural testicular differentiation (*nr0b1*, *nr5a1b*, *sox9a.1*, *cyp11a1*, *amh*, *cyp11b2.1*, *pax2a*, *igf2*, *hsd3b1*, *cyp17a1*, *dmrt1*) or ovarian differentiation (*cyp19a1a*, *nup62*, *casp3*, *fig1a*, *gdf9*, *sox24*, *aldob*).

RESULTS: The 17αMT immersion treatment induces a large range of effects in the resulting gonadal phenotype i.e., from total masculinization (perfect testicular structure) to no effect at all (perfect ovarian structure) with different degree of intersexual gonads. Gene expression patterns in the left and right gonads of females treated with 17alphaMT reveals that most of the genes investigated exhibit some left / right differences in their expression profiles. Theses gene are clustered in different left and right subgroups and a better correlation can be observed between expressions of different genes belonging to the same left or right subgroups than between the L/R expressions of same genes. However, a few genes like the male marker *hsd3b1* (*3betaHSD*) or the female marker *cyp19a1a* (*aromatase*) do not exhibit any L/R expression asymmetry.

CONCLUSION: These results demonstrated that rainbow trout larvae gonads exhibit a clear lateral asymmetrical response to a masculinizing treatment. This suggests that this observed L/R asymmetry is the result of an early asymmetry of the gonad during the sex differentiation period.

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Sex control strategies for the masculinization of common carp (*Cyprinus carpio L*) and tench (*Tinca tinca*)

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BACKGROUND: The most promising method for sex control in fish is the genetic production of monosex progenies by crossing sex-reversed fish with normal fish. In many species of cultured fish, females exhibit higher growth rates than males and attain larger sizes. Rearing of all-female carp and tench progenies can increase production yield from 7-8% and 15-20 %. The present study used gynogenetic offspring of both species to investigate the sex control by oral administration of different treatment with measuring the concentration of circulating steroids in the water of the recirculation systems and in blood plasma.

METHODS: In term of carp masculinization experiment incorporating oral administration of 17α-methyltestosterone (17α-MT) into the feed at 100 mg kg^{-1}. In term of tench, the oral administration of (17α-MT; 100 mg kg^{-1}), tamoxifen ((TA) 50, 100 and 150 mg kg^{-1}) or combination of (MT+TA, 100+100 mg kg^{-1}) for various durations were applied for masculinization. In addition, effect of water pollution on masculinization and concentrations of circulating steroids in the blood plasma were examined by liquid chromatography (HPLC/MS/MS) on an LCQ quadrupole ion trap mass spectrometer equipped with (APCI).

RESULTS: In term of common carp the percentage of reversed males varied from 61.5 to 97.4% among groups fed (MT-F) and from 85.2 to 100% among groups exposed to water polluted by MT without MT feeding. The proportions of inverted males between treatments were not significantly different. The blood plasma concentration of 17α-MT was significantly higher (41.7 ± 30.1 to 97.5 ± 49 $ng.ml^{-1}$) in the MT-F groups than in the MT-NF (2.5 ± 0.1 to 81 ± 42.7 $ng.ml^{-1}$). The levels of 11-ketotestosterone (11-KT) ranged from 5.1 ± 0.2 to 52 ± 7.1 $ng.ml^{-1}$ in MT-F groups and 5.6 ± 2.4 to 88.4 ± 9.2 $ng.ml^{-1}$ in MT-NF groups. Mean level of steroids in the control groups ranged from 2.6 ± 0.1 to 130.4 ± 46.4 $ng.ml^{-1}$ for 11-KT; 1.0 ± 0.2 to 36.7 ± 15.4 $ng.ml^{-1}$ for T and from 4.0 ± 0.1 to 11.35 ± 0.6 $ng.ml^{-1}$ for E_2, respectively. The water in the recirculation systems was polluted with 17α-MT at the level of 0.33 – 2.68 $\mu g.l^{-1}$ during the sampling period. Reversed males of common carp did not have abnormalities in development of sperm ducts. This permits the stripping sperm of reversed carp neomales for mass production of all-female progenies. In terms of tench, low masculinizing effect (20-25%) could be demonstrated for groups fed (MT) and also (TA). For groups that were exposed to the water polluted by (MT) moderated (10-15%) masculinization effect was observed. Significantly higher proportion of inverted males (40-45%) in terms of tench was observed among group fed diet containing (100 mg MT+100mg TA).

CONCLUSION: In terms of common carp, under the present culture conditions, not only oral administration of 17α-MT, but also direct exposure to water polluted with 17α-MT can cause a high level of masculinization of gynogenetic offspring of common carp. In terms of tench the significantly highest level of masculinization could be obtained by oral administration of 17α MT in combination with tamoxifen.

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Post-sex differentiational sex reversal in female common carp (Cyprinus carpio)

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BACKGROUND: It has been believed that gonochoristic fish can not change their sex of gonad after gonadal sex differentiation because of the loss of bipotentiality. Recently, it had been shown that inhibition of estrogen biosynthesis induce complete sex reversal in adult female tilapia (>1-year-old), indicating that sexual bipotentiality is maintained far beyond the embryonic labile period. To investigate the gonadal plasticity in other groups of gonochoristic fish, we address the potential ability of 'post-sex differentiational' sex reversal in common carp.

METHODS: Genetically controlled female (XX) population of common carp was produced by gynogenesis. One-year old all-female carp were fed with Fadrozole hydrochloride hydrate (0.2mg/g diet), an aromatase inhibitor (AI) for 24 weeks. Fish were sacrificed biweekly from the start of treatment and subjected to histological analysis.

RESULTS: At the start of the treatment, ovarian cavity had been formed and gonad contained oogonia and oocytes at early stages of oogenesis. At week 8, testicular lobule-like structure and cysts of spermatocytes began to appear. Spermatozoa were initially observed at week 16 and they spread to whole gonad in some individuals at week 18. On the other hand, there remained ovarian cavity throughout the entire period of experiment.

CONCLUSION: In this study, testicular differentiation in ovary was induced by depletion of endogenous estrogen in common carp, while the treatment was performed in immature fish. This observation shows that sexual bipotentiality of ovary is maintained in common carp as reported in tilapia at least before puberty.

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Early sex reversal during the embryonic development in the Nile tilapia

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BACKGROUND: The phenotypic sex of fish could easily be controlled using exogenous sex-steroids. It is well known that hormonal treatment with a masculinizing or feminizing sex-steroids hormone during the hormono-sensitive period lasting from a few days to a few months after hatching partially or totally reversed the phenotypic sex in fish. On contrary only a few information existed about hormonal sex-reversal effect during the embryonic development, before the "classical" hormono-sensitive period.

METHODS: Experiment were conducted on embryos originating from crosses between XX females and XX males (theoretically 100% XX) for the masculinizing treatment with 750 and 1000 $\mu g\ l^{-1}$ 17 α-methyltestosterone (17MT) and on embryos originating from crosses between XX females and YY males (theoretically 100% XY) for the feminizing treatment with 100 and 500 $\mu g\ l^{-1}$ 17β-ethynylestradiol (E2). Shortly after natural reproduction in tanks (<12h00) eggs were removed from the mouth of the female, and equally distributed into 4 batches : 1 control incubated in pure water, 1 control incubated in 0.5% ethanol solution and 2 batches incubated at the 2 experimental doses of hormone. All experiment were realized at 27°C and lasted during all the embryonic development (5days). At the age of 3 months 100 fish (MBW = 3g) per batch were sacrificed and the sex ratio determined with the aceto-carmin squash method.

RESULTS: After the feminizing treatment with E2, mean survival rates 5 days after hatching ranged from 13.1 % (500 $\mu g\ l^{-1}$ E2) to 63.9% (water control group). The masculinizing treatment also induced a significant ($p < 0.05$) decrease of survival rates from 53.2 % in the water control group to 31.5% for the 1000 $\mu g\ l^{-1}$ MT treated group. Incubation of XY eggs into 100 and 500 $\mu g\ l^{-1}$ E2 significantly ($p < 0.05$) skewed sex ratios towards females (from 50.6 to 79.0%) whereas incubation of XX eggs into 750 and 1000 $\mu g\ l^{-1}$ MT significantly ($p < 0.05$) significantly skewed sex ratios towards males (from 50.6 to 79.0%).

CONCLUSION: Our results proved for the first time the effectiveness of hormonal sex reversal treatment during the embryonic development and the possibility to control the differentiation pathway before hatching. As our treatment occurred long before the initiation of sexual differentiation, our results suggest a possible effect of sex-steroids hormones on other tissues (i.e. brain) than gonads. These results support the hypothesis that sexual differentiation in fish may occur during the early embryonic stages and the possible involvement of brain on sex differentiation.

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Stocking density at early developmental stages affects growth and sex ratio in the European eel (*Anguilla anguilla*)

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BACKGROUND: The process of sex differentiation in eel might be influenced by stocking density. This hypothesis is mainly supported by a wide range of ecological studies. However the interaction of other environmental factors in wild might obscure the interpretation of the data being necessary to design experiments under controlled conditions of culture density. In the present study, we have combined surface and volume densities to test the effect of four stocking densities on the growth and survival of European eels during the transition from the glass to the elver stage.

METHODS: Four constant density conditions were tested during the transition from the glass to the elver stage for 90 days (Period 1). The test conditions combined the weight of fish per unit surface or volume (surface density or volume density) resulting in four experimental groups: low surface density (0.5 kg/m2) and low volume density (5 kg/m3) (group S0.5V5); low surface density (0.5 kg/m2) and high volume density (10 kg/m3) (group S0.5V10); high surface density (2 kg/m2) and low volume density (5 kg/m3) (group S2V5); and high surface density (2 kg/m2) and high volume density (10 kg/m3) (group S2V10). Subsequently, fish from the S0.5V5, S2V5, and S2V10 groups were transferred to low density conditions (0.1– 0.4 kg/m2 or 0.1– 0.3 kg/m3) for another 21 months (630 days; Period 2). After Period 1, growth in each group was estimated by DNA and RNA analysis in muscle. Sex ratios, and stage of gonad development, was evaluated histologically after Period 2.

RESULTS: After Period 1, fish maintained at high surface density, regardless of the volume density, showed higher standard growth rates (SGR) and RNA/DNA ratio in muscle than those cultured at low surface density. The percentage of mortality was similar between the groups (34.2%– 41.8%), except for the S2V10 group that showed 83.3% of mortality. At the end of Period 2, most fish (about 95%) exhibited fully differentiated gonads, but different sex ratios were observed in each group. Thus, the S2V5 group showed a higher proportion of females (36.1%) and a lower proportion of males (56.8%) than the S0.5V5 group (11.4% and 72.5%, respectively), while all survivor fish from the S2V10 group developed into females. The gonadosomatic index and SGR were related with sex, being higher in females than in males.

CONCLUSION: These results suggest that glass eels maintained at high surface density during the first months of growth tend to develop into females. The data also indicate that growth and sex ratio are linked processes during eel development, with growth seeming to be sex dependant rather than being influenced by the density conditions in which glass eels are maintained.

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Administration of fadrozole to induce sex reversal in Atlantic halibut, *Hippoglossus hippoglossus* L.

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BACKGROUND: The differentiation of the female gonad occurs in Atlantic halibut postmetamorphic larvae and is under endocrine control by the female sex steroid estradiol-17-Beta (E2). E2 is converted from testosterone by aromatisation, which is controlled by the enzyme P450 aromatase. Inhibition of this enzyme before gonadal differentiation may affect phenotypic sex and result in producing the development of neo-males.

METHODS: Three experiments were conducted: one pilot experiment at Bodø University College and two at the Institute of Marine Research, in Austevoll research Research station Stationand third experiment in Bodø research station were conducted . The objective was to test the efficiency of the aromatase inhibitor Fadrozole on sex reversal in Atlantic halibut. using aromatase inhibitor, fadrozole. Fadrozole was administrated dietary in the diet, at in doses: of 500 mg/kg feed in experiment 1, and 100 mg/kg and 700 mg/kg of feed in experiments 1 2 and 23, respectively. In experiment 1, 550 fry of average size 26.5 mm total length (TL) were fed the experimental diet for 60 days. Six hundred halibut fry of average size 34 mm TL and 1,200 fry of average size 30 mm TL were fed with experimental diet for 17 and, 42 days in experiment 1 2 and experiment 2 3 respectively. Methyltestosterone (5 mg/kg feed) was used as a positive control in experiments 1 2 and 23. In experiment 3, five hundred and fifty fry of average size 26,5mm TL were used. Fadrozole was administrated dietary (500mg/kg of feed) for 60 days. Mortality has beenwas checked daily. Experiments are in progress. Sex ratio will be determined visually and histologically after the experimental fish have reached a size total length exceeding 150 mm.

RESULTS: In third experiment 1, significant differences in growth between fadrozole and control groups were observed after 60 days of treatment of experiment. Average length of fish in the fadrozole group was 56.8 ±13.2 mm and average weight was 2.1 ±1.3 g, while in the control group average length and weight were 72.7 ±17.2 mm and 4.4 g± 2.6 g, accordinglyrespectively. There were no differences in mortality during the course of the study.

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***In vitro* induction of gonadal sex change in a protogynous fish, three-spotted wrasse (*Halichoeres trimaculatus*)**

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BACKGROUND: Protogynous hermaphrodite fish change sex from the female to male phenotype at a certain stage of the life cycle. Sex steroid hormones, especially estrogens, are thought to regulate gonadal restructuring (ovary-to-testis) throughout protogynous sex change. However, the molecular mechanisms underlying sex steroid-regulated gonadal sex change have not been clarified. Recently, we succeeded in inducing *in vitro* spermatogenesis in the three-spotted wrasse (*Halichoeres trimaculatus*) during organ culture of ovary with a serum-supplemented medium. This culture system appears to be a good model for further study of the mechanisms controlling gonadal sex change. In the present study, we further optimized and used this culture system to determine effective steroid dose in more detail and investigate the importance of apoptosis during sex change *in vitro*.

METHODS: Ovarian tissue from sexually immature three-spotted wrasse was cultured in Leibovitz L-15 medium supplemented with 0.5% bovine serum albumin (BSA) or 10% fetal bovine serum (FBS) using the floating tissue culture method developed for eel testicular tissue. Tissue was exposed to 3 X 10^{-9} M 17α-methyltestosterone (MT) or 11-ketotestosterone (11KT) for a few weeks at 26°C. The effects of various concentrations (10^{-9}-10^{-6} M) of MT, 11KT and estradiol-17β (E2) were also examined. Furthermore, the detection of apoptotic cells during the culture was performed using the terminal-deoxynucleotidyl-transferase-mediated dUTP nick-end labelling (TUNEL) method.

RESULTS: In all experimental groups, including the control (no steroid), degeneration of oocytes and initiation of spermatogenesis was observed. MT and 11-KT accelerated the complete transition of ovarian tissue *via* spermatogonia to formation of spermatogenic crypts and sperm. There was no difference between supplementation with BSA and FBS, thus we chose the BSA-supplemented medium for the later experiments. The effects of androgens on inducing spermatogenesis showed a dose dependent manner, and 10^{-7} M of MT was most effective. E2 treatments reduced the incidence of oocyte degeneration, and prevented the occurrence of spermatogenesis. TUNEL-positive oocytes were observed and their abundance increased during the culture period. Moreover, androgens appeared to accelerate the apoptosis of oocytes.

CONCLUSION: These results show that the restructuring from ovary to testis in the wrasse could be induced *in vitro* with a serum-free medium. Gonadal sex change can be triggered during basic *in vitro* culture due to a lack of endogenous factors (especially, estrogens) for female sexuality, and can be accelerated by androgens. Furthermore, oocyte apoptosis may be an important mechanism effecting gonadal sex change.

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In vitro culture of rainbow trout gonads during sex differentiation and gonadogenesis

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BACKGROUND: Gonad gene expression is currently explored *in vivo* during sex-inversion by steroids or antagonists. In order to evaluate the direct effect of steroids on the embryonic gonad and to analyze some gene functions during sex differentiation, an *in vitro* culture has been set up for the embryonic gonads of rainbow trout, *Oncorhynchus mykiss*.

METHODS: Gonads have been collected around 100 days after fertilization from all-male and all-female populations. They were transferred on 10 mm cell culture inserts in 24 wells multidishes (10-12 gonads/well). Three culture media (300 µl/well, pH 7.8, pO 310 mOM) added with 2% steroid free Ultroser (Biosepra) have been tested: MCDB 105, M199 and modified L15. Gonads morphology and function before and after culture at 12°C were compared. Histological examination of gonads was performed and mitotic (BrDU) and apoptosis (TUNEL) activities analyzed. The expression of six genes have been recorded using real time RT-PCR: Germ Cell Less (*gcl*), Vasa (*ddx4*) , Follistatin (*fst*), Anti-Mullerian Hormone (*amh)*, Aromatase, (*cyp19A1*), and 11-ß-hydroxylase (*cyp11b1*). Finally, 11-ß-hydroxylase and aromatase enzymatic activities have been analyzed by following tritiated testosterone metabolism and by using a tritiated water assay, respectively.

RESULTS: Gonad histological structure was conserved after culture for 7 to 9 days. The best results were obtained with MCDB medium. However, the rate of cell mitosis decreased after 9 days while the apoptosis increased. *Gcl* and *ddx4* expressions were higher in ovaries than in testes and almost unchanged in both gonads after culture with MCDB for 9 days. *Fst* expression was lower but more stable in testes than in ovaries, while the opposite occurred for *amh*. *Cyp11b1* expression was higher in testes while *cyp19A1* expression was higher in ovaries and both of them decreased drastically after culture. About 60% of the ovarian aromatase activity was maintained after culture for 7 days in MCDB, when only 40% was maintained in M199 and L15 media. In the testes, 11beta-hydroxylase activity was maintained after culture for 7 days whatever the medium.

CONCLUSION: Embryonic gonads of rainbow trout can be cultured *in vitro* for a short period using MCDB. Their structure is conserved and gene expression is maintained, although reduced for some genes. Without specific hormonal supplementation, expressions of genes controlling steroidogenesis are decreased but their corresponding enzymatic activities are less affected, suggesting that protein turnover could be low. This *in vitro* model could be used to test the direct effect of sexual steroids on embryonic gonads or even to explore a putative action of pituitary hormones. Preliminary experiments designed to test these hypotheses will be discussed.

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An *in vitro* testis system to study the effects of xenoestrogens on spermatogenesis in fish

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BACKGROUND: Many field studies revealed evidence for endocrine disruptions in freshwater fish populations due to effluents from industries and municipal sewage. Both types of effluents are estrogenic and act in the wild as 'estrogen-like' exposure inducing feminization in males. In the testis, morphological analyses of exposed fish showed an ovotestis defined as female reproductive tissue within the testis. The ovotestis is the visual demonstration of the physiological response of the synergism of endogenous 17beta-estradiol and environmental concentration of 'estrogen-like' compounds, called xenoestrogens. The molecular pathway underlying this abnormal testis remains to be scientifically explained.

METHODS: Testes from wild gudgeon (*Gobio gobio*) were dissected in sterile conditions immediately after fish death. One testis was directly plunged in Bouin's solution as control. The other testis were placed in a Petri dish containing Leibovitz's L-15 medium and sliced in approximately 1-3 mm long. Each piece was placed in a single well of culture plate containing a Millicell CM insert filter. Testis pieces were under Leibovitz's L-15 medium improved as described by Miura et al. (1991). In order to induce spermatogenesis, the Leibovitz's medium were supplemented with 10 ng ml^{-1} 11-ketotestosterone. The culture plates were placed in an incubator at 12°C and the medium was changed every 48 hours. Each week, samples were taken and fixed in 4% paraformaldehyde. Light microscopy was performed on 5 µm tissue culture section and stained with hematoxylin and eosin.

RESULTS: Histo-morphological analyses of testis fragments at the beginning of the experiment were mostly formed by spermatogonia type A and B with early spermatocytes. This architecture was maintained during the whole 21-day experiment. Testis fragments supplemented with 11-ketotestosterone, a potent androgen in teleost, clearly demonstrated a progression of the spermatogenesis compared with the beginning of the experiment. However, the complete spermatogenesis was not reached at the end of the experiment. An evaluation of the cell proliferation remains to be determined in order to validate the *in vitro* testis system. The next step in the experiment will be to evaluate the effects of 17beta-estradiol and ethinylestradiol at environmental concentrations on spermatogenesis.

CONCLUSION*:* An *in vitro* testis system isolated from the *in vivo* is a good starting point to identify the mechanisms by which xenoestrogens can disrupt spermatogenesis in fish.

Miura T., Yamauchi K., Takahashi H. and Nagahama Y. (1991) Hormonal induction of all stages of spermatogenesis in vitro in the male Japanese eel (*Anguilla japonica*). Proc. Natl. Acad. Sci. **88**, 5774-5778.

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Brain aromatase expression, activity and immunohistological analysis during termolabile sex determination/differentiation period in pejerrey fish. Differences in brain cell proliferation

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BACKGROUND: In pejerrey gonadal sex is strongly determined by temperature (all males and all females are produced at 29ºC and 17ºC, respectively). In this species, as in other non-mammalian vertebrates, estradiol plays an important role in ovarian differentiation. Also, estradiol is very important in brain development and sexual differentiation of the dimorphic brain areas in mammals, but little is known on the role of estrogens in the development of fish brain. In this context, the objective of this work was to investigate the expression and activity of brain aromatase during the period of temperature sex determination/differentiation and also the differences in brain cell proliferation in fish raised at masculinizing and feminizing temperatures

METHODS: Brain aromatase expression was measured weekly by quantitative RT-PCR on total RNA extracted from the head of larvae reared at feminizing and masculinizing temperatures during the temperature-sensitive window (0-5 weeks) and the gonadal differentiation period (5-13 weeks). Aromatase activity in the head was also measured using the tritiated water assay. Brain and pituitary gland aromatase ontogeny was analyzed by inmmunohistochemistry. Brain cell proliferation was analyzed by immunocytochemistry during early termolabile sex determination period on brain slices of fish exposed for a short time to bromodeoxyuridine (BrdU). These brains were also analyzed using immunofluorescense against PCNA.

RESULTS: Brain aromatase expression was significantly higher at the masculinizing temperature during the sex determination and gonadal differentiation periods. A similar trend was observed when aromatase activity was analyzed in fish heads. Larvae at the masculinizing temperature exhibited higher aromatase activity at 5-6 weeks, just previous to the gonadal sex differentiation period. Aromatase-positive cells were firstly evidenced in the pituitary gland (2-3 weeks) and then in cells lining the ventricles (4 week), as observed in adults, without clear differences between temperature treatments. There was a clear sex/temperature-related difference in cell proliferation during the fourth week, where larvae maintained at feminizing temperature exhibited a clearly higher brain cell proliferation than those at the masculinizing temperature. A large majority of the new-born cells were observed in periventricular areas.

CONCLUSION: These results suggest that temperature exerts a strong effect on brain aromatase expression and that estradiol may be required for the masculinization of larval brain. Our findings also indicated the likely existence of a sex-specific mechanism modulating neurogenesis during this period and the possibility that it participates in the construction of sexually dimorphic brain circuitry.

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Effects of temperature on gonadal aromatase gene expression during sex differentiation in the European sea bass

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BACKGROUND: Aromatase has been implicated in the process of temperature sex differentiation (TSD) in an increasing number of fish. In this regard, there is evidence that in many fish species exhibiting TSD, exposure to elevated water temperatures during early development results in suppression of gonadal aromatase enzymatic activity and induces masculinization.

METHODS: To test whether aromatase is involved in TSD in the European sea bass, fish were reared at high (21ºC) or low (15ºC) temperature during early developmental stages. Samples from gonads were taken at different times before histological sex differentiation including 120 days post fertilization (DPF), 135 DPF and 155 DPF. Gonadal aromatase gene expression was assessed by semiquantitative PCR and final sex ratios were verified histologically at the end of this process. Since sex differentiation in the sea bass depends on length, a canonical discriminant analysis with fish length and aromatase gene expression as independent factors was performed to assign gonadal sex to each histologically undifferentiated fish.

RESULTS: The results showed that differences in aromatase gene expression were first detected in fish ≥ 4 cm SL, well before the first signs of histological sex differentiation (~ 9 cm SL), being significantly higher in putative females than in putative males. Rearing at 21ºC decreased the number of fish with high aromatase levels and the total number of females from 90% to 56% when compared to the groups at 15ºC. However, aromatase gene expression levels in putative males and females at 21ºC did not differ from those in putative males and females at 15ºC, respectively.

CONCLUSION: This study shows that aromatase can be used as a reliable marker to predict future ovarian differentiation in the European sea bass. Moreover, it also demonstrates that high temperature regimes during early developmental stages, including the critical period for sex differentiation, induced a decrease in the number of fish with high aromatase gene expression levels resulting in male development.

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Thermolabile sex determination in Hd-rR medaka Oryzias latipes: gender sensitivity, thermal threshold, critical period, and *DMRT1* expression profile

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BACKGROUND: In medaka *Oryzias latipes*, the first species of fish in which a main sex determining gene (*DMY or DMRT1bY*) was documented, sex is generally considered to be strictly determined by the animal's genotype. However, recent studies have indicated the possibility that medaka shows some degree of temperature-dependent sex determination (TSD). In this study we examined the developmental time and thermal threshold for TSD, gender differences in temperature sensitivity, the fertility of thermally sex reversed fish, and the effect of temperature on the expression of two major sex determination/differentiation genes (*DMY/DMRT1bY and DMRT1*) in the Hd-rR strain of medaka.

METHODS: Fertilized eggs were exposed from either shortly after fertilization (8-16 cells; embryonic stages 5-6) or from middle embryogenesis (heart development stage; stage 36) until hatching (stage 39) to temperatures ranging from 17ºC to 34°C. Secondary sexual characteristics, gonadal histology, progeny testing, sex-linked body coloration and gene expression were used to determine phenotypic and genotypic sex.

RESULTS: Sex determination was unaffected by low or high temperatures in genotypic (XY) males. In contrast, genotypic (XX) females treated from stages 5-6 showed increasing rates of sex reversal into phenotypic males at temperatures above 27°C up to 100% at 34°C. Thermal manipulation of sex was ineffective after stage 36, indicating that gonadal fate in medaka is determined considerably earlier than histological differentiation (stage 39). High temperature induced *DMRT1* expression in genotypic females and this was observed as early as from stage 36. Sex-reversed males had histologically normal testes, were capable of sexual courtship and, with the exception of fish from 34°C, sired viable progeny when mated to fertile females.

CONCLUSION: The results show that the process of gonadal sex determination in Hd-rR medaka is under the control of genetic and environmental (temperature) factors at a very early stage of embryonic development. High temperature induces (premature) expression of *DMRT1* gene in genotypic females. The *DMY/DMRT1bY* gene was shown to be sufficient (at low temperatures) but not absolutely necessary for testicular differentiation in medaka. The finding of TSD at environmentally relevant temperatures suggests that part, if not all, of the masculinizing role of *DMY/DMRT1bY* could readily be taken over by (high) environmental temperature. Finally, our results indicate that a brief exposure to high temperature early in life could impair the fertility of medaka as adults.

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Purification and assay of *Arapaima gigas* vitellogenin: potential use for sex determination

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BACKGROUND: Sex determination of breeders of *A. gigas* is not possible by morphological criteria. As reproduction in captivity relies on the constitution of male and female pairs, the development of sexing methods is of great importance to increase fry production in a such valuable Amazon species.

METHODS: Anesthetized *A. gigas* juveniles (average weight 881g) were injected intra-peritoneally with a single dose ($5mg.kg^{-1}$) of 17-ß Estradiol. Fifteen days later, fish were anesthetized and blood was sampled in the caudal vasculature in heparinised tubes and centrifuged at 10 000 g for 5 min. Plasma was recovered, aliquoted and immediately frozen at -80°C. One blood sample was taken from every fish prior the Estradiol injection as negative control.Vtg purification was performed by electroelution after polyacrilamide gel electrophoresis (PAGE). This Vtg preparation was used as antigen to obtain specific polyclonal antiserums on rabbits. Antibody specificity and affinity was tested by Enzyme Immuno-Assay methodology (EIA).

RESULTS: Using SDS-PAGE in non-reducing conditions and with Coomassie blue satining, the Estradiol treatment induced two major bands (Vtg_1 and Vtg_2) with 247 and 120 kDa apparent molecular masses respectively which were absent from control plasma. These bands correspond to 2 different vitellogenin molecules since they also migrate as two separate bands under SDS-PAGE with ß-mercapto-ethanol. The electroeluted Vtg preparations were used as coating and standard antigen to set up a specific EIA for each vitellogenin. Antibody-Antigen working concentrations, have been determined by cross dilution tests and they are similar for both vitellogenins, ($500\ ng.ml^{-1}$ for coating and 1:50 000 for antibody dilution).
This assay allowed us to quantify plasma Vtg in different plasma samples with a sensitivity around $30\ ng.ml^{-1}$.

CONCLUSION: We have set up a Vtg EIA for *A. gigas*, which can detect very low Vtg concentrations in the plasma. As Vtg is a female specific molecule in normal rearing conditions, we might be able to sex the 3 to 4 year-old fish since it has been reported that most of the fish can reach sexual maturity between 3 and 4 years. At this time Vtg levels in females must be higher than the assay sensitivity. To confirm this first results blood sampling of pre-adult *A. gigas* specimens has been undertaken. As killing fish from breeding stock was not possible, we will confirm sex determination by Vtg measurement during the first reproduction period and thus validate this methodology. The feasibility of early sexing (when fish are still immature) with sexual steroids like 11-Keto Testosterone, Estradiol and Testosterone will also be evaluated.

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Ontogeny of sexual development in the roach (*Rutilus rutilus*) and is interrelationships with growth and age

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BACKGROUND: The roach (*Rutilus rutilus*) has become a sentinel species for the study of sexual disruption in wild fish populations in UK Rivers that occurs as a consequence of exposure to endocrine disrupting chemicals (EDCs). However, little is known about the process (and timing) of normal sexual development in this species. Here, we analysed the ontogeny of sexual development in the roach and assessed how growth rate and fish size impacted on the timing of both sexual differentiation and sexual maturation.

METHODS: Sexual development was analysed (through gonadal histology) in 531 fish, derived from 3 populations of roach maintained independently under different husbandry regimes. For population 1, 196 fish were collected at 10 time intervals between 68 and 473 days post-fertilisation (dpf). For population 2, 183 fish were collected over 6 time intervals between 64 and 728 dpf; and for population 3, 152 fish were sampled over 13 collection times between 28 and 400 dpf. Fork length (mm) and wet weight (g) were recorded for each fish sampled and used to calculate individual condition factor and specific growth rates for each population.

RESULTS: Ovarian differentiation in roach was first recorded at 68 dpf and this preceded testicular differentiation (first recorded at 98 dpf). In contrast, sexual maturation occurred at an earlier age in males (300 dpf) compared with in females (368 dpf). No significant differences in body size (length or weight) were recorded between male and female roach until the fish were 415 dpf. Studies on the 3 populations of roach collectively, and growing at different rates, showed that the timing of sexual differentiation was highly variable and more related to fish size (length) than to fish age. Time to sexual maturation was also variable between populations. Subsequent to their first year of life, seasonality appeared to have the strongest influence on the timing to sexual maturation (size was less well associated with gonadal status). In the fastest growing population, sexual differentiation had occurred by 112 dpf, and sexual maturation by 368 dpf. An interesting observation was that fish growth rate during early life appeared to affect the subsequent gender balance of the population, biased to females in more rapidly growing fish.

CONCLUSION: Somatic growth rates in roach during early life affect the timing of sexual development and may also affect subsequent gender assignment. This work has shown that in addition to their utility for studying effects of EDCs on wild fish populations, roach are a practicable species for studies on the effects of EDCs on sexual development/function in the laboratory.

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Sexually dimorphic differentiation of gonadal somatic cells during sex differentiation in the teleost fish, tilapia, *Oreochromis niloticus*

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BACKGROUND: Conservative phenomena in vertebrate gonadal sex differentiation include the entry of embryonic germ cells into meiosis during gonadal sex differentiation in females but not, however, in males. Using genetic males (XY) and females (XX) of tilapia—a well-characterized model for gonadal differentiation in a teleost fish—we have conducted a study into the mechanisms of sex differentiation.

METHODS: To further understanding of the sexual dimorphic mechanisms during gonadal sex differentiation, we established an in vitro culture system for the entry of germ cells into meiosis using a teleost fish, *Oreochromis niloticus*. Next, to identify the gene products involving the entry of germ cells into meiosis or arrest of germ cells in gonial stage, we performed subtractive hybridization screening using this in vitro culture system.

RESULTS: In vivo, XX gonads showed the first appearance of meiotic cells approximately 35 days after hatching (DAH). By contrast, the meiotic cells of XY gonads became differentiated after 70 DAH. Gonads from the fry at 23 DAH were introduced into an in vitro organ culture system. In XX gonads, the appearance of meiotic cells—which is expressed as the meiotic prophase-specific gene DMC1—was observed after 21 days of culture. In XY gonads, however, no meiotic cells were seen after 21 days. This result indicates that germ cell differentiation in this culture system progresses in a similar manner in vivo, depending upon genetic sexes. Next, we performed subtractive hybridization screening using this in vitro culture system to identify the gene products expressed in XY and XX gonads dominantly. Finally, 15 gene products were obtained. Eleven out of 15 gene products were expressed in XX gonads dominantly. Male-related gene 2 (MR2), belonging to the TGF-beta superfamily, became expressed in the somatic cells in XY gonads when XX germ cells differentiated into meiosis. When functional XX sex reversal was induced with androgen, MR2 became expressed in XX gonads, such as in XY gonads. In details, MR2 is first expressed in germ cell-surrounding cells during sex differentiation and then becomes expressed in Sertoli cell-surrounding cells during testicular differentiation.

CONCLUSION: In this study, we established an in vitro culture system for sex differentiation using a teleost fish, *Oreochromis niloticus* and performed subtractive hybridization screening using this in vitro culture system. Finally, 15 gene products were obtained. One of Male-related genes, MR2, showed expression profile correlated with the formation of testicular structure. Taken together, this suggests that MR2 is involved in the testicular differentiation, especially, the formation of testicular structure.

Gametogenesis and Gamete Biology

Gametogenesis and Gamete Biology
(Part I : Spermatogenesis)

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Physiology and behavior of stripped and testicular sperm in *Perca fluviatilis* L. 1758

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BACKGROUND: Basic knowledge of seminal plasma composition, sperm production indices, and the behavior of sperm relative to environmental conditions can be used as indicators to aid understanding of intra- and inter-specific variations.

METHODS: Osmolality and the Cl^-, K^+ and Na^+ and Ca^{2+} contents of the seminal plasma were measured with an osmometer and ISE/flame photometry, respectively. Sperm velocity and motility rate were analyzed after recording sperm motility under a dark-field microscope. The effects of various factors upon sperm behavior including K^+ (KCl: 5.0, 20.0 and 50.0 mM), Ca^{2+} ($CaCl_2$: 0.5, 2.5 and 5.0 mM) and osmolality (Sucrose: 100, 200 and 300 mOsmol Kg^{-1}) were tested after determining the optimum dilution ratio (1:25, 1:50 and 1:100) in an immobilizing solution (IS: NaCl 200 mM, $NaHCO_3$ 2.38 mM, osmolality 380 mOsmol Kg^{-1}). In other experiment, effects of osmolality (Sucrose: 100, 200 and 300 mOsmol Kg^{-1}) and potassium (KCl: 5.0, 20.0 and 50.0 mM) were investigated to compare the behavior of testicular and stripped sperm. Buffered distilled water, containing 20 mM Tris, was used as control. The pH of solutions was checked and adjusted to 8.0. The sperm dilution ratio was 1: 50.

RESULTS: Mean values for sperm volume (ml), sperm density ($\times 10^9$ spz ml^{-1}), seminal plasma osmolality (mOsmol Kg^{-1}), along with Na^+, Cl^-, K^+ and Ca^{2+} concentrations (mM), were 2.6±0.5, 29.2±3.2, 298.1±5.1, 131.0±2.2, 106.8±2.4, 10.7±0.6 and 2.4±0.1, respectively. At 15 sec post-activation, the sperm velocity (μm s^{-1}) and motility rate (%) were 115.5±1.3 and 91.9±1.3, respectively and decreased significantly during subsequent time periods following sperm activation. Optimal sperm motility was observed when the sperm was pre-diluted in IS at a ratio of 1:50. The pre-diluted sperm showed optimal motility at 2.5 mM Ca^{2+} and 50 mM K^+ and when osmolality was 100 mOsmol Kg^{-1}. An osmolality of 300 mOsmol Kg^{-1} or above totally suppressed sperm motility. Both sperm velocity and motility rate were lower in stripped sperm compared to testicular sperm in terms of osmolality effects. In a medium containing K^+ (5-20 mM), both sperm velocity and the percentage of motility were higher in stripped sperm ($P<0.05$).

CONCLUSION: The present study demonstrated (a) high individual variation in terms of sperm volume and density, (b) behavioral differences of sperm to relation to the dilution ratio of sperm in IS as well as concentration of K^+, Ca^{2+} and of osmolality, and (c) behavioral differences of testicular and stripped sperm in relation to osmolality and potassium effects. A hypo-osmotic shock is predominantly required for the initiation of sperm motility in perch. This work was supported by GACR no. 524/06/0817, by MSM 6007665809, by NAZV QF 418 and by EU no. COOP-CT-2004, 512629.

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17α, 20β-dihydroxy-4-pregnen-3-one inducing substances, 11β-hydroxysteroid dehydrogenase and trypsin, are important factors for control of spermatogenesis in Japanese eel

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BACKGROUND: Spermatogenesis is a complex process and this is controlled by numerous endocrine factors.17α, 20β-dihydroxy-4-pregnen-3-one (DHP) is known as maturation-inducing steroid in male and female. However, recently, it has been found that DHP has the ability to induce the initiation of meiosis in gametogenesis.

METHODS: To elucidate the molecular mechanisms underlying the action of DHP, we attempted to clone cDNAs encoding genes whose expression was induced by DHP in eel testis, using cDNA subtraction and differential screening. We identified 25 genes that were differentially expressed by DHP stimulation. Among these clones, eel 11β-hydroxysteroid dehydrogenase short form (e11β-HSDsf) and eel trypsinogen were present. Using *in vitro* culture system for eel, we analyzed the function of these molecules.

RESULTS: The northern blot analysis showed that transcripts of e11β-HSD and eel trypsinogen in testis were induced by DHP stimulation. The recombinant e11β-HSDsf had 11β-dehydrogenase activity, metabolizing cortisol to cortisone, and 11β-hydroxytestosterone to 11-ketotestosterone (11-KT). In vitro experiments revealed that DHP treatment enhanced both of these 11β-dehydrogenase activities of eel testis. In testicular organ culture, cortisol enhanced the spermatogonial proliferation induced by 11-KT. However, excess cortisol inhibited this proliferation. On the other hand, cortisone did not affect 11-KT induced spermatogenesis. These data of suggest that optimal levels of cortisol induced spermatogonial mitosis by increasing 11-KT productions. Furthermore, positive feedback control of 11-KT production and modulation of cortisol levels to protect testes from excess circulating cortisol.
Trypsinogen/trypsin also showed an important role for spermatogenesis. Histochemistry showed that trypsinogen was expressed in Sertoli cells surrounding proliferated spermatogonia, spermatids and spermatozoa. Using anti-eel trypsinogen antibody and serine protease inhibitors, the spermatogenesis induced by DHP was reduced. The relatively low dose of trypsin (1-100 nM) induced DNA synthesis and Spo11, a molecular marker of meiosis, expression of germ cells. The relatively high dose of trypsin (10-100μM) induced a part of spermiogenesis. These results suggest that trypsin induce initiation of meiosis and spermiogenesis.

CONCLUSION: These data indicate that these two molecules are important for controlling spermatogenesis.

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Effects of trace elements on spermatogenesis and androgen production in fish

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BACKGROUND: Some trace elements such as Cd and As are known as water pollutants. Previously, we investigated the relationship between gonadal development and the accumulation of various chemicals in fish inhabiting Vietnam, and four trace elements (Pb, Mo, As and Rb) levels were at significantly high levels in fish which have abnormal testis. To clarify the influence of trace elements on testis in fish, we investigated the direct effects of Pb, Mo, As and Rb on spermatogenesis and the effects of As on steroidogenesis using *in vitro* testicular organ culture of Japanese eel (*Anguilla japonica*).

METHODS: To investigate the effects of trace elements on spermatogenesis, testicular fragments of Japanese eel were cultured in 1 ml of culture medium with or without 10 ng/ml of 11-ketotestosterone (KT) and 0.1-10 uM Pb ($PbCl_2$), 1-100 uM Mo (Na_2MoO_4), 10-1000 uM Rb (RbCl) and 0.1-100 uM As (Na_2HAsO_4) for 6 days. After culture, testicular fragments were treated with 5-bromo-2-deoxyuridine (BrdU) for analysis of proliferation of germ cells by quantifying their BrdU incorporation. In addition, to investigate the effects of As on KT synthesis in testis, testicular fragments were incubated in eel Ringer solution with or without 1 IU/ml human chorionic gonadotropin (hCG) and 0.0001-100 uM As for 18 hr. The concentrations of KT in Ringer solution were measured by time-resolved fluoroimmunoassay.

RESULTS: After 6 day culture, treatments with any trace elements alone did not affect the histology and BrdU incorporation in germ cells in comparison with control group. Treatment with KT increased BrdU incorporation in germ cells, whereas, treatment with 0.1 uM Pb, 10-100 uM Mo, 10-1000 uM Rb and 10 uM As significantly decreased KT-induced BrdU incorporation. Furthermore, treatment with 100 uM As and KT caused necrosis of testicular fragments. KT synthesis in testis was significantly increased by treatment with hCG. In contrast, As decreased the concentration of KT in medium in a dose dependent manner; the most effective dose of As was 0.1 uM. However, high dose of As (10-100 uM) did not affect KT synthesis in testis.

CONCLUSION: We demonstrated that Pb, Mo, Rb and As inhibit KT-induced germ cell proliferation. These results suggest that Pb, Mo, Rb and As can exert direct inhibitory effects on spermatogenesis in fish inhabiting Vietnam. Additionally, we showed that As inhibited hCG-induced KT synthesis. Interestingly, the most effective dose of As was 0.1 uM, and higher doses did not affect KT synthesis. From these results, As may also interfere with spermatogenesis via inhibiting steroidogenesis in testis, and these inhibitory effects may occurred at low contamination levels of As.

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The role of trypsin-like protease in sperm on fertilization in fish

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BACKGROUND: Fertilization is an important process for the continuance of the species. In most of fish species, sperm fuses with an ovum through micropyle on the egg chorion because sperm does not have acrosome, an organelle that consists of various proteases. However, we found from our previous studies that trypsin, members of the serine protease family, exists in sperm in Japanese eel (*Anguilla japonica*). These results suggest that trypsin plays an important role in fertilization of fish. Therefore, we investigated how trypsin acts on fertilization in fish using sperm of eel and kelp bass (*Epinephelus bruneus* Bloch), in which techniques for artificial fertilization have already been established.

METHODS: To determine the distribution of trypsin in sperm of eel and kelp bass, we performed immunofluorescence using the anti-eel trypsin antibody. In addition, enzymatic activity of trypsin of sperm membrane proteins was assayed fluorimetrically using the trypsin-specific substrate Boc-Phe-Ser-Arg-MCA. Moreover, to detect proteins exhibiting protease activity, zymography was performed using gelatin-containing SDS-PAGE. Furthermore, to investigate whether protease that exists in sperm acts on fertilization, artificial fertilization was performed using kelp bass sperms incubated with serine protease inhibitor, 1-100 uM PMSF or AEBSF, or 5 ug/ml anti-eel trypsin antibody for 2 h at 4 ˚C, and then the fertilization rates were measured.

RESULTS: In immunofluorescence using anti-eel trypsin antibody, the signal was mainly detected on the surface of sperm membrane, in both eel and kelp bass. In addition, enzymatic activity of trypsin was found in sperm membrane proteins in both fish. In zymography, trypsin-like proteases were detected as bands of 85 and 95 kDa in eel, and 110 and 140 kDa in kelp bass sperm membrane proteins, however their enzymatic activity were inhibited by PMSF, inhibitor of serine protease including trypsin. As for the result of artificial fertilization using sperm of kelp bass, treatment with PMSF or AEBSF significantly decreased the fertilization rates in a dose dependent manner. Furthermore, treatment with anti-eel trypsin antibody also significantly decreased the fertilization rates similarly as treatment with protease inhibitor.

CONCLUSION: We found that trypsin-like protease is located on the surface of sperm membrane and associated with fertilization. These results suggest that two types of trypsin-like proteases exist in sperm and act on substances that exist in egg chorion or are secreted from egg. Therefore, we demonstrated that trypsin-like protease which exists in sperm membrane plays an important role in fertilization of fish whose sperm does not have acrosome.

