10th International Symposium on Reproductive Physiology of Fish

Detailed Programme

Sunday – 25 May 2014

Real Marina Hotel

14:00 Registration

18:00-18:30 Welcome reception

Monday – 26 May 2014

Olhão Municipal Auditorium

08:30 Registration

09:00-10:35	Opening ceremony and lecture
09:00	Opening ceremony
09:30	Sumpter, John (UK) OPENING LECTURE Fish reproduction: from comparative endocrinology to environmental toxicology Chair: Scott, Alexander

10:35-10:55 Coffee break

10:55-12:20	Chemical Communication and Reproductive Behaviour Chairs: Scott, Alexander and Saraiva, João
10:55	Keller-Costa, Tina (Portugal) STATE OF THE ART LECTURE Chemical communication in tilapia
11:20	Soyano, Kiyoshi (Japan) Pheromonal and hormonal control of final oocyte maturation in groupers
11:35	Romallo, Martín (Argentina) Hormones and agonistic behaviour: what the South American cichlid fish, Cichlasoma dimerus, tell us about them
11:50	Ibarra-Zatarain, Zohar (Spain) Study of behavioural profiles in Senegalese sole (Solea senegalensis) broodstock to characterise stress coping styles in relation to reproductive success
12:05	Patil, Jawahar (Australia) Sterile Judas carp – surgical sterilisation does not impair, growth, endocrine and behavioural responses of male carp

12:20-14:00 Lunch time

Monday – 26 May 2014

14:00	Schulz, Rüdiger (Netherlands) KEYNOTE LECTURE Regulation of (zebra)fish spermatogenesis Chair: França, Luiz Renato

14:35-16:00	The Testis Chairs: França, Luiz Renato and Cerdà , Joan
14:35	Chauvigné, François (Spain) STATE OF THE ART LECTURE Germ line activation of the luteinizing hormone receptor directly drives spermiogenesis in the flatfish Solea senegalensis
15:00	Huertas, Mar (USA) Evidence for a lack of high affinity steroid binding protein in the sea lamprey
15:15	Pandolfi, Matías (Argentina) Male reproductive physiology in a cichlid fish with biparental care: the chanchita Cichlasoma Dimersus
15:30	Le Gac, Florence (France) Characterisation of putative spermatogonial stem cells in trout and fish: effects on potential regulatory pathways
15:45	Hayashi, Makoto (Japan) Identification of cell surface marker for enrichment of type A spermatogonia in rainbow trout

16:00-16:20 Coffee break

16:20-17:20	The Testis (continued) Chairs: França, Luiz Renato and Cerdà , Joan
16:20	Lacerda, Samyra (Brazil) Lentiviral transduction of male germline stem cells in Nile tilapia (Oreochromis niloticus)
16:35	Boj, Monica (Spain) Aquaporin-1aa and 8b are differentially localized in gilthead sea bream (Sparus aurata) spermatozoa and play distinct roles during the activation of sperm motility
16:50	Pérez, Luz (Spain) The ion puzzle which makes the eel sperm motile
17:05	Melo, Michelle C. (The Netherlands) The speed of pubertal development in Atlantic salmon (Salmo salar) post-smolts is modulated by salinity and photoperiod

17:20 Poster session I

Monday – 26 May 2014

17:20 Poster session *I*		
Chemical Communication and Reproductive Behaviour		
N°	Name & Country	Title of the Poster
P001	Fatsini, Elvira (Spain)	Observations of the courtship of mixed wild and captivity breed Senegalese sole (Solea senegalensis) broodstock
The Testis		
P003	Baeza, Rosa (Spain)	Water temperature effect on fatty acid mobilization in male European eels during sexual maturation and relationship between fatty acids, steroids and sperm quality parameters
P005	Crespo, Diego (The Netherlands)	Paracrine mediators of Fsh effects in the adult zebrafish testis: searching for candidates
P009	Lokman, Mark (New Zealand)	Effects of dose and route of administration of human chorionic gonadotropin on testicular development in immature male shortfinned eel, Anguilla australis
P011	Morais, Roberto (The Netherlands)	Amh inhibits and Igf3 promotes spermatogonial differentiation – does Amh modulate Igf3 effects?
P013	Marina Morini (Spain)	Progestin receptors expression in European eel testis through spermatogenesis
P015	Kazeto, Yukinori (Japan)	Evaluation of spermatogenesis and milt quality in the Japanese eel matured by recombinant eel gonadotropin injections
P017	Procópio, Marcela S. (Brazil)	Development of Wistar and GFP rat germ cells transplanted to tilapia (Oreochromis niloticus) testes
P019	Shaliutina-Kole□ov□, Anna (Czech Republic)	Oxidative status of seminal plasma and spermatozoa in different fishes
P021	Zhou, Linyan (China)	Blocking of progestin action disrupts spermatogenesis in Nile tilapia (Oreochromis niloticus)
Sex Determination and Differentiation		
P025	Fernandino, Juan (Argentina)	N-myc downstream regulated 3 (ndrg3): characterization during primordial germ cell proliferation and regulation by androgen in medaka
P027	Hliwa, Piotr (Poland)	Gonadal sex differentiation of the endangered cyprinid fish, lake minnow Eupallasella percnurus (Pallas, 1814)
P029	Kitano, Takeshi (Japan)	Loss of follicle-stimulating hormone receptor function causes masculinization and suppression of ovarian development in genetically female medaka
P031	Luckenbach, Adam (USA)	Characterization of molecular sex differentiation and the sexually labile period of gonadal development in sablefish
P033	Guigen, Yann (France)	Both aromatase isoforms are expressed in all gonads during sexual differentiation in zebrafish
P035	Nakasone, Kiyoshi (Japan)	Estrogen produced by the ovary stimulates the meninges to express a novel heme-binding protein gene, hebp3, in a female-biased manner in medaka
P037	Santinelli, Veronica (Greece)	Sex differentiation and hermaphroditism in sharp-snout sea bream, Diplodus puntazzo, in captivity
P039	Chakraborty, Tapas (Japan)	Role of alternatively spliced isoforms of IFN□2 in gonadal development of medaka, Oryzias latipes
P041	Sun, Lina (China)	Transdifferentiation of differentiated ovary and gene expression profiles in Nile tilapia during aromatase inhibitor induced sex reversal
P043	Wang, Deshou (China)	Comparative transcriptomic analyses of gene expression profile in southern catfish (Silurus meridionalis) gonad
P045	Zhang, Yan (Japan)	Expression profile of amhy and amha genes at high and low temperatures during early larval development in pejerrey, Odontesthes bonariensis