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Changes in expression profiles of genes related to sexual maturation during spermatogenesis in testes of early-maturing male Atlantic salmon parr, salmo salar

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BACKGROUND: The initiation of sexual maturation and spermatogenesis are complex processes that require the highly coordinated regulation of a number of key genes. The precise endocrine mechanisms involved in the sexual maturation of fish are poorly understood. Therefore, we investigated the expression of genes encoding proteins involved in sex steroid biosynthesis and the anti-Müllerian hormone (AMH) homolog during early sexual maturation in male Atlantic salmon parr.

METHODS: One-summer-old male Atlantic salmon parr were sampled from the prepubertal stage in December until spermiation in October. Testicular expression of AMH, Ff1b, steroidogenic acute regulatory protein (StAR), cytochrome P450 cholesterol side-chain cleavage enzyme (P450scc), 3beta-hydroxysteroid dehydrogenase (3beta-HSD), cytochrome P450 17alpha-hydroxylase/17,20-lyase (P450c17), cytochrome P450 11beta-hydroxylase (P45011beta) and 11β-hydroxysteroid dehydrogenase (11β-HSD) was analyzed by RT-PCR.

RESULTS: The expression of genes encoding Ff1b, StAR, 3beta-HSD, P450c17 and 11beta-HSD was upregulated when spermatogenesis was initiated in early June. During the course of spermatogenesis expression profiles of the genes encoding Ff1b, P450scc, P450c17, P45011β, 11beta-HSD were similar; transcript levels being relatively low during early stages, then strongly increasing during spermiogenesis. 3beta-HSD transcript levels were relatively high in early spermatogenesis and progressively increased during spermiation. StAR mRNA levels increased strongly during spermatogenesis. In contrast, transcript levels of AMH were found to be relatively high in immature testes during spring, but started to decrease when spermatogonial proliferation started while AMH gene expression remained high in nonmaturing males. During the course of spermatogenesis AMH expression declined to its lowest levels during spermiogenesis.

CONCLUSION: These results indicate that coordinated *de novo* transcription of genes encoding StAR as well as the key enzymes 3beta-HSD, P450c17 and 11beta-HSD is required for the production of sex steroids during the initiation of spermatogenesis. In addition, the profile of AMH gene expression strongly suggests that AMH suppression plays a crucial role in the process of spermatogenesis.

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Hormonal profile in adults and juveniles of critically endangered sturgeon (Acipenser sturio) adapted to hatchery in France

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BACKGROUND: In response to the dramatic decrease of its population, the European Atlantic sturgeon, has been totally protected since 1982 in France and since 1997 in all Western Europe. In the aim of artificially sustaining the wild population by restocking with fingerlings, a confined broodstock is being established with young adults caught from the wild in the Gironde estuary (France). Young adults and breeders are now maintained under experiment in the hatchery of CREA (Cemagref, France). In this species, very few data are available concerning the physiological parameters linked to the duration of gametogenesis, periodicity of spawning, and associated hormone levels.

METHODS: Thirty five fish were reared in recirculating water system and held in brackish water (14-16°% salinity). They were fed natural food composed of frozen shrimps. Age was determined using a section of pectoral fin ray. Blood samplings were performed over a five-year duration. Specific ELISAs were performed to measure plasma VTG and sex steroid levels (estradiol, testosterone, 11-ketotestosterone and 17α, 20βP).

RESULTS: From the steroid hormones measured, T exhibited the highest levels in most fish, reaching 40ng/ml in one maturing male and nearly 20 ng/ml in one female. T plasma levels presented seasonal variations with a marked increase at the beginning of spring. Females presented a marked drop of estradiol (up to 33 ng/ml) in spring. VTG plasma level, in females, increased for months, with levels as high as 30 mg/ml, correlated to the presence, in ovary of fully vitellogenic follicles. However, hormonal stimulation performed at this stage in female gave poor ovulation results. 11KT plasma levels remained low in both sexes all year round, except in male breeders, where a marked increase was observed, reaching a maximum at 6.6 ng/ml in one spermiating male, and correlated to a peak of testosterone. 11KT was also found in the plasma of mature female, but at lower levels than in mature males (2 ng/ml), in correlation with a peak of testosterone.

CONCLUSION: Our findings allow a better understanding of the reproductive cycle of this species : plasma parameters allow identification of sexes and can be used as predictive factors to determine the stage of development of the gonad, and anticipate imminence of spawning. The candidate to MIS in female sturio might not be 17α, 20βP.

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Induction of sexual maturation in the male Japanese eel (*Anguilla japonica)* by continuous administration of various hormones using an osmotic pump

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BACKGROUND: Repeated weekly injections of human chorionic gonadotropin (HCG) induce spermatogenesis and spermiation in the sexually immature Japanese eel. However, more efficient and reliable methods for induction of sexual maturation are required in order to establish techniques for the mass production of eel seedlings. The present study examined the effects of HCG, salmon pituitary extract (SPE) and gonadotropin-releasing hormone analogue (GnRHa) administration on the sexual maturation of the male Japanese eel; the newly introduced osmotic pump (OS) that can release constant amounts of hormones for a long period was used for the abovementioned examination.

METHODS: (1) A single OS loaded with various amounts of GnRHa (0.94, 1.86 and 3.75 μg/day), SPE (0.56, 1.13 and 2.25 mg/day) or HCG (50 IU/day) was implanted into each male eel. Forty-two days after the implantation, their gonads were sampled, and the gonad somatic index (GSI) was calculated. (2) The eels were administrated HCG by using various devices: injection (330 IU/injection/week), cholesterol pellet (CH) (1500 IU/pellet) and OS (50 IU/day). After 35 days, the milt volume and GSI were measured. (3) The eels were implanted with the OS loaded with various doses of HCG (1, 5, 10, 25, 50, 75 and 100 IU/day). After 42 and 45 days, the milt volume, sperm motility and GSI were measured.

RESULTS: (1) The GSI values increased in the eels administrated with SPE (2.25 mg/day) and HCG (50 IU/day) but not in the eels administrated with GnRHa. (2) The GSI values increased in all the groups that received HCG via various devices. The GSI values of the eels that received HCG by OS were higher than those observed in the CH group and were similar to those observed in the injection group. The milt volume of the OS group was similar to that of the injection group. (3) The GSI values and milt volume of the eels significantly increased at a dose of 5 IU/day and up to 50 IU/day in a dose-dependent manner; however, these remained constantly high at doses of 75 and 100 IU/day. Sperm motilities were not significantly different among the eels that received various doses of HCG, with levels ranging from 15% to 40%.

CONCLUSION: The present study indicates that the HCG administration using OS is effective in inducing spermatogenesis and spermiation in the male Japanese eel. The most effective induction of spermatogenesis and spermiation were observed at a dose of 50 IU/day. This may be used as an efficient and reliable method for the artificial maturation of the male Japanese eel instead of the repeated weekly injections of HCG.

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Reproductive cycle of the Venezuelan catfish "Sierra Negra" (*Oxydoras sifontesi*)

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BACKGROUND: *Oxydoras sifontesi* has an annual gonadal cycle closely related to changes in the environmental conditions of Venezuelan floodplain; which can be divided into four periods: preparatory (January-March), prespawning (April-May), spawning (June-July) and postspawning (August-December). As most members of the Doradidae family, *O. sifontesi* has considerable potential for sustainable aquaculture. The present work aims to describe the endocrine changes that occur during the gonadal development of *O. sifontesi*.

METHODS: Adults male and female *O. sifontesi* were sampled bimonthly from the Portuguesa River (Portuguesa State, Venezuela) between May 2004 and April 2005. Gonads were removed and fixed in Bouin's solution and processed for light microscopy. Blood samples from the caudal vasculature were obtained in order to determine sexual hormones concentration. Plasma concentrations of estradiol (E_2) in female and testosterone (T) in male were determined by ELISA commercial kits. Macroscopic and microscopic criteria were used to state the ovaries and the testis maturity stage.

RESULTS: There was a significant variation in the GSI and sexual steroid hormones according to the sex or the endocrine condition of the fish. In both females and males, highest values of GSI were observed during the prespawning period (April-May) and lowest values in March (preparatory period) and July (spawning period). In females, high concentrations of E_2 were observed at stages IV (April) and V (May). In males, high levels of T were registered in May (stage V) and July (stage VI). Examination of histological characteristic of gonads at different stages, suggest that *O. sifontesi*, like many freshwater teleosts, seems to be a synchronous spawner.

CONCLUSION: Changes in plasma levels of E_2 and T were well correlated with gonadal development and GSI. The high concentration of E_2 observed in female before spawning is in accordance to the established role for this hormone in initiating and maintaining vitellogenesis. While in male, the high concentration of T might participate in the stimulation of secondary sexual behavior, or could be used as a precursor for other steroids.

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Identification of molecular marker for type A spermatogonia in rainbow trout

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BACKGROUND: In fish, spermatogonia are classified into two types; undifferentiated type A spermatogonia and differentiated type B spermatogonia. Type A spermatogonia, which contain spermatogonial stem cells, have been proved to be a suitable material for germ cell transplantation in fish. In mammals, there are various molecular markers to identify type A spermatogonia, making it possible to isolate type A spermatogonia from whole testicular cell suspension. To date; however, there are not enough molecular markers to distinguish type A spermatogonia and type B spermatogonia in fish. Therefore, in this study, we aimed to identify a molecular marker for type A spermatogonia in rainbow trout.

METHODS: We first established a method to isolate stage-specific testicular germ cells from rainbow trout possessing the green fluorescent protein (Gfp) gene driven by trout vasa regulatory regions (pvasa-Gfp trout), using Gfp-dependent flow cytometry (FCM). With this method, we successfully identified cell fractions, which contain mainly type A spermatogonia or type B spermatogonia. Next, we compared gene-expression profile of each fraction with cDNA microarray displaying 16006 genes derived from several salmonid species. Genes, showing high and specific expression in the cell fraction containing type A spermatogonia, were identified and used for further characterization, such as real-time PCR and in situ hybridization.

RESULTS: From microarray analysis and real-time PCR, we found that rainbow trout notch1 homologue showed high expression in cell fraction, which contains type A spermatogonia. In situ hybridization of notch1 using rainbow trout testis showed notch1 expressed only in type A spermatogonia but not in somatic cells or differentiating testicular germ cells, including type B spermatogonia, spermatocytes, spermatids and mature sperm.

CONCLUSION: We concluded that notch1 gene could be a powerful molecular marker for type A spermatogonia. This marker would help us to isolate type A spermatogonia, which can be used for germ cell transplantation. Moreover, the combination of Gfp-dependent FCM and microarray analysis of pvasa-Gfp trout can be a useful method to identify stage-specific genes in fish.

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The occurrence of spontaneously spermiating eels in captivity

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BACKGROUND: Unlike many other fish, eels (*Anguilla* spp.) do not normally undergo gonadal development in captivity. Therefore, gonadal maturation is often induced artificially using injections of gonadotropin-containing substances. We recently stumbled across an exception to this dogma as two Japanese eels (*A. japonica*), one of which had been long-term reared in fresh water and the other in seawater, matured without exogenous hormone treatment. Here, we report on the appearance of the gonads from these spontaneously spermiating eels.

METHODS: A 7-year-old feminized eel was provided by the Aichi Fisheries Research Institute, and reared in ambient fresh water (26 degrees) under natural light conditions for 1 year. A further 8 eels of 3-years of age were purchased from a commercial company and then reared in ambient seawater (10.3-27.5 degrees) under natural light conditions for 2.5 years. On dissection and macroscopical analysis, gonad tissue of the feminized eel (an intersex; I-1) and one of the male eels (also an intersex; I-2) was greatly enlarged and had unusual lumps containing spermatozoa. To assess fertility, testicular tissue was minced into small pieces, and milt was collected and analyzed for motility, pH and spermatocrit. In addition, sperm was maintained in artificial seminal plasma at 4 degrees, and used to fertilize ovulated eggs obtained from artificially matured females. The fertilization rate was subsequently recorded and compared with that obtained from an artificially matured male.

RESULTS AND CONCLUSION: Gonadosomatic indices of I-1 and I-2 equated to 29.4 and 18.4%, respectively, whereas that of normal males was < 0.2%. Histologically, the ovarian compartment of I-1 contained oocytes in the oil droplet stage, whereas the testicular compartment had germ cells in all stages of spermatogenesis. Similarly, in gonads of I-2, male germ cells in all stages of development could be observed, together with a large number of early vitellogenic oocytes. The milt pH, spermatocrit and motility were 8.0, 90.7 % and 53% in I-1 and 8.1, 82.4 % and 87.1% in I-2, respectively. The fertilization rate for I-1 was low (5.3%) and larvae were not obtained. Sperm from I-2, however, had fertilizing capability as reflected in the acquisition of hatched larvae, 17 of which eventually underwent metamorphosis into glass eel. This value compares with a total of 22 glass eels obtained from a crossing with a different (artificially matured) male, but the same female. These findings suggest that aberrant sex determination, leading to an intersex condition, can prevent the block on gametogenesis in eels, resulting in the production of fertilizable sperm.

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Meiosis can be completed in zebrafish lacking the DNA mismatch repair gene *mlh1*

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BACKGROUND: Proper formation of chiasmata during meiosis I is necessary for subsequent spindle attachment and segregation of chromosomes in anaphase. The molecular mechanisms of meiosis are still largely unknown, but numerous genes are known to be involved, among which many DNA mismatch repair genes. One of them, mlh1, colocalizes with presumptive sites of crossing-over, although its exact action remains unclear. Mlh1 mutant male and female mice are infertile, showing problems during meiosis and hypogonadism. Also, mlh1 mutant male zebrafish (in mixed AB/TL background) are infertile showing arrest in metaphase I. On the other hand, mutant females are fertile. Since a mixed genetic background – such as AB/TL in the present case – can sometimes affect the phenotypic expression of a mutation, we re-analyzed testicular histology of the mutant after backcrosses with the TL parental line.

METHODS: A mutant mlh1 zebrafish was isolated by tilling after ENU-induced mutagenesis. This mutant (in AB/TL background) was back-crossed two times with TL wild-type animals to reduce heterogeneity and other possible ENU-induced mutations, and subsequently incrossed to obtain homozygous mutants. Twelve wild-type (TL) and eleven mutant males identified by PCR-based genotyping were anesthetized, weighed and testes were removed and fixed in 4% glutaraldehyde. After fixation testes were weighed, embedded in plastic (2-hydroxyethyl metacrylate), and prepared for morphological analysis.

RESULTS: Different from mice, the testis weight and the gonadosomatic index were significantly increased in the zebrafish mutants ($p < 0.05$). All wild-type animals presented normal spermatogenesis. On the other hand, all mutants showed a high incidence of atypical meiotic figures and an apparent accumulation of secondary spermatocytes. Different from previous observations in mice and AB/TL *mlh1* mutant zebrafish, where spermatogenesis arrests in metaphase I, some normal meiotic figures, a few spermatids, and very few spermatozoa were observed.

CONCLUSION: Surprisingly, in all mutant animals spermatogenesis can be completed qualitatively, although only very few spermatozoa are formed. This is the first observation that germ cells from animals lacking MLH1 protein can complete the two meiotic divisions and form sperm. This suggests that alternative cellular mechanisms must exist to direct spindle attachment and chromosome segregation.

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The effects of IGF-I on spermatogenesis *in vitro* in the zebrafish (*Danio rerio*)

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BACKGROUND: IGF-I is a well-conserved growth factor that in vertebrate testis can promote germ cell proliferation/differentiation, acting alone or as a permissive factor with androgens, for example. It is known that IGF-I has endocrine, paracrine and autocrine effects. Although some questions have been answered about the possible mechanisms of IGF-I action on the testis, many aspects of its function need to be clarified. Based on that, we decided to investigate the role(s) of IGF-I on spermatogenesis using zebrafish as a vertebrate model.

METHODS: Testes were removed from fifteen zebrafish and divided in two (half-testis was considered to be one fragment). For each IGF-I concentration, five or six replicates consisting of two fragments were placed on agarose blocks covered with a nitrocellulose membrane and agarose blocks were placed in a 24-well plastic tissue-culture dishes. The medium consisted of Leibovitz-15 supplemented with BSA and HEPES. Culture was carried out for 7 days with various IGF-I concentrations (0, 12.5, 25 and 50 ng/mL) in order to evaluate the *in vitro* effect(s) of IGF-I on zebrafish spermatogenesis. Fragments were fixed in 4% glutaraldehyde, embedded in plastic, and prepared for morphological and morphometric analysis.

RESULTS: After one week all germ cell types were present in testis tissue cultured in all groups. Although not statistically significant, early spermatogonia tended to accumulate in tissue cultured without IGF-I. A clear stimulatory effect of IGF-I was visible with respect to an increase in the relative (%) amount of primary spermatocytes, reaching statistical significance ($p < 0.05$) in the presence of the highest IGF-I dose. Also the relative quantity of spermatids was significantly increased, while the apparently lower incidence of apoptosis at the higher doses of IGF-I (25 and 50 ng/mL) approached but did not reach significance. Taken together, these data suggest that in zebrafish IGF-I alone can stimulate spermatogonial differentiation into spermatocytes and spermatids. Our preliminary findings are different from the results obtained for the Japanese eel, where IGF-I rather acts as permissive factor, or from trout, where IGF-I stimulated spermatogonial proliferation but not their entry into meiosis, but similar to results found in newt.

CONCLUSION: We showed that the tissue culture is a suitable model to study IGF-I effect(s) on spermatogenesis. Also, this is the first report to show IGF-I effect(s) on zebrafish spermatogenesis *in vitro*, such as promoting entry of spermatogonia into meiosis. Further studies will be necessary to elucidate, in more detail, the mechanism(s) of IGF-I action in this vertebrate species.

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Motility characteristics of spermatozoa in cod (*Gadus morhua*) and hake (*Merluccius merluccius*)

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BACKGROUND: Comparatively to acquisition in an increasing number of fish species, very little is known about activation conditions and motility characteristics of spermatozoa in cod and hake, two species of large fishery and/or aquaculture interests.

METHODS: Sperm samples were collected by stripping ripe reared cods and wild caught hakes. Video microscopy combined to stroboscopy were used to record spermatozoa at high resolution right after their activation by sea water (SW) and during the whole period of motility. Successive video images were used for measurement of covered distance (which evaluates individual velocity), of percentage of motile cells or of linearity of tracks. Higher magnification images were used to analyze flagella in terms of wave amplitude, wave length or curvature and their modifications with time after activation. Stroboscopic illumination was used to measure the variation of the beat frequency during the swimming period.

RESULTS: We devised a diluent preventing motility activation (SW:DW in a ratio 1:2 in cod). This was confirmed when varying osmotic pressure (OP) of the swimming medium (SM): motility occurs from 700 to 1550 mOsm/kg. By diluting seminal fluid (SF) by SW in various ratio, we observed that motility is allowed above a SF:SW ratio of 1:4. In all cases, addition of protein (Bovine Serum Albumin in this case) was found necessary to prevent sticking for observation of free swimming spermatozoa.
The swimming period is characterized by a decrease of the percentage of swimming cells (initial value of 95 % in cod) down to zero after few minutes. Similar decrease is observed for beat frequency (initial value = 52 Hz for cod and 57 Hz for hake) as well as for velocity (initial value = 130 µm/s for cod and hake). The total period leading to full immotility was estimated as 8 min for cod and 2.5 min for hake. The flagellar waves mostly show a decrease in amplitude and in number during the motility period. The diameter of the sperm trajectories varied only slightly during this motility period in the case of cod (from 50 to 150 µm), but decreased abruptly in hake (from 480 to 40 µm) which leaded to tight circling of spermatozoa later in the motility period and consequently restricted their efficient progressiveness.

CONCLUSION: As in the majority of marine fish species, motility is triggered by cod and hake spermatozoa microenvironment (OP increase) and the motility period is limited to minutes range. The efficient progressive period is even more limited as the high velocity (above 50 µm/s) period is restricted to about 100 sec in cod and hake. A rapid decrease in flagellar beat frequency is partly responsible of this briefness, but detailed observations of the changes in the wave shape of flagella showed that efficient waves become more and more restricted to the part of the flagellum proximal to the head. This is also true for wave amplitude which decreases as a function of time: the combination of these various factors contributes to drastically limit to the earliest period of motility (less than 100 sec) the ability for spermatozoa to efficiently progress towards egg in order to reach it for fertilization. All these spem motility features allow to better define conditions for artificial propagation.

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Interspecies transplantation of spermatogonia in marine teleosts

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BACKGROUND: Cell transplants from one species to a related but more common species that was easier to rear in captivity could help boost the wild populations of threatened or commercially valuable species. Recently, we have developed a novel germ cell transplantation technique to create salmon broodstock that could produce functional trout gametes. We are presently focusing on applying this technique to the seed production of economically important marine teleosts, such as bluefin tuna.

METHODS: Sciaenids were selected as model species of pelagic egg spawners for developing the interspecies germ cell transplantation technique in marine teleosts. Larvae/juveniles of Nibe croaker (Nibea mitsukurii) were used as recipients because year-round spawning and small-scale seed production of this species has been established in our laboratory. Spermatogonia were used as donor cells because it has been shown that, in Salmonids, they can differentiate into functional gametes in both male and female recipients when they are transplanted into sexually undifferentiated individuals. Donor cells were prepared from immature testes of either 3 to 6-month-old Nibe croaker or Giant croaker (Argyosomus japonicus). Freshly isolated testes were dissociated with 0.5% trypsin. Prior to transfer, cells were labeled with fluorescent dye (PKH26; Sigma). The transplantation of donor testicular germ cells was performed intraperitoneally using glass micropipettes.

RESULTS: The primary goal of this study was to investigate the suitable developmental stages of recipient larvae/juveniles for transplantation. We found that the addition of BSA in the recovery tank at 1 g/L effectively reduced mortality after handling for transplantation. The total length (TL) 4-mm larvae was the youngest recipient with high survival rates (>80%) at 24 hours after handling. We also found that when TL 15-mm juveniles were used as recipient, both of intra- and interspecific donor cells were avoided immune rejection for at least 3 weeks in the peritoneal cavity of recipients, suggesting that the immune system was still immature at this stage. Therefore, germ cell transplantation is ‘physically’ and ‘immunologically’ possible from TL 4- to 15-mm larvae/juveniles of Nibe croaker. Next, the presence of PKH26-labelled cells prepared from Nibe croaker in the allogenic recipient gonads were investigated at 3 weeks post transplantation. As a result, when TL 10-mm juveniles were used as recipient, PKH26-positive cells were identified in the gonads of 5 out of 136 (3.6%) recipients.

CONCLUSION: Here we demonstrated that the germ cell transplantation protocol, originally devised for hatched fry of salmonids, can be applicable to marine teleosts with several modifications. This is the first step towards confirming donor-derived gametes production by recipient gonads. For this purpose, proliferation and differentiation of donor-derived germ cells in the recipient gonads will need to be studied.

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Identification of germ cell-specific cell-surface antigen by spermatogonial EST analysis: Aim to purify viable germ cells from non-transgenic fish using specific antibody

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BACKGROUND: Recently, we have developed a germ cell transplantation technique using green fluorescent protein (GFP)-labelled primordial germ cells (PGCs) or spermatogonia of transgenic rainbow trout carrying a *Gfp* gene driven by *vasa* gene promoter. Donor germ cells microinjected into the peritoneal cavities of newly hatched embryos migrated to and colonized the genital ridges of the recipient embryos. Furthermore, donor-derived germ cells proliferated and differentiated into mature eggs and sperm in the allogeneic gonads. However, donor germ cells isolated from transgenic fish that contain the *Gfp* gene would not be appropriate for applications where fish would be released into the natural environment. A method for labeling fish PGCs and spermatogonia without introducing a transgene would therefore be useful. Therefore long time objective of this study is purification of viable germ cell from non-transgenic fish using specific antibody. In this study, we aimed to identify cell-surface antigen, which is specifically localized in germ cells, as the first step towards preparation of germ cell-specific antibody.

METHODS: Testes were excised from transgenic rainbow trout (*Oncorhynchus mykiss*) possessing the *Gfp* gene driven by trout *vasa*-gene regulatory sequences. After enzymatic dissociation of testes, A-type spermatogonia were purified from dispersed testicular cell suspension by GFP-dependent flow cytometry. Next, we constructed cDNA library using 1×10^7 cells of purified A-type spermatogonia, and determined 5,206 clone sequences which were chosen randomly. Candidate clones, which were expected to encode cell-membrane proteins were identified from 5,206 sequences by *in silico* analysis. Germ cell-specific expression of each clone was examined by RT-PCR and *in situ* hybridization analyses.

RESULTS: From the *in silico* analysis, 82 clones were identified as putative membrane proteins out of 5,206 clones. RT-PCR analysis revealed that 20 clones out of 82 clones were specifically expressed in germ cells. By *in situ* hybridization analysis of the 20 clones, we found clone #4017 was specifically expressed in PGCs, spermatogonia, oogonia, and primary oocytes. Moreover, this amino acid sequence contained distinct one transmembrane domain.

CONCLUSION: We identified clone #4017 as a germ cell-specific cell-surface antigen by spermatogonial EST analysis. Furthermore, clone #4017 mRNA was specifically expressed in fish germ cells that had an ability to colonize the genital ridges of recipient fish, such as PGCs and spermatogonia. Currently, production of recombinant protein of clone #4017 is under progress.

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Sperm biological characteristics in European hake (*Merluccius merluccius*)

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BACKGROUND: European hake has a very important economical value in Europe. However, total annual landings declined from 120 000 to 50 000t. The knowledge of its reproductive biology is required for fisheries management but also for aquaculture diversification. This study shows some sperm characteristics of European hake.

METHODS: Sperm was collected from 23 ripped males (737 ±665g and 44.8 ±13.0cm) caught during winter in the Bay of Biscay (France). Sperm first features were assessed after 48h at 4°C. Sperm density was estimated by counting in a Malassez chamber. Sperm motility was assessed using a two step dilution procedure. Changes in the percentage of motile cells, velocity and flagellar beat frequency with time were recorded using video microscopy combined to stroboscopy. Storage capacities of sperm samples kept at 4°C or cryopreserved, using Modified Mounib extender and 10% DMSO, were recorded. In both cases the percentage of motile cells was estimated. Total adenylate energy charge (AEC) was measured using HPLC.

RESULTS: The mean sperm volume was 3.2 ± 5.3 ml. Mean sperm concentration was 8.6 ± $3.0.10^9$ spermatozoa.ml^{-1}. Activation by transfer in sea water (SW) occurred synchronously for all spermatozoa. The initial percentage of motile cells decreased from 90/70% to 50% after 100sec. The total swimming period lasted about 3min. The initial flagellar beat frequency and velocity respectively decreased from 57Hz and 130µm/s just after activation to 30Hz and 70µm/s, after 100 sec. When sperm was activated with 50% sea water, the initial velocity was lowered but the swimming period lasted up to 8min. After 48h at 4°C, the mean AEC level was 0.711 with a large individual variability from 0.17 to 0.96. After 4 days at 4°C, sperm motility was very variable, ranging from 0 to 75%; after 8 days, 2 of these 6 individuals tested showed 50% motility. When cryopreserved, the percentage of motile cells at thawing ranged 11.8% to 29.6%. No significant relationship was found between individual sperm traits and fish characteristics except for the body length which was positively correlated with the volume of stripped sperm ($P<0.01$).

CONCLUSION: For the first time, some European hake sperm characteristics are reported here. Further investigations are needed in order to improve the knowledge of reproductive biology in this species and to settle gamete management methods.

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Morphology, biochemistry and physiology of chondrostean fish sperm: a comparative study between Siberian sturgeon (*Acipenser baerii*) and sterlet (*Acipenser ruthenus*)

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BACKGROUND: Both in teleostean and chondrostean fishes, many studies have shown inter- and/or intra-specific differences in terms of sperm morphology, biochemistry and physiology. We present a comparative study of these parameters in two sturgeon species, *A. baerii* and *A. ruthenus.*

METHODS: Spermatozoa morphology was studied using SEM (JSM 6300 and JSM 7401 S in cryo-regime) and TEM (JEOL 1010). Osmolality, pH and ionic composition (Ca^{2+}, Na^{+}, K^{+} and Cl^{-}) of seminal plasma were measured with an osmometer, a pH meter and Ion Selective Electrodes or flame photometry, respectively. Sperm density was estimated by counting with a Heamocytometer chamber under a microscope. Sperm motility was tested in buffered distilled water (20 mM Tris-HCl, pH 8.0) and recorded using a 3-CCD video camera, mounted on a dark-field microscope. Sperm velocity and percentage of motility were analyzed with a micro image analyzer. Data analyses were carried out by SPSS 10.0. Principal Component Analysis (PCA) was used to assess variations in terms of morphology of sperm.

RESULTS: We observed wide morphological variations between spermatozoa of the 2 species. The average length and width of the spermatozoon head were 7.01±0.83 and 1.12±0.12 in *A. baerii* and 5.14±0.42 and 0.82±0.04 µm in *A. ruthenus*, respectively. In both species, ten posterolateral projections deriving from the acrosome were observed. The nucleus consisted of electrodense homogeneous nuclear chromatin, except for three mingled endonuclear canals. The flagellum originating from the centriolar apparatus had a typical 9+2 organization. A PCA explained both inter-specific and intra-specific morphological variations fairly well. Of the total accumulated variance of *A. baerii* and *A. ruthenus*, 41.45 and 41.77 % was accounted for by parameters related to the head and midpiece of the sperm and the length of the flagellum, respectively. Mean values for density of sperm, seminal plasma pH, osmolality (mOsmol Kg^{-1}), along with Ca^{2+}, Na^{+}, K^{+}, Cl^{-} ions concentrations (mM) were $0.61\pm0.37\times10^{9}$, 8.16±0.18, 77.20±52.28, 0.24±0.06, 31.39±10.21, 3.51±1.10, 14.00±4.30 in *A. baerii* and $0.40\pm0.28\times10^{9}$, 8.13±0.19, 50.74±6.27, 0.16±0.11, 20.11±3.78, 1.26±0.54, 6.11±0.60 in *A. ruthenus*, respectively. Significant differences were observed in terms of Na^{+}, K^{+} and Cl^{-} concentrations in the seminal plasma as well sperm velocity ($P<0.05$). The percentage of motile sperm did not show significant difference between species ($P>0.05$).

CONCLUSION: The present study revealed large inter- or intra-specific differences in terms of morphology which could be valuable taxonomically. In addition, the observed biochemical and physiological differences should be considered for development of artificial reproduction and sperm cryopreservation.

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Sperm motility activation in the euryhaline tilapia *Sarotherodon melanotheron heudelotii* (Dumeril, 1859) acclimatized to fresh, sea or hypersaline waters

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BACKGROUND: *Sarotherodon melanotheron* is an estuarine tilapia naturally distributed in West Africa from Senegal to Congo. In Senegal, the sub-species *S. m. heudelotii* was found to reproduce successfully at salinities ranging from *c.* 0 (Guiers Lake) up to 120 (Saloum estuary). In order to better understand adaptive mechanisms enabling reproduction in such different environments, the effects of osmolality and ions were examined on motility of spermatozoa from males acclimatized to fresh (FW), sea (SW) or hypersaline (HW) waters.

METHODS: Fish used descended from a Senegalese population reared in fresh water in the "GAMET" facilities since 10 years. Ten months before observations, three groups of 10 males (243-402 g) were stocked together with females in recycling water systems at salinities set at *c.* 0 (FW), 35 (SW) or 70 (HW). Sperm was collected by squeezing testes and stored in tubes on ice. Motility parameters were assessed *vs.* time after activation, using video images recorded through a microscope (dark field) equipped with a stroboscopic illuminator and a camera. Velocity and % of motile spermatozoa were extracted from successive video frames with an image analyzer. Motility was initiated by 1:100 sperm dilution in a drop of swimming medium on a microscope slide. The effects of osmolality and ions were tested using synthetic sea salt or sucrose solutions of different osmolalities as swimming media. Media were added with EGTA or $CaCl_2$ for evaluating the Ca^{2+} requirements for activation of spermatozoa.

RESULTS: The osmolality of seminal fluid was slightly higher in HW-reared than in FW-reared males (340-360 *vs.* 305-330 $mOsmol.kg^{-1}$). The percentage of motile spermatozoa in synthetic sea salt solutions was highest (80-100%) at 1-370, 300-970 and 450-1600 $mOsmol.kg^{-1}$, for fish reared in FW, SW and HW, respectively. Sperm from FW-reared males showed a similar response in sucrose solutions. By contrast, sperm motility in sucrose was strongly depressed for SW-reared males, and almost fully for HW-reared males, at all osmolalities tested. The addition of $CaCl_2$ to sucrose caused all responses to rise close to the levels observed in synthetic sea salt solutions, while full removal of Ca^{2+} with EGTA almost blocked motility.

CONCLUSION: To our knowledge, this is the first study describing the osmolality and ions dependence of sperm motility for fish acclimatized to FW, SW and HW waters. The range of osmolality that enabled sperm activation, shifted and broadened as the maintenance salinity of broodfish increased. Hence, sperm motility was enough (even though not maximal) to ensure egg fertilization at the maintenance salinity. The requirement of extracellular Ca^{2+} for activation of sperm motility increased when the maintenance salinity of broodfish was higher.

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Sperm characterisitics and motility in *Pangasianodon hypophthalmus* (Sauvage, 1878) and *Pangasius djambal* Bleeker, 1846 (Pangasiidae, Siluriformes)

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BACKGROUND: In-depth knowledge of sperm physiology is a prerequisite for defining reliable methods of artificial fertilisation and gamete management in fish culture. Data on sperm characteristics and motility remain scarce in catfishes, particularly in pangasiids in spite of their economic importance.

METHODS: Males of *P. hypophthalmus* and *P. djambal* were injected with 0.3-mL.kg^{-1} Ovaprim in order to increase the volume of milt collected by striping 20-24 h later. Sperm was stored in tubes at 4°C, either fresh or diluted in an immobilizing solution. Sperm characteristics were assessed in 2-18 males, depending on species and observation. The variations of the percentage of motile cells, flagellar beat frequency and sperm head velocity were assessed versus time after motility initiation, using still video images recorded through a microscope equipped with a dark field condenser, a stroboscopic illuminator and a camera. A double dilution procedure was applied, composed successively of a 1/50 dilution in an immobilising medium allowing the dispersion of spermatozoa, followed by a 1/100 dilution in a drop of swimming medium (distilled or tap water) set on a microscope slide. This resulted in the immediate, synchronous and full activation of spermatozoa. The same procedure was used to assess the percentage of motile cells when using NaCl or sucrose solutions of different osmotic pressures as immobilization or swimming media.

RESULTS: In comparison to the osmolatity of intratesticular sperm (260-314 mOsmol.kg^{-1}), that of the seminal fluid was lower and more variable in sperm collected by stripping of males (190-282 mOsmol.kg^{-1}). This indicated a possible pollution of sperm by urine during stripping that may have activated spermatozoa at collection. Hence, in both species, potential motility of spermatozoa was much shorter for semen collected this way than for semen collected directly in an immobilising solution (290-350 mOsmol.kg^{-1}, 1/5 dilution). After activation in fresh water, the motility parameters followed similar patterns in both species, with a rapid, sigmoid-shaped decrease for the flagellar beat frequency and percentage of motile cells, and a continuous and rapid decrease of spermatozoa velocity. The total duration of motility (forward movement) was short in both species, ≤ 20-25 sec in *P. hypophthalmus* and 30-35 sec in *P. djambal*.

CONCLUSION: A drop in osmotic pressure appeared as the main trigger for initiating spermatozoa motility, which was particularly short in both species. Sperm preserved at 4°C in a saline medium (NaCl 155-175 mM, pH 7.5-8.5) survived longer than in the other media tested here. However, even in these conditions, sperm of *P. hypophthalmus* and *P. djambal* could not be preserved for more than 1 and 2 days, respectively.

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Germ cells transplantation using the Nile tilapia (*Oreochromis niloticus*) as a recipient experimental model

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BACKGROUND: Although germ cell transplantation is well characterized in rodents such as mice and rats, there are very few studies utilizing this approach for lower vertebrates. Tilapias (*Oreochromis niloticus*) and frogs (*Rana catesbeiana*) are two economically important vertebrate species that present cystic arrangement of spermatogenesis which develops at similar temperature (25ºC) in both species. Tilapias and frogs are also excellent experimental models for studies developed in laboratory conditions. In this regard, our main objective in the present study was to investigate the viability of adult tilapias as a recipient for syngenic (tilapia to tilapia) and xenogenic (frog to tilapia) germ cells transplantation.

METHODS: The depletion of endogenous spermatogenesis in tilapias was performed successfully with busulfan in association with the temperature of 35ºC, according to protocols specifically developed in our laboratory. The germ cells to be transplanted were obtained from fifteen sexually mature tilapias and twelve frogs that had their testes enzymatically digested with collagenase, DNAse, and trypsin, according to standard protocols. These cells were selected and enriched utilizing percoll gradient and labeled with the red fluorescent cell linker (PKH26; Sigma). Twenty adult tilapias received through the common spermatic duct a total of ~10^7 donor germ cells/mL. Recipient fishes had their testes analyzed by light, fluorescence and confocal microscopies, 15 minutes, 14h and 1 to 8 weeks post-transplantation.

RESULTS: *Syngenic transplant:* a) at 14h post-transplantation PKH26 labeled germ cells were observed contacting the recipient Sertoli cells; b) from two to six weeks, labeled cells formed evident spermatogenic cysts showing varied sizes and different stages of development; c) from seven to eight weeks, round and small PKH26 labeled cells were eventually detected in the seminiferous tubule lumem and they resembled released spermatozoa that were originated from transplanted spermatogonia. *Xenogenic transplant:* at the time intervals investigated forwhile (1 to 4 weeks), PKH26 labeled frog germ cells were observed in tilapias testes and these cells were either isolated or eventually formed cysts-like structures, this later was present particularly at 3-4 for weeks post-transplantation.

CONCLUSION: The results found showed for the first time that transplanted germ cells are able to colonize, proliferate and differentiate in the tilapia testes. These data also suggested that tilapias might represent a potential experimental model to investigate the spermatogonial stem cell biology and spermatogenesis in lower vertebrates.
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Motility activation and subpopulation analysis in *Solea senegalensis* spermatozoa

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BACKGROUND: The analyses of sperm motility have enhanced both the knowledge of sperm biology and sperm quality assessment. Computer Assisted Sperm Analysis (CASA) has been successfully applied to the study of fish sperm motility, allowing to obtain repeatable data and to track individual spermatozoa. Once these spermatozoa are characterized it is possible to group in homogeneous subpopulations with similar motility patterns which can be related to treatments, male differences or sperm physiology. This technique can be useful in the selection of males based on sperm characteristics. *Solea senegalensis* is a good candidate for the application of this technique due to the problems detected in male broodstock related with sperm production and quality. This analysis could have further implications for the selection of appropriate husbandry conditions during reproductive season.

METHODS: Sperm was colleted weekly from 44 fish maintained separately into 6 tanks during 3 months (May-July). Characterization of motility using CASA software was performed in two trials. In the first trial, the effect of pre-dilution solutions and the composition of the motility activating solutions were analyzed. In the second trial, the effect of different artificial seawater osmolarities was analyzed in male populations maintained in contact with females and isolated. In all studies both consecutive sperm extractions and male variability were taken into account. Motility was acquired at 15, 30, 45 and 60 s after activation.

RESULTS: In the first trial five spermatozoa subpopulations were identified. Three were present in most of all cases (SP1: slow/non-linear; SP2: fast/non-linear; SP3: fast/linear). The subpopulation pattern varied with time and activating treatment, observing an increase of SP1 and a decrease of the motility parameters for all subpopulations with time. Statistical analysis indicated that predilution in a non-ionic solution prior to the activation of sperm with sucrose produced the highest percentages of motile spermatozoa. Results from the second trail indicated that solutions equal or higher than 800 mOsm/kg produced the highest percentage of motile spermatozoa, VCL and VSL. Sperm consecutive extractions and female interaction revealed no significant effect on the analysed sperm motility parameters.

CONCLUSION: This study offered an interpretation of the subpopulation patterns and the differences between activating treatments based in studies on osmotic stress. The analyses demonstrated the existence of a heterogeneous sperm population in all samples. In conclusion, CASA and subpopulation analysis would allow a better understanding of the physiology of *Solea senegalensis* spermatozoa.

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After finishing of motility, common carp (*Cyprinus carpio*) sperm is able to re-initiate a second motility period and to fertilize eggs

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BACKGROUND: After a first round of motility, fish spermatozoa can become activated for a second time in return for a certain period of rest in a non-swimming medium; this time period is required to restore initial levels of energy. Once activated, trout spermatozoa lose their fertilizing capability very quickly, owing to their brief duration of motility. If activated in a physiological solution, however, motility can be eventually re-initiated. Our goal was to compare motility performances (sperm velocity and motility rate) and fertilizing ability of carp sperm in two situations: freshly activated sperm and sperm which had finished swimming and was "revived" after incubation in a K^+-rich non-swimming solution.

METHODS: Pools of stripped sperm and ova were used for these experiments. In a first series, sperm was activated in carp activation solution (CAS; 45 mM NaCl + 5 mM KCl + 30 mM Tris, pH 8) or hatchery water (HAS), both containing 0.1% BSA. Two minutes post activation (when 100% spermatozoa had finished motility) spermatozoa were incubated for 10 minutes in an immobilizing solution (IS) containing 200 mM KCl. Activated sperm in CAS, was reactivated with the same activation solution (CAS) after incubation in IS. But, sperm which has been firstly activated in HAS, was reactivated in both CAS and HAS after incubation in IS. In both cases, the sperm motility was observed and recorded using a dark-filed microscope to evaluate the sperm motility. Sperm velocity and percentage of motility were analyzed with a micro-image software. Sperm: egg ratio was adjusted to 2×10^5: 1 in both situations either freshly activated sperm or incubated/reactivated sperm. Eggs samples were fertilized as follows: 2×10^5 spermatozoa per egg were activated with 10 ml CAS and/or HAS, then 4000 eggs were added at 10, 20, 30 or 120 sec post sperm activation and followed by stirring on a shaking table for 2 min. The fertilized eggs in *triplicates* were transferred into experimental trays for further incubation.

RESULTS: For fresh sperm activated only once, the hatching rate was 66.96±1.94% in CAS and 75.48±3.65% in HAS at 10 sec and 1.05±0.36% in CAS and 0.00±0.00% in HAS at 120 sec post activation, respectively. For sperm firstly activated in CAS and reactivated in CAS, the hatching rate was 61.66±2.93 and 40.61±4.85% at 10 and 30 sec post reactivation, respectively. But, the hatching rate of sperm which has been incubated in IS after activation in HAS and thereafter reactivated in CAS and HAS, were measured as 25.76±3.82 and 8.73±6.65% at 10 sec and 0.20±0.39 and 0.46±0.33% at 30 sec post activation, respectively. Firstly activated sperm showed significantly higher percentage of motility and sperm velocity compared to reactivated sperm. Sperm incubation in IS improved the structural damages such as blebs or curling of the flagellum observed in freshly activated sperm diluted in CAS.

CONCLUSION: To our knowledge, this is the first observation showing that fish spermatozoa (devoid of acrosome in carp) can be revived for a second motility round and that such spermatozoa are also able to fully fertilize eggs, meaning that fish spermatozoa are not dead at the end of their first motility period. The present study was supported financially by USB RIFCH no. MSM6007665809 and GACR no. 524/06/0817.

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Evaluation of semen and urine of pike (*Esox lucius* L.): Ionic compositions and osmolality of the seminal plasma and sperm volume, density and motility

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BACKGROUND: Understanding of semen characteristics such as production and density, seminal plasma composition and spermatozoa motility are helpful to optimize methods of artificial reproduction. The aim of the present study was to (a) determine ionic compositions and osmolality of urine and seminal fluid of stripped (SPZs) and of testicular (SPZt) sperm as well sperm density and volume and (b) to study the effect of urine on sperm motility in pike (*Esox lucius* l.).

METHODS: In present study, sperm of 37 matured pike males was used. Sperm density and volume measured and expressed as ml and billions of spermatozoa per ml ($\times 10^9$ spz ml^{-1}), respectively. Ionic compositions (Na^+, K^+, Ca^{2+} and Mg^{2+}) and osmolality were measured Ion Selective Electrodes/flame photometry and an osmometer, respectively. The motility of spermatozoa was observed and recorded using a dark filed microscope with stroboscopic illumination and a video camera. Sperm velocity and percentage motile spermatozoa were extracted from five successive video frames with Micro-image software (version 4.0.1. for Windows). To study the sperm motility, the activation of both stripped and testicular sperm was induced in distilled water and in urine, containing 0.1 % BSA on a glass slide. BSA was added to present sperm heads from sticking to the glass slide.