17:20 Poster session *I* (continued)		
Reproductive Technologies and Selective Breeding		
N°	Name & Country	Title of the Poster
P047	Garcia, Carlos (Brazil)	Motility parameters of cryopreserved sperm from sex-reversed dusky grouper Epinephelus marginatus evaluated by computer-assisted sperm analysis (CASA)
P049	Holtz, Wolfgang (Germany)	Sex discrimination in rainbow trout (Oncorhynchus mykiss) using genetic markers
P051	Katayama, Naoto (Japan)	Germ cell-specific excision of the loxP-flanked transgene in rainbow trout: toward the establishment of cell ablation method in farmed fish
P053	Mylonas, Constantinos (Greece)	Parentage assignment and relative female/male participation in the spawning period and differential mortality during rearing in red porgy, Pargus pargus and common pandora, Pagellus erythrinus
P055	Viveiros, Ana (Brazil)	Sperm quality after dilution and freezing processes in Prochilodus lineatus and Brycon orbignyanus (Characiformes)
P057	Zupa, Rosa (Italy)	In vitro effects of recombinant gonadotropins on gonad maturation in pre-pubertal Atlantic bluefin tuna Thunnus thynnus (L.)
Brain & Pituitary		
P059	Cano-Nicolau, Joel (France)	Looking closer at the organization of aromatase-positive radial glial cells in the brain of zebrafish
P061	Chaube, Radha (India)	Cloning and characterization of brain and ovarian cytochrome P450 aromatase genes in the catfish Heteropneustes fossilis: sex, tissue and temporal variations in gene expression
P063	Etzion, Talya (Israel)	The development of the kisspeptin system in zebrafish brain
P065	Kah, Olivier (France)	Kisspeptins in the brain of the European sea bass: expression, regulation and putative functions
P067	Kawabata, Yukika (Japan)	Possible involvement of male-specific, androgen-induced hypothalamic vasotocin expression in aggressive behavior of medaka
P069	Medrado, Andreone (Brazil)	Brain in the endangered Neotropical catfish species – Steindachneridion parahybae (Siluriformes: Pimelodidae): main nucleus related to reproduction during ontogeny
P071	Sevilhano, Thais (Brazil)	Isolation of the □- and ß-subunit genes of luteinizing hormone (LH) of the giant Amazonian fish pirarucu (Arapaima gigas)
P073	Weltzien, Finn-Arne (Norway)	Plasticity of luteinizing hormone beta-expressing gonadotropes show that hormone production in the teleost pituitary is unexpectedly complex
P075	Zanuy, Silvia (Spain)	Molecular characterization of European sea bass sialyltransferases
The Ovary		
P077	Fairgrieve, Marian (USA)	Exploring possible roles of gonadal kisspeptins in the reproductive development of sablefish Anoplopoma fimbria
P079	Gomez, Ana (Spain)	Two teleost specific USF2 are involved in fshr regulation in European sea bass
P081	Guzmán, José (USA)	Characterization of genes regulated by follicle-stimulating hormone during early oogenesis in salmon
P083	Higuchi, Kentaro (Japan)	Changes of expression and cellular localization of insulin-like growth factor I in ovaries during ovarian development of yellowtail
P087	Nyuji, Mitsuo (Japan)	Characterization and gene expression analyses of gonadotropin receptors in greater amberjack (Seriola dumerili) ovaries