RESULTS: The average volume of SPZs was 1.2±0.8 ml. Sperm density was higher in SPZt ($33.7\pm5.3\times10^9$ ml^{-1}) than SPZs ($22.6\pm3.5\times10^9$ ml^{-1}) ($P<0.05$). The Na^+ (122.6±8.9 mM), Cl^- (126.7±6.6 mM) and K^+ (34.5±5.2 mM) concentrations in the seminal fluid of SPZt were significantly higher than measured in the seminal fluid of SPZs: Na^+ (115.5±8.9 mM), Cl^- (116.2±6.6 mM) and K^+ (24.8±3.9 mM) ($P<0.05$). Both SPZt and SPZs showed the higher concentrations of the ions compared to the urine (41.5±27.9 mM), Cl^- (34.8±21.5mM) and K^+ (6.1±3.6 mM) ($P<0.05$). But, no significant differences was observed in case of Ca^{2+} ($P>0.05$). The average osmolality of urine and seminal fluid of SPZs and SPZt were 67.9±35.5, 272.6±21.4 and 358.1±76.5 mOsmol kg^{-1}, respectively ($P<0.05$). Finally, both SPZt and SPZs showed significantly higher sperm velocity and percentage of motility after dilution in urine than in distilled water along the activation period ($P<0.05$).

CONCLUSION: Differences observed in terms of quantitative and qualitative characteristics between stripped and testicular sperm is due to contamination of stripped sperm by urine during stripping. The osmolality is regulatory factor for triggering sperm motility in pike. Therefore, low osmolality of the urine triggers the initiation of sperm motility, but direct collection of sperm in an immobilizing solution with the same or higher osmolality of the seminal plasma provide us with opportunity to avoid spontaneous activation due to contamination by urine. The observed differences in terms of sperm motility parameters after diluting in urine and distilled water should be related to osmotic effect on cellular mechanisms involved in initiation of sperm activation. Thanks are also expressed to USB RIFCH No: MSM 6007665809 and GACR no. 524/06/0817 for financial support.

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Cell-specific gene expression during European sea bass (Dicentrarchus labrax) spermatogenesis

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BACKGROUND: A major disadvantage of sea bass culture is the presence of precocious males that mature precociously. To understand the molecular basis of male precocity it is necessary to quantify gene expression during testis maturation. In this regard, here we have optimized a newly developed technique, Laser Microdissection Pressure Catapulting (LMPC), coupled with real-time quantitative PCR (Q-PCR) that allows determination of the precise expression pattern of genes in specific cell populations throughout testis development. We quantified the expression of several genes, including a germ cell marker, steroidogenic enzymes, several sex steroid receptors, and testis-specific genes. Knowledge of the expression levels of these genes will be useful to investigate their role in male spermatogenesis and their possible implication in male precocity.

METHODS: Adult males sea bass at different stages of spermatogenesis were used. LMPC was used to identify and extract specific cell types populations during germ line progression from spermatogonia to spermatozoa. Gene expression levels were determined by Q-PCR. Gene selection was based in their putative implication in testis development and function. The expression pattern of genes was compared during the spermatogenic progression.

RESULTS: LMPC coupled with a very efficient RNA extraction from tiny amounts of tissue and Q-PCR was used to study the relative expression patterns of about a dozen genes. For instance, the specific germ cell vasa was detected in all cell types confirming the successful identification and catapulting of germ cells. Estrogen receptor β1 (ERβ1) and estrogen receptor (ERβ2) had the highest and similar expression patterns, decreasing as spermatogenesis advanced. On the contrary, androgen receptor (AR) was only expressed in spermatozoa at very low levels. Ovarian aromatase was not detected in any cell type.

CONCLUSION: LMPC coupled with Q-PCR proved to be a useful method to quantify the expression of key genes in specific cell types during spermatogenesis. Some of the results include the observation that vasa was only detected in germ cells. AR was detected only in spermatozoa, suggesting a role in late spermatogenesis. ERα was only observed in spermatogonia whereas ERβ1 and ERβ2 were identified in most cell types, with a progressive decline as spermatogenesis advanced, suggesting that E2 is more implicated in the first stages of spermatogenesis. Knowledge of the expression patterns of these relevant genes is important for our understanding of spermatogenesis in sea bass and also a deeper insight in the phenomenon of male precocity. This approach allows obtaining RNA of good quality that can be used for microarray-based gene discovery.

Gametogenesis and Gamete Biology (Part II : Hormonal and Environmental Regulation of Gametogenesis)

P 105

In vivo and _in vitro_ ovarian sensitivity to gonadotropin in Eurasian perch _Perca fluviatilis_ maintained under constant photothermal conditions

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BACKGROUND: While reproductive cycle may be induced by a photothermal manipulation in Eurasian perch females, the resulted egg quality is not suitable to produce viable larvae for intensive culture conditions. It is not clear whether the inhibition of reproductive process by constant photothermal conditions of rearing is related to an inadequate hormonal stimulation and/or refractoriness of intrinsic ovarian features.

METHODS: In an *in vivo* setting, two groups of females reared in natural or constant conditions of temperature and photoperiod were investigated for gonad development, sex steroid (testosterone-T, 17β-oestradiol-E2 and 11keto-testosterone-11KT) dynamics and aromatase activity in January, February and March. Two days before each sampling date, a group of females reared in constant conditions was injected by hCG (human Chorionic Gonadotropin: 100 UI/kg) and evaluated for the same hormonal and gonad parameters. In addition, *in vitro* steroidogenesis capacity for each group of females was determined at each sampling date by a six-hour period of stimulation of ovarian tissues by different doses of hCG and/or IGF-I (Insulin-like Growth Factor-1).

RESULTS: The overall results concerning gonad histology indicate that the initiation of exogenous vitellogenesis is the major stage limiting the oocyte maturation in non-mature females submitted to constant photothermal conditions. This was associated with lower levels of circulating steroids (T, E2 and 11KT) comparing to mature females under natural conditions as an indication of a reduced steroidogenesis capacity. Nevertheless, non-mature females stimulated with hCG injections exhibited the same steroidogenesis status than mature females in March, as did aromatase activity between January and March, indicating that steroidogenesis process was not substantially affected by constant photothermal conditions. Such finding was confirmed by the *in vitro* stimulation by IGF-I, which induced comparable steroidogenesis responses in all ovarian groups, especially in February and March. In contrast, the *in vitro* stimulation by hCG resulted in a higher steroidogenesis capacity for mature ovarian tissues than for non-mature ones. The highest steroidogenesis responses were observed in IGF-I treatments and steroidogenesis capacity decreased with the time whatever the treatment, the dose and the rearing conditions.

CONCLUSION: The results of the current study show that the inhibition of reproductive cycle in Eurasian perch females by constant photothermal conditions of rearing may be related to a lower endocrine stimulation, which prevents the oocyte maturation at the time of initiation of exogenous vitellogenesis.

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Slow release GnRHa therapy prevented atresia during vitellogenesis and induced ovulation of captive wreckfish (Polyprion americanus)

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BACKGROUND: Due to its high growth rate, wreckfish *(Polyprion americanus)* may be a good candidate for aquaculture provided it can reproduce in captivity. In the wild, juveniles live near the surface, but migrate deeper as they get older. Puberty occurs at a total length (TL) of 60-90 cm. Spawning occurs at depths of 450-850 meters. Gametogenesis description and steroid level may allow defining adequate stimulation of reproduction in captivity

METHODS: Wild-caught wreckfish of TL between 59 to 90 cm were maintained in tanks or cages in natural conditions in France (Brest Bay, Brittany) and in controlled condition tanks in Greece (Heraklion, Crete). When possible, females were sexed by biopsy and males by sperm stripping. Blood samples and ovarian biopsies were collected monthly for 4 years. Estradiol (E2) and 11 keto-testosterone (11-KT) were assayed in the plasma, while oocyte stages were assessed after clearing fixation of ovarian biopsies and further, by histology. GnRHa was applied either by injection or implantation. Ovulated eggs were obtained by stripping and were fertilized artificially with sperm collected from two males.

RESULTS: Three females greater than 85 cm in TL had shown cyclic annual variations of E2 and maximum oocyte diameter for the 4 years. Both parameters had shown peak values by the end of winter (E2:1.5 ng ml-1; oocyte diameter: 800 µm) so that the reproductive period may take place in spring. However, the rapid drop of E2 was followed by the development of a general atresia of vitellogenic oocytes. The application of 100µg kg^{-1} LHRHa by slow release implant to females when E2 was still at a high level or females showing no atretic vitellogenic oocytes (diameter 800µm), had allowed sustaining vitellogenesis for 1.5 month up to its end (diameter: 1400µm). Then a last GnRHa supplementation of 33 µg kg^{-1} triggered, within 7 days, a peak of E2 up to 4 ng ml^{-1} immediately followed by a rapid hydration and ovulation. Eggs were collected by stripping, they were translucent and presented several oil droplets; their mean diameter was 2300µm. After artificial fertilisation, the embryos developed within 4 days at 15°C.

CONCLUSION: Wreckfish did not reproduce spontaneously in captivity, even after GnRHa stimulation, probably due to inadequate tank size, light environment, hydrostatic pressure and/or temperature stability, which are different from spawning environment in the wild. The captivity conditions resulted in complete vitellogenesis in Greece, but not in France, whereas spontaneous final oocyte maturation did not occur in either locations. Administration of GnRHa implants prevented ovarian atresia and was able to sustain vitellogenesis through the maintenance of high E2 levels. In addition, it induced multiple cycles of ovulation. Although generally large eggs show slow development, wreckfish eggs had a rapid development, similar to that of smaller eggs spawned by most marine fishes.

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Determinism of the quality of reproduction in Eurasian perch, Perca fluviatilis: a multifactorial study

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BACKGROUND: In Eurasian perch, Perca fluviatilis, a photothermal control program of the reproductive cycle has been defined. However, spawning is still of poor quality and numerous environmental and nutritional factors are likely to be involved. The objective of this study was to determine and rank the importance of these factors and their interactions.

METHODS: Eight factors (temperature (T) and photoperiod (P) variations, initial nutritional status of fish (I.N.S.), handling, dawn simulation, light intensity (L.I.), food type and feeding) were tested according to a 2^{8-4} fractional factorial design (16 combinations). In March 2005, 16 groups of 13 Eurasian perch breeders (2+) were let to initiate gametogenesis. To do so, T and P were decreased from 23°C and 16L:8D to 14°C and 8L:16D within 9 weeks respectively. Then, the 16 treatments were applied. According to treatments, T was decreased to 4°C or 8°C, dawn simulated or not, L.I. set at 100 or 1000 lux and P increased to 13L:11D after 3 months or kept constant to 8L:16D. Fish were handled every day or not, fed with pellets or fish, at two different levels. After 16 weeks, all fish were blood-sampled to assess plasma testosterone and 11 keto-testosterone (11KT) concentrations. Then, T was increased to 13°C within 2 weeks in all tanks. Three weeks later, all fish were blood-sampled and sperm quality (number of spermatozoa produced per male (NbSpz), concentration, motility, velocity) was assessed. Finally, all fish were killed for gonad histology and GSI determination.

RESULTS: At the end of the experiment, 95% of females and 98% of males displayed gonad development. However, no spawning was obtained and gonad development was very heterogeneous in females. In males, sex steroids concentrations were affected by numerous factors (T, P, I.N.S., dawn simulation and L.I.). At the beginning of T increase, 11KT concentration was the highest (about 5 $ng.mL^{-1}$) when chilling T was set at 4°C, I.N.S. was high and photoperiod increased to 13L:8D. NbSpz and sperm concentration appeared to be mainly affected by I.N.S. and L.I. Both parameters were the highest (1700.10^9 $spz.male^{-1}$ and 60.10^9 $spz.mL^{-1}$, respectively) when I.N.S and L.I. were the lowest. Sperm motility and velocity (about 100% and 180 $\mu m.s^{-1}$) were in the same range as wild perch and were not affected by any tested factor.

CONCLUSION: This multifactorial study allowed for the first time to rank the effects of numerous factors and interactions on quality of reproduction in fish. It was namely shown that I.N.S and L.I. had the most important impact on sperm quality. A first qualitative model of the quality of reproduction in male perch is proposed. Further experiments should be undertaken to better understand the determinism of gamete quality in females.

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Effects of the photoperiodic and thermal kinetics on the induction of the reproduction cycle in the perch *Perca fluviatilis*

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BACKGROUND: Perch is an annual laying fish; the ovogenesis and the development of the gonads begin in summer, and spawning intervenes in April, under the influence of the temperature and the photoperiod. Artificial thermal and photoperiodic variations make it possible to control the cycle of reproduction (Sulistyo 1988, Migaud 2004). The purpose of this work is to define the influence of the thermal and photoperiodic kinetics and the amplitude of the reduction in the photoperiod on the induction of the gonadogenesis in a controlled environment.

METHODS: Male and female perch weighing on average 114 g were placed (56 fish per tank) in 500L tanks allocated in two rooms (8 tanks per room) at a temperature of 20°C and an illumination of 17h of light (200 lx) and 7 h of darkness. From March 14, 2006 (T0), after 6 weeks of acclimatization, a reduction of the photoperiod was applied according to 4 modes (2 tanks per mode): from 17L/7DO to 17L/7D (0h), to 16L/8D (1h), to 13L/11D (4h) or to 9L/15D (8h), in a 6 weeks time in one room, and 16 weeks time in the other. From April 11, the temperature was decreased, from 20°C to 7°C, in 6 and 16 weeks respectively. At T0 then every 4 weeks, 5 fish were taken of each tank, anaesthetized using 2-phénoxyéthanol for blood sampling, then euthanized, measured, weighed, and the gonads, the liver and viscera were weighed. The sexual steroids (œstradiol in the females, testosterone in the males) were assayed by ELISA in plasma, and the gonado-somatic index IGS (weight of the gonads/total weight) was calculated.

RESULTS: In constant and long daylength (17L/7D), the temperature reduction does not lead to any gonadogenesis induction. Decreases of the photoperiod by 4 or 8 hours cause a maximal development of the gonads, similar in the two kinetics (6 or 16 weeks) applied. In both sexes, sub-optimal gonadic development and secretion of sexual steroids are observed after a 1 hour reduction of the photoperiod when the photoperiodic and thermal variations are carried out in 16 weeks (but not in 6 weeks).

CONCLUSION: These observations indicate that the photoperiod is the principal factor of induction of the gonadogenesis. The starting role of the reduction in the duration of the day is illustrated here. The development gonadic is not synchronized according to the kinetics of the photoperiodic variations, and its maximal amplitude is obtained for a reduction in the photoperiod ranging between 1 and 4 hours. The interaction between the thermo-photoperiodic kinetics and the amplitude of the photoperiod fall is highlighted here. These observations will contribute to a better control of the induction of the reproduction cycle of the perch in a controlled environment and out of season.

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Combination of photoperiod and temperature manipulation and LHRHa treatment permits prolonged spawning of Atlantic salmon *Salmo salar*

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BACKGROUND: Atlantic salmon aquaculture in Tasmania, Australia is based on a single stock that spawns over a short season of only 2-3 weeks in the austral autumn. The Tasmanian salmon farming industry is also highly reliant on accelerated hatchery production of under-yearling smolts. The present study examined methods to advance and extend the spawning season with a view to further extending under-yearling smolt production.

METHODS: Sexually maturing 2+ female Atlantic salmon were held at natural temperatures (NT; *c.* 15-20°C) and exposed to either a natural photoperiod (NP; 42°S) or an advanced photoperiod (AP; L:D 9:15) from the austral summer solstice. In late summer, a proportion of the AP fish were also exposed to an advanced temperature regime (AT; *c.* 6°C below natural temperature). Groups of 10 fish within each photothermal regime were then treated with either the commercial LHRHa/domperidone preparation Ovaprim™ (O) (20mg D-Arg6,Pro9-Net LHRHa + 10mg domperidone per ml propylene glycol; Syndel Laboratories Ltd., Vancouver, B.C.) or propylene glycol vehicle (V) only. Thereafter, ovulations and egg fertility and survival were observed.

RESULTS: Ovulations commenced first in the AP-AT-O fish (09 March) and were completed after 24 days (02 April). Thereafter, ovulations occurred in the AP-AT-V fish (30 March – 16 April) followed by the AP-NT-O (09 April – 20 April), AP-NT-V (16 April – 04 May) and the NP-NT-O (07 May – 14 May) fish groups. The last fish to ovulate were the in the NP-NT-V group, which ovulated over a 17-day period between 22 May and 08 June. Mean egg fertility exceeded 80% in all but the AP-AT-O fish where mean fertility was significantly reduced (*c.* 52%). Subsequently, mean eyed egg survivals ranged from *c.* 50% to 90% in the AP-AT-O and NP-NT-O treatment groups respectively but there were no significant treatment-related differences in percent survival.

CONCLUSION: Photoperiod advance, thermal advance and LHRHa treatment each had the separate capacity to advance the timing of median ovulation in Atlantic salmon by *c.* 14 to 28 days. The cumulative effect of photo-thermal advance and LHRHa treatment was an advance in time of median ovulation of 71 days over that in vehicle-injected fish under natural temperature and photoperiod. Consequently, the total duration of the ovulatory period was increased more than 4-fold from 3 weeks to 3 months. Meanwhile, eyed egg survivals remained at commercially acceptible levels throughout.

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Temperature influences on reproductive development and gamete quality in Atlantic cod (*Gadus morhua*)

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BACKGROUND: Reproductive endocrine homeostasis in fish is responsive to changes in temperature. The longer term consequences of altered temperature profiles on the reproductive cycle, gamete quality, and the endocrine processes regulating these events have been little studied. Information about responses to temperature changes on reproductive development in coldwater marine species, like the Atlantic cod, is particularly warranted.

METHODS: Three groups of repeat maturing cod were held at a common temperature of 7-8°C during ovarian growth (September-February). At the approach of the spawning season (March-April), temperature was changed to 4°C or 12°C for two of the groups, whereas the third group remained at 8°C. Sex steroid profiles of testosterone (T) and oestradiol-17b (E2), and timing of ovulation was used to evaluate temperature influence on reproductive development. Fertilization rate, early cell cleavage (4-64 cell stage) and the proportion of floating egg was used as indices of egg quality.

RESULTS: Temperature treatment influenced both temporal changes and peak plasma sex steroid concentrations. In fish exposed to 12°C, peak E2 plasma concentration was reduced compared to those held at 4°C and 8°C. Peak E2 plasma concentrations in fish exposed 4°C was, however, delayed by four weeks in comparison with those held at 8°C and 12°C. These differences were accompanied by corresponding shifts in the timing of ovulation. The proportion of normally cleaved eggs was significantly lower in the 12°C group (12%) than in the 8°C (85%) and 4°C (96%) groups. Also the proportion of floating egg (4°C; 73%, 8°C; 64% and 12°C; 35%) and fertilization rate (4°C; 43%, 8°C; 34% and 12; 23%) decreased with increasing temperature.

CONCLUSION: The results indicate that sex steroid synthesis, secretion and (or) metabolism in Atlantic cod are influenced by temperature treatment during the spawning season, and that the temperature experienced at this stage of the reproductive cycle also influences the timing of ovulation. Temperature also affects gamete quality and may have significant influences on total reproductive output.

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Effects of temperature on oocyte development and regression of mudskipper (Periophthalmus modestus)

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BACKGROUND: The mudskipper Periophthalmus modestus, is an amphibious fish that inhabit intertidal mud flats in East Asia. Although this species plays an important role in the ecosystem of tidal flats, little is known about its reproductive biology. In our previous studies, we found that the fish spawns from late spring (May) to summer (August). Oocyte development starts with the increase of temperature in early spring and progresses to regression during high temperature period in summer. In the present study, we exposed pre-vitellogenic and mature fish to various temperature conditions to examine the effects on oocyte development and regression.

METHODS: Pre-vitellogenic and mature female fish were collected from Ariake Sound, Japan in early February and in mid-May, respectively. Exp.1) Pre-vitellogenic fish were divided into three temperature groups (18, 24 and 30^{O}C), and were reared for 80 days. Exp.2) Mature fish were divided into two temperature groups (24 and 34^{O}C), and were reared for 21 days. In both experiments, the daylength was kept at 14 hours light – 10 hours dark. The ovaries were removed from the body cavity and weighed, and the gonadosomatic index (GSI) was calculated. Pieces of the ovaries were fixed in Bouin's solution for histological observation.

RESULTS: Exp.1) In the 24 and 30^{O}C groups, GSI increased on day 50 and initiation of vitellogenesis was confirmed. After 80 days, the ovaries of both groups had many oocytes at the tertiary yolk and final maturation stages. In contrast, vitellogenic oocytes were not observed in the 18^{O}C group. Exp.2) Throughout the experimental period, the ovary of the 24^{O}C group contained many oocytes at the vitellogenic and maturation stages. On the other hand, in the 34^{O}C group, GSI decreased on day 14 and oocyte regression was observed. Moreover, the regression was accelerated on day 21 and a large part of the ovary was occupied by perinucleolus stage oocyte.

CONCLUSION: In pre-vitellogenic fish, GSI increased and vitellogenesis commenced in the high temperature treatment (24 and 30^{O}C) but not in the low temperature treatment (18^{O}C). The oocyte development of P. modestus is induced by a rise in temperature. However, high temperature (34^{O}C) caused regression of ovaries in mature fish. Therefore, the regression mechanism of spawning by high temperature exists. These results suggest that environmental temperature have an important role in the regulation of gonadal development and spawning in this species. In both experimental groups, fish were kept at the same photoperiod (14L-10D). Our future studies will focus on the effects of photoperiod on oocyte development and regression of this fish.

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Photoperiod manipulation can stimulate or inhibit pubertal testis maturation in Atlantic salmon (*Salmo salar*)

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BACKGROUND: Early puberty in males is a significant problem in commercially farmed fin-fish species. Photoperiod manipulation can modulate the incidence of precocious puberty. Studies on the regulation of the start of spermatogenesis in salmon address a knowledge caveat in vertebrate reproduction in general, and improve our knowledge on the basis of the initiation of pubertal testis development in a species relevant to fin-fish aquaculture.

METHODS: Previously immature, 3 years old salmon were maintained in sea cages at the Institute of Marine Research under ambient light conditions (NL). An initial control sample was collected on January 8. Starting February 1, half of the animals were exposed to additional constant light (LL); the other half remained under NL conditions. Samples were collected February 18, March 19, April 25, and June 11, when the experiment was terminated and maturation was clearly visible in all males of the NL group. Plasma androgen levels were quantified. Testis development was studied morphologically (routine histology and immunocytochemical approaches to quantify proliferation and apoptosis). We moreover determined the levels of selected mRNAs by quantitative real-time PCR assays in pituitary (β-subunits of FSH and LH) and testis (receptors for FSH and LH) tissue.

RESULTS: Exposure to LL inhibited (~40% of animals) or accelerated (~60% of animals) testis development, in comparison with the NL-exposed control group. Throughout all samplings, we found that LL-mediated inhibition arrested germ cell development at the stage of undifferentiated spermatogonia, associated with a low level of germ cell proliferation. Also plasma androgens and the levels of all mRNAs quantified were low, in part even below the levels found in the initial control sample. In the February and March samplings, most parameters behaved in a similar manner in maturing NL- and maturing LL-exposed fish, in general displaying elevated levels compared to immature males. However, LL-mediated acceleration of testis development became apparent in the April and June samples where, compared to males maturing under NL conditions, LL-treated fish showed higher testis weight and proliferation activity, earlier presence of postmeiotic germ cells, and elevated plasma androgen and LH (but not FSH) receptor mRNA levels. However, there was no difference between maturing NL- and maturing LL-exposed males as regards gonadotropin mRNA levels in the pituitary or germ cell apoptosis.

CONCLUSION: Photoperiod manipulation generates experimental groups with largely diverging states of maturation that are well suited to study the regulation of testis maturation. It appears that the period shortly after changing the photoperiod regime will be particularly interesting for elucidating further the regulatory background of early stages of male puberty.

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Effects of different feeding and light regimes on physiology, endocrinology and incidence of early sexual maturation in three families of cultured Atlantic salmon (*Salmo salar*)

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BACKGROUND: Early sexual maturation is a common and costly problem in salmon farming. Although increased photoperiod is effective in reducing the incidence of early maturation, it is not yet clear how this occurs and many factors are involved. We have studied the effects of different feeding- and light regimes in caged salmon from different genetic background to better understand interactions between environment, growth, hormonal changes and maturation.

METHODS: Approx. 2000 fish (males/females 50/50) were distributed into a normal- and a low feed group between September 2003 to January 2004, when each group were redistributed into a natural- and a continuous light group until July. In addition, in January, 100 fish were transferred into a separate cage where they were subject to repeated blood samples once a month. All fish were on normal feed from January to September 2004, when they were sacrificed. Maturation %, weight, length, condition factor, fat % in gut and in muscle and plasma levels of 11-ketotestosterone (11kT), testosterone (T) in males and estradiol (E2), T in females were measured.

RESULTS: More males matured than females. There was lower incidence of maturation in fish kept on low feed. However, only light treatment significantly reduced the number of mature fish. There are clear differences in maturation in fish from different genetic background. Low fed fish were smaller than normal fed fish in January. However, low fed fish, if light treated, caught up with normal fed fish following natural light, or even those following continuous light. This effect was strongest in females. Low fed fish had less fat than normal fed fish both around gut and in muscle in January. In July, almost all groups had similarly high fat content both around gut and in muscle as normal fed fish in January. However, fat content around female gut in all July groups were similarly low as in the low fed group in January. 11kT and T levels in males and E2 and T levels in females increased in mature fish, while they remained low in immature fish. All males matured among blood-sampled fish.

CONCLUSION: Continuous additional light inhibited maturation and stimulated growth, regardless of feeding regime, which generally only had minor non-significant effects. The differences in maturation between families suggest clear genetic effects, but our knowledge about functional differences between these families is still small. Decreased deposition of fat around gut could be an indicator for lower incidence of maturation later during the season. The use of steroid levels for predicting maturation is, however, limited, since we didn't find any clear event early during the year that can be used as a cue for maturation.

P 115

The influence of polychlorinated biphenyl (PCB) on computer analysed sperm motility of common carp (Cyprinus carpio L.) in vitro

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BACKGROUND: The aim of this study was to evaluate the impact of low- (Delor 103) and high-chlorinated (Delor 106) biphenyl mixtures on common carp sperm motility. Polychlorinated biphenyls (PCB) are lipophilic industrial chemicals which cause abnormal development and negative physiological effects of the reproductive system in many vertebrates including fish. The still existing presence/contamination of PCBs in water besides their strong accumulative properties in living organisms, may also have a negative influence on sperm motility - the important factor responsible for successful reproduction.

METHODS: The semen from 8 spermating male carp (Cyprinus carpio L.) was firstly diluted 200-fold in a basal solution consisting of KCl 200 mM, Tris 30 mM, pH = 8. In the second step, just before registration 1 µl of diluted sperm were placed on the microscope slides and quickly mixed with 19 µl of distilled water (control group) or 19 µl of water containing the following concentrations of Delor 103 or Delor 106: 1; 5; 10 and 50 ng ml^{-1}. Sperm movement was recorded, then the videotapes were computer-analysed using a Hobson sperm tracker. The following parameters were analysed: VCL – curvilinear velocity; VSL – straight line velocity; VAP – angular path velocity, each parameter in µm s^{-1}.

RESULTS: Using computer - assisted sperm analysis it was found that all Delor 103 concentrations used in the experiment significantly decreased VCL and VSL velocities of carp sperm. In the case of average path velocity (VAP) only the highest tested concentration (50 ng ml^{-1}) of Delor 103 caused significantly decrease ($P<0,05$). In experiment with high-chlorinated biphenyl Delor 106 the lowest tested concentration (1 ng ml^{-1}) had no effect on sperm motility. The significant decrease in VCL, VAP and VSL velocities of carp sperm was found at the doses of 5, 10 and 50 ng ml^{-1}.

CONCLUSION: The obtained results showed that polychlorinated biphenyls similarly to other dangerous pollutants (for example: heavy metals) are able to influence the final phase of reproduction in fish affecting spermatozoa motility.

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Gonad gene expression profiles of GH receptor, IGF-I receptor and IGF-I in male Atlantic salmon through sexual maturation

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BACKGROUND: The GH-IGF-I system has been implicated in the control of gonadal development and growth in male Atlantic salmon. Plasma GH levels increase in sexually maturing males. Furthermore, injections of androgens raise plasma IGF-I levels. This indicates an interaction of the growth and maturation axis, although the precise functions of GH and IGF-I during sexual maturation remain unclear.

METHODS: Real-time Q-PCR was used to measure the relative gene expression of the growth hormone receptor (GHR), insulin-like growth factor-I receptor (IGF-IR), and insulin-like growth factor-I (IGF-I) in sexually maturing Atlantic salmon male testis. From August 2003, fish were exposed to either a natural photoperiod (NL), or NL combined with continuous light from January 2004 (LL) or 8h light:16h dark from June 2004 (8:16) until December 2004.

RESULTS: Plasma GH and IGF-I levels in the LL and 8:16 groups did not deviate from the NL group, with one exception, the LL group GH levels exceeded that of the NL in April. Plasma GH peaked in October 2004, at the time when most male had running milt. An additional smaller peak was noted in February. Plasma IGF-I levels dropped in February and declined again in October 2004. Gonad gene expression was more affected by the photoperiod than the plasma hormone levels. The LL group had significantly higher expression of GHR and IGFI in May than the NL group. In the NL group, GHR and IGFIR expression tended to be down-regulated in Jan/Feb 2004 and during late summer, compared to the rest of the experimental period. However, the IGFI gene expression was down-regulated only during the late summer period.

CONCLUSION: GHR and IGF-IR gene expression appears to be up-regulated during spring, an event that is reinforced by LL treatment. IGF-I appears to be down-regulated during the same period, but not in the LL group. The shift seen in the seasonal gene expression profiles of the growth axis genes will be correlated and discussed in relation to stage of gonadal development (germ cell proliferation, meiosis and spermiation), plasma steroid levels and the gene expression of pituitary gonadotropic hormone subunits.

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Mechanisms controlling the onset of puberty in female Atlantic cod – effects of photoperiod (*Gadus morhua*)

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BACKGROUND: Farming of Atlantic cod is a rapidly growing industry, but its sustainability is negatively affected by a high incidence of early sexual maturation. Photoperiod treatments can delay pubertal development in cod, but the protocols in use are not fully effective in arresting sexual maturation. The current study investigates some of the mechanisms that control onset of puberty in cod, in order to further your knowledge of the interaction between artificial photoperiods and sexual maturation.

METHODS: Fifteen months old cod (mean body weight 807g) hatched and first fed at IMR, were exposed to one of four photoperiod regimes from July 2004 until December 2005 in triplicate tanks: natural light (NL), continuous light in covered tanks (LL), NL to December 2004 and thereafter LL (NL-LL group) or LL to December 2004 and thereafter NL (LL-NL group). Monthly, 30 individuals were sampled from each group for growth measurements, blood plasma, gonads and pituitaries. Ovaries were weighed and sub-samples fixed for histology and oocyte diameter determination, and ovulation noted. The mRNA abundance of gonadotropin subunits in pituitaries and FSHR, LHR, P450 aromatase and 20beta-HSD in gonads are analyzed by newly developed real-time quantitative PCR assays. Plasma testosterone and estradiol are analyzed by ELISA.

RESULTS: Most females (>90%) in the NL group became sexually mature at an age of two years between January and April 2005, while none of the LL females had reached sexual maturity at that time. In most individuals in the LL group, oocytes did not develop beyond the previtellogenic stages and gonadosomatic index (GSI) stayed low (< 2%). However, increasing GSI values and ovulation was noted in a few individuals the LL group during the summer months. Pubertal development resumed rapidly following transfer from LL to NL in December as indicated by increasing GSI's and oocyte diameters, and ovulation from April to July. Transfer from NL to LL in December advanced the termination of the spawning period.

CONCLUSION: Continuous light appeared to arrest oocyte development in the previtellogenic stages during the normal spawning season, but some oocyte development and ovulation occurred during the subsequent months. Rapid pubertal development resumed shortly after cod were released from LL inhibition to NL during mid-winter, resulting in out-of-season ovulation during late spring. This response pattern is a well-suited model for studying the regulation of expression of genes that control onset and completion of puberty in cod. The NL-LL treatment, on the other hand, precociously ended the spawning season without reducing the incidence of maturation. Plasma steroids and mRNA abundance is currently analyzed, and will be presented and discussed in relation to the various light treatments and their effect on pubertal development.

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Selection for muscle lipid content modifies puberty and gametogenesis in rainbow trout

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BACKGROUND: In salmonids the timing of puberty is known to be linked to growth and metabolic status. In this work, we compared the occurrence of puberty and the kinetics of gametogenesis in males and females from two divergent lines of spring-spawning rainbow trout selected for muscle lipid content.

METHODS: The study was conducted on fish obtained after 3 generations of mass selection for high (fat line, FL) or low (lean line, LL) muscle lipid content. Two-way selection was performed on pan-size fish by using a non destructive measure of muscle lipid content (Distell Fish fat Meter), as described in Quillet et al., 2005. In both lines, growth, estimation of muscle (fat meter) and visceral (weight) lipid content and reproductive parameters (GSI, gonad histology) were studied at 4 different times in 1 to 2-year old males and females.

RESULTS: FL fish were characterized by a higher muscular lipid content associated with a lower visceral fat content while a similar growth was observed in both lines. In one year-old males, precocious puberty frequency was significantly higher in FL fish (24% *vs* 7% in LL fish). At 2 years-old, the maturity rate was the same in FL (93%) and LL (87%) males while it was significantly higher in FL females (95% *vs* 54% in LL fish). Furthermore, histological examination of gonads performed on 1 to 2-year old individuals showed that active spermatogenesis and vitellogenesis started earlier in FL fish. Muscle lipid content changed over the reproductive cycle in both lines. Maximal values were observed at early spermatogenesis and vitellogenesis for both lines (June). This increase was linked in part to a positive influence of sexual maturation (presumably through anabolizing steroid hormones). During full gametogenesis and gonadal growth (November), the muscular lipid content decreased faster in FL (28%) compared to LL fish (7%), whatever the sex. In contrast the visceral fat index decreased similarly in both lines (29% in FL fish *vs* 21% in LL fish).

CONCLUSION: Higher muscle lipid content positively influences sexual precocity and advances the initiation of gametogenesis during the first reproductive cycle of rainbow trout.

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Reproduction of Ballan wrasse (*Labrus bergylta*) in Norway

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BACKGROUND: The Ballan wrasse (*Labrus bergylta*) is a protogynous batch-spawning hermaphrodite found on coastal rocky reefs between Morocco and Norway. There is currently an interest in farming this species in Norway, where it is used as a biological control for ectoparasitic sea-lice in the salmon farming industry as well as a culinary commodity in some regions. At present there is very little knowledge about its reproductive physiology. The aim of this work, therefore, is to describe the reproductive cycle of Ballan wrasse in Norway and initiate a trial breeding program as a platform for future aquaculture research.

METHODS: Ballan wrasse were trapped or gill-netted monthly for 20 months in western Norway (60°6'0"N, 5°10'0"E). Total length, bodyweight and gonadosomatic index (GSI) of both male and female fish were recorded. All fish were checked for morphological indications of sex change. Blood-plasma samples were collected from each fish and stored at - 80° C. Gonad samples were fixed in either neutral buffered formalin (females) or Bouins fixative (males) before being embedded in paraffin and sectioned (4 µm). Light microscopy is being used to stage gonad development. Plasma levels of testosterone, 11 ketotestosterone and estradiol-17ß are being analysed by ELISA.
Sexually mature fish were held under an artificial light regime and fed a moist diet for the breeding trials. Spawned eggs were collected on mats and transferred to incubation tanks until hatching. The resulting larvae were fed enriched live-feed until weaning at approximately 60 days post hatch.

RESULTS: The size of wild caught fish ranged between 24.5 to 47 cm (body-length) and 0.19 to 1.60 kg (body-weight). Initial results indicate that both male and female fish have multiple stages of gamete development present during gametogenesis, consistent with other batch-spawning fish. Gonadosomatic index ranged between 0.41 to 9.19 (females) and 0.05 to 1.24 (males). Maximum and minimum GSI was recorded in May and November, respectively. Sex change appeared to occur during winter.
The pilot captive breeding of Ballan wrasse led to the successful production of approximately 40 000 larvae and yielded 6000 juvenile fish. Spawning occurred naturally inside the tanks without the need for intervention.

CONCLUSION: The peak spawning period of Ballan wrasse in western Norway appears to be between May to June when photoperiod peaks and water temperature is increasing. This reproductive cycle is relatively pliant to artificial manipulation, as indicated by the natural fertilisation of spawned eggs under pilot culture. Further results and conclusions will be discussed.

P 120

Effects of different temperatures on the mitotic activity of somatic and germ cells in the testis of adult tilapias (*Oreochromis niloticus*)

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BACKGROUND: Studies developed recently in our laboratory have shown that different temperatures alter the duration of spermatogenesis, which is faster in tilapias kept at higher temperatures (30-35 °C) in comparison with tilapias kept at 25 °C, which is considered the physiological temperature for the reproduction of this species. Because there is no data available in the literature regarding the effects of temperature on the mitotic activity of somatic and germ cells, the main objectives of the present work were to investigate the influence of this important parameter on the proliferation rate of Leydig (LC), peritubular myoid (PMC), Sertoli (SC), and differentiated spermatogonial (type A and type B) cells in Nile tilapias.

METHODS: Twenty-nine adult tilapias (*Oreochromis niloticus*) kept at the temperatures of 20 °C, 25 °C, 30 °C, 35 °C received one single intracelomic thymidine injection (~1µCi/G/BW), as a marker of cells that were synthesizing DNA at the time of thymidine injection. The fish were sacrificed approximately two hours after injection and testis fragments were fixed in 4% glutaraldehyde, embedded in glycol methacrylate, and routinely prepared for histomorphometrical and autoradiographical analyses. Approximately one thousand somatic cells (LC, PMC, and SC) were analyzed for each animal. We also determined which spermatogonial cyst (type A or primary; B1/B2; B3/B4/B5; and B6/B7) was associated with thymidine labeled Sertoli cell. The percentage of cysts (400 per animal) containing proliferating spermatogonia was also evaluated.

RESULTS: In general, tilapias kept at higher temperatures (30-35 °C) showed lower ($p<0.05$) rate of SC and LC proliferation than fish kept at the temperatures of 20-25 °C. Positive correlation ($r=0.63$; $p<0.01$) was observed between SC and LC proliferation rate, suggesting a functional interaction between these two somatic cell types. No clear trend was found for the proliferation rate of PMC, and the percentage of spermatogonial cysts with mitotic cells was very similar ($p>0.05$) at the different temperatures investigated.

CONCLUSION: Although we still do not have an explanation for the results found, we are currently investigating other functional aspects of spermatogenesis in tilapias kept at different temperatures. For instance, we have already found that the frequency of cysts containing spermatogonia is higher ($p<0.05$) at 20 °C (where the SC proliferation rate is significantly increased), in comparison to 35 °C. In conclusion, the data already found suggest that the somatic cells, particularly SC and LC, are more responsive to temperature variation than germ cells.
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Sperm characteristics in *Barbus barbus* as a function of nutrition throughout the reproductive season

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BACKGROUND: Improvement in broodstock nutrition and feeding greatly improves the gamete quality both sperm and eggs. In addition, the gamete quality varies across the spawning season in fish species. Sperm morphology, volume, density and motility and osmolality of the seminal plasma were compared in three feeding groups of *Barbus barbus* during the reproductive season.

METHODS: The groups were fed with three different diets: Group A fed only a commercial diet (Karpico containing 33 % crude protein and 6% fat), Group B fed 78 % of commercial diet (78 %) and 22 % of frozen natural food (chironomid larvae) and Group C fed 56 % of commercial diet and 44 % of frozen natural food (chironomid larvae) under controlled conditions. Total daily food ratios were 1.5 % of total body weight of fish in each group. This feeding regime of broodfish started in September 2005 and continued to May 2006. No significant difference was observed in terms of weight and length of the sampled fish ($P>0.05$). The stripping was carried out three times, at the beginning of March, April and May 2006. Sperm morphology was studied using scanning and transmission electron microscopy. Sperm volume and density were measured. The osmolality of the seminal plasma was measured by means of Vapour Pressure Osmometer. Sperm motility was evaluated for velocity of sperm and percentage of motile sperm after diluting in buffered distilled water (30 mM Tris-HCl, pH 8.0) at dilution ratio 1:1000.

RESULTS: Scanning and transmission electron microscopy clarified that a spermatozoon is uniflagellated and is differentiated into a head without acrosome, a midpiece and a flagellum. Feeding did not influence on sperm volume and density, osmolality of seminal plasma and percentage of motile sperm ($P>0.05$), but significantly affected sperm morphological characteristics (except of anterior and posterior parts of midpiece) and sperm velocity ($P<0.05$). Groups fed artificial diet Karpico and frozen natural food (Groups B and C) showed similar pattern of sperm characteristics during the reproductive season as compared to Group A. Almost all parameters were changed either between or within groups throughout the reproductive season ($P<0.05$), suggesting different optimal time for sperm collection between groups. The best time for sperm collection is estimated March in Group A, but April in Groups B and C, when the osmolality of the seminal plasma measured around 289 mOsmol Kg^{-1} along with the maximal sperm motility parameters.

CONCLUSION: Some characteristics changed between feeding groups throughout the reproductive season. Feeding influenced sperm morphological parameters (except of anterior and posterior parts of midpiece) and sperm velocity. Spermatogenesis, hydration and cell decomposition are three major parameters control the sperm characteristics throughout the reproductive season. Thanks are expressed to USB RIFCH no. MSM6007665809, GACR no. 523/06/P142 and 524/06/0817.

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Changes of serum levels of 17-β estradiol and testosterone and expression of their receptors in male *Spinibarbus denticulatus* during reproductive cycle

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BACKGROUND: In teleosts, gonadotropin (GtH) and sex steroid hormones are two sorts of key regulators influencing the physiological process of reproduction in brain-pituitary-gonad axis (BPG axis). Though gonadal-dependent changes of serum GtH II and gonadal steroids have been described in many teleost species, involvements of steroid receptors in interaction and interrelationship among GtH II and gonadal steroid hormones have been rarely discussed.

METHODS: Serum GtH II, 17-β estradiol (E_2) and testosterone (T) levels were determined by the radioimmunoassay (RIA). Expression patterns of sex steroid receptors were analyzed using real-time PCR.

RESULTS: In male *Spinibarbus denticulatus,* serum GtH II levels kept rising during recrudescing stages and achieved a significant peak value at late recrudescing stage prior to spawning; then declined significantly at the fully recrudesced stage. Serum testosterone (T) levels was low at early recrudescing stage and increased significantly at late recrudescing stage. However, serum E_2 levels in male fish showed no significant changes throughout the recrudescencing stages.AR was highly expressed in BPG axis during male reproductive cycle. AR expression in brain was increasing during recrudescing stages and reached a maximum value at late recrudescing stage. On the contrary, AR in testis was highly expressed at early recrudescing stage and then decreased significantly at late recrudescing stage. However, AR in pituitary was significantly high at fully recrudesced stage. ERα had always kept a rather poor abundance in BPG axis during reproductive cycle. Expression pattern of ERβ1 in brain was similar to that in testis with a high level at early recrudescing stage and a significantly low level at late recrudescing stage. But in the pituitary, ERβ1 was highly expressed at the beginning of recrudescence, then declined sharply at late recrudescing stage, and increased significantly to another peak at fully recrudesced stage.

CONCLUSION: During the recrudescing stages, GtH II facilitated secretion of gonadal steroid hormones to induce the final maturation. Meanwhile, E_2 and T also exerted the positive effects on GtH II release. However, the feedback effects of gonadal steroid hormones on GtH II turned negative when gonad was fully recrudesced. Steroid hormone receptors at least partly mediated the feedback actions on GtH II.

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Two new TGF beta members are restricted to the gonad and differentially expressed during sex differentiation and gametogenesis in trout

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BACKGROUND: It is well admitted that gonadal sex differentiation and gametogenesis imply reciprocal interactions between somatic cells and the germ cell lineage. These interactions are mediated by paracrine factors that are tightly regulated to ensure an appropriate coordinated action. To identify such paracrine factors in trout, global analyses of the gonadal transcriptome was carried out throughout the development and the maturation of the gonad.

METHODS: Transcriptome analyses, based on trout cDNA microarrays, was performed using RNA samples extracted from the gonad selected from histological analyses. A phylogenic study was carried out to determine the evolutive relationships of the genes. Quantitative real time PCR (rtQPCR) and *in situ* hybridizations were performed to determine the tissue distribution, the gene expression patterns, and the cellular localization of the transcripts of interest.