Monday – 26 May 2014

17:20 Poster session *I* (continued)		
Reproduction and Environment		
N°	Name & Country	Title of the Poster
P089	Abdalla, Raisa (Brazil)	Influence of acidic pH, aluminium and manganese on the activity of hepatic antioxidant enzymes of Astyanax bimaculatus (Characiformes: Characidae) males
P091	Bazzoli, Nilo (Brazil)	Reproduction and spawning of the endangered characid Brycon orthotaenia downstream a hydroelectric dam, São Francisco River, Brazil
P093	Golshan, Mahdi (Czech Republic)	Sexual differentiation in fertilized eggs of medaka following exposure to endocrine disrupting hormones
P095	von Krogh, Kristine (Norway)	Steroid exposure influences seasonal variation in reproductive gene expression in pituitary primary culture from Atlantic cod (Gadus morhua)
P097	Mishra, Abha (India)	Teratogenic effects of organophosphorous pesticide, chlorpyrifos, on freshwater catfish, Heteropneustes fossilis (Bloch).
P099	Ortiz-Zarragoitia, Maren (Spain)	Widespread occurrence of the intersex condition and associated molecular responses in thicklip grey mullet (Chelon labrosus) from the Basque coast (south east bay of Biscay)
P101	Rizzo, Elizete (Brazil)	Reproductive biomarkers responses induced by xenoestrogens in the characid fish Astyanax fasciatus inhabiting a South American reservoir: an integrated field and laboratory approach
P103	Scott, Alexander (UK)	Immunoassays are not immune to errors - examples from two studies measuring sex steroids in river water
P105	Técher, Didier (France)	Assessing reproductive and developmental toxicity in zebrafish after long-term exposure to gallic and pelargonic acids
Reproduction and Growth		
P107	Cardenas, Rodolfo (Mexico)	The pituitary adenylate cyclase-activating peptide (PACAP) in the gonads of Chirostoma humboldtianum
P109	Kleppe, Lene (Norway)	Candidate genes for sterility in Atlantic salmon (Salmo salar)
P113	Patil, Jawahar (Australia)	Gravid-spot as an external indicator of embryonic development in a live bearing fish Gambusia holbrooki
P115	Pérez Sirkin, Daniela (Argentina)	Food restriction stimulates final maturation of spermatogenesis and induces variation in leptin-A expression in the cichlid fish Cichlasoma dimerus
P117	Rizzo, Elizete (Brazil)	Insulin-like growth factors in gonad maturation of a Neotropical fish
P119	Samarin, Azin M. (Czech Republic)	Effect of delayed spawning on egg viability rates and the occurrence of malformations and ploidy anomalies in pike perch (Sander lucioperca L.)
P121	Shpilman, Michal (Israel)	Production, gene structure and characterization of two orthologs of leptin and a leptin receptor in tilapia
P123	Almeida, Fernanda (Brazil)	Puberty variances (male x female) drive harvest weight in the Amazonian tambaqui (Colossoma macropomum)

Tuesday – 27 May 2014

08:30	Schartl, Manfred (Germany) KEYNOTE LECTURE Transcriptional regulation, rewiring and neo-functionalization in the evolution of a novel sex determining gene: the case of dmrt1bY in medaka Chair: Guiguen, Yann

09:05-10:05 Sex Determination and Differentiation Chairs: Guiguen, Yann and Tanaka, Minoru	
09:05	Nishimura, Toshiya (Japan) Primordial germ cells exhibit sexually different characters by sex determination gene-independent mechanism in medaka
09:20	Wu, Guan-Chung (Taiwan) Tug-of-war of soma fate decision in the hermaphroditic black porgy
09:35	Yarikipati, Prathibha (India) Significance of transcription factor, Pax2 and the signaling molecules, Wnt4/5 in the gonadal differentiation of the catfish, Clarias batrachus
09:50	Uchikawa, Taku (Japan) Gonadal soma-derived growth factor sppresses the expression of follicle-stimulating hormone receptor in medaka

10:05-10:25 Coffee break

10:25-12:20 Sex Determination and Differentiation (continued) Chairs: Guiguen, Yann and Tanaka, Minoru	
10:25	Ribas, Laia (Spain) STATE OF THE ART LECTURE Overview of external influences on the process of sex differentiation in zebrafish gonads
10:50	Díaz, Noelia (Spain) A transcriptome study on temperature and estradiol effects on European sea bass (Dicentrarchus labrax) gonads
11:05	Yamamoto, Yoji (Japan) Genetic versus temperature-dependent sex determination in pejerrey: evidence from the testis-determining gene, amhy
11:20	D'Cotta, H□□na (France) Up-regulation of dax1 in the brain of phenotypic and genetic males during sex differentiation
11:35	Vizziano Cantonnet, Denise (Uruguay) Characterisation of molecular sex differentiation in the Siberian sturgeon, Acipenser baerii
11:50	Gennotte, Vincent (Belgium) The sensitive period of sex differentiation begins at the embryonic stage in Nile tilapia: new insights on the mechanisms of early feminization
12:05	Marivin, Elisa (France) Further characterization of rainbow trout Shbgb during sexual differentiation

12:20-14:00 Lunch time

Tuesday – 27 May 2014

14:00	Yoshizaki, Goro (Japan) KEYNOTE LECTURE Transplantation of salmon germ cells intro rainbow trout recipients: can iteroparous trout repeatedly produce gametes derived from semelparous salmon? Chair: Rosenfeld, Hanna

14:35-16:00 Reproductive Technologies and Selective Breeding Chairs: Rosenfeld, Hanna and Carnevali, Oliana	
14:35	Goméz, Ana (Spain) STATE OF THE ART LECTURE Gonadotropin actions in European sea bass: endocrine roles and biotechnological applications
15:00	Davie, Andrew (UK) Identifying sex-associated markers in farmed fish using RAD sequencing
15:15	Norberg, Birgitta (Norway) Tools for the development of a selective breeding strategy for Atlantic halibut (Hippoglossus hippoglossus L.): genetic sex markers and large scale all female production
15:30	Labbé, Catherine (France) DNA methylation of fish germ cells and the risk of alteration after cryopreservation
15:45	Grant, Bridie (UK) Tools and techniques to support ballan wrasse (Labrus bergylta) broodstock management

16:00-16:20 Coffee break

16:20-17:30 Reproductive Technologies and Selective Breeding (continued) Chairs: Rosenfeld, Hanna and Carnevali, Oliana	
16:20	Wong, Ten-Tsao (USA) STATE OF THE ART LECTURE Production of infertile zebrafish by disrupting primordial germ cell development: transgenic and non-transgenic approaches
16:45	Lee, Seungki (Japan) Production of offspring derived from frozen whole fish kept in freezer
17:00	Mylonas, Constantinos C. (Greece) Comparison between GnRHa injections and implants on spawning kinetics and egg/larval performance parameters in meagre Argyrosomus regius
17:15	Kookaram, Kazem (Iran) Effect of oral administration of GnRHa+NANO particles of chitosan in oocyte development of goldfish, Carassius auratus

17:35 Round Tables			
I – Sex Determination		***II*** – Int. Society for Fish Endocrinology	
17:35	Wang, Deshou S. (China) STATE OF THE ART LECTURE CRISPR/CAS9, a versatile tool to study sex determination and for sex control in teleosts	17:35	Round table to discuss the possible launch of a Int. Society for Fish Endocrinology
18:00	General Discussion		

Wednesday – 28 May 2014

08:30	Levavi-Sivan Berta, (Israel) KEYNOTE LECTURE Novel fish hypothalamic neuropeptides that regulate fish reproduction Chair: Swanson, Penny

09:05-10:00	Brain and Pituitary Chairs: Swanson, Penny and Habibi, Hamid
09:05	Ueda, Hiroshi (Japan) STATE OF THE ART LECTURE Neuroendocrine control mechanisms of olfactory imprinting and homing migration in Pacific salmon
09:30	Hiraki, Towako (Japan) Female-specific expression of neuropeptide B in the medaka brain
09:45	Zmora, Nilli (USA) Understanding the roles and modes of action of kisspeptin neurons in reproduction

10:00-10:25 Coffee break

10:25-12:25	Brain and Pituitary (continued) Chairs: Swanson, Penny and Habibi, Hamid
10:25	Kah, Olivier (France) STATE OF THE ART LECTURE Estrogens modulate cell proliferation in the brain of teleosts
10:50	Smith, Olivia A. (USA) The gonadotropin-inhibitory hormone system in zebrafish: ontogeny, interactions, and functional roles in the hypothalamus-pituitary-gonad axis
11:05	Fontaine, Romain (France) Neuroanatomical and genetic bases for the dopaminergic inhibition of the gonadotrope function in zebrafish
11:20	Guzmán, José M. (USA) Development of approaches to induce puberty in cultured female sablefish
11:35	Gothilf, Yoav (Israel) The zebrafish pineal gland, a central clock under spotlight

11:50	Sponsored by the Society for Endocrinology Thomas, Peter (USA) KEYNOTE LECTURE Membrane progesterone receptors: a critical review Chair: Miura, Takeshi

12:25 Hand-over of lunch bags

13:30 Social Programme

Thursday – 29 May 2014

08:30	Hiramatsu, Naoshi (Japan) KEYNOTE LECTURE Ovarian yolk formation in fishes: molecular mechanisms underlying formation of lipid droplets and vitellogenin-derived yolk proteins Chair: Andersson, Eva

09:05-10:05	The Ovary Chairs: Andersson, Eva and Bobe, Julien
09:05	Chakraborty, Tapas (Japan) Estrogen receptor □2 is required for germ cell migration and ovarian differentiation in genetically female medaka
09:20	Inbaraj, Rajamanickam M. (India) MIS receptor variations and expression in the reproductive cycle of Labeo rohita with a special reference to Cyp19 gene
09:35	Wargelius, Anne (Norway) A comparison between egg transcriptomes of cod and salmon reveals species specific traits in eggs for each species
09:50	Kazeto, Yukinori (Japan) Mass production of Japanese eel recombinant follicle-stimulating hormone and luteinizing hormone by a stable expression system: they fully induced ovarian development at a differential mode

10:05-10:25 Coffee break

10:25-12:20	The Ovary (continued) Chairs: Andersson, Eva and Bobe, Julien
10:25	Ijiri, Shigeho (Japan) STATE OF THE ART LECTURE Molecular mechanism of maturation-inducing hormone production during final oocyte maturation in the Japanese eel
10:50	Cancio, Ibon (Spain) Seasonal dynamics of ribosomal RNA components production and accumulation during oogenesis in fish; cautiously investing in reproduction
11:05	Fostier, Alexis (France) Isolation, in vitro culture and nucleic acid transfection of rainbow trout granulosa cells
11:20	Ryu, Yong-Woon (Japan) Very low-density lipoprotein is primary carrier of neutral lipids to ooplasm lipid droplets in teleosts
11:35	Damsteegt, Erin L. (New Zealand) How do eggs get fat? Insights into triacylglyceride uptake in the oocytes of the shortfinned eel Anguilla australis
11:50	Yilmaz, Ozlem (France) Multiple vitellogenin yolk precursers in European sea bass (Dicentrarchus labrax)
12:05	Bouleau, Aurélien (France) Maternally-inherited npm2 mRNA is crucial for early developmental success in zebrafish