RESULTS: Trout cDNA microarray analyzes of gene expression in differentiating gonads and maturing testis at different stages identify two distinct differentially expressed transcripts encoding for new members of the TGF beta superfamily that were named GSDF-1 and GSDF-2 with reference to one recently described gonadal growth factor [Sawatary et al (2006)]. These putative growth factors harbor a cysteine rich region at the C-terminal end that is homologous but not identical to the TGF beta domain consensus sequence. The phylogenetic analysis indicated that are translated from paralogous genes that have evolved from an common ancestral gene in Salmonids. A single-copy orthologous gene is present in other fish genome such as zebrafish and fugu. In contrast to GSDF-1 that is expressed in differentiating and immature gonads, both in Sertoli cells of the testis and in granulosa cells of the ovary, GSDF-2 is strictly restricted to the male gonad of adult animals. In microarray analysis, expression values of both transcripts globally decreased with gonadal rapid growth but the dilution of the expressing cells as gametogenesis progresses may explain part of this regulation. Nevertheless, a significant but transitory increase of GSDF-2 was observed at the earliest stage of testis maturation and, in spermiating animals, expression of GSDF-1 remained high only in Sertoli cells localized in the vicinity of non dividing spermatogonia.

CONCLUSION: The gonad specific expression profile of GSDF-1 and testis-specific expression of GSDF-2 suggest that these two genes encoding new members of the TGF beta superfamily are likely involved in gonadal sex differentiation and gametogenesis control. Further studies are currently in progress to better characterize the physiological significance of these genes taken into account that GSDF-2 is a divergent paralog resulting probably from the specific salmonid genome duplication.

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Effect of di-(2-ethylhexyl)phthalate on zebrafish (*Danio rerio*) fecundity

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BACKGROUND: Phthalates are esters of the phthalic acid. This family of chemical compounds is used in the plastic industry as plasticizers. Recently, the European Commission has banned the use of DEHP from toys and PVC products which are directed to be used in closed environments, moreover, the Food and Drug Administration has drastically limited his use in foodstuffs. While DEHP has low lethal effects, even at low doses it affects several organs such as lungs, kidneys and gonads. Even if, up to date, many studies have demonstrated that DEHP can negatively interfere with the endocrine system in most mammals including humans, the effects on aquatic organisms are still unclear.

METHODS: In the present study the effect of di-(2-ethylhexyl)phthalate on the reproductive biology of adult females of the fresh water teleost zebrafish was evaluated. The attention was focused on the effect on ovary maturation, fecundity and embryo's vitality. To this propose zebrafish adult females were exposed for three weeks, in semi-static conditions, to nominal 0,2, 2, 20 e 40 μg/L concentrations of DEHP. To test the possible estrogenic potency of DEHP, one group was exposed to 17β-Ethynylestradiol (EE_2, 25 ng/L), as positive control. At the end of the exposures, the effects of DEHP on fecundity and hatching was evaluated for the next two weeks.
By real time PCR the ovarian gene expression of peroxisome proliferator-activated receptor (PPARα) and the aromatase P450 ARO was tested.

RESULTS: After the three weeks exposure, the Gonadal Somatic Index (GSI) increased significantly in females exposed to 0,2, 2, 20 μg/L of DEHP and EE_2, respect to the control. No increase was observed in the group exposed to the highest dose of DEHP. Changes on oocyte classes frequency were observed in females exposed to DEHP with a significant reduction of post-vitellogenic oocytes in those females exposed to the highest doses and to EE2.
Fecundity, in terms of number of fertilized eggs, was reduced to 50% in females treated with 0,2 and 2 μg/L of DEHP, to 10% in females treated with 20 and 40 μg/L of DEHP and to 20% in females exposed to EE2. A negative effect of DEHP and EE2 was also observed on the hatching rates, as well as on embryo mortality. In addition, the DEHP induced a dose response increase of ovarian PPARα concomitant with a significant decrease of P450 ARO gene expressions.

CONCLUSION: The results obtained clearly evidenced the ability of the DEHP to interfere with the ovary maturation, fecundity and embryo vitality; the effects observed were similar to those evidenced by the exposure to EE_2. The negative effect of DEHP on zebrafish reproduction may be related with the increase of PPAR that induced a decrease of the P450 ARO, the enzyme responsible for the conversion of T to E2.

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Generation of aromatase-GFP transgenic medaka (*Oryzias latipes*) lines with fluorescence in the somatic cells of the ovaries

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BACKGROUND: Estrogens play important roles in female sex differentiation and other reproductive functions in fish. Cytochrome P450-aromatase (aromatase), a steroidogenic enzyme, catalyzes the conversion of androgens into estrogens, and is expressed pre-dominantly in the ovarian follicle cells. Aromatase transgenic lines that express green fluorescence protein (GFP) in the ovarian follicular cells may provide excellent opportunities to study molecular mechanisms underlying gametogenesis in fish.

METHODS, RESULTS, and CONCLUSION: To generate aromatase transgenic lines, we cloned 8.5 kb 5`-upstream region of the gonadal-form of medaka aromatase into phrGFP II-I expression vector and microinjected the circular construct into the cytoplasm of fertilized eggs at the one-cell stage. All the injected embryos that survived were maintained and subjected to the sibling mating at F0 generation. Homozygous lines were established at F4 generation. Co-expression of both endogenous aromatase and transgene GFP in the same cell were confirmed by immunohistochemical studies. A weak expression of GFP fluorescence was observed in the ovary on the day of hatching, and the intensity increased gradually thereafter. Aromatase/GFP signals were strong in both theca and granulosa cells of the immature ovaries and, in the mature ovaries, expression was more pronounced in the granulosa cells surrounding vitellogenic oocytes. In the testis, aromatase/GFP expression was observed in the mitotic germ cells and the clusters of spermatogonia. In each tissue examined, GFP expression was overlapped with endogenous aromatase expression, providing excellent opportunities for investigating sites of estrogen production and signaling in fish.

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Molecular characterization and seasonal changes in gonadal expression of a thyrotropin receptor in the European sea bass

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BACKGROUND: The thyroid stimulating hormone (TSH) is a glycoprotein synthesized and secreted from thyrotrophs of the anterior pituitary gland. It acts by binding to and activating its specific receptor, the TSHR, to induce the synthesis and secretion of thyroid hormones. Recent studies conducted in diverse fish species, suggest a direct role of TSH on gonadal physiology. Nevertheless, up to now, there is no information on the regulation or function of TSHR in the gonads in any fish species.

METHODS: A specific probe for the sea bass *TSHR* (*sbsTSHR*) was obtained by RT-PCR and used to screen a cDNA testicular library. A phylogenetic analysis was done by the neighbor-joining method with full length protein sequences. The expression of the *sbsTSHR* in gonads and somatic tissues of adult fish was studied by RT-PCR using specific primers. Sea bass testis total RNA was used for performing 3'-rapid amplification of cDNA ends (RACE) using a gene-specific primer annealing to the transmembrane domain. Finally, changes in ovary and testis *TSHR* expression, associated with the sea bass female and male reproductive cycles, were measured using real-time PCR.

RESULTS: Screening of a cDNA testicular library in combination with a PCR based approach allowed the isolation of the full-length sea bass *TSHR* cDNA. *sbsTSHR* is 3587 bp long and has an open reading frame of 2340 bp that codes for a 779 aa polypeptide. The mature protein displays typical features of the members of the glycoprotein hormone receptor family and shows the highest amino acid sequence identity with the TSHRs of other fish species. Phylogenetic analysis clearly demonstrates that the *sbsTSHR* belongs to the *TSHR* cluster. By RT-PCR analysis we demonstrate the extra-thyroidal expression of *sbsTSHR* in numerous tissues of the sea bass. Also, two transcripts that differ in the length of their 3'untranslated regions were found. They reflect the use of alternative polyadenylation cleavage sites. Seasonal changes in *sbsTSHR* mRNA levels in female and male sea bass during the first ovarian and testicular recrudescence suggest that in females the TSHR could participate in active vitellogenesis and in the regulation of gamete maturation and ovulation, whereas in males, the TSHR would be involved in the regulation of processes that occur during the early stages of the gonadal development and also of gamete maturation and spermiation.

CONCLUSION: A sbsTSHR has been cloned from the testis of the European sea bass. The results of this work provide the basis for future studies concerning the function of TSHR in this species.

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Purification and characterization of the Follicle-Stimulating Hormone (FSH) from Sea Bass (*Dicentrarchus labrax*)

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BACKGROUND: The follicle-stimulating hormone (FSH) is a heterodimeric glycoprotein secreted by the pituitary gland. The FSH is one of the key hormones in the control of reproduction in vertebrates. In the present study we report the purification and characterization of the FSH from sea bass, a perciform marine fish.

METHODS: FSH was isolated from a total of 1400 sea bass pituitaries by ethanol extraction (40-85%) and anion exchange chromatography (TSK DEAE-5PW), followed of reverse phase (RESOURCE 15RPC) HPLC. The obtained fractions were analysed by SDS-PAGE, western blot, mass spectrometry and N-terminal amino acid sequencing.
The biological activity of the purified hormone was analyzed using a cell line (HEK 293) stably expressing the sea bass FSH receptor (FSHR). Receptor activation by FSH was indirectly measured by recording changes of the luciferase activity, promoted by a rise of cAMP. Primary gonad tissue cultures were used to study the steroidogenic potency of this hormone.

RESULTS: Pure fractions of FSH dimer, glycoprotein alpha (GP-alpha), FSH-beta and LH-beta subunits were obtained. The molecular mass, estimated by mass spectrometry, was of 28.5 KDa for FSH dimer, 12.6 KDa for GP-alpha, 13.6 KDa for FSH-beta and 15.3 KDa for LH-beta. Purified sea bass FSH was able to activate the sea bass FSHR in a doses dependent manner, although it did not activate sea bass LHR exhibiting specificity for its receptor. In gonad tissue cultured “in vitro”, the purified FSH stimulated steroid release (Estradiol and 11-ketotestosterone) in the culture media in a doses and time dependent manner.

CONCLUSION: A pituitary sea bass FSH has been purified. This glycoprotein is biologically active, stimulating its receptor and promoting steroid release. This FSH will be a key tool to study gametogenesis in teleost fish.

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Effects of photoperiod, light intensities and colour on gamete development and plasma hormones in Atlantic cod (*Gadus morhua* L.)

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BACKGROUND: Early sexual maturation is a major problem during on-growing of farmed Atlantic cod. Continuous light (LL) treatments can delay or stop sexual maturation, but is not fully effective in outdoor situations. In order to develop more efficient protocols for outdoor use, two experiments with different photoperiods, light intensities, and colours (white and blue) were run in seawater tanks and sea cages to reveal the effects on light perception as monitored by diel plasma melatonin profiles, and sexual development monitored by gonadal growth, germ cell development and plasma sex steroid levels.

METHODS: Atlantic cod was exposed to different light treatments, covering the first spawning season under ambient light. Gonadal growth (gonadosomatic index; GSI) and development were monitored throughout the trial. A histomorphometric method was developed by analysing the volume fractions of different germ cell stages across the testis. This method was applied to quantify effects of the different light treatments on germ cell development. Plasma melatonin was analysed in both experiments, and plasma levels of testosterone and 11-ketotestosterone are currently analysed in the sea cage experiment.

RESULTS: Fish under LL in seawater tanks and sea cages had significantly lower GSI values than ambient light controls during the normal spawning time. Fish under LL in indoor tanks did not show major gonadal growth during the experimental period up to 3 years of age, whereas some gonadal growth was noted under LL in outdoor tanks. In contrast, LL in sea cages delayed gonadal growth by around 3 months compared to ambient light controls. In spite of low GSI values in the LL groups in tanks, full spermatogenesis was noted in all individuals. Controls under ambient light had higher night-time plasma melatonin levels than LL fish, both in indoor tanks, outdoor tanks and sea cages. The different LL treatment varied in their effect on gonadal growth and development, with the highest intensities being most efficient. Blue light appeared to be less effective than "white" light in delaying gonadal growth and development. In spite of the differences in gonadal growth and development, no differences were found in the diel plasma melatonin profiles among the various LL treatments of different intensity or colour.

CONCLUSION: LL treatments delayed gonadal development by up to three month in indoor and outdoor seawater tanks, although without fully arresting spermatogenesis. LL treatments or long day treatment was less effective when applied in sea cages. This could maybe be due to lower light intensities of the LL treatment in the sea cages compared to the tanks. Plasma melatonin levels were affected by the LL and long day treatments, but different light intensities or colours did not affect melatonin levels differently despite the different responses in terms of gonadal growth and development.

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Effects of gonadotropin-releasing hormone agonist (GnRHa) administration on male germ cell apoptosis in Atlantic bluefin tuna (*Thunnus thynnus thynnus* L.) kept in captivity

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BACKGROUND: In the last years, the expansion of the Atlantic BFT fattening industry has stimulated an increase of fishing pressure and, consequently, a strong depletion of the wild stock. In this study, carried out in the framework of a research project aimed to the control of the reproduction of wild-caught, cage-reared BFT, the effect of GnRHa administration on the apoptosis of male germ cells was analysed.

METHODS: In 2004 and 2005, a total of 19 male BFT kept in captivity in floating cages for 1 to 3 years, were administrated a GnRHa-implant to produce effective doses of 40 to 100 μg GnRH Kg^{-1}, while 17 were left untreated as Controls. For comparison, wild fish captured in the natural spawning grounds in the Mediterranean were used. Testes slices were fixed in 10% buffered formalin and embedded in paraffin wax. The detection of apoptotic cells was carried out according to the TUNEL method.

RESULTS: Captive-reared bluefin tuna were at different stages of spermatogenesis. No difference in reproductive state was observed between controls and GnRHa-treated fish, as specimens at early and late spermatogenesis, as well as spent individuals were found in both groups. No fully mature fish, with seminiferous lobules filled with spermatozoa, were observed among captive fish. The diameter of seminiferous lobules was not different between control and GnRHa-treated fish, while it was significantly lower in caged fish than in wild spawners. TUNEL-reaction demonstrated the presence of apoptotic cells in all the specimens analysed. Apoptosis involved both spermatogonia and primary spermatocytes. The percentage of spermatocysts containing apoptotic cells, as well as the mean number of apoptotic cell per cyst, were significantly lower in wild than in captive fish and, among the latter group, both the parameters were significantly lower in GnRHa-treated than in untreated Controls.

CONCLUSION: The present study indicates that, although the GnRHa administration in male BFT kept in captivity did not result in evident positive effects on testicular maturation, it was effective in reducing the apoptosis rate of germ cells.

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Gametogenesis and Gamete Biology
(Part III : Biotechnology)

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Egg quality in captive and wild seabass (*Dicentrarchus labrax*): a proteomic analysis

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BACKGROUND: Fish egg quality is highly variable and can be affected by several environmental factors and husbandry practices. In addition, differences between captive and wild populations can sometimes be observed. However, the molecular bases of egg developmental competence (e.g survival at several stages of embryonic and larval development) are poorly understood. Indeed, egg components such as maternally-inherited proteins support early development and their abundance in the eggs are possibly affected by negative environmental factors and husbandry practices.

METHODS: Ovulations of 16 wild females and 28 domesticated (3dr generation) females were stimulated by application of DTRP6 LHRHa at a similar post-vitellogenic stage. Eggs were collected by stripping and subsequently fertilized in vitro. Fertilization rate and embryonic survival rates at specific stages were subsequently monitored in order to fully characterize egg quality for each individual female. For each spawn, egg cytoplasmic proteins were delipidated by application of a two phase extraction medium containing polar and apolar solvants (Butanol /Diisopropylether; 4/6). Samples were then subjected to isoelectric focusing on 3-10 linear PH gradient 17 cm strip. The second dimension migration was run through 12% SDS polyacrylamide gels. 2D protein expression patterns were analyzed using Image Master2D Platinum after Coomassie blue coloration. Differential spots were then analyzed by MALDI TOF/TOF mass spectrometry. Corresponding protein sequences were identified from MSDB database by Mascot software (Matrix science). For candidate proteins, an antibody directed against a portion of the sequence was raised in rabbits.

RESULTS: Wild origin eggs showed higher fertilization rates (62.8%) than domestic ones (41.4%, $p<0.001$). Similarly, a higher hatching rate was observed in wild females (32.3% vs 16.1%, $p<0.001$). Among the 100 spots that could be detected on 2D gels, several domestication-linked or quality-linked spots were identified. Among them, one spot was identified by MS/MS a Nucleoside Diphosphate Kinase. On western blot of seabass eggs, a single band was observed at the expected size of 18 kDa. Analysis of the abundance of this protein in eggs of varying quality is currently in progress.

CONCLUSION: The use of large scale analytical methodologies such as proteomics to decipher the molecular bases of egg developmental competence is promising. The identification of specific proteins exhibiting a differential abundance in eggs of varying quality will allow the identification of the mechanisms triggering egg quality defects.

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Effects of hormone injections on tolerance to cryopreservation of ayu spermatozoa

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BACKGROUND: The ayu (*Plecoglossus altivelis altivelis*, Osmeridae) is one of the main freshwater cultured fish in Japan. After thawing, percent motility of cryopreserved ayu spermatozoa collected in the early and the middle stages in the spawning season showed low values, and increased rapidly at the end of the season. To elucidate the factors that enhance tolerance to cryopreservation of ayu spermatozoa, we examined the effect, on post-thaw motility of the spermatozoa, of salmon pituitary extract (SPE) or 17α-hydroxyprogesterone (17α-OHP) injected into ayu males.

METHODS: The hormone was injected for three consecutive days in the early and the late stages of the spawning season. In both stages, spermiating males were divided into three groups: (1) SPE (0.2mg/gBW/day), (2) 17α-OHP (10μg/gBW/day), and (3) Ringer control. Milt collected from males by pressing the abdomen on the day after the last injection was diluted with a cryopreservation extender comprising 10% methanol and 90% fetal bovine serum. The mixture was dispensed into 150μl acrylic straw tubes, cooled at -50.1±0.8ºC/min to -50ºC, immediately plunged into liquid nitrogen and stored for one hour. The spermatozoa were then thawed and diluted with an activating solution (DW buffered with 20mM HEPES-NaOH at pH 7.5) to evaluate post-thaw motility.

RESULTS: Injections in the early stage. Percent motility of fresh spermatozoa from males injected with SPE or 17α-OHP, was significantly higher than from those injected with Ringer control. Comparative post-thaw motility (percent motility of post-thawed spermatozoa/that of fresh spermatozoa ×100) was also higher in the SPE and 17α-OHP groups than in the control. **Injections in the late stage.** No significant difference in percent motility of fresh spermatozoa was observed among the three groups. Comparative post-thaw motility in the hormone treated groups, especially in the SPE group, was higher than in the Ringer control. In a pair-wise comparison between the two stages of each group, comparative post-thaw motility of the late-stage spermatozoa was consistently higher.

CONCLUSION: The results indicated that hormone injections that promote testicular maturation enhanced the tolerance of the sperm cells to cryopreservation. The mechanism that regulates tolerance to cryopreservation needs to be clarified in further studies.

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Biological activities of single-chain recombinant goldfish follicle-stimulating hormone and luteinizing hormone

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BACKGROUND: In a previous study, we demonstrated the production of recombinant follicle-stimulating hormone (rFSH) and luteinizing hormone (rLH) of goldfish by using baculovirus in silkworm *Bombyx mori* larvae, and confirmed biological activity using *in vivo* bioassays. However, the rate of production and association of α and β subunits of the recombinant hormones is not clear, since each subunit is made separately in the silkworm larvae system (co-infection method). In the present study, we produced single-chain recombinant FSH and LH using fusion genes containing α subunit cDNA and β subunit cDNA for each hormone (single-chain method), which we expect to reduce some process of hormone production. Biological activity of those recombinant hormones was analyzed by *in vivo* bioassays.

METHODS: The fusion genes of α subunit cDNA and β subunit cDNA of FSH or LH were constructed, and introduced into the baculovirus, which was infected into silkworm larvae after propagation of the recombinant virus in silkworm culture cells. Hemolymph of silkworm larvae containing single-chain rFSH or rLH were injected into male goldfish *Carassius auratus*, female bitterling *Rhodeus ocellatus cellatus*, and male Japanese eel *Anguilla japonica*. Biological activity was assessed by milt production in goldfish, ovulation in female bitterling, and spermatogenesis in Japanese eel.

RESULTS: Injection of hemolymph containing single-chain rFSH or rLH (20μl/gBW) induced milt production in male goldfish. Low dose (5μl/gBW) of rLH induced ovulation but rFSH had weak potency in bitterling, and both rLH and rFSH induced ovulation at high dose (20μl/gBW). Both rLH and rFSH induced spermatogenesis in male eels by 6 times injection at 7 days interval (2μl/ml). Spermatozoa were observed in rLH-injected fish and spermatid in rFSH-injected fish.

CONCLUSION: The present study demonstrates various biological activities of the single-chain recombinant goldfish FSH and LH in male goldfish, female bitterling, and male Japanese eel, as well as that of recombinant hormones produced by the co-infection method. Gonadotropins produced in this study promote not only the final phase of gametogenesis (milt production and ovulation) in goldfish and bitterling, but also the early phase of gametogenesis (spermatogenesis) in Japanese eel. It is suggested that the single-chain recombinant fish gonadotropins could be applied for the induction of gonadal development in aquaculture fishes.

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A preliminary approach for nuclear transfer in goldfish: reduction in the variability of egg quality, optimization of egg manipulation

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BACKGROUND: Promising embryo developments were reported in several fish species after embryonic and somatic nuclear transfer. Fertile adults were occasionally obtained although at a very low rate. It is admitted that no reliable technique has been developed yet to ensure a repeatable development rate. One reason is that the success of nuclear transfer depends on many cellular and physiological factors that have to be considered together with technological skill. In this work, we propose a step by step approach led on normal embryos to circumvent some possible failure factors in goldfish nuclear transfer: initial egg quality, egg manipulation conditions (chorion removal), egg activation control, and efficiency of nucleus removal.

METHOD: Several experiments on *in vivo* and *in vitro* ageing of post-ovulation eggs were set up: conservation time ranged between 1 and 5 hours, *in vitro* conservation temperature ranged between 4 and 20°C. Viability of 24h-old embryos and 72h-old hatchlings developed at 20°C were used as egg quality descriptors. Three protease concentrations (0.5, 1, 1.5mg/ml) in combination with different incubation medium at 20°C were tested to reduce the chorion removal time. As eggs are activated upon dilution of cœlomic fluid, inhibitors such as protease- and proteasome-inhibitors (soybean trypsin inibitors STI 0.5 and 1mg/ml; MG132 5 and 20 µM) were tested. Hoechst labelling of female DNA was attempted to assist egg enucleation. The testing of an enucleation procedure was performed in the first 20 min after egg fertilization. Female pronucleus removal was revealed by the inability of haploid embryos (where only the male pronucleus was left) to reach the 2 cells stage.

RESULTS: After ovulation, egg quality was better preserved in the female genital tract than *in vitro*. After stripping, *in vitro* egg storage in cœlomic fluid at 4°C for up to 3 hours yield better development rate after fertilization than did egg storage at 12,16 or 20°C. However, whatever the temperature, *in vitro* conservation always impaired the egg activation pattern: blastodisc formation was slower, and polar body extrusion was more difficult to observe. We confirmed that STI prevents egg activation with the same efficiency as female cœlomic fluid. On the contrary, MG132 did not prevent or slow down egg activation. Chorion removal time was reduced from 5min to 1-2min when protease was used at 1mg/ml in goldfish ringer. In the enucleation procedure, we failed to label female DNA prior to pronucleus removal. The "blind" procedure we had to use (enucleation under the polar body) led to enucleation rates not higher than 18% of the manipulated eggs.

CONCLUSION: The procedure proposed here should improve egg manipulation in nuclear transfer experiments: eggs should be stripped just before use, they can be diluted and manipulated without activation, and the chorion removal can be performed far before blastodisc formation. Enucleation remains to be improved.

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Culture conditions for maintaining the survival and mitotic activity of rainbow trout transplantable type A spermatogonia

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BACKGROUND: Germ-cell transplantation is a powerful tool for studying gametogenesis in many species. We previously showed that spermatogonia transplanted into the peritoneal cavity of trout hatchlings were able to colonize recipient gonads, and produced fully functional sperm and eggs in synchrony with the germ cells of the recipient. An in vitro-culture system enabling spermatogonia to expand, when combined with transplantation, would be valuable in both basic and applied biology. To this end, we optimized culture conditions for rainbow trout spermatogonia.

METHODS: Testes were collected from pvasa-Gfp transgenic rainbow trout at 8–10 months of age; at this stage, the germ cells are mostly type A spermatogonia. First, we optimized the basal culture conditions for whole testicular cells. Second, the influence of eliminating testicular somatic cells on mitotic activity of spermatogonia was investigated. Third, the effects of several soluble factors on promoting spermatogonial mitotic activity were investigated. Finally, to ascertain whether cultured spermatogonia could maintain their ability to colonize recipient gonads, we performed the allogenic transplantation of spermatogonia after 1 month of culture.

RESULTS: Spermatogonial survival and mitotic activity were improved during culture in Leibovitz's L-15 medium (pH 7.8) supplemented with 10% fetal bovine serum at 10°C compared with culture under standard conditions for salmonids. Elimination of testicular somatic cells promoted spermatogonial mitotic activity. In addition, insulin, trout embryonic extract and basic fibroblast growth factor promoted the mitosis of purified spermatogonia in an additive manner. Mitotic activity increased nearly seven-fold over 19 days of culture compared with growth factor-free conditions and was maintained for >1 month. Furthermore, the cultured spermatogonia could colonize and proliferate in recipient gonads following transplantation.

CONCLUSION: These results suggest that optimum culture condition established in this study could allow spermatogonia to mitose and maintain their activity to colonize recipient gonads at least for 1 month in vitro. This study represents the first step towards establishing a transplantable spermatogonial cell line for use in surrogate broodstock technology and cell-mediated gene-transfer systems.

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Peptide vaccination against the gonadotropin receptors in rainbow trout: a promising method to control the onset of puberty

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BACKGROUND: Precocious sexual maturity is a drawback encountered in fish aquaculture and reliable methods to control the onset of puberty are required. In mammals, previous studies demonstrated that targeting specific regions of LH and FSH receptors, through the immune pathway, induced impairment of adult fertility and sexual maturity. We aimed to study the effect of immunization against specific regions of each receptor on sexual maturity in rainbow trout.

METHODS: Filamentous phages displaying different overlapping decapeptides of the extra cellular region of the FSH receptor (FSHR-A and FSHR-B) and of the LH receptor (LHR-C and LHR-D) were engineered and used as peptidic vaccines in rainbow trout. Male and female trout already in their first reproductive cycle, received either adjuvant alone or one of the 3 following vaccines: anti-LHR (C+D), anti-FSHR (A+B), or a combination of both vaccines (anti-FSHR+LHR; males only), through multiple injections over an 8 weeks period. The consequences of immunization on gonadal development were analysed by measuring the gonad weight and by histological analysis of gametogenesis, 13 days and 10 weeks after the final injection. Serum testosterone (T) was also determined by radioimmunoassay (RIA).

RESULTS: As compared to control, males immunized against LHR alone or in combination with FSHR, showed a decrease in mean GSI. In the anti-FSHR+LHR group, evolution of the spermatogenetic process appeared inhibited, as observed by histological analysis of the gonads 13 days after the end of treatment: 30% of the males presented only spermatogonia (against 0% in adjuvant or anti-FSHR group), and only 40% of the males had reached the stage of initiation of spermiogenesis and first appearance of spermatozoa (against 90% in adjuvant or anti-FSHR group). Inhibitory effects of immunization were not longer detected 10 weeks after the final injection. In females, the evolution of the vitellogenesis process also appeared inhibited after immunisation against FSHR and LHR. Vaccination against FSHR, and to a less extent against LHR, had a global inhibitory effect on plasma testosterone levels in male as in female trout.

CONCLUSION: We show for the first time that the antireceptor vaccination strategy could also have specific antagonist effects on gametogenesis in fish. Nevertheless, the immunization protocol has to be improved to be more potent and practicable in aquaculture.

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Cryopreservation of testicular neomales and stripped males sperm of European perch (*Perca fluviatilis* L.)

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BACKGROUND: Breeding of monosex population appears to be advantageous and its production is based upon sex reversal of females to neomales by means of externally administered hormones (methyltestosterone) and use of X-chromosomes-bearing sperm of the neomales for production of an all-female population. The goal of our study was to attempt to freeze the sperm of perch neomales and to compare fertilization capacity with stripped normal male sperm in perch, *Perca fluviatilis*.

METHODS: Testicular sperm of neomales (TSN) was used for the study because of necessity to confirm the original of sex (XX) according to gonad morphology by dissection after killing. Gonads of 7 neomales (body weight 30-50 g) were cut into pieces and squeezed through a nylon mesh. The released testicular sperm was pooled and kept on ice (0-4 °C). As control, stripped sperm of normal males (SSNM) was also pooled and stored at 0-4 °C. TSN and SSNM was diluted in a 300 mM glucose solution at dilution ratio 1: 6. DMSO was added as a cryoprotective in final concentration 10%. Both sperm was pipetted into 0.5 ml straws and frozen in a Styrofoam closed box (3cm above the N_2 level) for 10 min and then transferred into N_2. For the fertility test, frozen TSN and SSNM were thawed on 40 °C water bath for 8 sec. Every sample was used for eggs of 4 females as followed: 1g eggs (451) + 12×10^5 and/or 2.4×10^5 TSN/SSNM per 1 egg + 1 ml hatchery water, stirring on a shaking table for 1 min. Eggs were then stocked into trays of an experimental incubator and hatching rate estimated from 451 eggs per tray. Sperm velocity and motility rate were analyzed with an image analyzer.

RESULTS: Sperm concentration of the fresh TSN and the SSNM were estimated 45.3×10^9 and 37.8×10^9 spz ml^{-1}. Both sperm velocity and motility showed a significant decrease in the TSN (134.6 µm s^{-1} and 12.8%) compared to the SSNM (203.2 µms^{-1} and 94.7%) at 10 sec post sperm activation ($P<0.05$). Hatching rate of fresh TSN and of fresh SSNM were 42.5 and 49.3 % at 12×10^5 spermatozoa per egg, respectively. At the same sperm: egg ratio, no significant difference was observed between hatching rate of frozen/thawed SSNM and fresh SSNM ($P>0.05$). Frozen/thawed SSNM exhibited higher hatching rates at 12×10^5 than 2.4×10^5 spermatozoa per 1 egg (37.2% *vs.* 29.1%). Hatching rate achieved by use of the frozen/thawed TSN was 7.3 % at 12×10^5 spermatozoa per 1 egg and did not show any significant difference with 2.4×10^5 spermatozoa per 1 egg (6.6%) ($P>0.05$).

CONCLUSION: The present study demonstrated that (a) stripped sperm of normal perch could be successfully frozen, but further studies are needed to optimize the procedure. (b) In terms of neomale sperm, squeezing for collection of testicular sperm is not recommended for cryopreservation due to low sperm velocity, motility and subsequently hatching rate.

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Powder coconut water (ACP-104™) as extender for semen cryopreservation of Brazilian migratory fish species

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BACKGROUND: Coconut (*Cocus nucifera* L.) water, as semen extender during cooling preservation, was first tested in goats, in 1986. Since then, coconut water has been tested in many mammalian species with great success. During the last decade, researchers of State University of Ceará (UECE) have developed a technology to dehydrate coconut water and produce a stable and standardized powder coconut water (ACP). The use of ACP as fish semen extender has been poorly tested. Semen cryopreservation of fish semen has been extensively studied by researchers of Federal University of Lavras (UFLA), and efficient protocols have been developed. Therefore, the aim of this study was to compare ACP-104™ with a previously approved extender for semen cryopreservation of three Brazilian migratory fish species, piracanjuba *Brycon orbignyanus*, curimba *Prochilodus lineatus* and piapara *Leporinus obtusidens*.

METHODS: This study was carried out at Hydroelectric Company of Minas Gerais (CEMIG) and Laboratory of Animal Sciences of UFLA, Brazil. Six males of *B. orbignyanus* and *P. lineatus* and three males of *L. obtusidens* received a single injection of carp pituitary extract to induce spermiation. ACP-104™ was tested in combination with methylglycol or DMSO (dimethyl sulfoxide) as cryoprotectant and compared with BTS™ (*B. orbignyanus* and *L. obtusidens*) or glucose 5% (*P. lineatus*) and methylglycol as cryoprotectant. BTS™ (Beltsville thawing solution, Minitub) contains glucose 79.9%, sodium citrate 12.7%, EDTA 2.7%, $NaHCO_3$ 2.7%, KCl 1.6% and gentamycin sulfate 250 μg/mL. Diluted semen was then aspirated into 0.5 straws, placed in a nitrogen vapor vessel (Cryoporter™ LN_2 dry vapor shipper) at -170°C for 24 h and plunged into liquid nitrogen for storage. Straws were thawed in 60°C water bath for 8 sec. Sperm motility was evaluated immediately after thawing and NaCl 50 mM was used as sperm motility activating solution.

RESULTS: ACP-104™ as semen extender was efficient during the cryopreservation process. Post-thaw sperm motility of semen cryopreserved in ACP combined with methylglycol was similar ($P>0.05$) to semen cryopreserved in the previously approved extender in all the three fish species. However, when ACP was tested in combination with DMSO, post-thaw motility was similar to the previous approved extender only in *B. orbignyanus*. In *P. lineatus* and *L. obtusidens*, semen cryopreserved in ACP and DMSO produced very low post-thaw sperm motility.

CONCLUSION: Powder coconut water can be used as fish semen extender during cryopreservation process, maintaining high post-thaw sperm motility.

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Pre-freezing incubation of intratesticular semen of rainbow trout (Oncorhynchus mykiss) XX-males

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BACKGROUND: Female rainbow trout grow faster than male does. To produce all-female progenies, genetic females are sex-reversed into males (XX-males) through hormone diet during larval stages and are used as semen donor during artificial reproduction. However, most of these XX-males does not have a complete and functional sperm duct and does not release semen during hand-stripping. Thus, XX-males have to be sacrificed in order to obtain intratesticular semen by dripping or by testicular maceration. The left over intratesticular semen could be frozen to maximize the use of a single XX-male, but intratesticular semen of this trout submitted to the freezing-thawing process, produces low post-thaw motility rate. In this study we tested the hypothesis that intratesticular semen needs further maturation before freezing.

METHODS: XX-males (n=5) were anaesthetized in a solution containing benzocaine 1:10000 until swimming movement stops. Then XX-males were sacrificed by spinal cord transsection and bled. Intratesticular semen was collected by dripping to avoid blood contamination and placed all together in a beaker to produce a pool of semen. The pool was diluted in seminal plasma obtained from normal XY-males by centrifugation (3000 rpm for 2 h) in three semen:plasma dilution ratios: 1:0 (undiluted control), 1:6 and 1:8. Immediately after dilution, half of each semen volume was rediluted (1:3) in a freezing medium containing 80% of glucose 5.4.%, 10% egg yolk and 10% DMSO, aspirated into 0.5-mL straws, frozen in nitrogen vapor at -170ºC (dry-shipper CP 100 Taylor Wharton) for 12-16 h and stored to liquid nitrogen. The other half was first submitted to a pre-freezing incubation period of 1:30 h at 4ºC and then rediluted in the same freezing medium and frozen. Sperm motility was subjectively evaluated as percentage of moving cells, in an optical microscope, after thawing in a 70ºC water bath for 3 seconds.

RESULTS: Post-thaw sperm motility of undiluted control semen (1:0) submitted or not to the pre-freezing incubation period was very low (0-6%). Post-thaw sperm motility of semen diluted 1:6 and 1:8 in seminal plasma increased from 16-18% when frozen immediately after dilution, to 50-60% when submitted first to the pre-freezing incubation period and then frozen.

CONCLUSION: Intratesticular semen of rainbow trout XX-males can be successfully frozen in a freezing medium containing glucose, yolk and DMSO, after a pre-freezing incubation period of 1:30 h in seminal plasma (1:6) at 4ºC.

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Development of oocyte viability molecular signature (OVMS) assays for zebrafish (Danio rerio) oocyte cryopreservation studies

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BACKGROUND: Cryopreservation and cryobanking of germplasm of aquatic species offers many benefits to the fields of aquaculture, conservation and biomedicine. Although successful cryopreservation of spermatozoa has been achieved with many fish species, cryopreservation of fish oocytes is still in progress. One of the problems associated with fish oocyte cryopreservation is that there are no reliable simple methods for confirming fish oocyte viability after cryopreservation.

METHODS: Stage III zebrafish (ZF) oocytes were exposed to cryoprotectant (CPA) 2M methanol or 2M DMSO (in Hank's) for 30min at 22°C before they were loaded into 0.5 ml plastic straws and placed into a programmable cooler. Oocytes were cooled from 20ºC to -7.5ºC at 2ºC/min, manually seeded at -7.5ºC and held for 5min, then cooled from -7.5ºC to -40°C at 0.5ºC/min and -40ºC to -80°C at 10ºC/min. Samples were then plunged into liquid nitrogen and held for at least 10 min, and thawed by immersing the straws into a 27ºC water bath for 10 seconds. Thawed oocytes were washed twice in Hank's medium. Oocytes were then fixed in 4% paraformaldehyde overnight at 4°C, rinsed three times in phosphate buffered saline, twice in methanol, and stored at -20°C in methanol until used. Samples were treated with proteinase K and hybridized at 58°C with 50% formamide. Two-colour whole-mount in situ hybridization was carried out with fluorescein- and digoxigenin-labelled RNA probes (*met2* and *cyclinb*) to detect the conservation of the polarization of maternal transcripts inside oocyte cytoplasm. Samples were mounted in 100% glycerol and observed under an Eclipse E1000 Nikon microscope.

RESULTS: The results obtained showed a drastic variability in the number of the polarized cells in controls or after cryopreservation treatments, and a very low correlation between trypan blue and OVMS assay viability tests. No polarization of maternal transcripts was retained in ZF oocyte in the absence of CPA treatment prior to cooling and thawing. DMSO was most appropriate in retaining the polarization of the maternal transcripts tested. However, the variability in the percentages of retained polarized transcripts in repeated experiments indicates that an optimization of sample preparation, culture conditions, CPA treatments, and cryopreservation procedures are needed.

CONCLUSION: These data demonstrated that the polarization of maternal transcripts inside the oocyte could be used as a new and original method to monitor oocyte viability after manipulation and/or cryopreservation and will help in protocol refinement. OVMS assay is therefore a nice complementary approach for the evaluation of oocyte viability both before and after a cryopreservation procedure, and may substitute to visual and staining methods, and to heavier functional evaluations.

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Expression of biologically active red seabream gonadotropins in a baculovirus system : comaprison of two different strategies regarding their expression

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BACKGROUND: Recent advances in recombinant gene technology have enabled the production of biologically active fish gonadotropins (GTHs: FSH and LH). However, an efficient expression method for the production of GTHs has not yet been developed. In this study, we attempted to produce red seabream GTHs in silkworm larvae using two different baculovirus expression systems.

METHODS: To synthesize recombinant red seabream heterodimeric GTHs, two strategies were employed. The first utilized a dual vector expression strategy, in which each GTH subunit (α, FSHβ, and LHβ) was expressed from a separate comparative vector in a single host. The second method used a bicistronic expression strategy, in which both GTH subunit cDNAs were expressed from the vector. Either a dual or a bicistronic expression vector inoculated into silkworm larvae of the 5th instar early stage. Five days after inoculation, the hemolymph of the larvae containing recombinant FSH and LH was collected, and examined of biochemical property and biological activity.

RESULTS: Western blot analysis revealed that by using a dual vector expression system, each subunit of FSH and LH was successfully synthesized and secreted into the hemolymph, allowing the synthesis of heterodimeric molecules. In the case of the bicistronic vector coexpressing GTH subunits, the yields of FSHα and LHα subunits was very low, whereas those of both FSHβ and LHβ subunits were high. The biological activities of GTHs produced by a dual vector system were comparable to those of native GTHs in *in vitro* steroid hormone production. Both recombinant FSH and LH stimulated the *in vitro* 11-ketotestosterone production in sliced testis of red seabream. Furthermore, recombinant LH stimulated the *in vitro* production of estradiol-17β by ovarian follicles in a concentration-dependent manner. Conversely, GTHs derived from a bicistronic vector system had no effect on *in vitro* steroid hormone production.

CONCLUSION: We succeeded in efficiently producing biologically active red seabream GTHs using a dual vector baculovirus-mediated expression system. We also demonstrated that the expression of the α-subunits of FSH and LH relative to those of the β-subunits were very low using a dual vector expression system. The reasons for this unexpected finding are unclear; however, it may be related to the positioning of the α-subunit in the vector, since a similar trend was seen in the case of using a dual vector expression system. The baculovirus-expression systems described here have already been used. However, to our knowledge, this is the first attempt to compare the two vector systems. Further studies are needed to develop a method of producing recombinant GTHs in large quantities.

P 141

Improved storage conditions for unfertilized rainbow trout (Oncorhynchus mykiss) eggs

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BACKGROUND: Salmon eggs are commonly shipped in plastic bags inflated with air or oxygen. The objective of this study was to establish ways of optimizing storage and shipping conditions for unfertilized rainbow trout eggs.

METHODS: Freshly collected unfertilized rainbow trout eggs pooled from 9 strippings were supplemented with 125 IU penicillin and 0.125 mg streptomycin and stored at 2 $\pm$ 1°C in 3 L-PE bags inflated with O_2, CO_2, N_2, air or exhaled air (40 bags each). In half of the bags of each treatment group 120 eggs were stored loose, in the other half the same number of eggs was stored sealed in PE foil of 0.03 mm thickness (Toppits, Melitta, Germany). In 5 of the 20 samples per group at a time after 5, 10, 15 and 20 days of storage the pH was determined and the eggs were fertilized with homogenous pooled cryopreserved semen and incubated to the eyed stage.

RESULTS: Within one day the bags filled with CO_2 were deflated and all the eggs were dead. The pH had dropped dramatically from 8.2 to 6.5. Apparently CO_2 diffuses through PE foil both out of the external bag and into the sample. There was no difference to speak of between samples stored under air or exhaled air (47% overall fertility in both after 20 days). Contrary to the findings of other authors, O_2 was less suited than air (21 vs. 47% after 20 days). N_2 was less suited than air in the case of loose eggs (0 vs. 40%), when the eggs were sealed in PE foil, however, N_2 was even slightly superior to air (59 vs. 54%). Evidently the effect of different gas atmospheres was mitigated by the enveloping PE foil serving as a semipermeable protective membrane prolonging the viability of eggs. Under air and under N_2, after 20 days of storage, fertilization rates of 54% and 59%, respectively, were observed, which is significantly higher than what was accomplished with loose eggs ($P < 0.05$, Scheffé- test).

CONCLUSION: When sealed in PE foil, unfertilized rainbow trout eggs may be stored under air or N_2 at 2 $\pm$ 1°C for up to three weeks maintaining a fertilization capacity of well over 50%. This approach proved to be superior to the conventional practice of storing and shipping eggs loose in oxygen or air inflated bags.

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Production of biologically active recombinant Follicle-Stimulating Hormone (FSH) from Sea Bass (*Dicentrarchus labrax*) in different expression systems

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BACKGROUND: The follicle-stimulating hormone (FSH), a heterodimeric glycoprotein hormone secreted by the pituitary gland, is one of the key hormones in the control of reproduction in vertebrates. The present study reports the production and characterization of biologically active recombinant sea bass FSH in insect cells, *Spodoptera frugiperda* (Sf9) and in Chinese Hamster Ovary (CHO) cells.

METHODS: For the expression of recombinant sea bass FSH dimer, the full length alpha and FSHbeta subunit cDNAs were directionally subcloned into the pcDNA3 and pFASTBAC1 transfer vectors. Recombinant viruses (Bacmid-alpha and Bacmid-betaFSH) were generated, and later coinfected (MOI of 3) in Sf9 insect cells in order to produce and release the FSH dimer into the culture media. In addition, Chinese Hamster Ovary (CHO) cells were cotransfected with pcDNA3alpha and pcDNA3betaFSH constructs, using the lipofectamine reagent, with the aim of developing stable CHO clones expressing the sea bass FSH. After subsequent culture of infected Sf9 cells and CHO clones in serum-free medium during four and nine days, respectively, supernatants were collected and their ability to induce the production of cAMP in a cell line (HEK 293) stably expressing the sea bass FSH receptor and to promote steroid production in culture of gonad tissue were tested.

RESULTS: Both expression systems successfully produced recombinant sea bass FSH (rsbFSH-CHO and rsbFSH-Sf9). These proteins were able to stimulate the sea bass FSHR in a doses dependent manner. Receptor activation by FSH was measured indirectly by changes in luciferase activity, promoted by a rise of cAMP. Both rsbFSH induced steroid production in ovary tissue in a doses dependent manner.

CONCLUSION: Both recombinant forms of FSH are bioactive and able to promote steroid production by the ovary. Their availability represents an important advantage for the development of future studies about FSH function in teleost fish.