12:20-14:00 Lunch

Thursday – 29 May 2014

14:00	Dufour, Sylvie (France) KEYNOTE LECTURE Origin, fate and roles of duplicated genes in teleost neuroendocrine control of reproduction: new insights from the eel Chair: Zanuy, Silvia

14:35-16:00	Reproduction and Environment Chairs: Zanuy, Silvia and Ueda, Hiroshi
14:35	Habibi, Hamid R. (Canada) STATE OF THE ART LECTURE Study of reproductive impairment by environmental contaminants using genomic and metabolomics approach
15:00	Golshan, Mahdi (Czech Republic) Recent findings illustrating anti-androgenic activity of vinclozolin that cause impairment in male fertility in goldfish
15:15	Katsiadaki, Ioanna (UK) Is the contraceptive pill as oestrogenic to fish as we think?
15:30	Anastasiadi, Dafni (Spain) Methylation levels of key genes in European seabass (Dicentrarchus labrax) exposed to different thermal conditions
15:45	Palstra, Arjan P. (The Netherlands) In- and outdoor reproduction of first generation common sole under a natural photothermal regime: temporal progression of sexual maturation determined by plasma steroids, genome sequencing and pituitary gonadotropin expression

16:00-16:20 Coffee break

16:20-17:30	Reproduction and Environment (continued) Chairs: Zanuy, Silvia and Ueda, Hiroshi
16:20	Andersson, Eva (Norway) STATE OF THE ART LECTURE The impact of environmental factors on the gonadotropic system in Atlantic salmon (Salmo salar)
16:45	Peñaranda, David S. (Spain) Temperature as modulator of the steroidogenic process in European eel: migratory implications
17:00	Lafont, Anne-Gaëlle (France) Characterisation and regulation during reproduction of four nuclear corticosteroid receptors in the European eel, Anguilla anguilla
17:15	Harding, Louisa B. (USA) Effects of estrogenic chemicals on transcripts for gonadotropin subunits and genes in gonadotropin-releasing hormone signaling and circadian rhythm pathways in female coho salmon, Oncorhynchus kisutch

17:30 Poster session **II**

20:00 Conference dinner at Real Marina Hotel

Thursday – 29 May 2014

17:30	Poster session *II*	
The Testis		
N°	Name & Country	Title of the Poster
P002	Assis, Luiz (Brazil)	Effects of Insl3 (insulin like peptide 3) on spermatogonial dynamics in adult zebrafish
P004	Boj, Monica (Spain)	Endocrine control of aquaporin expression during gilthead sea bream (Sparus aurata) spermatogenesis
P006	Ichida, Kensuke (Japan)	Enrichment of transplantable germ cells using magnet-activated cell sorting in rainbow trout
P010	Melo Dias, Gisele (Brazil)	Characterization of germ cells and isolation of type A spermatogonia from the testes of Prochilodus lineatus (curimbatá)
P012	Morini, Marina (Spain)	Sperm protein for egg activation: PLCZ1 expression in European eel testis through spermatogenesis
P018	Safian, Diego (The Netherlands)	Fsh modulates the insulin growth factor binding proteins (Igfbps) expression in zebrafish testis
P020	Szentes, Katalin (Hungary)	Morphology and ultrastructure of Asian sea bass (Lates calcarifer) spermatozoa
Sex Determination and Differentiation		
P022	Rosenfeld, Hanna (Israel)	The relative role of LH and FSH during sex change in a protandrous hermaphrodite model species, the gilthead sea bream (Sparus aurata)
P024	Gómez, Ana (Spain)	Differential expression patterns of two sox9 genes in the teleost fish Dicentrarchus labrax
P026	Goto, Rie (Japan)	Visualization and migration of primordial germ cells in a marine fish, the Japanese anchovy, Engraulis japonicas
P028	Honji, Renato (Brazil)	Sex differentiation in the endangered Neotropical species Steindachneridion parahybae (Siluriformes: Pimelodidae): a cytogenetic and morphological study
P032	Mañanós, Evaristo (Spain)	Steroid levels and gonadotropin gene expression during larval development and gonad differentiation in Senegalese sole (Solea senegalensis), reared under intensive and mesocosm conditions
P034	Muncaster, Simon (New Zealand)	Aromatase inhibitor induced sex change in the temperate wrasse Notolabrus celidotus
P036	Wang, Deshou (China)	The essential role of figla in the ovarian differentiation in tilapia was demonstrated by TALENs
P038	Sarida, Munti (Japan)	Adoptosis and anti-müllerian hormone gene expression during gonadal sex differentiation in pejerrey Odontesthes bonariensis
P042	Di Landro, Santiago (Uruguay)	Amh expression during sex differentiation of the Siberian sturgeon, Acipenser baerii
P044	Wu, Limin (China)	Rspo1/□-catenin signaling pathway involving in the ovarian differentiation in a teleost, tilapia (Oreochromis niloticus)