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P 143

Chromatin fragmentation and oxidization during short- and long-term storage of sex reversed rainbow trout sperm

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BACKGROUD: Cryopreservation produces damages to spermatozoa at different levels: motility, plasma membrane integrity and functionality, ATP content or fertility rates. In the last few years different studies point out that cryopreservation could also induce nuclear DNA fragmentation, which could be related to oxidative processes. This fact should be take into account in the evaluation of sperm storage protocols, especially when are designed for gene banking. Trout sex reversed males lack spermiducts and testicular spermatozoa are used for fertilization. Testicular cells are more sensitive to damages, particularly the oxidative stress. Our objective was to detect if short and long-term storage promote DNA strand breaks or nucleotide oxidization in spermatozoa from sex reversed rainbow trout.

METHODS: Sperm from 8 sex reversed males was separately incubated in maturation solution for 2h. Two aliquots were stored at 4ºC for 5 days or cryopreserved in liquid nitrogen. Chromatin fragmentation was analyzed using the single cell gel electrophoresis assay (Comet assay), without any previous treatment and after a previous digestion with the E.Coli enzymes Endonuclease III and FPG. These enzymes cut DNA in oxidized citosines and guanines respectively.

RESULTS: An increase of DNA damage was detected with both conservation procedures. Endonuclease III and FPG did not increase the DNA fragmentation in fresh sperm, showing the absence of oxidized nucleotides before storage. Samples refrigerated at 4ºC during 5 days showed a significant increase of DNA fragmentation after enzymatic digestion, especially after FPG treatment. Cryopreservation (long-term storage) increases the percentage of cells with fragmented DNA, but previous treatment with nucleases did not increase the rate of DNA damage.

CONCLUSION: The digestion with specific endonucleases, previous to the performance of the Comet assay, allows us to detect the presence of oxidized nucleotides in trout spermatozoa. Cold storage (short-term) promotes significant oxidative damages in the nuclear DNA that affects guanines in a higher extent than citosines. On the other hand, cryopreservation causes DNA fragmentation, but apparently do not affect the degree of oxidization, suggesting possible apoptotic events.

Gametogenesis and Gamete Biology
(Part IV : Oogenesis)

P 144

Expression of nitric oxide synthase isoforms in the ovary of *Heteropneustes fossilis* (Bloch.) during follicular development, and oocytes maturation

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BACKGROUND: In mammalian ovary, nitric oxide (NO) is well documented as autocrine / paracrine factor involved in ovarian development and maturation but its role in fish ovary is not yet described. The aim of present study was to demonstrate the presence of nitric oxide synthase (NOS), an enzyme involved in NO production and to study the role of NO in oocyte maturation in *H. fossilis*.

METHOD: Localization of different forms of NOS–like molecule was demonstrated by immunohistochemistry in fish ovary collected during previtellogenic, vitellogenic, and postvitellogenic phases. The presence of NOS isoforms was confirmed by western blot analysis. The production of NO was evaluated by biochemical estimation of stable NO metabolite nitrate/ nitrite level. The *in vitro* effect of either sodium nitroprusside (SNP) (10μM – 500μM), NO donor, or L-NAME (100mM), NOS inhibitor, on 17α, 20β-dihydroxyprogesterone (17α, 20β-P) (0.1μg) induced oocyte germinal vesicle breakdown (GVBD) was studied in 12 hr culture.

RESULTS: The immunohistochemical studies demonstrated the presence of eNOS and iNOS in granulosa and thecal cells of early vitellogenic follicles. The immunoreactivity of eNOS was more intense than iNOS. The immunoreactivity of nNOS was observed in oocytes of previtellogenic follicle. Western blot analysis confirmed the presence of three types of NOS in the ovary (nNOS, eNOS and iNOS). Total nitrate / nitrite concentration was higher in previtellogenic phase which increased further in early vitellogenic phase and decreased during postvitellogenic phase. Nitric oxide decreased the incidence of GVBD in 12 hr culture at higher dose. The L-NAME could not enhanced the maturation rate induced by 17α, 20β-P.

CONCLUSION: Both immunohistochemistry and western blot analysis showed the presence of NOS-like molecules in the ovary of fish. The differential expression of three NOS isoforms and variation in NO production during different stages of follicular development suggest a role of NO in modulating follicular development in *H. fossilis*. The *in vitro* study showed the inhibitory action of NO in oocyte maturation. The present study provides sufficient evidences for the presence of NO in the fish ovary and experimentally providing its implication in oocyte maturation.

P 145

Relationships between egg fatty acid and metal composition and maternal traits in two walleye (Sander vitreus) populations

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BACKGROUND: The quality and quantity of egg nutrient reserves will influence offspring viability and thus, both maternal and offspring fitness. Numerous studies have examined relationships between egg size and maternal age or size in fishes but, relatively little is known of how egg chemical composition varies with maternal traits. We examined the variation in egg fatty acid and metal composition with respect to maternal traits in two walleye populations.

METHODS: Ovulated females were sampled from spawning sites in the Bay of Quinte, Lake Ontario (Ontario, Canada) and in the south basin of Lake Winnipeg (Manitoba, Canada). Both egg and maternal tissue samples were collected and analyzed for a variety of characteristics. Eggs and somatic tissues were analysed for water and lipid contents. Egg fatty acid profiles were determined by gas chromatography and concentrations of 6 metabolically important metals were determined by inductively coupled plasma atomic emission spectrometry.

RESULTS: Egg total lipid concentrations were similar in the two populations, although Lake Winnipeg walleye produced smaller eggs. In Lake Ontario walleye, larger females produced eggs with higher percentages of docosahexaenoic acid (DHA) and arachidonic acid (AA), lower percentages of eicosapentaenoic acid (EPA) and lower EPA/AA ratios. Percentages of palmitoleic acid also decreased with maternal size while those of the most abundant monounsaturated fatty acid, oleic acid, were unrelated to maternal size. In Lake Winnipeg walleye, egg fatty acid profiles were not strongly related to any maternal trait examined. Lake Winnipeg walleye eggs also had much lower levels of AA than eggs from Lake Ontario. There were few consistent relationships between egg metal concentrations and any of the maternal and egg characteristics examined. Egg calcium concentrations declined with egg size in Lake Ontario walleye and with egg lipid concentration in Lake Winnipeg walleye. Egg manganese concentrations declined with maternal somatic mass in both populations.

CONCLUSION: Our results indicate that larger females may produce ova with superior essential fatty acid profiles in some populations but not others. The dichotomy between maternal size effects on percentages of oleic and palmitoleic acids may reflect the important role of oleic acid in membranes. Generally, measures of egg chemical composition were more strongly and consistently related to ontogenetic traits (i.e., maternal size and/or age) than to indices of maternal body condition (i.e., indices of reserves available for reproduction).

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High transcript level of a fatty acid-binding protein but not of vitellogenin receptor during ovarian follicle atresia of a teleost fish (Solea senegalensis)

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BACKGROUND: Atresia, a follicular degeneration and resorptive process, is a normal physiological event of vertebrate ovarian morphogenesis. In teleost fish, atresia is involved in normal ovarian growth and postovulatory regression, mostly in females that are not able to carry out maturation or ovulation after vitellogenesis. The presence of atretic follicles is also frequently associated with environmental stress or changes in hormone levels during vitellogenesis. Histology of teleost fish gonadal tissue is routinely used to describe and quantify morphological stages, including the number of vitellogenic and atretic follicles, but no molecular markers are currently available to study the molecular mechanisms underlying or regulating the balance between follicular development and atresia in teleosts.

METHODS: Full-length cDNAs coding a new fatty acid-binding protein (FABP), and two isoforms of very-low-density lipoprotein/vitellogenin receptor (VtgR), one containing the *O*-linked sugar domain and the other not, were characterized in sole (*Solea senegalensis*) ovaries after screening of an ovarian cDNA library. Reverse-transcriptional polymerase chain reaction (RT-PCR), in situ hybridization, and real-time quantitative PCR (RT-qPCR) were performed to study the pattern of gene expression and the correlation between the number of copies of the target transcripts in the ovary and the percentage of ovarian follicles at different developmental stages determined on histological sections.

RESULTS: Phylogenetic analysis indicated that a new FABP group, including the characterized Senegal sole sequence, and specific to teleost fish, clustered in a sister group of FABP3/FABP7. RT-PCR revealed high levels of *vtgr* transcript splicing variants in the ovaries and, to a lesser extent, in somatic tissues, while *fabp* was highly expressed in the ovaries, liver, and adipose tissue. Low levels were also detected in heart, muscles, kidneys, and testicles. In situ hybridization analysis showed a high level of *vtgr* and *fabp* mRNAs in previtellogenic oocytes, while no hybridization signals were detected in the larger vitellogenic oocytes. *Fabp* transcript expression was strongly up-regulated in somatic cells surrounding atretic follicles. RT-qPCR demonstrated that ovarian transcript levels of *vtgr* and *fabp* had a significant positive correlation with the percentage of follicles in previtellogenesis and atresia, respectively.

CONCLUSION: These results suggest that the expression level of *vtgr* transcripts may be used as a precocious functional marker to quantify the number of oocytes recruited for vitellogenesis and that *fabp* mRNA may be a very useful molecular marker for determining cellular events and environmental factors that regulate follicular atresia in Senegal sole and perhaps in other teleost fish.

P 147

Time of first embryonic cleavage in the rainbow trout (*Oncorhynchus mykiss*): parental effects and correlations with the quality of subsequent development

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BACKGROUND: Early evaluation of developmental ability of *in vitro* fertilized embryos could help to improve the reliability of reproductive biotechnologies. Among other criteria, the time lag from insemination to first embryonic cleavage, or "Time of First Cleavage" (TFC), was shown in some mammals to be correlated with the ability of the embryo to develop *in vitro*. In rainbow trout, direct observation of the first embryonic cleavages cannot be performed on living eggs, due to the opacity of the chorion. Therefore, a method based on the fixation and coloration of serial samples from a single egg batch was performed to examine the chronological profile of cleaved egg proportion (CEP), and to evaluate statistically the mean time of first embryonic cleavage (TFC). We used this method to investigate the link between mean TFC, parental gamete origin, and egg quality.

METHODS: Experiments were carried out on fish from three different strains, spawning either in autumn, winter or spring. Fish were checked twice a week for spermiation and ovulation. Eggs from individual females were sampled as soon as ovulation was detected, and 9 days later. They were inseminated either with the sperm of individual males or with a pool of sperm and subsequently incubated at 10°C. For each cross, a sample of eggs was fixed every 30mn from 6H30 to 10H30 post-insemination. After coloration, the proportion of eggs at 2 cell or later stages was determined by observation under a binocular microscope. In parallel, eggs from the same cross were incubated until yolk sac resorption to evaluate embryonic survival and malformation rates.

RESULTS: A significant maternal effect on CEP profile and mean TFC was observed in both autumn- and spring-spawning strains, whereas a less pronounced paternal effect was revealed by interactions with the maternal effect. TFC was not correlated with the survival rate at yolk-sac resorption, but with malformation rate. Egg batches showing the shortest mean TFC thus exhibited the lowest malformation rates. Post-ovulatory oocyte ageing, known to impair egg quality, clearly delayed the time of first cleavage. No differences in mean TFC could be observed between genetic strains.

CONCLUSION: Our data show that CEP profile and mean TFC exhibit a noticeable variability mainly resulting from a maternal effect. Such variability in the early embryonic developmental time course, observed both at the population and individual level, probably accounts in part for uneven results observed in polyploidy induction technologies. From a practical point of view, however, this method is probably too heavy to be used as it is in current practice to evaluate the quality of individual spawns.

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Apoptosis in Zingel asper (Percidae) ovarian and testicular development

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BACKGROUND: The endemic Rhodanian Percidae Zingel asper lives in the Rhone Basin. At the beginning of the 20th Century it was found in this entire Basin. Its distribution is now over 17% of the previous 380 km area. The entire population is only represented by a few thousand individuals that are scattered in several isolated populations. This fish is the subject of a conservation program. Understanding of the reproductive physiology of Zingel asper is important to control artificially its reproduction in view of re-introducing it. This work aims at investigating the susceptibility of Z. asper ovarian follicles and testis cells to apoptosis at different stages of development.

METHODS: The study was carried out on fish obtained by artificial fertilization in March 2000, and bred in external tanks (Allex, Drôme, France). After dissection of each specimen, gonads were removed, fixed in Bouin's fluid, dehydrated in ethanol, cleared in butanol prior to be infiltrated and embedded in paraffin. The 5 µm sections were fixed on superfrost slides. Apoptotic were identified using in situ detection by the terminal-deoxynucleotidyl-transferase-mediated dUTP nick-end labelling (TUNEL) method and two immunohistological methods, using an antibody directed against caspase-3 and a monoclonal antibody directed against DNA single-strands. Sections were photographed using a Nikon Eclipse E400 microscope equipped with a DXM 1200 Nikon digital camera.

RESULTS: In females, TUNEL method showed apoptosis only among follicle cells in increasing follicles, postovulatory follicles, but never in atretic follicles. In controls for which DNA was intentionally fragmented by nuclease treatment, no positive TUNEL reaction was observed in the nuclei of oocytes. Using immunohistological methods the only labelled cells were follicular cells. In oocytes, postovulatory and atretic follicles apoptosis was not observed. In males, both TUNEL and immunohistology had similar results. Apoptosis was found in post spermiation testes. A lot of Leydig cells, some Sertoli cells and, in few cysts, type A spermatogonia, were apoptotic. Both in males and females, negative controls in which antibodies were absent and tissue controls (brain) were always negative.

CONCLUSION: Our results indicate that apoptosis is not involved in postovulatory regression and follicular atresia in female Zingel asper. In male, apoptosis takes place in mature cysts after sperm release. Degeneration entails Sertoli and Leydig cells.

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Histological study of Zingel asper (Percidae) sexual maturation: patterns of ovarian and testicular development

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BACKGROUND: The endemic Rhodanian Percidae Zingel asper lives in the Rhone Basin. At the beginning of the 20th Century it was found in this entire Basin. Its distribution is now over 17% of the previous 380 km area. The entire population is only represented by a few thousand individuals that are scattered in several isolated populations. This fish is the subject of a conservation program. Understanding of the reproductive physiology of Zingel asper is important to control artificially its reproduction in view of re-introducing it.

METHODS: The study was carried out on 32 males and 53 females that were obtained by artificial fertilization in March 2000, and bred in external tanks (Allex, Drôme, France). The fish were observed from the juvenile stages to adulthood. Patterns of ovarian and testicular development were defined from histological observations. After dissection of each specimen, gonads were removed, fixed in Bouin's fluid, dehydrated in ethanol and cleared in butanol prior to paraffin infiltration and embedding. The 5 µm sections were stained with modified azan. They were photographed using a Nikon Eclipse E400 microscope equipped with a DXM 1200 Nikon digital camera.

RESULTS: Ovaries and testes of Zingel asper are paired, elongated and fusiform dorsocaudal organs. The two lobes of each gonad joined together to form a duct that extended to the urogenital papillae. Up to 10 months old, most of the males and females were immature: testis showed only type A spermatogonia and, in ovaries, no inclusion was observed in cytoplasms of oocytes. At 10 months and after, the presence of type B spermatogonia in the lobules of testis and fatty droplets into the oocytes signalled the beginning of gametogenesis. At 20 months, histological female sections showed the onset of vitellogenesis. In Mars 2002, 24 months after the fertilization, spermiogenesis was observed in males and advanced vitellogenesis in females. The total maturation occurred in 26 months old fish in May 2002. During this period, in males, the cyst opened and released spermatozoa into the lumen of lobules. In females, the end of oocyte maturation was characterized by the coalescence of lipid and yolk globules followed by ovulation. In 28 months old fish, the post-spawning stages are characterized, in males, by testes showing evidence of sperm resorption and, in females, by oocytes resting. During the third year, histological observations pointed out the same evolution of adult gonads than during the second year.

CONCLUSION: The reported data indicate that sexual maturity is reached in captive Zingel asper after two years. Annual reproductive cycles were similar in both males and females. These observations demonstrate that spawning occurs in May.

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Juvenile intersexuality in the cyprinid fish *Labeo victorianus*

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BACKGROUND: Comprehensive knowledge of sexuality in fishes is considered vital for understanding of behaviour patterns, breeding periodicity, growth rates, body colour and shape or sexual dimorphism. Information on sex development has also been applied in fish culture to either initiate sexual change or to improve growth of a particular sex. No data on sex development and differentiation in any African *Labeines* are available. The present study investigates gonadal development and sex differentiation in *Labeo victorianus* under lake and pond environments.

METHODS: Live juvenile fish were obtained from Lake Victoria and ponds at Kajjansi, Uganda by seining; (169 from the ponds and 178 from the lake) at different stages of gonadal development. The fish were anaesthetised with 2-phenoxyethanol, their fork length measured and gonads fixed in Bouin's solution for 24 hours before transfer to 70% ethanol followed by standard light microscopy tissue processing techniques. Structural measurements were made on scanned sections from at least five fish per microscopic stage using Sigma Scan Pro Image Analysis Software.

RESULTS: All the gonads first developed oogonia, which underwent meiotic division and developed through chromatin nucleolar oocyte to the perinucleolus oocyte stage. In gonads that were to develop into ovaries, oogenesis continued leading to formation of more perinucleolus oocytes and subsequent stages. In gonads destined to develop into testes, the perinucleolus oocytes underwent asynchronous atresia. Concomitant with onset of oocyte atresia was active division of gonocytes and appearance of several spermatogonial nests encompassed by the spindle shaped somatic cells. This stage marked the onset of spermatogenesis.

CONCLUSION: Sex differentiation in *L. victorianus* initially follows an oogenic pathway. Development of the testes seemed to be an inversion of the female gonad. This was exhibited by atresia of pre-vitellogenic oocytes and concomitant appearance of spermatogonia. Spermatogenesis ensued from the start of oocyte atresia and progressed to rupture of spermatozoa cysts even in gonads that still contained atretic oocytes. Although results from this study revealed the presence of gametes of both sexes in the same gonad during development, the gonads were not yet functional as oocytes in predestined males did not progress beyond the perinucleolus oocyte stage. *L. victorianus* can, therefore, not be considered to be hermaphroditic but undifferentiated gonochorist.

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Oogenesis in Silverfish, *Chirostoma humboldtianum*. A structural and ultraestructural study

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BACKGROUND: The structural and ultrastructural features of gonads from endemic Mexican fish have received few attention. This study describes the oogenesis of *Chirostoma humboldtianum*, both, histological and ultrastructural levels.

METHODS: fish were collected from the Zacapu lagoon in the Federal State of Michoacan, Mexico. After collection, the fish were first anaesthetized with MS 222, and then measured and sacrificed by transversal section. For light microscopy, the ovaries were removed and fixed in paraformaldehyde 2% or Bouin solution for 24 hours. The samples were dehydrated and were included in paraffin, cut at 1-5 micrometres and stained with hematoxylin and eosin or PAS. The sections were mounted with Entellan. For electron microscopy, other samples were fixed in glutaraldehyde 4% in cacodylate buffer (pH 7.4) or glutaraldehyde and paraformaldehyde mix in phosphate buffer. This samples were postfixed with osmium tetraoxide, dehydrated with alcohol, included in plastic resin (EPON), cut with an ultramicrotome and contrasted with uranyl acetate and lead citrate. Observations were done in a Jeol 100 X or a Jeol 1200 EX electron microscope.

RESULTS: *Ch. humboldtianum* has an asynchronic ovary, and as such, most phases of oocyte development are found in the same ovary. The complete process of oogenesis was divided in five stages: oogonium and folliculogenesis, primary growth, cortical alveoli and lipid inclusions, vitellogenesis and maturation. The structural and ultrastructural features in Chirostoma are common with other teleost fish. However, the presence of big filaments, which start to appear in the vegetal pole at the end of primary growth is a peculiar adaptation. During primary growth, abundant ribosomes, rough endoplasmic reticulum and mitochondria are grouped in the cytoplasm. At the end of this stage, the Z1 layer of chorion is developed, while microvilli start to be evident as well. In the cortical alveoli and lipid droplets phase, intense PAS positive vesicles are observed in the peripherical cytoplasm, and the lipid droplets take a more central position. In vitellogenesis, the proteic yolk accumulates in a centripetal way while the chorion is completely formed with 3 layers. In maturation, , meiosis is resumed, the germinal vesicle migrates to the animal pole and the nuclear envelope breakdown; the oocyte increases in size and contains some oil droplets and a big fluid mass of yolk. On the outside, filaments can be observed.

CONCLUSION: The oogenesis in silverfish is similar to other species of teleosts. However, the presences of big filaments around oocyte since previtellogenesis, is very peculiar of these species. Should be interesting to study if that adaptation modifies the relationship between oocyte and follicle cells.

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Ethinylestradiol (EE2) differentially interferes with insulin-like growth factor I (IGF-I) in liver and gonads of male and female tilapia

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BACKGROUND: Growth and sexual differentiation are closely interlinked in fish but no reports exist on the potential interference of estrogen(s) and the growth hormone (GH)/insulin-like growth factor I (IGF-I) system that plays a central role in the regulation of growth and differentiation during development. The main source of (endocrine) IGF-I is the liver but it also occurs in extrahepatic sites, such as the gonads.

METHODS: A balanced population of tilapia (*Oreochromis niloticus*) was fed during 10-40 days post fertilisation (DPF) with ethinylestradiol (EE2) at the optimal dosage to induce functional feminization in most fish. Fish at 50, 75, 90 and 165 DPF were investigated using radioimmunoassay for serum IGF-I and real-time RT-PCR for IGF-I and estrogen receptor α (ER α) mRNA in liver and gonads.

RESULTS: EE2 treatment led to a shift in sex ratio (86.5% females vs 47.2% in control) and to severe growth impairment in fish of both sex. Serum IGF-I was significantly (-31%) decreased in EE2-treated fish at 75 DPF and later recovered. IGF-I mRNA was significantly reduced in male and female liver at 50, 75 and 90 DPF and recovered at 164 DPF. Erα mRNA in liver was transiently (50-90 DPF) induced. In the gonads, no alteration in the expression of IGF-I was detected at 50 DPF. At 75 DPF, there was a significant decrease in IGF-I mRNA in the ovary and at 90 DPF in testes. At the later stages, IGF-I mRNA was almost at the normal level. At 50 DPF, a significant raise in Erα mRNA was found. The increase in Erα mRNA persisted until 90 DPF in testes. In contrast, in ovary, a significant decrease in Erα mRNA was obtained at 75 DPF. Later, Erα mRNA was at the normal level in both sex.

CONCLUSION: Growth impairment by estrogen(s) in fish may be due to direct suppression of liver IGF-I synthesis and release. Furthermore, two options apply to explain the impairing effects of estrogens on growth, differentiation and function of fish gonads. They may be exerted via suppression of IGF-I production in liver resulting in a lowered level of endocrine IGF-I and/or by the reduction of autocrine/paracrine IGF-I expression within the gonads.

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Maternal mRNA differences in unfertilized rainbow trout (*Oncorhynchus mykiss*) eggs from batches exhibiting variable embryonic survival

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BACKGROUND: The number of offspring that survive the embryonic period of development in fishes can be highly variable. In rainbow trout, some females produce egg batches in which more than 20% of the offspring do not survive for 12 hours after fertilization. The purpose of this study was to compare differences in the maternal mRNAs for genes stored in unfertilized eggs from female rainbow trout characterized by having high or low early embryonic survival.

METHODS: Messenger RNA from pooled 100 egg aliquots derived from four female rainbow trout categorized as fertile (>95% embryonic cleavage at 12 hours post fertilization [hpf]) and four subfertile (<80% embryonic cleavage at 12 hpf) females were used. The mRNA was converted to cDNA by reverse transcription and hybridized onto 2-channel cDNA microarrays (GRASP, Centre for Biomedical Research, Univ. of Victoria). Each microarray used egg cDNA samples derived from a fertile and a subfertile female to determine differences in gene expression. Statistical analysis was performed to identify microarray targets that differed between eggs from fertile and subfertile females. The mRNAs for selected genes were quantified using real-time RT-PCR to validate the microarray results.

RESULTS: It was determined that 104 microarray targets had significantly ($p<0.05$) higher amounts of mRNA in unfertilized eggs from fertile females compared to subfertile females. Not all of the microarray targets represented known genes. Fortunately, many of the genes with the greatest differences in expression were known. Four of these genes, transglutaminase, guanylate kinase, metallothionein, and apolipoprotein E had mRNA amounts close to or more than a 2-fold higher relative to eggs from subfertile females. Only 6 microarray targets gave the opposite result, that is, greater mRNA amounts in subfertile females compared to fertile females. In all cases, the increment was approximately 0.5-fold higher than comparable eggs from fertile females. A notable known gene was septin 5.

CONCLUSION: This study shows that the mRNAs for a considerable number of genes, in unfertilized eggs from females that produce egg batches with compromised survival, are reduced in quantity. The apparent reduction in the maternal mRNA for these genes could be directly related to the reduced numbers of embryos that survive in these batches. It is possible that one or more keys genes are not being translated sufficiently to support fertilization or subsequent early embryonic development. Alternatively, some other independent process (e.g., apoptosis) could indirectly affect the reduction of these maternal mRNAs in some eggs. Regardless, this information provides a starting point for the study of genes that are critical for successful fertilization and early development in fishes.

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Two growth hormone receptors in Nile tilapia (*Oreochromis niloticus*): molecular characterization, tissue distribution and expression profiles in gonad during reproductive cycle

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BACKGROUND: It is intriguing to note that fish growth hormone receptors (GHRs) could be clustered into two clades and further studies have proved that two types of GHRs co-exist in one teleost species. In addition, it is well known that GH may be related to the early phases of oogenesis by regulating GHR gene expression in vertebrate. Whether two types of GHR co-exist in Nile tilapia (*Oreochromis niloticus*) or whether the two GHRs bear any roles in reproduction is unclear**.**

METHODS: Two cDNAs encoding growth hormone receptor (GHR) were identified from Nile tilapia liver tissue by GeneRacer^{TM}. The expressions of two GHRs in different tissues were compared by real-time PCR and Northern blot. Furthermore, the expression patterns of GHRs in gonad during reproductive cycles were analyzed using real-time PCR.

RESULTS: Two cDNAs, one consisting of 1908 bp and the other of 1728 bp, encoding for putative 635- and 575-amino acid GHR (designated ntGHR1 and ntGHR2, respectively), shared 34.4% identity in nucleotide sequence and 29.6% in deduced amino acid sequence. ntGHR1 and ntGHR2 showed distinctly different features in structure, ntGHR2 lacked one pair of extracellular cysteines (C5, C6) and four intracellular tyrosine residues (Y1, Y3, Y6, and Y7) which were conservative in GHR1. Northern blot analysis indicated a single 6.0-kb transcript of ntGHR1 and a single 4.0-kb transcript of ntGHR2 in liver. Real-time PCR analysis showed that both ntGHR1 and ntGHR2 mRNAs were presented in all tissues tested and expressed extremely highly in liver. Significantly higher expressions of ntGHRs in ovary were detected at sexually matured stage, while expressions of GHRs in testis were significantly higher at sexual recrudescent stage.

CONCLUSION: The sequences analysis and Northern blot analysis showed that ntGHR1 and ntGHR2 are two distinct GHRs rather than alternative splicing products of a single gene. Different features in structure between ntGHR1 and ntGHR2 suggest their various biological functions. The ntGHRs mRNA levels in testis were significantly higher at sexual recrudescent stage and then decreased significantly at sexually matured and regressed stages, indicating that GH might be potentially important for testis functions during the early stage of spermatogenesis.

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Gonadal development and spawning rhythm in mudskipper (Periophthalmus modestus)

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BACKGROUND: Periophthalmus modestus (family Gobiidae) is an amphibious mudskipper fish that live in the tidal flat of East Asia. In the Ariake Sound in Japan, the reproductive season of the fish starts from mid-May to early August. Although this species has been known as a multiple spawner, the timing of maturation and spawning are not well-understood. In the present study, we investigated the seasonal changes in gonadal development and the changes in developmental stages of oocyte during the spawning season in order to elucidate the mechanizms underlying reproductive and spawning cycles in the mudskipper. In addition, we examined the timing of acquisition of maturational competence in oocyte using in vitro culture system.

METHODS: Mudskipper were collected every month between April 2005 and April 2006 from a tidal flat of the estuary of Kashima River (Saga, Japan). Ovaries were removed from the body cavity and were used for calculation of gonadosomatic index (GSI) and histological observation. To obtain information on gonadal development during spawning season, fish were collected every day from June 13th to 30th in 2006 from the tidal flat. The ovaries were devoted for the in vitro culture of oocyte. The oocytes were incubated in two concentrations of hCG (10 and 100IU/ml) and DHP (10 and 100ng/ml). After incubation, the maturational competence of oocyte was checked by microscopy observation.

RESULTS: GSI value was relatively low from mid-August to late April, then increased rapidly and reached maximum level in mid-May. The peak of GSI was observed four times during the reproductive season. The GSI peak occurred at the intervals of ~13days between mid-May and early June. GSI started to increase during the spring tide and reached its peak during the middle tide. The GSI peak was accompanied by the increased rate of occurrence of oocytes at the tertiary yolk and the mature stages. However, the GSI peak in late June was delayed and observed after the middle tide. In the oocytes in vitro culture experiment, final oocyte maturation (FOM) was observed both in the hCG and DHP treatment groups with oocyte diameter of >400μm.

CONCLUSION: In the present study, the changes in GSI and oocyte development showed a regular cycle of ~13 days interval in the early spawning phase. These results suggest that mudskipper exhibits a tide-dependent cue for oocyte development and spawning. However, there is no similar rhythm for the acquisition of maturational competence of oocyte. The maturational competence is acquired only in the oocyte >400μm. These indicate that FOM and spawning in natural conditions depend on the endocrine changes that are induced by environmental cue such as tidal variability.

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Differential Expression and Regulation of Gonadotropins and Their Receptors in the Japanese eel, *Anguilla japonica*

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BACKGROUND: The objectives were to measure the pituitary expression of the gonadotropin subunits (glycoprotein-α, FSH-β, LH-β) and ovarian expression of LHR and FSHR of Japanese eel during induced gonadal development and after sex steroid treatments.

METHODS: Eels received weekly injections of salmon pituitary homogenates for 14 to 17 weeks in order to induce ovarian development. Sex steroid treated eels received injections of 0.75 mg or 3.75 mg sex steroid (T or E_2)/kg BW. The LH content of pituitary was measured by Radioimmunoassay, and the real-time quantitative PCR analyses were performed to measure the glycoprotein-α, FSH-β, LH-β, FSHR and LHR mRNA levels.

RESULTS: We reported the first molecular data on eel gonadotropin receptors. The partial sequences cloned covered two-third of the open reading frame and included most of the extracellular and transmembrane domains. Similar levels of pituirary FSH-β and LH-β subunit transcripts were found in the immature previtellogenic female eels. In contrast, ovarian FSHR mRNA was at least 42-fold higher than that of LHR. These data revealed that FSHR rather LHR would play a major role in the mediation of gonadotropin stimulation of the early stages of ovarian growth. Chronic treatment with fish pituitary homogenates, applied to induce eel sexual maturation, significantly stimulated pituitary LH-β mRNA but suppressed FSH-β transcripts. In the ovaries, both FSHR and LHR mRNA were significantly increased in the experimentally matured eels. Treatments with sexual steroids showed a stimulatory effect of estradiol-17β (E_2) on pituitary LH-β mRNA levels, while FSH-β transcripts were suppressed by E_2 or testosterone (T). In contrast, neither E_2 nor T-treatments had any significant effect on ovarian FSHR and LHR transcripts.

CONCLUSION: These were the first data on the sequence, expression and regulation of gonadotropin receptors, FSHR and LHR in the eel. Our study demonstrated that gonadotropins and their receptors are differentially regulated in control eels and during experimental maturation and steroid treatments. These data provide new foundation for basic and applied research on eel reproduction.

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Fish egg quality: effect of spawning induction in rainbow trout, *Oncorhynchus mykiss*, and relationship with egg transcriptome

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BACKGROUND: Fish egg quality can be defined as the ability of the egg to be fertilized and subsequently develop into of a normal embryo. Egg quality variability is usually considered to be influenced by external factors, genitors breeding conditions or biotechnologies. However, molecular mechanisms involved in these regulations remain poorly investigated. Besides, egg quality is mostly evaluated by only recording egg fertilization and embryonic mortalities rates. In the present study, the effect of spawning induction on rainbow trout egg quality has been investigated. Embryonic survival and malformations were accurately recorded and changes in egg mRNA content were explored using cDNA microarrays.

METHODS: Eggs were collected 5 days after ovulation from 25 naturally ovulating females (Control group: C) and 33 females that had been given a 60 µg/Kg-1 body weight intraperitoneal injection of [Des-Gly10, Darg6, Pro-NHEt9]-GnRH analog (Hormonally induced group: HI). Ten fully mature males were stripped and their sperm pooled for fertilization. Survival at eying and completion of yolk sac resorption (YSR) were monitored. Malformations of alevins at YSR were carefully recorded and classified in several types. Besides, batches of 60 unfertilized eggs were collected for RNA extraction from 4 control females and 11 hormonally-induced females. Nylon micro-arrays displaying 9152 distinct trout cDNA clones were hybridized with complex probes obtained from 3µg of each RNA sample. Significant differences in mRNA abundance were identified using Significance Analysis of Microarray (SAM) software. When possible, levels of mRNAs showing differential abundances were also quantified using real time PCR.

RESULTS: Survival was not affected by spawning induction at eying but was slightly lowered at YSR. In both cases, inter-females variability was strongly increased. Embryonic malformations at YSR were always limited in the C group (no more than 20%) while all types of malformations occurred at a high rate in some females of the HI group (up to 90%). Besides, the egg mRNA abundance of 7 genes was found to be significantly modified by spawning induction. More specifically, a dramatic increase of Apolipoprotein C1 (APOC1) and tyrosine protein kinase (HCK) egg mRNA abundance was observed. In addition, both microarray and real-time PCR analyses showed that prohibitin 2 (PHB2) egg mRNA abundance was negatively correlated with developmental success after fertilization.

CONCLUSION: The egg quality observed after spawning induction can be highly variable depending on females. This high variability is probably due to differences in the oocyte competency for maturation and ovulation of the females at the time of GnRH induction. Besides, some oocyte mRNA abundances were strongly modified after GnRH stimulation. The consequence of such changes on the egg quality will be discussed according to the identity of involved genes.

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Variation in egg quality after hormonally-induced ovulation in goldfish is more related to female variability than to post-ovulation ageing

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BACKGROUND: Variability in fish egg quality between spawns is a major drawback when the eggs are to be used for biotechnologies such as nuclear transfer or transgenesis. In this work, the variability of egg quality was explored at ovulation (female readiness to hormonally-induced ovulation) and during post-ovulation ageing in the female tract.

METHOD: Thirty females separated in two independent experiments were used. Fish raised at 14°C were conditioned for 6 days at 20°C before they were treated with 1ml/kg Ovaprim® to induce ovulation. Nine hours after Ovaprim® injection, ovulation was checked every hour by gentle stripping. After ovulation (T0), egg aliquots were collected every hour on each female. At each post ovulation time, eggs were fertilized with the same preserved sperm pool and incubated at 20°C in water. Embryo viability was checked 24hour after fertilization. Quality of the hatchlings (percentage of the normal hatchling number to the initial number of eggs named hatching rate) was recorded 3.5 days after fertilization.

RESULTS: *Response to Ovaprim® treatment*: Of the 30 females conditioned at 20°C, 23% ovulated spontaneously before hormonal injection and they were removed from the study, 20% were unresponsive to the Ovaprim® treatment, and 57% (n=17) ovulated between 10 and 14 hours after Ovaprim® treatment.
Egg Quality upon ovulation: Eggs collected in the first hour after ovulation supported an embryo development rate of 72.9% ± 25.8 (ranging from 21 to 95%). The same variability between females was observed for the hatching rate (54.2% ± 21.6 from 6 to 84%). Included in these data were some females collected at the very onset of ovulation which emitted only few eggs of the most variable quality.

Ageing after ovulation: From 1 to 2 hours after ovulation, egg quality of the worst females improved noticeably, leading to more than 12% increase in the mean quality values (85.3 ± 12.4 embryo viability and 66.4 ± 16.2 hatching rate). Afterward, no significant ageing trend could be observed up to 5 hours after ovulation. Each spawn displayed a specific quality variation, some being improved, others being altered with post ovulation time increase. It is only 10 hours after ovulation that egg quality dramatically decreased (41.1% ± 30 hatching rate), although 3 females still had a rate above 50%.

CONCLUSION: Post ovulation ageing in the 5 first hours after ovulation was not a major actor in egg quality alteration provided that eggs were not collected at the onset of ovulation. Since variation in egg quality had merely an intrinsic origin, efforts to improve spawn quality should therefore focus on female related factors such as quality of gametogenesis, synchronization of maturation, stress handling, or female lineage.

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Ovarian development of the spotted rose snapper (Lutjanus guttatus)

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BACKGROUND: The spotted rose snapper (Lutjanus guttatus) is a commercially important species with aquaculture potential. To advance research towards controlled spawning, more information is required on reproductive development of this species in captivity.

METHODS: On the 18 Oct. a group of 20 female wild caught snapper was transferred and maintained in optimum conditions in CIAD. At monthly intervals, from December through to June, 2-4 captive females and 6-10 wild females (Nayarit, Mexico) were sampled. The following parameters were determined for each fish: gonadal somatic index (GSI); size and nuclear position of 100 cleared oocytes from fresh samples (40x magnification), stage of development of 100 oocytes from histologically processed samples (5um sections, 100x) and synthesis of hepatic vitellogenin. Oocytes were staged as follows: primary growth, cortical alveoli, vitellogenesis V, VI, VII. Liver samples were fixed in RNAlater, RNA was extracted and cDNA synthesised, vitellogenin gene expression was evaluated by RT-PCR followed by agarose gel electrophoresis.

RESULTS: GSI exhibited little change; the only significant difference was between wild fish sampled in Jan. (0.5±0.1) and May (2.0±1.3). The histological analysis and observations of fresh oocytes were correlated ($r=0.93$ $P<0.05$), wild fish had vitellogenic oocytes in every month except Jan. while captive fish only exhibited stage V vitellogenesis in June. Oocyte development was asynchronous, characteristic of batch spawners. Wild fish exhibited the highest proportions of advanced vitellogenesis (VI and VII) in April and May and an ovulating fish was caught in June. Wild fish exhibited elevated levels of vitellogenin synthesis in months when vitellogenic oocytes were present, but these were only significantly higher than Jan. in Dec., Feb., April and May. The captive fish exhibited a decrease in vitellogenin synthesis from Dec. to Jan and a significant increase from April through to June.

CONCLUSION: Oocyte development appeared to be rapid, one month or less. In wild fish, spawning appeared to peak in May and April, extending into June. Vitellogenesis appeared to be delayed in captive fish, either due to environmental conditions or reproductive dysfunction. Unlike in wild fish, vitellogenin synthesis in captive fish started the month before vitellogenesis was observed in oocytes, possibly related to delayed development or dysfunction. The observed vitellogenesis in captivity would suggest that induced or even natural spawning may be possible.

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In vitro* induction of oil droplet accumulation into previtellogenic oocytes of Japanese eel, *Anguilla japonica

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BACKGROUND: Previtellogenic oocyte development in the Japanese eel, *Anguilla japonica*, is mainly characterized by the appearance and accumulation of oil droplets in the cytoplasm of the oocyte. The origin of the lipids in the oil droplets is unknown; regardless, lipoproteins, the transporters of lipids from one tissue to another through blood, are thought to play an important role in the accumulation of lipids into the oocytes. Very low density lipoprotein (VLDL) in particular, contains rich neutral fat which is the main component of oil droplets. Meanwhile, recent studies in our laboratory have shown that 11-ketotestosterone (11-KT), a major androgen in some teleosts, is involved in controlling previtellogenesis in Japanese eel. However, the mechanisms underlying 11-KT-regulated previtellogenic oocyte growth are not known. In the present study, we examined the effects of VLDL and 11-KT on the accumulation of oil droplets into previtellogenic oocytes of Japanese eel using an *in vitro* culture system of ovarian fragments.

METHODS: Ovarian fragments from female eel in the previtellogenic stage were cultured by a floating tissue culture method in Leibovitz L-15 medium supplemented with 0.5% bovine serum albumin, 0.16 □M bovine insulin and 10 mM Hepes, pH 7.4 at 20°C in humidified air. Tissue was treated with or without VLDL isolated from eel plasma (0.5, 1, 2.5 or 5 mg/ml) in the presence or absence of 11-KT (10 ng/ml) for 28 days. The cultured ovarian fragments were collected for histological analysis, and the surface areas of oil droplets and oocytes in cross section were measured.

RESULTS: In control (no treatment) ovaries, oocyte size was slightly increased compared to oocytes in freshly dissected tissue, and the surface area of oil droplets was unchanged. VLDL had no effect on oocyte size, but high concentrations of VLDL slightly increased the surface area of oil droplets. 11-KT significantly increased oocyte size, but led to abnormal cytology, evidenced by vacuoles in the cytoplasm, and by decreasing amounts of oil. However, following co-treatment of VLDL and 11-KT, the abundance of abnormal oocytes was decreased and the surface area of oil droplets was increased as VLDL concentrations rose. Especially under conditions of high VLDL concentrations, abnormal oocytes were not seen, and the number and size of oil droplets increased remarkably.

CONCLUSION: This study showed that accumulation of oil droplets in previtellogenic oocytes of Japanese eel could be induced *in vitro*. VLDL alone was not very effective in causing the accumulation of oil droplets, whereas 11-KT had a negative effect on oil droplet surface area. However, co-treatment of VLDL and 11-KT resulted in significant oil droplet accumulation. These data suggest that lipids in oil droplets originate, at least in part, from VLDL, and that 11-KT plays an important role in their transfer and/or accumulation into oocytes.

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Seasonality of reproduction in *Piaractus brachypomus* in South Bolivia

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BACKGROUND: The reproduction of Tambaquí (*Piaractus brachypomus*) is well documented. However, most of the studies took place in central Amazon with limited climate fluctuations throughout the year. Reproduction of Tambaquí in captivity has been reported to be restricted to December-January in South Bolivia, close to the Southern distribution limit for the species. Moreover, few objective selection criteria of female brooders for induced breeding are described in the literature, leading to poor success of reproduction when conditions are not permanently optimal.

METHODS: Two groups of 15 brood fishes, 10 females and 5 males were placed in two 100-m^2 ponds, one covered with a greenhouse, the other uncovered. During one year, individual aspect and diameters of oocytes were checked monthly by means of ovarian biopsy, while males sexual maturity was evaluated by sperm emission during a gentle stripping. Animals were weighed and measured and females were blood sampled for further analysis of vitellogenin plasma levels. An Enzyme Immuno-Assay was set up for *Piaractus brachypomus* vitellogenin and used in this study. Six females, three of each group, found with post vitellogenic oocytes were hormonally induced for reproduction. These females were used for a second induced breeding as soon as their modal oocyte diameters reached again their initial values. Viability of eggs and larvae were registered in all cases.

RESULTS: High variations in the oocyte diameter distributions and plasmatic vitellogenin levels were observed in both groups throughout the year. Oocyte recruitment and growth were observed all year round without periods of total sexual inactivity even during the cold and dry season, from May to September. Temperatures in both ponds differed to some extents while differences in oocyte diameter distributions between groups were not significant. After induced breeding, females developed a new batch of mature oocytes in about 10 weeks and responded positively to a second hormonal induction. Eggs fertilization and hatching rates as well as size of 24h-old larvae were equivalent in the two successive reproductions.

CONCLUSION: At this latitude, oocyte distributions compatible with successful induced breeding were observed during the warm and rainy season from November to March. Nevertheless, extending the reproductive season should be possible as indicated by the presence of mature oocytes throughout the year. However, limiting water temperature fluctuations by pond covering was not sufficient to extent substantially the reproductive season. Further studies on temperature, photoperiod and their interactions will be conducted to extend the period of sexual maturity. Females were found to be able to recover their full sexual maturity in less than 10 weeks after induced breeding during the warm season.

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Senegalese sole (*Solea senegalensis*) vitellogenin: Purification and development of an enzyme-linked immunosorbent assay (ELISA)

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BACKGROUND: Vitellogenin (VTG) is the major yolk protein precursor in fish. In female, it is well-established that high plasma VTG levels coincide with the period of most pronounced growth of the oocyte. Thus, plasmatic VTG is a direct indicator of oocyte growth and its quantification an important tool to understand the oogenesis process. The Senegalese sole (*Solea senegalensis*) has become a priority species for the diversification of European aquaculture. Although the reproduction of wild-caught soles is feasible in captivity, the descendants (F1 generation), are unable to produce eggs or show a marked reduction in its spawn frequency and quality. This fact is currently limiting the establishment of a captive reared broodstock in this species. The development of a VTG ELISA would be a useful tool to study the reproductive dysfunction observed in the Senegalese sole and thus, helping to establish a suitable protocol for its successful management in captivity.