Thursday – 29 May 2014

17:30 Poster session *II* (continued)		
Reproductive Technologies and Selective Breeding		
N°	Name & Country	Title of the Poster
P040	Silva, Mariana A. (Brazil)	Germ cell transplantation from the jundiá catfish (Rhamdia quelen) into the Nile tilapia (Oreochromis niloticus)
P046	Bernáth, Gergely (Hungary)	Methodical standardization of motility assessment and short-term storage in Eurasian perch (Perca fluviatilis)
P048	Cerdà, Joan (Spain)	Genetic variants in the aqp1ab gene are associated with egg quality in the gilthead sea bream (Sparus aurata)
P050	Holtz, Wolfgang (Germany)	Chilled storage of eyed rainbow trout (Oncorhynchus mykiss) eggs in sealed polyethylene (PE) bags
P052	Leal, Marcelo (Brazil)	Seminal plasma affects post-thaw sperm motility and curvilinear velocity but not membrane integrity of Prochilodus lineatus (Pisces, Characiformes)
P054	Nocillado, Josephine (Australia)	Application of recombinant DNA and oral delivery technologies to manipulate fish reproduction in aquaculture
P056	□arski, Daniel (Poland)	Can cortical reaction be a commercially applicable egg quality indicator in freshwater cyprinids?
Brain & Pituitary		
P058	Bogerd, Jan (The Netherlands)	Hypothalamic and pituitary factors controlling reproduction in Atlantic salmon
P060	Carvalho, Roberto (Brazil)	Isolation of the □- and ß-subunit genes of follicle-stimulating hormone (FSH) of the giant Amazonian fish pirarucu (Arapaima gigas)
P062	Chauvigné, François (Spain)	Development of a homologous enzyme-linked immunosorbent assay for Senegalese sole FSH using a recombinant chimeric gonadotropin
P064	Jesus, Lázaro (Brazil)	Gpha, fshb and lhb gonadotropin subunits in the South American fish Astyanax altiparanae (Teleostei, Characiformes): molecular characterization and their phylogenetic relationships
P066	Kah, Olivier (France)	Inhibitory effect of cadmium on estrogen signaling in zebrafish brain and protection by oligoelements (zinc)
P068	Liu, Xiaochun (China)	Molecular Identification and functional characterization of the GnIH/GnIHR signaling in protogynous orange-spotted grouper, Epinephelus coioides
P070	Oliveira, Catarina (Portugal)	Expression of kiss2 and kiss1r genes in sea bream (Sparus aurata) broodstock and ontogeny
P072	Weltzien, Finn-Arne (Norway)	Expression and putative function of kisspeptins and their receptors during early development in medaka
P074	Weltzien, Finn-Arne (Norway)	Electrophysiological differences between fshb- and lhb- expressing gonadotropes in primary culture
The Ovary		
P076	Degani, Gad (Israel)	Oogenesis control in multi-spawning blue gourami as a model for the Anabantidae family
P078	Gen, Koichiro (Japan)	Expression profiles of follicle-stimulating hormone and luteinizing hormone gene expression during oocyte development in cultivated yellowtail, Seriola quinqueradiata
P080	Grant, Bridie (UK)	Broodstock spawning performance of ballan wrasse (Labrus bergylta)
P084	Cancio, Ibon (Spain)	5S rRNA and TFIIIA in gonads and the molecular identification of sex and female reproductive stage in commercial fish species
P086	Mushirobira, Yuji (Japan)	Ligand binding properties of ovarian lipoprotein receptors in the cutthroat trout
P088	Parreira, Walquiria (Brasil)	Analysis of hormone profile in Astyanax bimaculatus females with positive response to reproductive stress protocol in fish farm

Thursday – 29 May 2014

17:30 Poster session *II* (continued)		
Reproduction and Environment		
N°	Name & Country	Title of the Poster
P092	Brambila-Souza, Gabriela (Brazil)	Reproductive physiology of Astyanax fasciatus (Teleostei: Characiformes) in captivity
P094	Kida, Bianca (Brazil)	Influence of acidic pH, aluminium and manganese in testicular steroidogenesis of Astyanax bimaculatus (Characiformes: Characidae)
P098	Moreira, Renata (Brazil)	Trophic transference of fatty acids in females of Astyanax fasciatus (Teleostei: Characidae), inhabiting a freshwater system with anthropogenic impact
P100	Rhody, Nicole (USA)	Influence of tidal cycles on the control of reproductive activity in common snook (Centropomus undecimalis)
P104	Servili, Arianna (USA)	Plasticity of the GnRH3 system in developing zebrafish
P106	Vílchez, Carmen (Spain)	Effect of thermal regime on vitellogenesis, ovulation and larval development of European eel
Reproduction and Growth		
P108	Hirai, Toshiaki (Japan)	Relationship between sexual plasticity and body growth in the ovary of koi carp (Cyprinus carpio)
P110	von Kürthy, Corinna (Switzerland)	Alternative reproductive tactics in snail-brooding cichlids diverge in energy reserve management
P112	Miura, Chiemi (Japan)	Controlled feeding rescues the growth reduction associated with spawning in farmed yellowtail (Seriola quinqueradiata)
P114	Pandolfi, Matías (Argentina)	L-tryptophan supplemented diet enhances brain serotonergic activity and reduces stress threshold: the role of serotonin in two South American cichlid fish
P116	Pinto, Patricia (Portugal)	Evolution of the Galinergic system and their possible role in sea bass reproductive function
P118	Rodrigues-Filho, Jandyr (Brazil)	Profile of sex steroids during spawning induction in dusky grouper Epinephelus marginatus
P120	Saraiva, João (Portugal)	Olfaction, growth and maturation in farmed sea bass Dicentrarchus labrax
P122	Skaftnesmo, Kai (Norway)	Small RNAs in immature and mature testis of Atlantic salmon (Salmo salar L.)

Friday – 30 May 2014

08:30	Joy, Keerikkattil P. (India) KEYNOTE LECTURE Catfish ovarian vasotocin system: distribution, regulation and functional implications Chair: Elizur, Abigail

09:05-10:15	Reproduction and Growth Chairs: Elizur, Abigail and Lokman, Mark
9:05	Miura, Takeshi (Japan) STATE OF THE ART LECTURE The role of gonads for growth in teleosts
09:30	Taranger, Geir L. (Norway) Links between somatic growth; growth related hormones and onset of puberty in Atlantic salmon reared under different feeding and photoperiod conditions in sea cages
09:45	Martins, Rute (Portugal) Transcriptome profiling of European sea bass brain and adipose tissue responses to photoperiod to identify puberty onset related genes
10:00	Hu, Wei (China) Effects of growth hormone gene over-expression on reproduction in the common carp Cyprinus caprio L. CANCELLED

10:15-10:35 Coffee break

10:35-12:30	Reproduction and Growth (continued) Chairs: Elizur, Abigail and Lokman, Mark
10:35	Sullivan, Craig V. (USA) STATE OF THE ART LECTURE Ovarian transcriptome reliably predicts egg quality in wild and domesticated striped bass (Morone saxatilis)
11:00	Nagler, James J. (USA) Plasma nesfatin-1 is not affected by long-term food restriction and does not predict rematuration among iteroparous female rainbow trout (Oncorhynchus mykiss)
11:15	Schmitz, Monika (Sweden) Sex steroids modulate the leptin system in vitro and in vivo in male Atlantic salmon (Salmo salar L.)
11:30	Miccoli, Andrea (Italy) Beneficial bacteria affect egg quality by the modulation of maternal factors involved in autophagic and apoptotic processes during Danio rerio development
11:45	Pérez Sirkin, Daniela I. (Argentina) Neuropeptide Y (NPY) increases gonadotropins (GTH) and growth hormone (GH) release and synthesis in the cichlid fish Cichlasoma dimerus
12:00	Berkovich, Nadia (Israel) The neuroendocrine control linking energy balance and reproduction in captive Atlantic bluefin tuna (Thunnus thynnus)
12:15	Wylie, Matt J. (New Zealand) Growth and gonadal development of F1 hapuku (Polyprion oxygeneios) under two different temperature regimes

12:30-14:00 Lunch

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14:00-15:30	Closing Lecture and Ceremony
14:00	Carrillo, Manuel (Spain) CLOSING LECTURE Puberty in sea bass: environmental control and endocrine aspects Chair: Piferrer, Francesc
14:45	General assembly
15:15	Zohar, Yonathan (USA) Concluding remarks and closing

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OPENING LECTURE

Chair: Alexander Scott

Monday – 25 May 2014, 09h30-10h30

FISH REPRODUCTION: FROM COMPARATIVE ENDOCRINOLOGY TO ENVIRONMENTAL TOXICOLOGY

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When I began my scientific career, most researchers studying the reproductive physiology of fish were comparative endocrinologists. At the time it was thought that reproductive processes in fish were very distant from those in the much better studied higher vertebrates, especially mammals – thus to understand the reproductive physiology of fish necessitated the study of fish. For example, I remember vividly long discussions about whether or not 'simple' vertebrates like fish had only one, not two, gonadotrophins. Our thinking has changed dramatically in the three decades since those discussions. The advent of molecular sciences, and the sequencing of whole genomes, has transformed our understanding of the reproductive physiology of fish, and in many ways turned our ideas upside down. We now know that fish are surprisingly similar to mammals. For example, they have more-or-less the same reproductive hormones, which are synthesised in the same tissues. These hormones have similar, if not identical, functions in fish and higher vertebrates. The hormonal control of reproduction is very similar throughout the vertebrates. Of course there are subtle differences, such as 11-ketotestosterone apparently being a major androgen in fish, but not mammals, but these differences seem minor in contrast to the strong similarities. The realisation that similarities outweigh differences has led to a reduction in interest in comparative endocrinology and physiology of fishes. Yet there are some major differences in the reproductive physiology of fish and mammals, and even between different groups of fish, that are still very poorly researched and understood. For example, what mechanisms account for some fish producing relatively few eggs each time they spawn, whereas others produce millions? While interest in comparative aspects of fish reproductive physiology has probably waned during the last few decades, other groups of scientists have become very interested in fish reproduction. This is particularly true of ecotoxicologists, who study the effects of chemicals present in the environment on wildlife. Because many chemicals end up in rivers, the study of how these chemicals can adversely affect the reproductive physiology of fish has become a major topic of investigation. For example, in the last decade many hundreds of papers have been published on the effects of the human pharmaceutical ethinyl estradiol on fish. It is now accepted that extremely low concentrations of this chemical disrupt fish reproduction. Other human pharmaceuticals, such as the synthetic progestogens, do likewise. Perhaps more surprising, so do some industrial chemicals, such as nonylphenol (albeit at higher concentrations). The realisation that these, and other, chemicals present in the aquatic environment can disrupt fish reproduction has led to legislation in the European Union to reduce these risks. Perhaps ecotoxicologists currently conduct more research on fish reproduction than comparative physiologists and researchers in aquaculture put together! My talk will try to summarise how our thinking has changed, and our knowledge improved, since the first International Symposium on Reproductive Physiology of fish.