METHODS: Synthesis of VTG was induced in male Senegalese sole by 7 intramuscular injections of 17β-estradiol (2 mg E_2 kg^{-1} bw) given every other day. VTG was further purified from plasma by selective precipitation (EDTA+$MgCl_2$) followed by anion exchange chromatography on a FPLC system. The suspected VTG was submitted to electrophoresis, and used to raise specific antibodies in rabbits. A F1 broodstock of 2-years old Senegalese soles (sex ratio, 1:1), was monthly sampled from May 2004 to November 2006 to obtain blood plasma to study VTG and sex steroid (E_2 and T measured by EIA) levels. Also, the spawning occurrence among consecutive spawning seasons was studied daily.

RESULTS: A specific and homologous ELISA was developed for the measurement of VTG in plasma of Senegalese sole. The standard curve ranged from 11.4 to 305.2 ng/ml (80-20% of binding), showing a sensitivity, measured at 50% binding, of 56.8 ng/ml. The ELISA allowed the description of the annual plasmatic profile of VTG in female Senegalese sole. Plasma VTG increased 4-fold from early winter to a peak value in April (2005) or February (2006), just few weeks before the beginning of the spawning period. A second plasma VTG rise was observed at late summer/early autumn. Both steroids showed annual patterns similar to VTG plasma levels. In spite of the annual peaks in plasmatic VTG and sex steroids, which would indicate two possible spawning seasons, only few and unviable eggs batches were observed in spring. It must be mentioned that wild-caught soles two spawning periods have been reported, coinciding in time with the rise in VTG of our F1 broodstock.

CONCLUSION: For the first time, Senegalese sole VTG have been purified by one step chromatography, suitable for the development of a sensitive ELISA. Our results suggest that, the Senegalese sole seem to go through a normal vitellogenesis but with the onset of the spawning season, most (spring) or all (autumn) developing oocytes may fail to undergo a proper final maturation, explaining the lack of spawning success.

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Reproductive disorders in the white mullet (*Mugil curema*) in two estuaries from Northwest Mexico

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BACKGROUND: In Mexico, several coastal lagoons and estuaries receive untreated domestic and industrial discharges which contain complex mixtures of contaminants. The aim of this study was to evaluate potential estrogenic contamination in two estuaries in Northwest Mexico: Urias (highly polluted) and Teacapan (apparently less polluted) through the quantification of vitellogenin transcription together with reproductive parameters of the white mullet (*Mugil curema*) as endpoints.

METHODS: Fish samples were collected from the two estuaries every two months. A partial sequence of the vitellogenin gene of the white mullet was isolated from the liver, and mRNA expression was analysed by conventional and quantitative RT-PCR. Initial PCR amplification for the vitellogenin gene was carried out with the following primers: vtg-F 5' AAR ACC TAT GTG TAC AAG TAT GAG G 3' and vtg-R 5' GTC TTC TTG AKG TTR AGC TG 3', rendering a product of 358 bp. The fragment was sequenced and gene-specific primers were designed (mcVtg-F 5' GTC AGC AGC AAA GTT CTC ATC AGC 3' and mcVtg-R 5' TCA AAC TTG ATG GGA GTC ACA AGC 3') to analyse Vtg by real-time PCR. At the same time, gonad samples were analysed by histology to determine sex ratio, gonadal development, and the reproductive cycle.

RESULTS: The reproductive peak was detected in April in both sites, but the gonadosomatic index was consistently higher in Teacapan. Sex ratios were leaning to the females in both estuaries (3:1), and one intersex gonad and gonadal malformations (such as dark pigmentation and impaired development) were found in Urias. A partial cDNA sequence of the vitellogenin gene of *M. curema* was obtained and deposited in GenBank (AY236420). Vitellogenin transcription was detected in males from both sites during and right after the rainy season, although it was slightly higher in Urias.

CONCLUSION: The information collected in this study suggests potential estrogenic contamination in both estuaries, since vitellogenin was expressed by males at similar levels in Urias and Teacapan. The analysis of reproductive parameters indicates reproductive impairment in fish from Urias possibly due to the complex mixtures of contaminants continuously discharged into the system. The effects of estrogenic contamination were exacerbated during the rainy season. Chemical analyses have revealed high levels of PAHs, chlorine pesticides, Pb, Cr, and Cd in Urias, and high levels of Hg in Teacapan, some of these chemicals have been proved to be estrogenic.

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Reproductive versus somatic growth in teleosts: Estrogen regulation of multiple vitellogenin, estrogen receptor, and growth hormone receptor genes in tilapia

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BACKGROUND: There is overlap of the reproductive and growth axes in teleosts, but the mechanisms by which they interact are largely unknown. Mozambique tilapia (Oreochromis mossambicus) express three types of vitellogenin (Vg), VgA, VgB, and VgC as well as two estrogen receptors (ER), ER-alpha and ER-beta, and two growth hormone receptors (GHR), GHR1 and GHR2. The objectives of this study were to describe the simultaneous and differential regulation of multiple Vgs, ERs, and GHRs by estradiol (E2) in liver and testes of male tilapia and to characterize their expression in females.

METHODS: Mature males were given a single injection of E2 at 5 ug/g body weight of hormone or vehicle alone. After 48 hours, plasma was collected for measurement of hormones and Vg, and liver and gonad samples taken for analysis of gene expression by real-time PCR. Mature female fish also were sampled and compared with untreated males.

RESULTS: Plasma Vg levels and hepatic expression of Vg A-C and ER-alpha genes increased significantly in E2-treated males, whereas E2 reduced gene expression of ER-beta, both GHRs, and insulin-like growth factor-I (IGF-I) in the liver. In the testes, ER-alpha transcripts were significantly increased by E2 while expression of the other genes was not. All three types of Vg transcripts were detected at low levels in the testes, but their expression was not altered by E2. Expression patterns of all genes analyzed in the liver of E2-treated males mirrored those seen in female fish. Transcript levels of ER-beta, GHRs, and IGF-I were higher in the ovary than in the testes. Plasma IGF-I levels were reduced in E2-treated males, but E2 did not alter plasma GH levels.

CONCLUSION: In male tilapia, E2 induced Vg production and hepatic upregulation of three Vg genes concomitant with induction of ER-alpha. In contrast, expression of ER-beta, which is the less abundant isoform in the liver, was not affected by E2. Patterns of gene expression in the liver of E2-injected males were similar to those seen in females, whereas those in the testes were not significantly altered by E2. It is highly likely that E2 induces vitellogenesis while sacrificing somatic growth. The shift of energy utilization away from growth and towards reproduction in the liver may be a key mechanism behind the sexual dimorphism often seen in fish. Estrogen-induced alterations of multiple facets of reproductive and growth regulation in male tilapia provide an excellent model for examining molecular mechanisms of the physiological transit between somatic and gonadal growth in female teleosts.

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Specific binding of exogenous sex hormones and chemical substances to sex hormone-binding globulin (SHBG) in carp (Cyprinus carpio) and rainbow trout (Oncorhynchus mykiss)

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BACKGROUND: Sex hormone-binding globulin (SHBG) is a plasma glycoprotein that able to bind specifically sex steroids. It transports sex steroids in the blood and regulates their metabolic clearance and access to target cells. Therefore, it is accepted that SHBG is one of the key factors that control the effectiveness of sex steroids. However, there is little information about the binding of exogenous sex steroids or chemical substances to SHBG *in vivo and in vitro*.

METHODS: Experiment 1: Using the steroid-stripped serum of rainbow trout (dextran-coated charcoal treatment), binding characteristics of SHBG to various steroids or chemical substances was investigated *in vitro*. After the incubation with various steroids or chemicals, serum samples were applied to gel filtration by FPLC, and the quantity of SHBG-binding steroids and chemicals were measured by ELISA. Experiment 2: Male immature carp were treated with 17β–estradiol (E2) by three different methods (repeated intraperitoneal injection, water immersion and oral administration) for 2 weeks. The serums of the E2-administered fish were applied to FPLC and ELISA analysis to measure the SHBG-binding E2 quantity.

RESULTS: Experiment 1: Androgens (testosterone and 11-ketotestosterone), estrogens (E2 and ethynylestradiol) and progestins (17α–hydroxyprogesterone and 17α,20β–dihydroxy-4-pregnen-3-on) have a high affinity to SHBG. But, the affinity of estrone, cortisol, and bisphenol-A to SHBG was very low or none. Experiment 2: In all three E2 administrations (repeated intraperitoneal injection, water immersion and oral administration), serum E2 concentration of male carp increased significantly. Specific binding of E2 to SHBG was confirmed in male carp treated by water immersion and oral administration methods. However, no E2 binding to SHBG was observed in fish treated by repeated intraperitoneal injection.

CONCLUSION: Experiment 1: Tested all native sex steroids except estrone bound specifically to SHBG. Ethynylestradiol, the synthetic estrogen, also have a high potency to bind SHBG. However, no specific binding to SHBG was observed in cortisol (non-sex steroid) and bisphenol-A (environmental estrogen). These results suggest that highly active sex steroids have a potent to bind SHBG. Experiment 2: Specific binding of E2 to SHBG *in vivo* was observed in fish treated with E2 by water immersion and oral administration, there was no binding in E2-repeated injections. These results indicated that continuous exposure of exogenous sex steroids for fish induce the specific binding of them to SHBG in blood.

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Effect of xeno-(estrogens) on zebrafish P450c17 (C17, 17 alpha-hydroxylase/17, 20-lyase) mRNA and protein expression in gonadal tissue

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BACKGROUND: Cytochrome P450c17 is a critical enzyme for the biosynthesis of gonadal steroids in vertebrates. Recent progress have been made with respect to the cloning and sequencing of this gene in zebrafish. However, little is known about the expression and localisation of this enzyme in the gonads as well as its regulation by (xeno)-hormones. The first objective of this study was to determine the expression and the cellular localisation of P450c17 in ovarian and testicular tissue in adult zebrafish. The second objective was to investigate the effect of (xeno)-hormones (estrogen and androgen) on its expression at both mRNA and protein levels.

METHODS: Adult zebrafish were exposed to either 17B-estradiol (E2,10 nM), 11B-hydroxyandrostenedione (11-OHA, 10 nM), ethynylestradiol (EE2 10 nM) or nonylphenol (NP 20, 100, 500 µg/L) during 7 days. Gonadal cyp17 mRNA levels, normalised to B-actin, were measured by a specific bDNA assay (N=6 fish/sex/group). Cyp17 protein expression was characterised using a polyclonal antibody raised against two synthetic peptides of the ovarian zebrafish cyp17 sequence (AAP41821). Specificity of the antisera was verified by western blotting and immuno-histochemistry experiments.

RESULTS: We confirmed that P450c17 gene is expressed in gonads of non-treated adult fish with a dominant expression within the testis. At the protein level, we further determined that P450c17 is a 47 kDa protein. In the ovary, P450c17 is mainly expressed in follicle cells surrounding stages I and II oocytes. In the testis, positive immuno-reactive cells were detected in interstitial cells, presumably Leydig cells. We showed that exposure to 11-OHA had no effect on cyp17 mRNA level in both ovary and testis while exposure to E2 led to a dramatic decrease of the testicular expression of cyp17 at both mRNA and protein level. Interestingly, exposure of male fish to the xeno-estrogens EE2 and NP also decrease the testicular cyp17 transcription although the effect of NP was slight as compared to the natural and synthetic estrogens.

CONCLUSION: For the first time, immuno-localization of the steroidogenic P450c17 enzyme is documented within the ovarian and testicular tissue in a cyprinid fish species. Additionally, we demonstrated that E2 as well as xeno-E2 negatively affected testicular steroidogenesis by inhibiting Cyp17 expression at both gene and protein level. This study provides new information concerning the molecular mode of action of (xeno)-estrogens on the steroidogenic pathways which rise further question about the endocrine and physiological consequences for organisms. Given that steroidogenic enzyme are known to be expressed in extra-gonadal tissues, additional works are in progress to characterize and to localize the cyp17 expression within the central nervous system.

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Effects of cortisol and 11-ketotestosterone on testicular development and sperm quality in male spotted wolffish

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BACKGROUND: Farming of spotted wolffish (*Anarhichas minor*) is constrained by variable sperm production of unpredictable quality, requiring maintenance of large numbers of brood-stock males, and a resulting in poor utilisation of hatchery facilities. Plasma androgens (11-ketotestosterone; 11-KT) that direct testicular development are low in spotted wolffish, compared to other fish species, possibly as a result of physiological stress associated with a suboptimal farming environment. Effects of stress are usually mediated by the hormone cortisol, and we have investigated the interactions between cortisol and androgens in the reproductive development of male spotted wolffish.

METHODS: The effects of cortisol and 11-KT on sperm production (sperm volume, spermatocrit, sperm motility evaluated by CASA, pH and ionic composition of seminal fluid) were investigated. Male spotted wolffish were treated with cortisol, 11-KT, or combinations of both, and sperm characteristics of treated groups were compared with those of sham-treated controls.

RESULTS: Cortisol treatment reduced the proportion of males that produced sperm (50 v 85%), whereas 11-KT treatment gave an increase in motile sperm cells. There were strong negative correlations between the concentrations of Na^+ and Mg^{2+} ($R^2 = 0.91$), and Na^+ and Ca^{2+} ($R^2 = 0.86$), in seminal fluid. Further, there were trends towards higher concentrations of Mg^{2+} and Ca^{2+}, and a lower concentration of Na^+, in seminal fluid of fish treated with cortisol (alone or combined with 11-KT) than in controls and those treated with 11-KT.

CONCLUSION: The results indicate that both the stress hormone cortisol and the male sex steroid 11-KT have an influence upon sperm production and seminal fluid characteristics in farmed spotted wolffish.

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Regulation of androgen and estrogen receptor subtypes by gonadal steroids in goldfish (*Carassius auratus*)

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BACKGROUND: The gonadal steroids, estrogens and androgens, are important regulators of reproduction and development. Specific androgen and estrogen receptors mediate their action in the gonads and other tissues. Three estrogen receptor (ER) subtypes and one androgen receptor (AR), which belong to the nuclear receptor super-family, have been demonstrated in goldfish. These receptors act as inducible transcription factors and are regulated by various hormones. The present study provides evidence that the expression of AR and ER subtypes are under the regulatory control of the gonadal steroids in goldfish.

METHODS: Goldfish at different stages of maturity (early-recrudescence and mid-recrudescence) were injected with various doses of estradiol, testosterone, or an endogenous non-aromatizable androgen, 11-ketotestosterone, followed by removal of liver and gonad tissue after 12, 24 and 36 hours of treatment. AR and ER subtype expression were investigated by means of quantitative real-time PCR, semi-quantitative RT-PCR and Western blotting.

RESULTS: Treatment with estradiol significantly increased expression of ER subtypes in the liver, testis and ovary in a dose- and time-related manner. In contrast, androgen treatment of early-recrudescent fish, down-regulated ER expression in female tissues, without having effect in male goldfish. Treatment with testosterone in mid-recrudescent goldfish increased the expression of ER in the ovary while 11-ketotestosterone had no effect. The observed seasonal response is likely due to increased aromatase (Cyp-19a) expression in mid-recrudescent females, allowing testosterone to be converted to estradiol. Treatment with androgens reduced AR expression in the male liver without effecting AR transcript level in female liver. Treatments with androgens increased the expression of AR in the male testis. In the ovary, however, treatment with 11-ketotestosterone decreased AR expression, while testosterone was without effect.

CONCLUSION: The findings demonstrate that expressions of ERs and AR are under complex regulation by gonadal steroids in goldfish. The extent of regulation is dependent on receptor subtype, season and reproductive maturity, dose, type of steroid administered, sex, and tissue. Seasonal variation could, in part, be due to variation in aromatase (Cyp19-a) expression. The observed changes in AR and ER subtypes in the liver and gonads have important implications in terms of control of vitellogenesis and gonadal development in goldfish. The results provide a framework for better understanding of steroid receptor regulation and hormonal control of reproduction in goldfish and other vertebrates.

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Identification and expression of nuclear progesterone receptors in zebrafish

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BACKGROUND: The nongenomic actions of steroids have been recognized and generally accepted recently. However, the identities of the receptors that mediate the actions of the steroids have been debated over the years. Some of the studies have suggested that nuclear receptors might mediate part of the non-genomic steroid actions. Progestin induced oocyte maturation in fish is one of the classical examples of non-genomic actions of steroids. In the present study, we identified the nuclear progestin receptors and examined expression of the receptors in oocytes and various tissues in zebrafish.

METHODS: The nuclear progestin receptors were identified by Blast searching of zebrafish genomic databases and the EST database. The identified EST clones were obtained and analyzed. The expression of the receptors was examined in oocytes and various tissues using RT-PCR and immunohistochemistry.

RESULTS: We have identified and localized three nuclear progestin receptor (PR) isoforms in zebrafish— PR-B, PR-A1 and PR-A2. The three unique cDNAs identified in this study had different transcription initiation sites encoded in three separate start codons (Met), products most likely created by different promoters and alternate splicing of same gene. The longest PR isoform, PR-B, had 618 amino acid residues containing the five typical steroid receptor domains. Compared to PR-B, PR-A1 and PR-A2 respectively lack 110 and 197 additional amino acid residues in the variable region (A/B domain) of the receptor at the N-terminus. The ligand binding domain of the zebrafish PRs shares high homology (68-83% aa identity) with known PRs of other vertebrate classes, and low homology (29-52% aa identity) with other steroid receptors including GR, AR and ERs of zebrafish. Tissue/cell specific expression of PRs was determined by Western blotting and immunocytochemistry using PR specific antibodies, and RT-PCR using isoform specific primers. The PR-B and A1 transcripts were relatively abundant in the ovaries, testis and brain, and scarce in the intestine and gills. PR-A2 transcript was expressed primarily in immature oocytes, while the transcript was barely detectable in most of tissues tested including brain, mature ovaries, testis, gill and intestine. In ovaries, the transcripts and proteins of PRs were produced predominantly in follicle cells surrounding the oocytes, whereas no signals were detected in denuded oocytes.

CONCLUSION: The multiple forms and different expression of PRs suggest multiplicity of progesterone's role in a number of target tissues in zebrafish. No signal of PRs in denuded oocytes suggests that nuclear PR is not involved in oocyte maturation and nongenomic action of progestin within the oocyte in zebrafish.

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Estrogenic activity of nonylphenol in marine fish, *Hexagrammos otakii* during oocyte development by evaluating sex steroid levels

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BACKGROUND: Nonylphenol (NP) is estrogenic in various aquatic animals. This compound has been found to be widely dispersed in the environment, including in waters, aquatic sediments, and groundwater. In many experiments with fish, estrogenic activity of NP mostly has focused on freshwater species using vitellogenin assay during vitellogenic stage. In this study, we investigated the estrogenic activity of NP on oocyte developments of marine fish, *Hexagrammos otakii,* using steroid hormone assay: measurement of estradiol-17β (E_2), testosterone (T) and 17α,20β-dihydroxy-4-pregnen-3-one (17α20βP). This species, known to be multiple spawner for the spawning period, inhabits coastal waters of Korea, Japan and China.

METHODS: The experimental fish were collected at the coastal waters of Oryukdo, Busan, Korea during the spawning period (November-December). The vitellogenic (0.80-82, 0.92-96 and 1.08-12 mm diameter) and fully matured oocytes (1.90-95 mm diameter) were *in vitro* exposed to NP at different concentrations (0.1, 1, 10 and 100 ng/ml) with or without 50 IU HCG (human chorionic gonadotropin). After 36 or 48 h incubation, the productions of E_2, T and 17α20βP were measured by radioimmunoassay. At the same time, we checked its histological observation to see its developmental status.

RESULTS: The levels of sex steroid were calculated to the ratio of E_2/T and E_2/17α20(OHP, as a parameter of estrogenic activity due to altered steroid levels. NP (0.1, 1 and 10 ng/mL) has estrogenic activity by increasing E2/T ratio in the oocytes of 0.80-82 and 0.92-96 mm. In the oocytes of 1.08-12 mm, which were fully vitellogenic stage, NP increased E2/T ratio only at 100 ng/mL. In the oocytes of 1.90-95 mm, NP increased E2/T ratio at 0.1, 10 and 100 ng/mL without HCG and increased E2/T ratio only at 100 ng/mL in the presence of HCG. NP also increased E2/17(20(OHP ratio at all concentrations tested (0.1, 1, 10, and 100 ng/mL) without HCG, and decreased E2/17(20(OHP ratio at 0.1 and 1 ng/mL in the presence of HCG.

CONCLUSION: In the stage of vitellogenic oocytes, NP increased E2/T ratio with oocyte of 0.80-82 and 0.92-96 mm. In the final oocyte maturation stage, NP increased E_2/T and E_2/17α20βOHP ratios without HCG. However, NP decreased E_2/17α20βOHP ratio at concentrations of 0.1 and 1 ng/mL in the presence of HCG, implying NP inhibition of gonadotropin-induced maturation. Taken together, these results suggest that NP exhibited estrogenic activity in ovarian development of *H. otakii*.

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Survey of contaminations by endocrine disrupting chemicals (EDCs) using wild grey mullet in Korea and Japan, and the cloning of estrogen receptor gene as new biomarker for effect evaluation of EDCs

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BACKGROUND: Some chemical compounds have estrogenic effects on aquatic organisms, and cause endocrine disruption in aquatic animals. However, the actualities of contamination by endocrine disrupting chemicals (EDCs) in coastal waters are not well-investigated in East Asia where industrialization is rapidly advancing. Thus we carried out the monitoring of the contamination of EDCs in coastal waters of Korea and Japan using wild grey mullet (Mugil cephalus). Moreover, the cloning of the estrogen receptor α (ERα) cDNA of grey mullet was performed in an attempt to develop a new biomarker for effect evaluation of EDCs.

METHODS: The influence of EDCs on grey mullet collected from five sites (Yeosu, Jeju, Tongyeong, Busan, and Ansan) in Korea and three sites (Nagasaki, Omuta, and Hakata) in Japan was evaluated by measurement of serum vitellogenin (VTG) concentration and histological analysis of the gonad. Serum VTG concentration was measured by Enzyme-Linked Immunosorbent Assay (ELISA) or Single Radial Immunodiffusion (SRID). The ERα cDNA fragment was cloned based on the PCR method. The cDNA encoding ERα of grey mullet was characterized, and the structure was compared with that of other fish.

RESULTS: In four sampling sites in Korea (Yeosu, Tongyeong, Busan, and Ansan) and in the two sampling sites in Japan (Nagasaki and Hakata), serum VTG concentrations of some immature and male grey mullet were detected at levels more than 1.0 μg/ml. Based from our previous studies, we consider that the value of 1.0 μg/ml or more is abnormal level. Testis-ova was observed in some individuals collected from Tongyeong, Busan, and Ansan in Korea and from Omuta in Japan. The obtained cDNA fragment of ERα in grey mullet was 2294 bps long, but the N-terminal region was unidentified in the deduced amino acid residues of this cDNA.

CONCLUSION: The results of our survey in Korea and Japan show that individuals with abnormal levels of VTG concentrations or with testis-ova existed in some areas of both countries, and these individuals were influenced of environmental EDCs. However, there was no correlation between having high concentration of serum VTG and the appearance of testis-ova. It is assumed that the mechanism of appearance of these two abnormalities is different. The deduced amino acid residues of ERα in grey mullet had a highly conserved region, DNA binding domain and ligand binding domain. We are currently preparing expression analysis of this gene. The results of the gene expression analysis in grey mullet will be applied for effect evaluation of EDCs.

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Steroid synthesis during ovarian follicle maturation in the Siberian sturgeon *Acipenser baerii*

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BACKGROUND: The role of steroid hormones during last phases of follicular ovarian maturation in the Siberian sturgeon has been poorly studied. The present works aims to identify the major metabolites produced by follicles during the maturation process in order to identify the potential maturing inducing steroid (MIS) for the species.

METHODS: Thirty follicles from four females (10 years old) with advanced follicle maturation (polarization index: from 5.1 to 10%) were incubated in SIS culture media (pH 7.95, OP=275 mosmol. kg^{-1}) with ^{3}H-17-hydroxy-4-pregnene-3,20-dione (~6µCi) and with or without 1.5 µg ml^{-1} of purified LH from A. baerii. Metabolites produced were analysed by TLC, HPLC and oxidation. The in vivo secretion of one of the metabolites identified (17,20β-dihydroxy-4-pregnen-3-one, 17,20ßP) was measured by RIA in plasma after stimulation by 5 µg kg^{-1} b.w. of GnRHa ([D-Ala^{6}, Des Gly^{10}]-LHRH Ethylamide). Maturation competence after GnRHa stimulation was tested with germinal vesicle break down (GVBD).

RESULTS: The pattern of metabolites analysed was similar for the four females assayed. The main metabolites observed in both control and stimulated follicles were: androstenedione, 17,20β,21-trihydroxy-4-pregnen-3-one (20βS), 17,20β-dihydroxy-4-pregnen-3-one (17,20βP) and the 17,20α-dihydroxy-4-pregnen-3-one (17,20αP). When progestagens were compared in basal and stimulated conditions the stronger radioactivity co-migrated and co-eluted with 20ßS. This steroid was further identified by oxidation. The *in vivo* assays showed that 17,20ßP was not always detected by RIA in the plasma of females that matured (GVBD) after GnRH stimulation. Lamentably, we had no the possibility to explore changes in circulating 20ßS.

CONCLUSION: The fact that the maturing follicles (migrating nucleus) of the four females assayed produced as major progestin the 20ßS and that 17,20βP did not increased clearly in females that matured after GnRH treatments suggest that 20ßS could act as the main mediator of oocyte maturation in follicles of the Siberian sturgeon as it has been shown in Sciaenidae fish.

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Evidence of endocrine disruption in wild roach population (*Rutilus rutilus*) in the Seine river

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BACKGROUND: Environmental pollutants, such as industrial and agricultural chemicals, heavy metals, drugs and products with hormonal activity may disrupt reproduction of aquatic wildlife such as fish.

METHODS: In the present study, the estrogenic response of wild roach (*Rutilus rutilus*) from 11 sites of the Seine river, exposed to complex mixtures of endocrine disrupting chemicals, was measured through plasmatic vitellogenin concentrations, plasma levels of sex steroids, aromatase levels in the brain and ovary, and gonadal histology.

RESULTS: Intersex fish, characterized by oocytes in the testes, occured at the prevalence of 4-5% of fishes. The number and pattern of oocytes within testicular tissue in intersex roaches was found to vary greatly. Severe intersexuality may decrease the fertility of the individual. The presence of plasmatic vitellogenin, a specific female protein, in male fish, result from an induction by xeno-estrogens. 90% of the studied sites showed vitellogenin amounts in male fish higher than 100 $ng.ml^{-1}$. The concentrations of sex steroids measured in fish plasma, depend on the sex of the fish, and its sexual maturity status. A positive correlation was observed between concentrations of vitellogenin and estradiol in females. The sex ratio was found to be significantly altered, indicating feminization of the roach population in the Seine river. The chemicals involved in endocrine disruption have been identified in bile, using a biological test, the YES assay, and chemical analysis. HPLC profiles suggested both natural and synthetic hormones as well as industrial chemicals as nonylphenol and ethinylestradiol.

CONCLUSION: In conclusion, many endocrine disturbances were observed in fish from all sites, suggesting an aquatic contamination by xeno-estrogens which interfered with immune and reproductive systems of fish. The study was supported by an european programme, interreg III.

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Sex steroids and steroid receptors in the male sea lamprey, Petromyzon marinus

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BACKGROUND: As part of the nuclear receptor superfamily of transcription factors, the steroid receptors have received much attention due to both their importance as a key evolutionary innovation within the vertebrate lineage, and in regulation of development and reproduction. The lamprey is one of the earliest-evolving extant vertebrates that has a functional hypothalamic-pituitary-gonadal axis that coordinates and controls reproduction. Studies of steroid receptors, their characteristics, and their functions in lampreys can provide us with further understanding of what such systems were like in the earliest of vertebrates.

METHODS: We combined classical binding experiments and immunoassays with molecular techniques to characterize androgens, progestogens, and their receptors in lampreys. Changes in steroid concentrations and receptor expression were investigated in response to injections of lamprey gonadotropin-releasing hormones (GnRHs). In vivo experiments using time-release steroid implants were performed to characterize steroid (and receptor) function in parasitic-phase and immature adult lampreys.

RESULTS: Steroid levels and the number of receptor binding sites change with stage of maturation and after injections of GnRHs. Steroids are found in much greater concentrations in testicular tissue than in blood plasma. Androgens and progestogens have multiple effects on male lamprey reproductive physiology, including effects on maturation, secondary sex characteristics, and pheromone production.

CONCLUSION: As one of only two extant Agnathan lines and as a vertebrate taxon that evolved over 500 million years ago, lampreys are a key group in which questions regarding the timing of vertebrate innovations, such as the steroid-receptor systems, can be examined. The unusual ligands that bind to lamprey steroid receptors may represent early hormones in vertebrate evolution. The role that the gonadal steroids and receptors play in regulating reproduction in lampreys may yield clues to the evolution of this important vertebrate innovation.

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Effects on steroidogenesis after exposure of previtellogenic Atlantic salmon (Salmo salar) oocytes to dioxin-like PCB congeners 126 and 77

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BACKGROUND: The effects of dioxin-like polychlorinated biphenyls on development of reproductive organs are not well investigated. PCBs are known to act as hormone mimics, either as agonists or antagonists. Effects of PCBs on genes involved in key and rate limiting steps in steroidogenesis are of interest.

METHODS: : Previtellogenic oocyte samples from Atlantic salmon were collected after anaestasia and cultured in vitro according to the floating agarose method. The samples were cultured in media containing essential nutrients and the test chemicals at different concentrations. The experiment was conducted in replicates with PCB126 (0.05,0.01 and 0.001 uM) or PCB77 (10, 1 and 0.1 uM) concentrations. Oocyte samples were collected on days 0 (control), 3, 7 and 14 after exposure. At each sampling time, 3 groups of tissue were collected for total RNA isolation, fixed in formaldehyde solution for histology and snap frozen in liquid nitrogen for immunochemical analysis. The effects on gene expression pattern were analyzed for gene expression (Steroidogenic acute regulatory StAR, CYP1A1, P450 side chain cleavage and CYP19 aromatase alpha) using validated real time PCR. Aromatase activity levels will be analyzed using tritiated water release assay method. The effects on protein expression patterns will be determined using ELISA and western blotting methods with specific StAR and CYPscc antibodies. Tissue fixed in formaldehyde will be sectioned and used for evaluation of morphological changes in localization of cells expressing the StAR and CYPscc with our specific antibodies using the immunohistochemical methods.

RESULTS: Using real-time PCR analysis, an up regulation of CYP1A1 was observed at the highest concentration on day 7 and 14 when exposed to PCB77. Steroidogenic acute regulatory protein gene expression was up regulated at the lowest exposure to PCB77 whilst down regulated at high exposure on day 7. P450scc was down regulated at all concentrations on day 7 when exposed to PCB77. CYP19 aro alpha; showed similar gene expression patters to StAR on day 7. CYP1A1 was down regulated at the low and middle concentrations of PCB126 while it was up-regulated at the highest concentration. In contrast to the PCB77 exposures the gene expression profiles of P450scc and StAR showed similarity when exposed to PCB126. CYP19 aromatase alpha; showed up regulation on day 3 at middle and high concentrations and at the highest concentrations on day 14.

CONCLUSION: Gene expression analysis has shown differential time- and concentration dependent transcriptional changes in the StAR, P450scc, CYP1A1 and CYP19 genes. Aromatase assay, protein analysis, and immunohistochemistry are currently ongoing in our lab. We hypothesize that differences in the profile of these key steroidogenic genes and protein responses, including over or down expression in exposed oocytes, will be novel, indicative, prognostic and predictive of chemical susceptibility and adverse endocrinological, and reproductive effects in exposed fish.

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Multiple estrogen receptor genes in Atlantic salmon (salmo salar)

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BACKGROUND: Estrogen receptor (Esr) has been isolated and characterized from lamprey to mammals. Two Esr subtypes have been identified in several vertebrates, denoted Esr1 (also called estrogen receptor α, ERα) and Esr2 (ERβ). Many fish species have two Esr2 subtype, named Esr2a (ERβa, Erβ1, ERγ) and Esr2b (ERβb, ERβ2). Esr1 differs from Esr2 in its ligand binding affinities to both natural and synthetic estrogens, transactivation properties, tissue distribution and cofactor recruitment in estrogen signalling. It has been proposed that estrogen signalling in mammals often occurs as a balancing act between Esr1 and Esr2, where Esr1 often plays the role of an agonistic principle whereas Esr2 is antagonistic.

METHODS: Variants of *esr* transcripts were sequenced as cDNAs using rapid amplification of cDNA ends (RACE). Transcription was monitored using semi-quantitative rtPCR using expression of ribosomal protein E27 as an internal control. Genomic sequences was obtained from cloned PCR fragments. DNA binding was monitored by electrophoretic mobility shift assay (EMSA) and activation in living cells monitored as GFP-Esr fusion proteins expressed in Cos7 cells. Protein-protein interactions was detected using yeast two-hybrid assays.

RESULTS: The current paper reports the isolation and cDNA cloning of Esr2a and Esr2b from Atlantic salmon. We have also isolated a third cDNA sequence, apparently a expressed pseudogene, resembling other *esr1* (ERα) cDNAs, but clearly different from the previously reported sequence from Atlantic salmon and therefore named *esr1b*. In addition, there may be two genetic variants of this pseudogene, both expressed in different tissues. The complexity is further increased by the apperance of several splice variants of both *esr1* and *esr2*. The over all tissue distribution of *esr1* transcripts were similar to *esr2*, and the expression level of the *esr1* genes weres notably higher in brain and liver than in other tissues examined. Expression of *esr1* in the liver of juvenile Atlantic salmon, treated by estradiol-17β (E2) and 4-nonylphenol (NP) was clearly induced (3 fold). In contrast, the *esr2* mRNA levels was not induced, but showed a marked reduction of gene expression in the liver.

CONCLUSION: Esr variants are expressed from four different genes in Atlantic salmon, two variants of *esr1* (a and b) and two variants of *esr2* (a and b). The *esr1b* will probably only give a truncated protein. Only *esr1* transcription was induced in juvenile animals after treatment with E2 or NP. Esr1 protein was present in both cytoplasma and nucleus and was found in nuclear specles after induction with E2. Esr1 and Esr2 formed homo- and hetero-dimers, but only after induction with E2.

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The regulation and potential modulation of the arachidonic acid pathway in zebrafish ovaries

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BACKGROUND: 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. There are several enzymes within the arachidonic acid pathway that contribute to the formation of PG, including phospholipases and their activating proteins, COX 1 and 2, as well as PG synthases. We have previously shown that ovarian PG levels rise around the time of ovulation and that fish exposed to PG inhibitors exhibit reduced levels of egg production. The objective of this study is two-fold. The first objective is to investigate the hormonal regulation of PG levels in the zebrafish ovary and secondly, we wish to investigate the mechanisms by which selected environmental compounds reduce egg production in exposed fish.

METHODS: We utilized in vivo, short-term exposure regimes in order to expose sexually-mature females or breeding groups of zebrafish to various compounds. The pharmaceuticals tested were non-steroidal anti-inflammatory drugs (NSAID), ethynyl estradiol, or fluoxetine; the sex steroids tested were maturation-inducing steroid (17a,20b-P) and estradiol. For objective 1, fish were exposed to compounds for 24 to 96 hours, while objective 2 fish were exposed for 7 days. The mRNA levels of selected genes of interest within the AA pathway were quantified by real-time PCR using Sybr Green methodologies. Egg production data and ovarian estradiol and PG levels were also included in the studies, as were genes of interest in the steroid biosynthetic pathway.

RESULTS: Our in vivo studies demonstrated that female zebrafish exposed to estradiol had significantly reduced mRNA levels of cytosolic phospholipase A2 (cPLA2), COX1, and PGE2 synthase, while 17a,20b-P only reduced COX1 and PGE2 synthase levels. The NSAIDs indomethacin and diclofenac tended to reduce the mRNA expression of PGE2 synthase and cPLA2. Two other pharmaceuticals, fluoxetine and ethynyl estradiol did not impact aspects of the AA pathway but did significantly reduce the expression of selected genes within the steroid biosynthetic pathway.

CONCLUSION: It is likely that ovarian PG production in the zebrafish is regulated by sex steroids. As expected, the mechanisms by which NSAIDs reduce egg production is due to the reduced levels of PG and this reduction may involve both enzymatic and mRNA inhibition. The reduced egg production caused by the drugs fluoxetine and ethynyl estradiol do not seem to involve the AA pathway; rather these drugs exert their actions on the steroid biosynthetic pathway.

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Cloning and characterization of 17β-HSD type 1, 3, 8 and 12 from tilapia

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BACKGROUND: 17β-hydroxysteroid dehydrogenases (17β-HSDs) are remarkably multifunctional enzymes that modulate the synthesis and metabolism of sex steroids in gonadal tissues. So far, twelve types of 17β-HSDs have been identified from vertebrates which showed different substrate specificities, biochemical properties and in particular, tissue distribution. In fish, 17β-HSDs are required for the production of estrogen and androgen. Therefore, different isozymes of 17β-HSDs might play essential roles in the process of sex differentiation and gametogenesis in fish.

METHODS: RT-PCR and subsequent RACE were carried out to clone 17β-HSD type 1, 3, 8, 12A and 12B cDNAs from tilapia gonads. These 17β-HSDs were also identified from medaka, tetraodon and fugu genomes by *in silico* analysis. Phylogenetic analysis was carried out based on the amino acid sequences of four types of 17β-HSDs from vertebrates. Tissue distribution analyses of four types of tilapia 17β-HSDs were carried out by RT-PCR and Northern blot using various tissues from adult fish. Enzymatic activities of four types of 17β-HSDs were characterized by thin layer chromatography.

RESULTS: Four types of 17β-HSDs were cloned from tiapia. Phylogenetic analysis showed that 17β-HSD1, 3, 8 and 12 clustered to different clades corresponding to their mammalian counterpart. Duplication of 17β-HSD12 is a unique phenomenon in teleost fish. Tissue distribution analysis revealed that 17β-HSD1 was exclusivly expressed in the ovary, while 17β-HSD3 was expressed dominantly in the testis. Interestingly, 17β-HSD12B was found to be expressed exclusively in the testis, which is completely different from 17β-HSD12A. The function of 17β-HSD12A is, as its mammalian counterpart, lipid metabolism, and therefore, it is expressed in many different tissues. 17β-HSD8 was ubiquitously expressed. Enzymatic assay indicated that 17β-HSD1 can catalyze the interconversion between estrone to 17β-estradiol and interconversion between androstenedione to testosterone. Similarly, conversion between estrons and 17β-estradiol can be catalyzed by 17β-HSD8. Meanwhile, 17β-HSD3 can efficiently convert the interconversion between androstenedione and testosterone.

CONCLUSION: Like their mammalian counterparts, 17β-HSD1 is ovary specific and is responsible for the synthesis of 17β-estradiol, while 17β-HSD3 is dominantly expressed in the testis and responsible for the production of testosterone. The expression pattern and enzymatic assay of 17β-HSD8 indicate its role in the metabolism and regulation of peripheral steroids. However, the function of the duplicated 17β-HSD12s in fish needs further characterization.

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Effect of sexual steroid implants on the early puberty (precocity) in juvenile male sea bass exposed to continuous light

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BACKGROUND: It has been demonstrated that continuous light (LL) inhibits the early puberty in 1-year old male sea bass, affecting testicular development and endocrine profile of some key hormones of the reproductive axis. The present study is focused on the effects of the exogenous administration of an aromatizable androgen (testosterone, T) and the precursor of a non-aromatizable androgen (precursor of the 11-ketostestosterone, 11-KTp) on the gonadal development of juvenile male sea bass maintained under environmental inhibitory conditions (i.e., continuous light photoperiod).

METHODS: Four month-old male sea bass previously exposed during 5 months to LL or to natural photoperiod received silastic implants containing 100 mg/g of T or 11KTp or blanck. Plasma and tissues were collected at specific times during 4 months after implantation. Plasma levels of T, 11-KT, and pituitary content of gonadotropin releasing factors (GnRHs) and luteinizing hormone (LH) were determined by specific ELISAS. The expression level of glycoprotein alpha (GP alphą, FSH beta, and LH beta-subunits mRNAs were also determined by dot blot.

RESULTS: LL provoked an important reduction of the gonadosomatic index (GSI), plasma 11KT and pituitary LH content respect to the controls (i.e., natural photoperiod). However, exogenous administration of sexual steroids clearly enhanced the plasma steroid levels and partially inhibited gonad development. Besides, those fish exposed to LL that received steroid implants, exhibited a significant rise of pituitary LH levels compared to controls. This suggests that 11 KTp or T administration could revert the inhibitory effects induced by the LL exposureas consequence of a along term increase of LH pituitary levels. Other noteworthy effects of LL on pituitary GnRHs levels and GTH sub-units expression levels were also observed and will be discussed.

CONCLUSION: The exogenous administration of sexual steroids was partially effective for reverting the inhibitory effects of LL on the early sexual maturation. However, the mechanism of action of LL on the reproductive axis still remains to be elucidated.

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Gametogenesis and Gamete Biology (Part VI : Yolk protein deposition and new findings in oocyte maturation)

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Differential synthesis and uptake of dual vitellogenins in Japanese medaka (*Oryzias latipes*)

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BACKGROUND: Recent studies verified the structural and functional multiplicity of piscine vitellogenin (Vg). Two types of Vg protein (VgA/B and VgC) have been discovered in Japanese medaka, however, quantitative variation in circulating Vg levels and modes of ovarian Vg uptake are largely unknown. The objectives of this study were to investigate duality of medaka Vgs with regard to: 1) changes in serum Vg levels during the reproductive cycle, 2) initiation of Vg uptake by oocytes, and 3) induction of Vgs by estradiol-17β (E_2).

METHODS: Females were reared in outdoor ponds where water temperature was ~12 to ~18(C during the pre-spawning (PRS) phase, ~18 to ~27(C during the spawning (S) phase, and ~5 to ~9(C during the post-spawning (POS) phase. They were sampled at irregular intervals to obtain blood and ovarian tissue. Males were acclimated to 16L:8D photoperiod at ~24(C and were: 1) exposed to waterborne E2 (0.1 (g /L) or control water for various durations (0.5 - 6h) followed by 24h exposure to control water before being sampled, or 2) exposed to E2 (0.1- 10 (g /L) or control water for 24h and immediately sampled.

RESULTS: Ratios of dual Vg levels (VgA/B:VgC) were approximately 2:1 and 7.5:1 at the beginning and end of the PRS phase, respectively. The latter ratio was maintained or slightly increased (~10:1) during the S phase, and then fell to ~2:1 during the POS phase. Immuno-cytochemistry using antisera against VgA/B (a-VgA/B) or VgC (a-VgC) revealed a-VgC immunoreactive granules in pre-vitellogenic oocytes (cortical alveolus stage) but no immunoreactivity was found by staining with a-VgA/B. Both antisera stained yolk globules in vitellogenic oocytes. Although VgC alone was detected in the 2h E2 exposure group, after the 4h and 6h E2 exposures both Vgs were detected at a fairly constant VgA/B:VgC ratio (~2.5:1). Both Vgs were detected in male fish from all of the E2 dosage groups except the control and the VgA/B:VgC ratios were fairly constant (~2:1).

CONCLUSION: This study verified variation between distinct reproductive phases in circulating ratios of dual Vgs in female medaka. Phase specific differences between plasma levels of VgA/B and VgC (VgA/B:VgC ratios) likely reflect different rates of hepatic synthesis and of uptake by oocytes for each form of Vg. This is the first report on selective uptake of VgC into oocytes during the cortical alveolus stage, indicating specialized mechanisms for endocytosis of each form of Vg.

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Insemination too soon after ovulation induced by a GnRH analog may lead to triploid offspring in rainbow trout, *Oncorhynchus mykiss*

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BACKGROUND: Some previous studies have suggested that development rate in rainbow trout may be lower when insemination is performed on the day of ovulation rather than 2 or 3 days later. Besides, in similar conditions, we have occasionally observed a high proportion of triploid fry in some offspring. Such observations raise the question of possible incomplete maturity of just ovulated eggs. Moreover, hormonal induction, which allows predicting accurately the time of ovulation, might also increase the risk of premature insemination.

METHODS: Experiments were carried out on 2 years-old fish from a fall-spawning strain. Four weeks before the expected peak of spontaneous ovulation known for this strain, 20 females were injected with a sGnRH analog (D-Arg6 sGnRH, 20 µg/kg), and 10 females were kept as control. About one half of the eggs from each female was sampled and inseminated as soon as ovulation was detected, and the other half was sampled and inseminated 7 days later. Eggs and fry were incubated at 10°C. Survival rates at various stages were monitored in each batch, up to hatch and yolk sac resorption. At that stage, the rate of morphological abnormalities was recorded, and ploidy level was checked by flow cytometry.

RESULTS: Survival rate at yolk sac resorption exhibited large variations in all batches, originating either from experimental or control females, and either from D0 or D7 inseminated eggs. However, it was significantly improved in fry from induced females when insemination was performed at D7 instead of D0. Moreover, 65, 80 and 90% of triploid fry were found in egg batches from 3 induced females inseminated at D0, whereas no triploid fry could be found anymore in batches from the same females inseminated at D7. In contrast, no triploid fry was observed in batches from control females inseminated at D0, but 1 control batch inseminated at D7 showed 20% triploid fry. Concerning the rate of morphological anomalies, no significant differences were found between any groups.

CONCLUSION: The present data show that insemination of rainbow trout eggs too soon after GnRHa-induced ovulation can lead in some offspring to a high proportion of triploid fry. These are not observed anymore when eggs from the same spawns are inseminated a few days after ovulation. The main interest of sGnRHa ovulation induction in rainbow trout is to synchronize ovulations in fish close to final stages of oocyte maturation, but still at various maturity stages. Triploid fry observed thereafter may result from abnormal fertilization of oocytes induced to ovulate before full completion of the maturation process in some females. In current practice, it can be recommended to wait a few days between induced ovulation and insemination to prevent the uneven occurrence of undesired triploid fry.

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Controlled accumulation of multiple vitellogenins into oocytes during vitellogenesis in the barfin flounder, *Verasper moseri*

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BACKGROUND: Involvement of two forms of vitellogenin (VgA and VgB) in the regulation of egg buoyancy has been elucidated in some marine teleosts spawning pelagic eggs. However, the accumulation by growing oocytes of these two forms of Vg and of the third type of Vg (VgC) has not previously been investigated because type specific assays for the different forms of Vg and their derived yolk proteins have heretofore been unavailable.

METHODS: Three forms of Vg, VgA, VgB and VgC (phosvitin-less Vg) and their derivative lipovitellin yolk proteins, LvA, LvB and LvC, were purified using combinations of immuno-precipitation and various orthogonal chromatography procedures. Specific antisera were raised against the purified Vgs and Lvs, and type specific enzyme-linked immunosorbent assays (ELISAs) were developed for the proteins. Concentrations and contents of the Vgs in serum and liver, respectively, and contents of the Lvs in ovarian follicles were measured by ELISA in female barfin flounder sampled throughout the year.

RESULTS: The established ELISAs for VgA (510 kDa), VgB (530 kDa), VgC (350 kDa), LvA (430 kDa), LvB (400 kDa) and LvC (350 kDa) were highly sensitive and showed very low cross-reactivity to the other types of Vg or Lv. The Lv contents measured in annually sampled follicles increased through October to February prior to the spawning season in April and May, keeping the proportional ratio (by weight) of LvA : LvB : LvC at about 9 : 15 : 1. In contrast, ratios of VgA : VgB : VgC concentration in serum changed from about 13 : 18 : 1 during active vitellogenesis in October to December to 32:10:1 during the postvitellogenic phase in February and March. A quite different tendency of the Vg ratios, e.g. 12:1:10 by weight in December, was observed for liver Vg contents.

CONCLUSION: Controlled generation of free amino acids (FAA), which is regulated by the disparate proteolysis of VgA- and VgB- derived yolk proteins during oocyte maturation, is considered to be essential for adjusting egg buoyancy via maturation-associated oocyte hydration in marine teleosts that spawn pelagic eggs. Our quantitative study clearly illustrates the constancy of the ratio by weight of the three Vg-derived Lvs accumulated in growing and postvitellogenic oocytes of barfin flounder. Moreover, our results suggest that this controlled accumulation of multiple Vgs is regulated primarily by rates of hepatic Vg synthesis and secretion and secondarily by mechanisms for receptor-mediated uptake of Vgs into oocytes.

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Characterization of rainbow trout IL-6 and possible relationship with reproductive function

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BACKGROUND: The cytokine, IL-6, is produced by a diverse group of cells including, T-lymphocytes, macrophages, fibroblasts, neurons, endothelial and glial cells. In mammals, the pleiotropic effects of IL-6 include the regulation of various immune and neuroendocrine processes including the control of immunoglobulin production, lymphocyte and monocyte differentiation, chemokine secretion, migration of leukocytes towards sites of inflammation, control of adipose tissue metabolism, development of the central nervous system, angiogenesis, as well as the synthesis of steroids in the gonads. However, very little is known about IL-6 in nonmammalian vertebrates.

METHODS: Primary cell cultures of trout macrophages were stimulated with *E. coli* lipopolysaccharide (LPS) and the resulting RNA was used for suppression subtractive hybridization (SSH) to look for LPS regulated genes. One partial cDNA obtained from SSH was similar to IL-6. Rapid amplification of cDNA ends (RACE) was used to obtain the full-length sequence and expression in various tissues including ovaries, follicles and follicle walls were analyzed by Northern blot, reverse transcriptase (RT-PCR) and quantitative PCR (QPCR).

RESULTS: The full sequence of the trout IL-6 homolog obtained from RACE was 1180 nucleotides encoding a predicted protein of 219 amino acids. While the complete sequence of the trout IL-6 is poorly conserved as compared to mammals (24-28% identity), the trout protein contains a distinct IL-6 family consensus pattern and the predicted characteristic α-helical tertiary structure of IL-6. On Northern blots, the expression of the IL-6 homolog in trout macrophages was highly up-regulated by LPS but not poly (I:C). Using RT-PCR, IL-6 expression was detected in trout spleen, gill, gastrointestinal tract, ovary, testes, and brain; however, the highest transcript levels were detected in the ovary. In more finely resolved QPCR analysis of the ovary, IL-6 expression increased significantly by the end of oocyte maturation and was highest in postovulatory ovaries.

CONCLUSION: Trout IL-6 is highly regulated in macrophages by LPS, and likely has an important immune function. However, the high expression and relationship of IL-6 with ovarian maturation and ovulation in trout, suggests that IL-6 also plays a role in reproduction in fish.

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Expression of membrane progestin receptors α and β in zebrafish

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BACKGROUND: Recently, a unique family of membrane progestin receptors (mPRα, mPRβ, and mPRγ) was identified, which may be responsible for mediating rapid, nongenomic actions of progestins in a variety of target tissues. Our recent results also suggest progestin binds both zebrafish mPRα and mPRβ and induces rapid non-genomic signaling in cells transfected with mPRa or mPRb. However, the biological functions of the receptors and their involvement in non-genomic actions of progestin in oocyte maturation in zebrafish are still unknown.

METHODS: The expressions of mPRa and mPRb were examined in oocytes and various tissues, using RT-PCR, real-time quantitative PCR, immunohistochemistry and immunofluorescence.

RESULTS: The transcripts of mPRα and mPRβ from zebrafish were shown to localize primarily to reproductive tissue and regulate within oocytes. The mPRα and mPRβ proteins were expressed most abundantly in the zebrafish ovary, as detected by Western blotting. Further examination of zebrafish oocytes using immunohistochemistry and immunofluorescence showed that mPR proteins are expressed at or near the oocyte membrane—a likely location for their proposed roles as MIS receptors. Examination of mPRα protein levels in developing oocytes and during the daily zebrafish reproductive cycle showed that mPRα protein levels increase daily in late-stage oocytes until the time of oocyte maturation, and then significantly decrease after ovulation.

CONCLUSION: These results further support the role of mPRα and mPRβ as the MIS receptors in zebrafish.

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17,20(,21-Trihydroxy-4-pregnen-3-one is an oocyte maturation-inducing steroids in protandrous yellowfin porgy, Acanthopagrus latus

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BACKGROUND: Fish oocyte development can be divided into two hormone-dependent phase, oocyte growth and oocyte maturation (OM). After oocyte growth is complete. The oocytes could be responsive to the maturation-inducing steroids (MIS) and complete oocyte maturation. The identity of the MIS in yellowfin porgy, a marine protandrous teleost of aquacultural importance, and its precise physiological role in OM are still unknown. The purpose of the study was to investigate the physiological MIS in yellowfin porgy. The characteristics of the oocyte maturation were also conducted.

METHODS: two-year-old females were injected with 2 successive doses of LHRH analog (10 and 40 (g per kg of fish). The ovarian tissue was obtained at 6-hr intervals for in vitro oocyte maturation test of varies sex steroids. A tritiated precursor, [3H]-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, acetylation reaction and finally recrystallization.

RESULTS: Both 17,20β-dihydroxy-4-pregnen-3-one (DHP) and 17,20β,21-trihydrorxy-4-pregnen-3-one (20βS) are the most effective steroids to induce *in vitro* maturation (e. g. germinal vesicle breakdown, GVBD) in oocytes cultured for either 24 hrs or one min. 20βS has a better potency than DHP in inducing oocyte maturation. 11-dexoycortisol, 17α-hydroxyprogesterone, and 20β-21-dihydroxy-4-pregnen-3-one also could significantly induce oocyte maturation at higher concentrations. The development of the oocyte maturation could be divided into hormone-insensitive stage (insensitive to gonadotropin and MIS), MIS-sensitive (respond to MIS) and spontaneous stage (GVBD in the hormone-free condition), respectively. Significantly levels of tritiated 11-dexoycortisol (a precursor of 20βS) and 20(S but not DHP were biosynthesized from [3H]-pregnenolone on the basis of HPLC and TLC. Similar TLC profiles were obtained with the tritiated products isolated from the HPLC/TLC 20(S fraction and standard 20(S after the acetylation reaction. Constant specific radioactivity of tritiated 11-dexoycortisol and 20(S by recrystallization was obtained in the tritiated metabolites isolated from HPLC fraction.

CONCLUSION: The present study provides the first evidence that 20(S is the MIS in yellowfin porgy. The steroid is produced by ovarian follicles of this species undergoing oocyte maturation and its identity confirmed by chromatography, derivatization, and recrystallization.

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Aquaporin and proteolysis of yolk and ovarian follicles play essential roles in the regulation of oocyte hydration during oocyte maturation in the Japanese eel

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BACKGROUND: The hormonal control of oocyte maturation has been well studied. However, there is only limited information on the mechanisms involved in oocyte cytoplasmic maturation, such as a hydration, lipid coalescence and clearing of the ooplasm, during oocyte maturation. In the present study, we examined the effects of $HgCl_2$ and bafilomycin A1 and the presence of ovarian follicles on the hydration of oocytes in the Japanese eel.

METHODS: Oocytes were obtained from fully matured female eels that were induced by repeated weekly injections of salmon pituitary extracts. For *in vitro* experiments, follicle-enclosed oocytes (follicles) and de-folliculated oocytes (denuded oocytes) were incubated in Leibovitz L-15 medium along with human chorionic gonadotropin (HCG) or 17, 20β-dihydroxy-4-pregnen-3-one (DHP) in the presence or absence of $HgCl_2$ and bafilomycin A1. Changes in water content were elucidated by measuring the follicle diameter. Morphological changes in oocytes were documented by photographs that were obtained using a binocular microscope.

RESULTS: The wet and dry weight measurements of the follicles reveal that water accumulation during oocyte maturation is the major factor contributing to the increase in follicle diameter; further, this suggests that measurements of the follicle diameter can be used as a hydration index. During *in vitro* oocyte maturation, both HCG and DHP caused an increase in the follicle diameter but not in the denuded follicle diameter. Moreover, HCG and DHP also stimulated lipid coalescence and clearing of the ooplasm in the follicles and denuded follicles. Addition of $HgCl_2$ (50, 100 and 200 μM) to the incubation media inhibited HCG- and DHP-induced increase in the follicle diameter in a dose-dependent manner. Inhibition by $HgCl_2$ was reversed by addition of β-mercaptoethanol. Addition of bafilomycin A1 (0.1, 1 and 10 nM) also inhibited HCG- and DHP-induced increase in the oocyte diameter in a dose-dependent manner.

CONCLUSION: The present study indicates that during HCG- and DHP- induced *in vitro* oocyte hydration, aquaporins facilitate water uptake via a water channel, and acidification of the yolk spheres, which activates yolk proteolysis, is essential for the water influx via osmotic mechanisms. Moreover, the present study also indicates that the ovarian follicles in the Japanese eel may play an essential role in HCG- and DHP-induced oocyte hydration.

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Effect of C21 steroids on germinal vesicle break down in sturgeon follicles *in vitro*

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BACKGROUND: In fish, maturation inducing steroids (MISs) regulate the resumption of oocyte meiosis and induce germinal vesicle break down (GVBD). Presumably, MIS belongs to the C21 steroids, comprising gestagens and glucocorticoids. In sturgeon, several C21 steroids are produced by gonads during final maturation and are able to induce GVBD *in vitro,* but the actual MIS is still not identified.

METHODS: Ovarian follicles were taken from three gravid sterlet females (*Acipenser rhutenus* L.) and incubated in Leibovitz's L-15 media (Life Technologies; pH adjusted to 7.8) with penicillin G (0.03 g 1^{-1}) and streptomycin sulfate (0.05 g 1^{-1}). The effect of different concentrations of progesterone (P4), 11-deoxycortisole (S) and 11-deoxycortisone (P21) on GVBD were quantified in an in vitro assay.
Triplicates of 10 ovarian follicles, each in 2.5 ml media were exposed to 600, 800, 1000 or 1200 nM of P4, S and P21 for 5 min, followed by 24 h incubation in steroid-free media. In a second experiment, 10 follicles (in duplicate) were exposed for 1 min. After incubation, follicles were fixed in formalin-ethanol solution and scored for GVBD. Controls were incubated in steroid free media.

RESULTS: After short-time exposure (1 and 5 min), all C21 steroids tested were able to induce GVBD in a dose-dependent manner in sterlet follicles. After 5 min of exposure, P4 was more effective in inducing GVBD than S and P21. Significant differences were observed between P4 and S at 600 nM (95.0% vs 56.0% GVBD in female 1; 95.8% vs 25.5% GVBD in female 2; 100% vs 64.2% in female 3) and between P4 and P21 at all concentrations. After 1 min of exposure, P4 was more effective in inducing GVBD than S at concentrations 600, 800 and 1000 nM (52.1% vs 0%; 100 vs 10%; 100 vs 27.8%) and P21 at all concentrations.

CONCLUSION: The results clearly demonstrate that P4 is more effective in the stimulation of GVBD than S and P21. These findings are congruent with previous studies in other sturgeon species, where P4 induced GVBD at lower concentrations than S, 17,20-beta-dihydroxy-4-pregnen-3-one and 17,20,21-beta-trihydroxy-4-pregnen-3-one after short expose. Taken together the data suggest that P4 is the most potent MIS and should therefore be consider the natural MIS in sterlet.

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Molecular characterization of a nuclear progestin receptor and regulation of its ovarian RNA expression by gonadotropin during maturation and ovulation in Atlantic croaker

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BACKGROUND: Cellular and molecular mechanisms underlying ovulation are uncertain for fish ovarian follicles. There is evidence suggesting that gonadotropin-dependent ovulation is mediated by increased production of maturation-inducing hormone (MIH) acting on a classical (nuclear) progestin receptor (nPR). The objectives of this study are to characterize nPR cDNAs from the ovary of Atlantic croaker and to determine their expression pattern during maturation and ovulation.

METHODS: Nuclear PR cDNA was cloned from ovarian RNA extracts using reverse transcription PCR followed by rapid amplification of cDNA ends. Human CG was used as homolog of LH and was administrated in vivo or in vitro to induce oocyte maturation and ovulation. Ovarian tissue fragments (containing the follicles) were collected at various times during hCG treatment for analysis of nPR RNA content using ribonuclease protection assay.

RESULTS: Only one nPR cDNA could be isolated from the Atlantic croaker ovary. It was 2517-bp in length (excluding the poly-A tail) and encoded a 676-aa predicted protein. The putative DNA-binding and ligand-binding domains of the protein showed high homology with those of known nPR. Fish injected with hCG underwent ovarian maturation and ovulation; and the levels of nPR RNA increased during germinal vesicle migration (GVM), remained elevated during GV breakdown, and declined at ovulation. Similar results were obtained for the in-vitro experiment; namely, hCG stimulated an increase in nPR RNA content that peaked during GVM and gradually decreased through ovulation.

CONCLUSION: The expression of nPR is up-regulated by gonadotropin prior to ovulation in ovarian follicles of Atlantic croaker. This observation suggests that LH-dependent ovulation is mediated by increased levels of MIH production as well as nPR expression. (This study was supported by National Research Initiative Competitive Grant no. 2002-35203-12239 from the USDA Cooperative State Research, Education, and Extension Service.)

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Changes in ovarian TGF-beta superfamily mRNA levels during natural oocyte maturation, and in response to 17,20beta-dihydroxy-4-pregnen-3-one treatment in vivo and in vitro, in rainbow trout

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BACKGROUND: Follicle maturation is a complex process regulated by the coordination of diverse hormones. The mechanisms and extent to which these different hormones interact to induce oocyte maturational competence, production of the maturation-inducing hormone (MIH), and the ability of the MIH to induce the resumption of meiosis is not well understood. Although members of the transforming growth factor-beta (TGF-beta superfamily, including activin and bone morphogenetic proteins (BMP), have been identified as important regulators of ovarian function in mammals, their actions in the fish ovary have received little attention.

METHODS: Blood plasma and ovarian tissues were collected from rainbow trout at various stages of the reproductive cycle, and following injection with sex steroids. Ovarian follicles from fish at various reproductive stages were incubated with graded doses of sex steroids and then collected for mRNA measurement. Ovarian expression of TGF-beta superfamily and related transcripts were measured by RT-qPCR. Sex steroids were measured in the plasma by radioimmunoassay.

RESULTS: Ovarian transcripts for BAMBI and activin A were found to increase and decrease respectively as the follicles progressed from obtaining competence to completing germinal vesicle breakdown (GVBD), which is an indicator of the resumption of meiosis. These changes in transcript levels coincided with a decrease in estradiol-17beta and an increase in 17,20beta-dihydroxy-4-pregnen-3-one (17,20beta-P) concentrations in the plasma. The progestin 17,20beta-P is the putative MIH in rainbow trout. In vitro treatment with 17,20beta-P increased BAMBI and decreased activin A mRNA levels in ovarian tissues. Injections of 17,20beta-P increased BAMBI but did not alter activin A mRNA levels. The responses to 17,20beta-P treatment were specific to this steroid.

CONCLUSION: Changes in mRNA levels of activin A and BAMBI during follicle maturation, and in response to the MIH, 17,20beta-P, suggest a role for TGF-beta superfamily peptides in the regulation of follicle maturation in rainbow trout.

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Effects of dietary DHA:EPA:ARA ratio on egg and larval quality of Eurasian perch (*Perca fluviatilis*) broodstock

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BACKGROUND: One of the major constraints for the development of Eurasian perch culture is the supply of good quality eggs. The importance of 3 fatty acids (FAs; docosahexaenoic (22:6n-3; DHA), eicosapentaenoic (20:5n-3; EPA) and arachidonic (20:4n-6; ARA) acids) in the reproduction and larval development processes has been demonstrated. It is important that eggs contain the correct balance of DHA/EPA to ensure proper larval development after hatching. ARA-derived series 2 prostaglandins are important in the modulation of ovulation, although EPA is a potent inhibitor of ARA-derived eicosanoid production. Regarding the chemical similarities of those 3 FAs, leading to competitive interactions, the optimal requirement for each FAs can not be considered alone.

METHODS: Three groups of 40 perch breeders were reared in order to test different ratios of dietary DHA/EPA/ARA (16% lipids) during 9 months (from March to November 2006). Two experimental diets, R1 (2/2/1) and R2 (17/12/1), were compared to one commercial food (R3) usually given to perch breeders. Survival and feed intake were studied along the experiment. Spawning and fertilization rates were checked in November, and larval robustness was evaluated by osmotic shock two days after hatching. The eggs and diets were homogenized in ten volumes of dichloromethane/methanol (2:1, v/v) using an ultraturax and total lipid extracted by the method of Folch et *al.* (1957). The dichloromethane layer was evaporated under nitrogen and the lipid weight obtained after 2h vacuum dessicator. Total lipid was transmethylated as described by Christie (1982). The fatty acid methyl esters were separated and quantified by gas-liquid chromatography. The quantification of lipid classes was performed using double-development high-performance thin layer chromatography (HPTLC) and scanning densitometry.

RESULTS: Survival was similar among the three diets. The spawning rate was high for R1 and R3 (85.7 and 73.3%, respectively) while only few spawnings were obtained for R2 (33.3%). On 12 egg strands, only one was fertilised in R2 (with a fertilization rate of 93%), while 38.9 and 72.7% of the spawnings were fertilised in R1 and R3, respectively, with similar fertilization rate on those eggs (88.6±10.4 and 87.4±15.1). Larval robustness was comparable between the diets R1 and R3 (74.2±25.0% and 69.0±24.2%, respectively). Lipid level in the eggs of the 3 diets was comparable (min. 4.6±1.4% for R2 and max. 4.9±1.4% for R1). FAs and lipid classes quantification are still on going.

CONCLUSION: Dietary DHA/EPA/ARA ratios may influence egg and larval quality of Eurasian perch. The diet poor in n-6 FA, especially ARA, led to very low spawning and fertilization rates.

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Urine of ovulated female masu salmon (*Oncorhynchus masou*) contains a priming pheromone which increases a plasma 17,20β-dihydroxy-4-pregnene-3-one level in mature male parr

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BACKGROUND: In salmonid fishes, mature female urine has been known to act as sex pheromones having primer and releaser effects. Recently, we have reported that ovulated female masu salmon secrete L-kynurenine as a releaser pheromone for spermiating male masu salmon parr. In this study, we tested a primer effect of ovulated female urine and L-kynurenine to males.

METHODS: Adult masu salmon were catheterized to collect the urine. Spermiating male masu salmon parr were divided into five groups (n=10 in each group; exposed to distilled water, immature female urine, mature male urine, ovulated female urine, and L-kynurenine [final concentration is 10^{-9} M]). Blood sample was taken from the caudal vasculature of each male and the expressible milt was collected before exposure and 3 hours after exposure. Each group was exposed once to 100 μl of odours. Plasma 17,20β-dihydroxy-4-pregnene-3-one (17,20β-P) levels were measured by enzyme-linked immunosorbent assay (ELISA). Plasma concentrations of 17,20β-P and milt volumes were analyzed by ANOVA followed using Fisher's PLSD test among test groups.

RESULTS: All groups showed no statistically significant effect on levels of expressible milt. Exposures to ovulated female urine and L-kynurenine, however, caused significant elevations in 17,20β-P concentration 3 hours after introducing odours.

CONCLUSION: In masu salmon, ovulated female urine and L-kynurenine induced a primer effect. The 17,20β-P level has been used as an indicator of the priming effect in mature male fish, including salmonids. We suggest that L-kynurenine has both primer and releaser effects. It is necessary to elucidate the detail function of L-kynurenine as a primer pheromone and further, we must identify other active components in ovulated female urine.

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Hormonally induced ovulation in Eurasian perch (*Perca fluviatilis*) by GnRHa with and without dopamine inhibitor

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BACKGROUND: Artificial propagation with hormonally induced ovulation is still not a common practice in females of Eurasian perch, *Perca fluviatilis*. However the synchronization of ovulation is often limited in artificial propagation of females without hormonal treatment. The successful and thrifty hormonal synchronization of ovulation by GnRHa is feasible method to future decrease fingerling production costs. These effects of different doses of GnRHa (with and without dopamine inhibitor) on the synchronization of ovulation, fecundity and hatching success were determined in this study.

METHODS: The experiment tested two hormonal preparations (Supergestran - GnRHa without dopamine inhibitor and Dagin - GnRHa with dopamine inhibitor) and their different doses as feasible hormonal treatment of induced ovulation in perch females. In total, 11 hormonal groups including 10 mature females (GSI=28.6 ± 3.5 %) were tested with one duplication. Groups S1, S2, S3, S4 and S5 were dorsal intramuscular injected by Supergestran with dose 6.25, 12.5, 25, 50 and 100 µg GnRH.kg^{-1} BW, respectively. Groups D1, D2, D3, D4 and D5 were injected by Dagin with dose 3.12, 6.25, 12.5, 25, 50 µg GnRH.kg^{-1} BW, respectively. Beside, one group (C = control group) was injected with physiological saline only. After ovulation, absolute and relative fecundity, time of latency (time between injection and spawning), synchronization of spawning and hatching success were found in each spawned females from each treatment. All realized values were compared among all treatments by ANOVA, Tucky's multiple comparison test ($P< 0.05$).

RESULTS: After this experiment, in total 170 (77.3 %) females were spawned from all treatments. Average fecundity was found as absolute: 24 375.4 ± 9862.6 ova or 54.3 ± 16.1 g of egg ribbon and relative: 135 200 ± 77 500 ova or 251.6 ± 62.2 g of egg ribbon per kg BW per female from all treatments. Fecundity was not statistically different among all groups. Statistically the longest time of latency (137.3 ± 62.8 hours) and the worst synchronization effect (17.5 % of spawned females till 5th day after injection) were found in females control group (C). On the contrary, statistically the shortest time of latence (48.0 ± 10.4 hours) and the best synchronization effect (100 % of spawned females till 3rd day after injection) were found in females group D1. Statistically differences were not found among other treatments (time of latency fluctuated from 54.5 ± 15.8 to 75.5 ± 4.5 hours). After egg incubation, low hatching rate was found in all treatments. The lowest hatching rate was determined in treatments D4 and D5 (15.0 ± 3.0 %). Higher hatching rate was evident in others treatments (fluctuated from 33.2 ± 5.8 % to 47.5 ± 3.0 %).

CONCLUSION: Results of this study showed that lower doses up to 25 µg GnRH.kg^{-1} BW of tested treatment were more suitable for using in artificial spawning of perch females. For future their practical using is necessary test way of artificial egg fertilization increasing hatching success. Thanks are expressed to projects: USB RIFCH no. MSM6007665809, NAZV no. QF 4118 and EU no. COOP-CT-2004, 512629.

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Effects of the reconstruction in the Shibetsu River on upstream migration behavior of chum salmon assessed by EMG radio telemetry

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BACKGROUND: Physical characteristics created by river environment affect the limited energy costs of adult anadromous salmonids, including chum salmon. Effects of physical characteristics like current speeds affected by the river reconstruction, re-meandering on migration of adult chum salmon are unknown.

METHODS: During the periods 2004-2006, 30 adult chum salmon tagged electromyogram (EMG) transmitter and depth/temperature (DT) logger tracked along both the canalized and reconstructed segment of the Shibetsu River, Japan. To estimate effects of the physical characteristics and sex on upstream migration of chum salmon, swimming speed calculated from EMG signals, ground speed, swimming efficiency index (SEI) and time of holding behavior were examined using statistical analysis. Critical swimming speed of 13 chum salmon were also measured using a swim chamber. Water depth and current speed were measured at the locations along the two segments every 20-100 m.

RESULTS: Swimming speed, ground speed and SEI were not significantly different between the canalized and reconstructed segment in 2004 and 2005. However, holding time of chum salmon in the reconstructed segment increased from 2004 to 2006 compared with the canalized segment. Further, longitudinal and lateral variations in water velocity and depth were observed in the reconstructed segment in 2005. Swimming speeds calculated from electromyogram (EMG) signals of chum salmon decreased during holding behavior and observed exceeding critical swimming speed (U_{crit}) during moving. Although groud speed and SEI were not significantly different between sex, swimming speed of male was significantly lower than that of female.

CONCLUSION: The effects of the reconstruction, re-meandering on upstream migration of chum salmon were examined using EMG transmitters and DT loggers for three years. Our data suggest that the reconstructed segment have been developing the longitudinal and lateral variations in water velocity and depth, and these physical characteristics may be suitable for holding behavior of chum salmon. Further, swimming speed exceeding U_{crit} prior to holding behavior may be exhaustive swimming and holding behavior may be important for muscle recovery and efficiency swimming to reduce the energetic cost during upstream migration. Thus, holding sites developed in the reconstructed segment may help to achieve a successful spawning migration of chum salmon. In addition, lower swimming speed of male chum salmon compared with female indicates that male chum salmon migrated to reduce muscle activity rather than female during upstream migration because of larger body size of male.

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Involvement of aquatic plants in the spawning behavior of goldfish and crucian carp

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BACKGROUND: Construction of artificial riverbanks and lake banks greatly changes the aquatic environment. One of the possible changes is the decrease of aquatic plants in the shallow water area, which affects spawning of fish species that use plants as their spawning substrate. In the present study, we examined the involvement of aquatic plants in the spawning behavior of goldfish (*Carassius auratas*) and crucian carp (*Carassius auratus* subsp.), which are known to deposit their eggs on aquatic plants.

METHODS: Spawning behavior was induced in goldfish and crucian carp by injection of prostaglandin F2alfa to females. Pairs of male and female goldfish or crucian carp were placed in 60 L glass tanks with artificial aquatic plants made of acrylic yarn. Spawning behavior was observed under the conditions where the aquatic plant was set, removed, and replaced.

RESULTS: Both goldfish and crucian carp actively performed spawning behavior towards the aquatic plant (goldfish: average, 36.5 times/30 min; carp: 56.7 times/60 min), whereas frequency of spawning behavior decreased when the plant was removed (goldfish: 5.5 times/30 min; carp: 2.2 times/60 min). Fish performed the spawning behavior at the corner of the tank in the absence of the plant. When the plant was replaced in the tank, both goldfish and crucian carp showed spawning behavior as actively as before the removal of the plant (goldfish: 38.1 times/30 min; carp: 32.1 times/60 min).

CONCLUSION: The present experiment demonstrates that the removal of aquatic plants largely suppresses the spawning behavior of goldfish and crucian carp. Active spawning behavior resumed after the replacement of the plant, which indicates that the removal of the plant did not affect the capacity of spawning behavior of the fish but only reduced the chance of spawning success. Such suppression of reproductive activity would happen in the natural environment where plants are reduced.

Influence of human activities on wildlife is commonly assessed by measuring the impact on the health of organisms. However, some environmental factors suppress the reproductive activities of animals without directly affecting the health of the animals. It is proposed that such possibilities should be considered for adequate environmental conservation.

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Efficiency of Gonazon™ in rainbow trout, the first officially approved inducer of ovulation in the EU

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BACKGROUND: Intervet recently developed a new product, Gonazon™ (containing azaglynafarelin, a GnRH analogue), the first product of its kind to receive EMEA (EU/Norway) approval to induce and synchronise ovulation of female salmonids. This paper gives an overview of studies performed with Gonazon™ in rainbow trout during the development process. For the first time, the effect of such a treatment is evaluated in carefully controlled studies.

METHODS: Two experiments evaluated the effect of different doses on ovulation induction (Exp. 1) and, subsequently, looked at the effect of the optimal dose on reproductive traits and fry quality (Exp. 2). Ovulation was checked every 8-9 days.
Exp. 1: Four doses (16, 32, 64 and 96 µg/kg BW) were tested and compared with two control groups (vehicle and saline); natural ovulation rate at the time of treatment was less than 4%.
Exp. 2: 32 µg/kg BW dose was compared with a saline control. Natural ovulation rate at the time of treatment was less than 10%. Growth of juveniles was assessed (in duplicate) until D42 after feeding in early, mid and late ovulation control groups *vs.* the treated group. Vertebral malformations were estimated by radiography.

RESULTS: Exp. 1: Ovulation was significantly advanced for all doses (72, 72, 96 and 80% *vs.* 8% in vehicle or 20% in saline). Number of eggs or weight of eggs/kg did not differ between groups except for the 16 and 96 µg/kg groups (15.3 vs. 12.0%). Egg weight was not significantly different between the saline and treated groups; the 96 µg/kg group had bigger eggs than the 16 µg/kg group (65.5 *vs.* 60.3 mg). The % eyed eggs was also not different between treatments (range of 68.4 to 83.0%).
Exp 2: Ovulation was advanced in the treated group (94.2 *vs.* 27.6% at D9). No difference was observed between the treated and the control groups for fecundity, egg weight, % fertilisation (78.6 *vs.* 87.0), % eyed eggs (81.9 *vs.* 71.3), hatching rate (79.4 *vs.* 87.9%) and rate of swimming fry (66.6 vs. 79.6%). Mean weight of juveniles was not different at resorption (111.2 *vs.* 112.5 mg) or at D42 (2.0 *vs.* 2.0 g). Incidence of malformations was unaffected by treatment (range of 6 – 19 in controls *vs.* 12%).

CONCLUSION: These studies have shown for the first time the effect of an hormonal inducer when taking into account the total population. Gonazon™ (32 µg/kg) was accepted by the EMEA for female salmonids. This low dose for intraperitoneal treatment can be linked to its longer terminal half-life in trout (5 hr) when compared with other GnRH analogues (typically measured in minutes). The approval provides breeders with an efficient product that respects regulations for animal and human safety, and that is produced according to Good Manufacturing Practices. In addition, Gonazon™, as prescribed by veterinarians, will ensure better traceability that is required by the end users (supermarkets, consumers, etc.).

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Reproductive strategy of pouting *Trisopterus luscus* (Linnaeus, 1758)

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BACKGROUND: In terms of fecundity in the current stock assessment methods two strategies could be distinguished, depending on this species can be classified as determined and indeterminate fecundity. To assess the annual fecundity accurately is necessary identify which of the two reproductive strategies presents our target fish species. Studies on reproduction of pouting are scarce until now, and generally have been assumed that this species is a determined spawner but no clear evidence of that has shown.

METHODS: Histological examination of ovaries from Trisopterus luscus from the coast of Galicia sampled on a monthly basis along 2004 was used to to identify different stages of oocyte development. Seasonal variations of gonadsomatic index (GSI) and hepatosomatic index (HSI) were estimated. Stereology has been applied to estimate atresia following Emerson method. The combined method using the gravimetric method and image analysis was used to assess both Number of developing oocytes (NDO) and batch fecundity.

RESULTS: Histological examination of the gonads revealed that pouting posses an asynchronous ovarian development organization. GSI and HSI presented significant difference between months. Regards oocyte development, there was no presence of hiatus between previtellogenic and vitellogenic oocytes. Atresia process was present along the year with a peak in last months of spawning period. In pouting a continuous oocyte size-frequency distribution was found for every maturity stage except in hydrated ovaries. Number of developing oocytes (NDO) shown a clear decreased throughout the spawning season and the ratio between NDO and batch fecundity was relatively high.

CONCLUSION: This study suggests that although pouting has an asynchronous ovarian development organization and although there is not categorical data of what kind of strategy the pouting selected our results shown some evidence to consider the pouting as a determinate fecundity species for annual fecundity assessment.

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Reproductive omission and "skipped spawning": detection and importance

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BACKGROUND: It is becoming increasingly obvious that, for potentially iteroparous fish, there may be optional, rather than essential, facets to their reproductive events. Options may include repeated spawnings within or between years as well as up- or down-regulation of gamete number. Reproductive omission or "skipped spawning" for oviparous teleosts can occur in previously reproductive fish (i.e. "adults") as an extreme expression of down-regulation. Such omission may occur irregularly or frequently in potentially long-lived fish, and could have a profound effect on maintenance of populations particularly for those under fishing pressure. Reproductive omission may be an important strategy for fish subject to periods of food scarcity. The regulation of reproductive periodicity is still poorly understood; the detection of reproductive omission is critical to understanding reproductive periodicity and effective spawning biomass.

METHODS: Morphological, morphometric and histological analysis of gonads from different taxa is used to differentiate fish that have never reproduced ("immature") from those that have reproduced at least once ("adults"). Attention is particularly directed at teleosts with different ovarian structure, e.g. cystovarian or gymnovarian. Further analyses attempt to differentiate adults that have recently reproduced and will repeat next reproductive season, from adults undergoing reproductive hiatus or temporary quiescence, the state leading to "skipped spawning" in some teleosts. Morphometric analyses has also been used on old ("historical") data sets and histology is particularly appropriate where post-ovulatory follicles can be identified.

RESULTS: It is relatively easy to identify non-reproductive females for those taxa that have cystovarian ovaries (i.e. gadids, pleuronectids) and group synchronous oogenesis, especially if histology is an option. Morphometrics may also identify non-reproductive adults of either sex and old data can be productively re-assessed to reveal patterns of reproductive non-participation. The occurrence of spawning omission can also be recognised for taxa with gymnovarian ovaries but compressed spawning time. Considerable difficulties can ensue in the identification of reproductive omission in taxa with extended batch-spawning and a poorly defined reproductive season and/or with asynchronous gametogenesis.

CONCLUSION: Guidance on identification of reproductive status, particularly reproductive omission, for different taxa is possible based on a set of criteria which may be applicable to old data. Re-assessment of reproductive periodicity for some potentially highly fecund species can be accomplished. Further information can be garnered on sensitive periods in the reproductive cycles which may be prone to varying levels of down-regulation including reproductive omission. Reproductive strategies which include reproductive omission are particularly relevant to the many species which are subject to extreme seasonal variability in feeding success. It is suggested that "skipped spawning" is both physiologically important, providing insights to control of gametogenesis, and economically significant for some populations in which spawning biomass may have been over-estimated by assuming full participation of all adults each year.

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Reproductive dynamic and strategy and oocyte recruitment process of European hake (Merluccius merluccius, L. 1758) in Galician shelf waters

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BACKGROUND: Spawning stock biomass (SSB) has been generally used as an indicator of spawning stock production but it is not exact because there are other factors like size, experience or condition that affect capacity of females to produce viable offspring that may eventually recruit to adult population or fishery. This ability is the Stock Reproductive Potential (SRP). To account for the long-term reproductive ability of the stock, fisheries management should be based on SRP. There are not many studies focused on Reproductive Potential of hake that is one of the most important commercial species in the Northeast Atlantic.

METHODS: Histological analysis of gonad was carried out. Biochemical composition was analyzed in gonad, liver and muscle in 50 female hakes as indicators of female condition status, these results were converted to energy values (kJ). Number of developing oocytes (NDO) and batch fecundity (BF) were also estimated. Relationship between NDO, BF and maternal attributes (length and gutted weight) and energetic condition was studied as well as variation of these parameters along the spawning season.

RESULTS: In histological analysis was observed that all oocyte developmental stages were present at the same time in the ovary and females in different maturity stage were observed simultaneously along the year. Hake population in Galician waters has a protracted spawning season with a peak of spawning from February to March. Proximate composition of tissues considerably changes along spawning season although population spawning synchrony masks temporal patterns. Both NDO and BF are affected by maternal attributes; nevertheless NDO is modulated by energy intake.

CONCLUSION: European hake from Galician Shelf waters is highly asynchronous species, not only at individual but also at population level and shows indeterminate fecundity. In this species larger females mobilize more oocytes during their spawning season, although this trend is modulated by their capacity to capture external energy. In other words, this work corroborates the importance of proximate composition as a short-term energy reserve for egg production in European hake from Galician shelf waters, but energy dynamic associated with egg production are different from those observed in cold water species.

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Japanese fat minnow (*Phoxinus lagowski*) males form a spawning school in a creek

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BACKGROUND: Japanese fat minnow *Phoxinus lagowski* is one of the key species to evaluate environmental changes under human activities. Fat minnow, a common fluvial cyprinid fish in northern Japan, is not known the physiology in maturation and spawning.

METHODS: Process of maturation of the fat minnow in a creek was monitored in distributions, gonadosomatic index (GSI), ovarian development, length of upper snout, plasma levels of sex steroid hormones, and spawning behaviors. An underwater CCD camera monitored spawning behaviors. A group of fat minnow was transferred to university tanks to examine the school formation in a Y-maze tank receiving two test water flows. Male group was placed in the lower common space where they could choose either water flow.

RESULTS: GSI increased in April with increase in water temperature and peaked in late May in both females and males as similar increasing profiles of plasma testosterone (T) and estradiol-17β (E2) for females, and T and 11-ketotestosterone (11-KT) for males. Spawning schools were observed in late May and early June at a gravel (2-10 mm length) bottom in the creek. Males formed a school; single female approached to the male school, dove into the bottom from the snout, and swam off. Males aggregated above the place to release milt. In the Y-maze experiments, males preferred the water from a tank that contained spermiating males. Ovulated females had 20% longer snouts; the dissected female snouts lengthened 20% in hCG solution.

CONCLUSION: In spring, sex steroids and GSI increased with increase in water temperature, and spawning period was defined in late May and early June. In a spawning school consisted of males, a female dove into gravel bottom and laid eggs. The female quickly swam off the gravel, and males spawned on the place. A female of cyprinid dace *Tribolodon hakonensis* in Japan spawn adhesive eggs with several males on gravel without diving into it in a similar period. These results reveal the first note of Japanese fat minnow in spawning period, steroid hormone profiles, spawning school formation of males, and spawning behavior of females.

P 200

Regulation of early sexual maturation in Atlantic salmon male parr: Effects of growth modulation during spring

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BACKGROUND: Age and size at first sexual maturation is a key component in life-history strategies of many fish species and has evolved to achieve maximal reproductive fitness. In Atlantic salmon, males may either mature as small parr in freshwater or later at large size after returning from the sea. There is increasing evidence that the incidence of early sexual maturation is influenced by somatic growth and / or energy stores, however the underlying endocrine mechanisms are still poorly understood.

METHODS: One-year-old Atlantic salmon were subjected either to a restricted feeding regime or a standard hatchery feeding (control) regime during eight weeks prior to the onset of gonadal growth in spring. Fish were sampled at the start of the experiment in early May, and after 3 and 6 weeks of treatment. Gonadotropin beta subunit (FSH beta, LH beta) and growth hormone gene expression (GH1, GH2) in the pituitary as well as Insulin-like growth factors (IGF-I, IGF-II) expression in the liver were measured by RT-PCR. Total fat content was analyzed and the incidence of sexual maturation was estimated at a final sampling in August.

RESULTS: Restricted growth conditions resulted in significant lower body weight, condition factor and adiposity levels, while no significant differences in GH(1 and 2) and IGF-(I and II) expression was observed. Maturation rate was reduced from 50% in controls to 26.4% in males on restricted feeding regimes. Males committed to early maturation had significantly higher FSH beta transcript levels at the beginning of spermatogenesis and significant higher condition factors and total fat content.

CONCLUSION: The results show a strong influence of growth conditions and energy stores on the incidence of early maturation in male Atlantic salmon parr, supporting the hypothesis that certain growth or energy thresholds have to be reached during the permissive period in spring in order for spermatogenesis to begin.

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Lipid content and fecundity in autumn- and winter spawning North Sea herring (Clupea harengus)

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BACKGROUND: Reserves are stored as lipid in the body after the spawning season. Throughout the maturation of the ovaries these lipid reserves and protein are used for the development of the oocytes. Not much is known about the relationship between the lipid content and fecundity in North Sea herring. This paper describes the results of lipid content and fecundity analysis of herring.

METHODS: In 2006 North Sea herring have been sampled throughout the maturation and spawning cycle, from May till September for the autumn spawning stock, and from May till January for the winter spawning stock or 'Downs' herring. Length, weight, sex, maturity, stomach fullness and age were measured. Ovary samples were fixed in 3.6% formaldehyde and analyzed using image analysis. Lipid content was directly measured using a 'Distell' fish fat meter. At the summer feeding grounds both herring stocks are mixed and spawning type of the herring was determined by microstructure analysis of the otoliths.

RESULTS: Throughout maturation and spawning the variation in lipid content was large for both spawning types. The seasonal cycle of lipid content differs between both spawning stocks. Lipid content of the winter spawners is on average higher compared to the autumn spawners, although the autumn spawners are on average larger both in length and weight.
Fecundity results are not yet available but will be at the time of the symposium.

CONCLUSION: In 2006 lipid content was very low at the onset of maturation. Spawning of the both the autumn and winter spawners was later in the year in 2006 compared to earlier years. This is in agreement with results of North Sea herring acoustic and larvae surveys.

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Timing and fecundity determination in Norwegian spring-spawning herring

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BACKGROUND: Spawning Stock Biomass is no longer considered a suitable proxy for reproductive potential in many fish stocks. It has become clear that the energy levels of individual fish within the stock can greatly affect egg production. There is also emerging evidence that a portion of fish within a stock may skip spawning if the requirements for successful reproduction (sufficient energy reserves, temperature etc.) are not met. Herring are a capital breeder, in that gonad development is heavily subsidised from stored reserves built up during the feeding season. The decision to proceed with gonad development is made many months before spawning takes place. This implies that energy levels during the time of early vitellogenesis may have a strong influence on potential fecundity and may also influence the decision on whether to skip spawning.

METHODS: We carried out a lab experiment on herring to locate the period in which food availability and energy levels have the greatest influence on potential fecundity and also when the decision to skip spawning is made. Four groups of captive wild herring were fed to satiation twice weekly for three months and 7 times weekly for one month. The month in which the fish received food 7 times per week was different for each group. The fecundity and condition was assessed for each group every other month until February. Wild Norwegian spring-spawning herring were also sampled in the summer feeding areas and in the overwintering areas. This was to assess the changes in fecundity throughout the year in relation to the changing energy reserves of the fish and to assess the proportion of skipped spawners in the population.

RESULTS: In the experimental study the group which was the last to be fed for 7 times weekly had fewer fish which had begun vitellogenesis in October. Come December however, there were no differences in fecundity, follicle size or the number of fish which had commenced vitellogenesis. The results from the field study showed a change in fecundity throughout the year with a large decrease in fecundity between sampling in August and November. The fecundity in November was not significantly different from the fecundity in the previous February. All samples collected during November showed signs of ovary maturation.

CONCLUSION: Our results from the laboratory experiment show that herring can quickly take advantage of an abundant food source and the timing of this food source (during the feeding season) is not important for ovary development and maturation. Our results from the field study are consistent with previous findings that herring have a higher fecundity at the beginning of ovary development and that this is down regulated during the Autumn months. All samples analysed showed the signs of maturation and hence no fish were considered to be skipping spawning for the coming year.

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Spawning, early life history and feeding studies in marine ornamental fishes

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BACKGROUND: Anthropogenic activities are creating new problems for the world's coral reefs causing profound changes in this delicate ecosystem with a consequent decline in biodiversity. The marine ornamental industry plays an important role in reef impairment since some fishermen use destructive fishing methods (sodium cyanide and dynamite) that allow them to catch as many fish as possible with a minimum of effort. Less than 1% of marine fish and invertebrates are commercially produced thus, the development of ornamental aquaculture is becoming crucial to provide an alternative supply of fish, and to acquire new information on the reproductive biology and life history of these species critical to understanding the response of natural stocks to anthropogenic effects

METHODS: Three different species of clownfishes, (*Amphiprion ocellaris, A. melanopus and A. clarkii*), the cleaner goby (*Gobiosoma evelynae*), the Sunrise Dottyback (*Pseudochromis flavivertex*), the Lemonpeel Angelfish (*Centropyge flavissimus*) and the Cuban hogfish (*Bodianus pulchellus*) were kept in 200 L breeding tanks filled with live rocks to simulate the reef habit. Photoperiod and temperature were manipulated using light timers and heat pumps to simulate seasonal changes. Reproductive behavior, embryo and larval development were described for all the mentioned species and feeding studies were performed using different types of live prey.

RESULTS: Environmental changes resulted in natural gamete release and a positive correlation between water temperature and number/size of spawns was observed. The reproductive cycle was closed for the clownfishes and Sunrise Dottyback using rotifers and *Artemia* as live prey. Food enrichment was essential for the larvae to reach metamorphosis. *Euplotes* sp naked ciliates were essential to close the reproductive cycle of the cleaner goby. First feeding Lemonpeel Angelfish and Cuban hogfish larvae did not accept Rotifers as first food; larvae were fed on different species of cultured copepods (*Acartia tonsa and Parvocalanus spp)* and wild caught, size-sieved zooplankton.

CONCLUSION: The main bottleneck in rearing ornamental fish larvae is the transition from endogenous to an exogenous feeding. Rotifers and *Artemia* are the most widely used live prey since they can be easily cultured in large quantities but they are naturally deficient in some fatty acids required for fish larvae and thus must be properly enriched before their administration. Moreover, many of the small larvae refuse to feed on rotifers at first feeding because they are too large or do not provide appropriate cues. The potentiality of naked ciliates and copepod nauplii as candidates for marine ornamental aquaculture was evidenced in this study.

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Results of maturation and ovulation in European eel females

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BACKGROUND: The hormonal treatments to mature female European eels produce final maturation in a time between 10 and 25 weeks, in treatments based on carp or salmon pituitary extract injections. The ovulation can be induced with one prime dose of pituitary extract and a final injection of 17α,20β-dihydroxy-4-pregnen-3-one (DHP). Correct timing during this process can affect the egg quality in terms of viability and survival. Treatments with testosterone and LHRHa has been successful in Japanese eels.

METHODS: Two experiments were performed to obtain maturation and ovulation in female European eels. In the experiment 1, 25 wild eels were treated with weekly injections of salmon pituitary extract (SPE, 20 mg/kg) and hCG (1,5 IU/g), and ovulation was induced with four protocols with different times between doses: A) Weekly SPE+ prime dose SPE (24 h later)+ DHP (35 h after prime dose), B) Weekly SPE+ prime dose SPE (48 h later)+ DHP (9 h after prime dose), C) Weekly SPE+ prime dose SPE (24 h later)+ DHP (12 h after prime dose), and D) Weekly SPE + DHP (11 h later).
In the experiment 2, 11 wild females (749 ± 36 g) and 18 farmed female eels (718 ± 24 g) were weekly treated with SPE and hCG, and induced to ovulate with protocols C or D. Another group of farmed eels was pretreated with LHRHa, testosterone and pymozide during 6 weeks, and later treated with SPE+hGC or LHRHa+pymozide. In both experiments fertilization assays were made with fresh sperm obtained from males weekly treated with hGC (1,5 IU/g). Egg incubation was performed in 5 l glass jars with seawater at 20 ºC and soft aeration from the bottom.

RESULTS: In the first experiment 18 females matured between 10^{th} an 17^{th} weeks of treatment. Final maturation was induced in 15 females, and 12 of them ovulated and their eggs were fertilized. The shorter protocols (C and D) originated most of the floating spawns. In experiment 2 ovulation was obtained in females, farmed or wild, injected with SPE+hCG, but not in the other groups. Five farmed females ovulated between 11^{th} and 17^{th} week of treatment, but just two spawns were floating. Four wild females ovulated between 10^{th} and 12^{th} week of treatment, and two of the spawns were floating. Eels treated sequencially with LHRHa+pymozide+testosterone and LHRHa+ pymozide did not show any sign of maturation. The fertilization assays originated cell cleavage in some cases, but blastula or gastrula stages were not observed.

CONCLUSION: Shorter ovulation protocols (1, 2 days) seems to be better to induce floating spawns and avoid overmaturation of the eggs. Latency time after DHP injection is related with the probability to obtain a floating spawn. Wild and farmed females do not show great differences in the response to the treatments.

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Hormonal maturation of female European eels at two temperature regimes

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BACKGROUND: In natural conditions European eels migrate across the Atlantic ocean, at depths around 300-400 m, where the temperature is lower than 10 ºC. In the spawning area, assumed at 200-600 m depth in the Sargasso Sea, temperatures are higher, around 18-20 ºC. Therefore, in the natural environment, the vitellogenesis of the females of this species occurs at low temperatures, while the spawning occurs at high temperatures.

METHODS: Once acclimated to seawater, wild female eels were treated with weekly intraperitoneal injections of carp pituitary extract (CPE, 20 mg/kg) and maintained in two different water temperature regimes: A) constant 20 ºC, and B) 10 ºC during the first 5 weeks, followed by a progressive increase, reaching 19-20 ºC at the maturation time. Five eels of each group were sacrificed at 0, 4, 8 and 12th weeks of treatment. Biometric parameters and samples of gonad were taken. Final maturation and ovulation was attempted with 6 females from the group B, injecting 17α,20β-dihydroxy-4-pregnen-3-one (DHP, 2 µg/g) the same or the next day to the weekly CPE injection. Additional dose of CPE was given in some cases. Fertilization assays were made using excess of sperm with motility higher that 50 %. Egg incubation was performed in Petri dishes with artificial seawater at 20ºC and serial observations were made.

RESULTS: Eye index increased along the treatment, without significant differences between A and B treatments. After 8 injections, the oocytes of all the females were in vitellogenic (VTG) stage, but oocytes in group A were in early VTG, while in group B they were in medium VGT. At this moment, GSI and oocyte diameter were significantly higher in B group, but after this week differences were not significant. Mortality was high in group B and there was no enough females to mature until ovulation. CPE treatment continued in 6 females from B treatment, and 5 of them ovulated between 16 and 20th weeks of treatment. The first ovulation occurred spontaneously in the tank, but the rest were induced by DHP injection. Latency time was shorter than 12 hours in all cases. Cell cleavage was observed in 3 of the 5 batchs of eggs, but although four and eight cells stages have been clearly observed, the development never continued after one stage similar to a morula.

CONCLUSION: A changing temperature regime can be used to induce hormonal maturation and ovulation in female European eels. Low temperatures during first weeks of treatment do not produce inhibition in the oocyte development but even apparently accelerate the vitellogenesis process. Differences observed between treatments should be evaluated in terms of blood and pituitary hormones. Embryo development fails due to unknown factors.

Supported by the Generalitat Valenciana (GV04A-508, ACOMP06/193, and a grant to D.S. Peñaranda), the Spanish Ministy of Education and Science (AGL2003-05362-C02-00, including FEDER funds). The eel farm Valenciana de Acuicultura, S.A. (Puzol, Valencia) provided most of fish used in these experiments.

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Effect of water volume and sperm density changes on monospermic and polyspermic ratio in Persian sturgeon (*Acipenser persicus*)

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BACKGROUND: Since the sturgeon fishes have several micropile pores in any egg, it is necessary to exactly determination the water volume and other related factors as well in order to the maximum efficiency achievement in artificial reproduction procedure.

METHODS: Within several groups of *Acipenser persicus* broodstocks, an appropriate male and female were selected and injected with sexual maturation hormones. The trail was carried out in two simultaneous experiments. In the experiment 1, 100 g eggs were located in the four containers and the sperm with proportions of 1:100 (1 ml sperm in 100 ml water), 1:200, 1:300 and 1:400 were added in 3 replications to each container. In the experiment 2, the four similar other egg container were exposed to the proportion of 1:100, 2:200, 3:300 and 4:400 in 3 replications. After 3 hours in 18-20 ˚C, 100 eggs were removed and preserved in 10% formalin. Counting the monospermic and polyspermic percentage has been considered only for the eggs containing 4 cells.

RESULTS: In experiment 1, the monospermic percentage mean ± SD for the proportions 1:100, 1:200, 1:300 and 1:400 were 74.33 ± 2.88, 79.33 ± 9.60, 81.66 ± 6.80 and 82.66 ± 2.30, respectively. The polyspermic percentage mean ± SD for this test were 24.66 ± 3.05, 17.33 ± 8.08, 15.33 ± 7.57 and 18.66 ± 7.23, respectively. In experiment 2, the results for monospermic percentage mean ± SD for the proportions 1:100, 2:200, 3:300 and 4:400 were 74.33 ± 2.88, 87.96 ± 1.76, 74.66 ± 12.34, and 82.00 ± 6.08, respectively. Also in this test, the polyspermic percentage mean ± SD included 24.66 ± 3.05, 10.26 ± 0.70, 25.00 ± 9.64, and 15.66 ± 5.50, respectively.

CONCLUSION: By increasing in water volume (2-4 times) with diminishing in sperm density (experiment 1), we can see a gradual improvement in monospermic percentage, as their differences are significant (P<0.05). The current proportion for the combination of sperm and water volume for Persian sturgeon is 1:100 and more dilution of this mixture to 1:400 can be advisable for optimizing the reproduction procedure. In the experiment 2, we found by increasing the water volume without any change in sperm density (holding the proportion of 1:100 in other treatments), the greatest monospermic percentage has been occurred in the proportion of 2:200 and then in proportion of 4:400 as their differences with 1:100 were significant (P<0.05). In both tests, it seems using more water volume for dilution of water solution and dilution of ceolumic compounds surrounding the eggs can lead more improvement in monospermic percentage and consequently more success in reproduction of this species.

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Physiological changes in Russian sturgeon (Acipenser gueldenstaedtii) after longterm reservation and final maturation

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BACKGROUND: Russian sturgeon is a diadromous fish. Its preservation in nature is possible only by modern technology in fish farms, including long-term holding and hormonal treatment. The investigation of physiological status of broodstock is important during holding and induced final maturation.

METHODS: Male winter form Russian sturgeon from Volga River were held in tanks from capture at spring till next spring (11-12 months) without feeding. After completion of gametogenesis spermiation was induced by LHRH-A treatment. Testosterone (T), 11-ketotestosterone (11KT) serum concentrations were determinated by ELISA, glucose (Gl) levels by glucoseoxidase enzyme method, total protein (TP) concentration by biuret method, cholesterol (Ch) levels by cholesterol esterase enzyme method. Blood samples were taken before holding from 4 males. After holding blood samples were taken twice from the same fish (n=11) before hormonal treatment and after induced spermiation.

RESULTS: In male sturgeon at the beginning of river migration a year before spawning (spring) T levels were 41.3±10.9 ng/ml, 11KT - 29.4±9.6 ng/ml, Ch – 2.8±0.3 mM/l, TP – 25.7±1.7 g/l, Gl – 1.7±0.3 mM/l. Next spring after long-term reservation the spermatogenesis was completed and T, 11KT, Ch and TP levels elevated significantly (T - 218.9±26.4 ng/ml, 11KT - 81.4±12.1 ng/ml, Ch – 6.8±0.3 mM/l, TP – 44.2±5.2 g/l), Gl levels did not change and was 1.4±0.3 mM/l. After induced spermiation T, 11KT, Ch and TP concentrations decreased significantly (T - 8.1±1.1 ng/ml, 11KT - 48.6 ±11.8 ng/ml, Ch – 3.2±0.3, mM/l, TP – 26.7±2.0 g/l) and Gl levels elevated (4.4±0.5 mM/l) in comparison with the concentrations before treatment.

CONCLUSION: We observed the increase of sex steroid levels and TP and CH after holding at the fish farm followed by the completion of gametogenesis. The data showed the possibility of gonad development without exogenous nutrition after exclusion of river anadromous migration. Consequently high adaptive plasticity of sturgeon allows to use modern technology for breeding and preservation of these fish in nature.

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Preservation of fish gene pool by cryoconservation

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BACKGROUND: At present the decrease in number of species, including those of fish, is observed caused by several factors such as destruction of natural complexes – biogeocenoses. One of the ways to preserve a particular species of animals is creation of gene banks, in particular deep freezing (cryoconservation) of sex cells (sperms). Sperms are the most adapted for cryoconservation as they contain densely packed genetic material, and the level of the vital activity when they are immobile is low.

METHODS: For carrying out the researches, the conventional cryobiology equipment and methods were used. Protective environment was prepared; sperms were collected by straining off; laboratory glassware, environment and activators were cooled down to 5-15°C (depending on species of fish); ejaculates were poured out into containers. The freezing process included the following stages: cooling of the thermal block, sinking into a Dewar vessel, transfer of the packages with sperms into liquid nitrogen.

RESULTS: Protective environment for the species of carp family (Cyprinidae) consisted of 600 ml of Tris-HC1-buffer in which were dissolved in turn 7,3 g NaCl, 0,04 g KCl, 2,3 g $NaHCO_3$, 1,15 g sucrose, 0,52 г $MgSO_4$, 0,15 г $CaCl_2$. 30 minutes before use, 150g of egg yolk and 120 g of ethylenglycol were added to the solution; the volume of the solution was augmented up to 1 litre by adding the Tris-HC1-buffer. For cryoconservation the sperms of fish were used in which more than 80% of cells maintained forward movement after activating by water. The sperm concentration was calculated in a Gorjaev chamber. The selected ejaculate was poured out into containers which were pressurised and placed in the ice bath. After pouring out the containers were taken out of the bath and placed in the cartridges or freezer disk. For granule freezing, the diluted sperms were taken in pipette and dripped on a Teflon plate or an aluminium foil which was placed on the distance of 4 cm above the nitrogen surface. The sperms were frozen according to the following program: 1^{st} stage - from 5^0C to -15^0C at a speed of $1\text{-}2^0$C per minute; 2^{nd} stage - from 15^0C to -70^0C at a speed of $15\text{-}20^0$C per minute; 3^{rd} stage – slow immersion into nitrogen. During artificial fertilisation the sperms were thawed (defrosted). The containers were taken out of liquid nitrogen and transferred to the water bath or the unfreezing device with temperature of $38\text{-}40^0$C while shaking until a liquid phase. Immediately after that, after detecting the moving spermatozoons, the unfrozen sperms were used for fertilisation.

CONCLUSION: Gonadotropic products preserved by deep freezing are one of reliable ways of reproduction of industrial or rare species of fish, further valuable from economic or scientific point of view. It gives the opportunity to preserve the properties of highly productive specimen, and that for virtually unlimited period of time. Besides, the cryoconserved production can be transported over various distances, while preserving all its properties. Cryoconservation of sperm products gives also a possibility to create sperm gene banks which will supply the sperms for breeding new species of fish.

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Advances on the techniques for the control of the European eel reproduction: spermiation induction, sperm quality evaluation and cryopreservation

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BACKGROUND: European eels do not mature in captivity, so the development of methods for the reproduction of this species is necessary to face the demands of elvers from eel farms, but also to reduce the pressure over the extremely reduced natural populations. Our group has developed several techniques trying to improve the control of reproduction of this species.

METHODS: First experiments were carried out to optimize the maturation-inducing hormonal methods for farmed males and wild females, establishing its timing to obtain good quality gamets. Spermiation can be obtained with weekly injections of human chorionic gonadotropin (hCG), and different hormonal treatments has been assayed with the females, resulting in the obtention of eggs and making necessary the synchronization of gamets production. With the intention of solving this problem, we faced the sperm cryopreservation. The study of physio-chemical characteristics of seminal plasma in good quality sperm samples was the basis for the design of cryopreservation media. Later, different factors as the ionic composition, pH, cryoprotectants, the sperm:freezing medium dilution factor, or the presence of protective proteins, as well as different freezing-thawing protocols, were considered trying to improve the spermatozoa survival postcryopreservation.
Different techniques were assayed to evaluate the sperm quality, looking for fast and accurate results to know the effect of hormonal treatments and cryopreservation processes: subjective valuation of the percentage of spermatozoa activated with sea water, evaluation of spermatozoa motility parameters by computer assisted sperm analysis (CASA), morphometry characterisation of spermatozoa by assisted sperm morphology analysis (ASMA) based on previous scanning electron microscopy measurements, and several fluorescent stainings.

RESULTS: Weekly administration of 1.5 IU hCG/g fish was the best treatment. Best sperm was obtained during weeks 8-11, as well as the highest percentage of motile cells (measured by CASA), an increase of alive cells (by Hoechst staining), coinciding with the increase of the spermatozoa head size (by ASMA).
Cryopreservation media (P1 modified) supplemented with FBS or L-α-phosphatydilcholine caused higher percentages of alive cells. Best results were obtained with low ratios (1:2) of sperm:cryopreservation medium dilutions. DMSO caused the lowest reduction in the head cells morphometry, resulting the best cryoprotectant in terms of posthawing survival, near 40%.

CONCLUSION: Once defined a good maturation-inducing hormonal treatment, good results on the sperm cryopreservation has been obtained. The evaluation of hormonal treatments and freezing-thawing protocols has been some of the first applications of CASA, ASMA and fluorescent stainings techniques in fish, and the first in the European eel.

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Hormonal induction of Atlantic dusky grouper (*Epinephelus marginatus*) broodstock

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BACKGROUD: Dusky grouper is a protogynous monandric hermaphrodite and only individuals with a large size (more than 9 Kg) are present as males in the wild. Due to this fact, it is difficult to maintain a sex balanced broodstock in captivity without considering sex ratio control. The use of hormonal induction as a reproductive strategy for a balanced broodstock management of this species has been successfully applied in individuals from the Mediterranean. The aim of this study was to verify if such a technique would be successful in Atlantic dusky grouper.

METHODS: Broodstock was constituted during the period of 2001-2003 from fish captured in the Atlantic Ocean, most of them in the Azores islands. First reproductive attempts in Portugal started in 2005 and 2006. Gonodal biopsy was done monthly to determine sex, gonadal maturation and oocyte size. Immature fish and young females were sex reversed using methyltestosterone implants two months prior gamete extraction. Females were induced with GnRHa when the diameter of oocytes was >325. Gametes were collected by abdominal massage. The frequency of ovulation and oocyte and sperm quality were recorded during the season. Part of the sperm samples were used in cryopreservation trials. Fertility rates were determined 2h after, and only fertilized eggs were incubated until hatch (48h later).

RESULTS: In 2005 one natural male, 8 females and two immature fish were studied. Of the three reversed individuals (young females), two (66%) were functional males. After a 9 months period, one permanent male was obtained. In the second year, 6 females, three males and two immature fish were studied. All sex-reversed fish (one female and two immature) were successfully reversed into functional males (100%). One female was natural reversed into male changing the sex-ratio to 6 males:5 females. Collected sperm of both years range from 30 µl to 300 µl and cell concentration varies between 3 to 6.5 $x10^9$ spermatozoa/ml. Duration of sperm motility was 35 min and decreased in cryopreserved sperm. Males produced sperm within 24 h after Lucrin Depot injection. A total of 4 and 8 spawns were obtained in 2005 and 2006, respectively. Relative fecundity was 66.8 and 114.7 for each year. In the second year, the frequency of ovulations was 1.6 per female, with a maximum of 3 spawns. In the 2005, 76% of eggs were fertilized, with 8-100% of hatching rate. In 2006, only 13% of eggs were fertilized, and no hatching occurred.

CONCLUSION: Young female and immature Atlantic dusky grouper can successfully be induced to functional males with methyltestosterone implants. Hormonal induction of females is also possible to perform as in Mediterranean dusky grouper. Sex ratio control is possible to achieve in dusky grouper with a close control of the broodstock in the beginning of Spring.

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Photoperiod regulation of sexual maturation in Haddock (*Melanogrammus aeglefinus*)

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BACKGROUND: The haddock (*Melanogrammus aeglefinus*) is considered to be a potential species for marine aquaculture like its close relative the Atlantic cod (*Gadus morhua*). However the attainment of sexual maturity prior to a suitable harvest size severely restricts the growth potential and hence profitability of this fish in commercial culture. This work investigated the environmental entrainment of sexual maturation in order to design management practices which could then be used to inhibit or prevent sexual maturation.

METHODS: In January 2003, 462, 9 month old haddock were individually tagged and divided between 3 photoperiod regimes (1: simulated natural photoperiod (SNP), 2: SNP until January 2003, then continuous illumination (LL), 3: SNP until July 2003, then LL). Individual growth performance and maturation status were inspected every three months until July 2004. At the same time 15 individuals per treatment were sacrificed and blood plasma removed for calcium (indirect measurement of vitellogenin), testosterone and 17β-estradiol analysis. Gonads were removed, weighed and fixed for histological analysis and livers were removed and weighed.

RESULTS: Sexually mature individuals were only observed in the SNP treated population. Male plasma testosterone concentration was the first indication of sexual maturation, elevating in October 2003. In January 2004, histological examination of gonads revealed active gametogenesis coinciding with elevations in GSI. At this time steroid levels peaked in both sexes (Testosterone, M:F 11.2 ± 1.3 $ng.ml^{-1}$: 6.4 ± 0.9 $ng.ml^{-1}$, 17β-estradiol, 2.4 ± 0.4 $ng.ml^{-1}$) and plasma calcium was significantly elevated in females. Spawning was recorded from the 10th March until 7th May 2004. Following which, all parameters (steroids, calcium and GSI) returned to pre spawning levels. Both LL treated populations recorded no significant elevations in sex steroids or calcium concentrations, GSI levels or histological development through the course of the entire experiment. Growth was stimulated (elevated SGR, TGC and improved FCR) by the transfer from SNP to LL while spawning significantly reduced growth in the SNP population resulting in a 40% improvement in weight in the immature populations at the trials end.

CONCLUSION: These results clearly demonstrate the mechanism for photoperiod entrainment of sexual maturation in haddock. The reduction in day length from summer to winter, prior to first maturation, is the environmental signal that initiates puberty. Therefore the application of artificial illumination to mask this signal results in the complete inhibition of sexual maturation. For the aquaculture industry such a management practice will be of great economic importance as it significantly improves the species growth potential.

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Photoperiod manipulation of yellow snapper (*Lutjanus argentiventris*) broodstock induced out-of-season maturation, spawning, and differences in steroid profiles

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BACKGROUND: Yellow snapper *Lutjanus argentiventris* (Family: Lutjanidae) is a candidate for commercial aquaculture. Its natural breeding season is May through October. Photoperiod manipulation is know to induce out-of-season maturation and spawning, but in tropical species this is not well studied and is controversial because differences in photoperiod are less pronounced than in the mid and higher latitudes.

METHODS: Yellow snapper broodstock were maintained at a 1:1 sex ratio in two large fibreglass tanks (7 tons each), which were covered with a cone-shaped roof. During November 2004, the photoperiod was adjusted to 8L:16D, and every two weeks daylight was increased, as follows: 10L:14D, 12L:12D, 14L:10D, then decreased, as follows: 12L:12D, 10L:14D, 8L:16D for a total of 98 days. Blood samples were taken at the end of each photoperiodic change. The plasma was tested for testosterone and estradiol. A second experiment began in October 2005, where increasing and decreasing photoperiods were applied, as stated before, except that the 14L: 10D treatment lasted 5 weeks. Relative fecundity was calculated as the number of eggs/ml/day, and the mean fecundity and standard error of each photoperiod were used to interpret results.

RESULTS: Data from 2004 showed mean relative fecundity ranging from 0 during the 8L:16D photoperiod to ~100,000 during the 10L:14D and 12L:12D periods and abruptly increasing to ~500,000 during the 14L:10D period. This very high value remained during the first two subsequent decreasing photoperiods, then declined and varied, and finally ceasing at the end of 8L:16D photoperiod. The steroid profile showed significant testosterone increases as photoperiod increased to 14L:10D (20 ± 2.6 ng/ml) when compared to the three lower photoperiods 8L:16D, 10L:14D, and 12L:12D (2, 10, and 17 ± 1.5 ng/mL, respectively. Testosterone progressively diminishing as photoperiod decreased. Estradiol levels were not significantly different, ranging from 3 to 12 ± 2.5 ng/mL Data from 2005 showed a remarkable reduction of fecundity.

CONCLUSION: During the two years of compressed photoperiod manipulation, yellow snapper matured and spawned out of season. Photoperiod manipulation influenced fecundity and it was clear that the endocrine pathways of reproduction are linked to reception of light.

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Hormonal Indicators of Kutum (*Rutilus frissi kutum*) in Sex Maturation Stages

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BACKGROUND: Due to importance of kutum (*Rutilus frissi kutum*) and decreasing of its stock, different researches have been carried out in Iran at national levels and with support of Fisheries Organization for increasing its stocks. One of the main ways for increasing stocks is appropriate propagation and releasing larva to Caspian Sea. Fish spawn annually according to an Iranian study and probably return to their river of birth. Temperature and river flow are the factors determining the entrance of fish into the rivers on the spawning migration.

METHODS: In this study, sampling used 3, 4, 5 and 6 year-old female fertile kutum caught from artificial reproduction station in Shirood, main migratory river of kutum in Caspian Sea. They were captured in March-April 2003 and 2004 during the final reproductive periods. Blood sample was taken before injecting pituitary extract and centrifuged at 2000g. Radioimmunoassay (RIA) was used to measure Progesterone according to Kagawa et al. *(*1994) Statistical software (SPSS, version 11.5 &13; Excel and Minitab 13.1) was used for all statistical analyses. Tuckey test was performed on Log-transformed data followed by Dunett's test for post-hoc comparisons of groups. Differences between groups were analyzed by one-way ANOVA for normally distributed data or Kruskal–Wallis nonparametric test when data failed tests of normality.

RESULTS: The results revealed that the maximum and minimum of progesterone level of kutum was recorded 2.1(ng/ml) in age 3, at stage of maturation IV in 2003, and 0.20(ng/ml), in different age groups, stages of maturation and in both years respectively. Statistical analysis showed that there was no significant difference between different age groups (p>0.05). The mean progesterone level of kutum in stage V (0.89 ± 0.07 ng/ml) was more than IV (0.24 ± 0.006 ng/ml) and there was significant difference between these two stages (p<0.05).The mean progesterone level in kutum in 2004 (0.43 ± 0.05 ng/ml) was decreased in comparison to 2003 (0.70 ± 0.07 ng/ml) and also there was significant differences between these values (p<0.05). The Pearson correlations between progesterone level with growth and fecundity indices was too weak (Pearson coefficient < 0.1, p>0.05), but there was an inverted correlation between progesterone and 17-β estradiol (Pearson coefficient = -0.54, p<0.05).

CONCLUSION: In this study the concentration of progesterone are low in kutum in stage II in comparison to other stages the level of progesterone has increased significantly in comparison to other stages in stage V, the results reveal that the mean alternations concentration of progesterone in stage IV are between 0.23 to 0.25 ng/ml, and the maximum and minimum level of this hormone in this stage were found to be 0.2 ng/ml and 0.33 ng/ml respectively. But the mean alternations in concentration of progesterone in stage V are between 0.6 to 1.1 ng/ml in different age (0.8 ng/ml), and the maximum and minimum levels of this hormone in this stage were observed to be 2.1 ng/ml and 0.5 ng/ml respectively. Therefore, progesterone is an indicator for stage of maturation and we can use progesterone concentration for distinguishing stage of maturation IV and V from each other. In addition, this study will suggested make a biochemical kit for determining stages directly in the field. According to the results and data presented here suggests that there is a physiological relation between fluctuation of progesterone level and stage of maturation in kutum. In the present study, we also found year-to-year differences in the changes in plasma level of progesterone.

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Intracytoplasmic Sperm Injection (ICSI) in three different genera of teleost

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BACKGROUND: In the past decade Intracytoplasmic Sperm Injection (ICSI) has gained interest due to its diverse applications, and it has been evaluated with varying levels of success in different mammalian species including humans. This technique could be used in finsfish for different purposes ranging from basic reproductive biology studies to the use of sperm preserved by methods other than freezing. We have performed ICSI in three different genera of teleost: zebrafish, Danio rerio, channel catfish, Italurus punctatus, and tilapia, Oreochromis niloticus.

METHODS: Injections were performed using a micromanipulator apparatus consisting of an inverted microscope equipped with two mechanical micromanipulator units. A pipette was used to hold the egg and other to inject the sperm. Due to the variability in size between eggs of different genera of fish, the holding pipettes were constructed with an internal diameter ranging from ~0.1 to ~2.0 mm. Eggs were placed in a 200-µL drop of Hanks' in the lid of a plastic culture dish. When eggs were correctly positioned with the animal pole facing outward, the injection pipette was pushed through the micropyle canal into the cytoplasm and a sperm was injected with ~7 pL of Embryo Medium containing 10% polyvinylpyrrolidone to prevent the sperm from sticking to the wall of the pipette.

RESULTS: Sperm injections yielded fertilization and development in zebrafish and Nile tilapia but not in channel catfish. From a total of 233 eggs injected through the micropyle in zebrafish, 4 (2%) developed normally and hatched. Of the 160 eggs injected in Nile tilapia 5 (3%) developed normally and hatched. Injections of 188 channel catfish eggs yielded no fertilization.

CONCLUSION: ICSI in fish is certainly possible but further studies in the mechanism of fertilization need to be addressed to improve its efficiency and incorporate this technique as another genetic tool for the improvement of finfish. The diversity of physiological and morphological characteristics among fish gametes requires the development of a technique for each species of fish. The different characteristics that must be considered when performing ICSI include: spawning period and conditions, gamete size and activation characteristics, adhesive properties of eggs, transparency of the chorion, size and localization of the micropyle, and the value of the species studied.

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Multiple spawning of the fat snook Centropomus parallelus using different dosages of LHRH analog

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BACKGROUND: The fat snook Centropomus parallelus is distributed along the Atlantic coast of the Americas. In Brazil the species is being studied for aquaculture purposes. The main objective of the present experiment was to verify the effect of different LHRHa dosages and multiple inductions to obtain consecutive spawnings in one reproductive season.

METHODS: The LHRHa hormone was administered in cholesterol pellets. From December 2001 to April 2002 five induction cycles were carried out, with 16 females and 32 males induced per cycle. Broodstock was maintained in four 8000-L tanks. Females were induced with dosages of 15, 35 and 50 (micro)g of LHRHa per kg. Males had a unique dosage of 25 µg of LHRHa per kg. After induction, two males and one female were placed in 1000-L tanks to obtain natural fertilization.

RESULTS: The spawning rates obtained were 24.1%, 60%, 65.5%; respectively for 15, 35 and 50 (micro)g/kg. The number of fertilized eggs per kg of female was 86,658, 181,328 and 383,026, respectively for 15, 35 and 50 (micro)g/kg. The third induction cycle, corresponding to February, resulted in the highest number of spawnings. From the 16 females, ten had three or four consecutive spawnings.

CONCLUSION: It was demonstrated that the female fat snook could be induced to spawn with a dosage of 35 (micro)g/kg and that up to four consecutive spawnings could be obtained per female in one reproductive period, with a 30-day interval between inductions.

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Domestication and GnRHa induced-spawning of meagre (*Agyrosomus regius*)

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BACKGROUND: Meagre (*Argyrosomus regius*) is a promising fast growing (1kg in first year of cage culture) species for aquaculture. Information on wild organisms indicates that puberty is at >8 kg. Protocols for domestication and spawning are required.

METHODS: Twelve meagre broodstock were caught of the Algarve coast, Portugal by Flying Sharks fish capture consultants (Email: info@flyingsharks.eu) and transported to IRTA. On arrival (25th October) fish were weighed, pit tagged, examined for parasites and given an antibiotic injection (10mg.Kg^{-1} Enrofloxacin). Parasites were stained in aceto-carmine, dehydrated and mounted in Canada balsam. Fish were acclimatised to two thermically-isolated 20m^3 tanks in a recirculation system and given a regimen of natural light (approx. 50 Lux) and temperature, dampened to 14-25°C. To control parasites, three consecutive daily formalin bathes (100ppm / 1 hour) were given weekly for the first three weeks, followed by an oral praziquantel treatment (5mg Kg^{-1} body weight, BW). Food, frozen sardines and squid in a 2:1 ratio were offered from 6 days after arrival (DAA). On the 26th March the maturational status, sperm activity and oocyte size, was assessed by canulation and abdominal pressure. Females with oocytes greater than 560µm were treated with either a GnRHa injection of 20µg.kg^{-1} or implant of 50 µg.kg^{-1}, males were treated with half the dose.

RESULTS: The mean broodstock weight was 20±7Kg (12-30kg). The fish were infested with monogenean, initial identification suggests two undescribed *Calceostoma* sp. After de-parasitizing treatments, no parasite problems were encountered. The first positive feeding response was observed 13 DAA. At temperatures of 18-21°C the fish comfortably (feed eaten when offered, no uneaten feed left) ate 2.5 % BW, as temperatures decreased and were then stabilized (with recirculation) at 14-16°C food intake was decreased to 0.8 % BW. Ammonia levels were stabilized at 0.1-0.3 mg.L^{-1} after start-up peaks of 1.2 and 0.5 mg.L^{-1}. Four females matured to exhibit oocytes greater than 560µm, during the two weeks after GnRHa treatment, two injected females spawned 7,022,727 fertilised eggs (3 spawns) and two GnRHa implanted females spawned 7,765,804 eggs (5 spawns). The two males present matured to give abundant sperm, motility > 90%, duration 5 minutes.

CONCLUSION: This is the first report on domestication and spawning of meagre, the protocols that were applied have enabled large adult meagre to be domesticated and spawned within a 6 month period.

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Characterization of physiological response of killifish embryos to water stress: evidence for stimulation of development and differential aquaporin gene expression

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BACKGROUND: The killifish, Fundulus heteroclitus, presents a semi-lunar reproductive cycle which is common in fish living in coastal areas in adaptation to tidal fluctuations. The tides in marshes induce water removal and air-exposition of the embryos during several days. In killifish, the hatching process is adapted to this environmental changes and, even if normal hatching occurs around day 12 after fertilization, under absence of external water embryos can wait from days up to one month within the chorion. In the present study, we investigated the physiological response of killifish embryos to water removal stress under laboratory conditions.

METHODS: Groups of 30 to 80 embryos collected from naturally spawning fish were incubated in Petri dishes, with or without seawater, maintained at approximately 100% ambient humidity for several days. Hatching, mortality, and water content and length of embryos were determined regularly. Water content was also monitored in the different embryo compartments: intact embryos, embryos without chorion, chorion, and yolk. Integrity of the chorion under desiccation was assessed by scanning electron microscopy and by SDS-PAGE. Gene expression of aquaporin-0 (AQP0), -1 (AQP1) and -3 (AQP3) was determined by real-time quantitative PCR (RT-qPCR) and whole-mount in situ hybridization.

RESULTS: Induction of water removal at blastula stage for 6 or 12 days accelerated development and hence the time of hatching when compared to control embryos. However, embryos at day 15 after fertilization maintained without water for 12 days did not hatch until water was added, while development was not stopped. None of the water removal treatments induced a significant effect on mortality rates, indicating the ability of embryos to resist water removal stress. Embryos under water stress were able to maintain the water content similar to that of controls, although a differential distribution of water between the different compartments of the embryo was observed. The chorion appeared to be the main biological barrier to prevent water loss despite that protein degradation probably occurred in the outer layer forming the chorion. RT-qPCR revealed that AQP3 transcripts were down-regulated in embryos maintained without water with respect to controls during gastrulation. The amount of AQP0 mRNAs in embryos which were localized almost exclusively in the developing eye, correlated with the increased growth, and thus these transcripts were overexpressed in late neurula and 18-somites stage embryos developing without water. In contrast, AQP1 gene expression remained unchanged between water stressed and control embryos during development.

CONCLUSION: Killifish embryos are able to develop without external water which is possibly allowed by the presence of a quite impermeable chorion, while water seems to be the signal for hatching. The physiological response of embryos to water removal stress is the acceleration of development, which might signify an strategy to survive in the wild. Interestingly, expression of AQP3 in the embryo is down-regulated during water removal which likely accounts to prevent water loss from the embryo.

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Basic management to spawn the cultured sturgeon, *Acipenser sturio* L, 1758, a critically endangered species

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BACKGROUND: Most sturgeon populations are threatened world wide. Though protected in France since 1982, the status of *A.sturio* has been deteriorating. The only chance to prevent the species from extinction depends on the capacity to build a functional cultured broodstock. The key condition is to succeed in breeding the fish, i.e. to provide the fish with suitable rearing conditions and appropriate management.

METHODS: The present study deals with two groups of fish: group-1 (N1=7) composed of older fish born in the wild (1984-1989), and to a lesser extent group-2 (N2=16) composed of fish born in the wild in 1994. Group-1 fish arrived in the hatchery between 1993 to 1997, and group-2 fish during 1995. There is only one female in group-1. Total absence of data and experience of the species has led us to adopt the following strategy with regard to methods, based on our experience with other sturgeon species, the ecology of the species, a step by step procedure, non-invasive practices, and conservative decisions. As a precaution, the fish were kept in brackish water (~15 ‰) all year round. Winter vernalization and natural daylight were applied. The range of water temperature was 10-12/23-25°C. The fish were fed frozen shrimps. The size and migration of the germinal vesicle (polarization index, PI), and the in vitro maturation competence (GVBD) of ovarian follicles were performed. Males were staged according to methods developed for Siberian sturgeon. Hormonal stimulation (CPE or LHRHa) was needed to obtain gametes. Priming was frequently applied to speed up maturation.

RESULTS: Alternate appetite and growth has been currently observed throughout the year. Five out of six males from group-1 matured in the period 1999-2006. 5 fish matured in 2000 and 2001. 4 of these fish matured in the two consecutive years. Only 1 or 2 fish matured in the remaining years. Depending on the individuals, 0 to 7 maturations were recorded within the experimental period. Only one male maturation was observed in group-2. Females (the older and 3 youngest specimens) exhibited partial maturation in 2002 and 2005 respectively. These maturations were characterized by an abdomen that was not swollen, difficulties in sampling ovarian follicles, some unusual drawings, heterogeneity in germinal vesicle migration, and moderate GVBD. Simulation of upstream migration was then applied by transferring the mature fish into fresh water for one week prior to new maturation control. Injected males produced good quality semen. Hormonal stimulation increased GVBD to 90-100% on five occasions from 2002 to 2006. This high level of GVBD also corresponded to high PI values (0.10-0.13) currently known to be too high. It is worth noting that PI values exhibited a large range (e.g. 0.08-0.16) for some spawners, illustrating an asynchronous advance in maturation. Further hormonal treatment did not bring about ovulation.

CONCLUSION: In order to improve the results, the following changes were made in 2006. Food deprivation (one month prior to spawning) was applied, light intensity was lowered and water temperature control was set to limit high summer temperatures to 18-20°C. Since the oogenesis cycle is non-yearly, any consequences would be most likely to occur in spring 2007 or, more probably, in 2008.

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Genetic diversity of Senegalese sole (Solea senegalensis) stocks reared in captivity

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BACKGROUND: The Senegalese sole (Solea senegalensis) is a promising new aquaculture species that commands high prices in a different market to bream and bass. Spawning broodstock groups have been difficult to establish, particularly with fish reared in captivity. Progeny from captive wild broodstock groups have been shown to have a drastic reduction in genetic variability indicating that few broodstock within the group actually participated in spawning. This study was undertaken to establish the genetic variability in 4 apparently different stocks, one wild stock and three stocks reared in captivity.

METHODS: A saline precipitation method was used to extract DNA from blood samples taken from each fish. Genetyping of each sample was completed with 8 loci microsatelite described for this species. The PCR products were analyzed in an automatic sequencer ABI 310 (Applied Biosystems). The alleles size was determined and revised with the software Genotyper Version 3.7. The genetic diversity of the different stocks was quantified in terms of number of alleles per locus and FSTAT, Ver. 2.9.3 software was used to identify significance of genetic relatedness of the different stocks.

RESULTS: The genetic characterization showed a heterogeneous population with alleles in disequilibrium compared to the expected proportions according to Hardy-Weinberg. The disequilibrium originated principally from two of the captivity-reared stocks that genetically exhibited no significant difference and that appeared to be predominantly siblings and half siblings from two families. The third captivity-reared and wild stock exhibited higher levels of genetic variation and appeared to be genetically significantly different populations.

CONCLUSION: This study confirms that unselected captivity-reared Senegalese sole may be genetically similar sibling groups and highlights the requirement to genetically characterise broodstock before forming potential spawning groups. The species characteristics of high fecundity and difficulties to establish spawning groups has resulted in substantial genetic loss in one generation and siblings and half siblings groups being held as broodstock. Such sibling broodstock groups may partially explain increased spawning problems in captivity-reared broodstock compared to captive wild broodstock.

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Effect of sexual genotype on the reproductive biology of Nile tilapia

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BACKGROUND: The production of all-males families of Nile tilapia (*Oreochromis niloticus*) with improved growth performances was achieved using breeders displaying a phenotypic sex different from the genotype (i.e. XX and YY males and XY and YY females). Although these fish were widely used in production, the reproductive biology of such breeders has received little attention.

METHODS: Six combinations of crosses were realized in duplicate between XX, XY and YY females and XX, XY and YY males. During 13 weeks, natural reproduction occurred in $4m^2/1.6m^3$ tanks at 27°C with a ratio of 1 males and 10 females per tank. Once a week eggs were removed from the mouth of the female, weighted, counted and incubated at 27°C until hatching (hatching rates assessment). At the end of the experiment, gonadosomatic index (GSI) was determined on 10 females from each genotype and spermatozoa concentration (number of spermatozoa per ml of sperm) and motility were assessed on 6 XY and YY males and 4 XX males.

RESULTS: The total number of spawns significantly varied with the type of cross : from 36 spawns for XX x XX cross to 9 spawns for YY x YY cross. Similarly, period between spawns significantly ($p<0.05$) increased from 10.0 days for XX x XX cross to 30.1 days for YY x YY cross. Hatching rates were correlated with the type of cross and ranged from 20.5 % for a YY x YY cross up to 68.0% for a normal XX female x XY male cross or XX x XX cross. Eggs originating from YY females displayed a mean weight significantly lower (3.2mg) than eggs from XX and XY females (4.2 and 4.0mg respectively). At the end of the experimental period YY females displayed a GSI 4-fold higher (15.7%) than XX females (4.2%) and a hypertrophied gonad and no gonoduct. Spermatozoa concentration significantly ($p<0.05$) increased with the number of Y chromosomes from 2.76 10^9 spermatozoa ml^{-1} for XX males to 5.90 and 39.17 10^9 spermatozoa ml^{-1} for XY and YY males respectively. On contrary, sperm motility significantly decreased from 10'30" for XX males to 6'12" and 3'38" for XY and YY males respectively.

CONCLUSION: Our results demonstrated that the reproductive biology was influenced by the sexual genotype of the breeders. Crosses between XX females and XX or XY males were the best in term of reproduction and hatching rates. On contrary crosses between YY females and YY males induced the worst results. The low reproductive performances of the YY genotypes could be explained by the abnormal morphology of the gonad for the female and the low activity of the spermatozoa of the YY males. This impact of the sexual genotype on the reproductive biology could also be related to the more aggressive behavior of the YY males or YY females, as it was also demonstrate for other tilapia species.

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