CLOSING LECTURE

Chair: Francesc Piferrer

Friday – 30 May 2014, 14h00-14h45

PUBERTY IN SEA BASS: ENVIRONMENTAL CONTROL AND ENDOCRINE ASPECTS

Carrillo, M., Felip, A., Escobar, S., Espigares, F., Rodríguez, R., Gómez, A. and Zanuy, S.
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Puberty is the process by which an immature animal acquires the ability to reproduce for the first time and its onset occurs soon after sexual differentiation and is characterized by the beginning of gametogenesis in both sexes. In fish, the mechanisms underlying puberty and its triggering actors are still largely unknown. In the European sea bass, the recent isolation of genes coding for kisspeptins and their receptors allowed for expression studies that are clarifying something more the general endocrine picture of puberty of this species. We present here new insights on when and how the onset of male sea bass puberty occurs, its dependence of a critical size and how it can be controlled by the photoperiod. The suppression of plasma temporal patterns of key reproductive hormones in fish exposed to continuous light, revealed the existence of a photolabile period in September. Moreover, delayed puberty in male sea bass is an important economic issue because marketing time coincides with its puberty onset. Constant long photoperiod was highly effective in delaying puberty, which was linked to a phase difference in the rhythms of hormones that regulate gametogenesis. Indeed, the study of hormone daily rhythms revealed that its core values had equivalence with the seasonal rhythms so that the daily could be considered as the functional units of the seasonal rhythms. Regarding size, apparently only large fish attain the ability to carry out gametogenesis while the small ones do not succeed. Could this imply that to initiate and conclude puberty fish need to achieve an optimal threshold in hormone production? Studies performed with fish of different size demonstrate that small fish exhibit lower plasma hormonal levels than large fish confirming this assumption. Besides, size-photoperiod combined studies demonstrate a differential role of kisspeptins; apparently while Kiss1 is more linked to the photoperiodic signaling, Kiss2 is mostly involved in the reproductive processes. Is it necessary to accomplish a critical body size/fat stores/energy level in order to launch puberty attaining the hormonal signal able to initiate gametogenesis? Preliminary data show that a long-term restricted feeding regime did not prevent the onset of puberty. Besides, hormonal analysis revealed an upregulation of the kisspeptin system and Fsh, suggesting that the fish is able to maintain the reproductive function even at the expense of other functions.

Supported by LIFECYCLE (FP7-222719-1) and PUBERCONTROL AGL2006-0472).

KEYNOTE LECTURE

Chair: Luiz Renato França

Monday – 26 May 2014, 14h00-14h35

REGULATION OF (ZEBRA)FISH SPERMATOGENESIS

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Spermatogonial stem cells (SSCs) either self-renew to produce new SSCs, or differentiate to produce spermatogonia that further develop into spermatozoa. While the equilibrium between SSC self-renewal and differentiation is critical for fertility, SSCs are rare, difficult to identify, and information on the regulation of their proliferation/differentiation behaviour is limited. In stem cell biology, it is generally assumed that when stem cells reside in a specific micro-environment called niche, they self-renew due to the signalling molecules present in the niche. Displacement out of the niche would result in the signalling balance being tipped towards differentiation. Considering the cystic type of spermatogenesis in fish, the SSC candidates are among the single type A undifferentiated (A_{und}) spermatogonia (as in all other vertebrates), which are enveloped by one or two Sertoli cells forming the niche. Under these conditions, a single type A_{und} spermatogonium going through a cell cycle would self-renew, and the resulting new type A_{und} spermatogonium then would have to recruit additional Sertoli cells, thereby forming a new spermatogenic cyst. Our recent work in zebrafish indicates that thyroid hormone plays a stimulatory role in this regard. In cystic spermatogenesis, type A_{und} spermatogonia usually do not leave, or are displaced out of the niche, so that supposedly the signalling in the niche has to change in association with switching from self-renewal to differentiation. Pituitary gonadotropins regulate spermatogenesis and these endocrine signals are translated by Sertoli and Leydig cells into short-range, paracrine signals (e.g. steroids, growth factors) to regulate SSC activity. Pioneering work in this regard has been done using a tissue culture system to incubate testis tissue from prepubertal Japanese eel rich in type A_{und} spermatogonia. A series of elegant studies showed that sex steroids produced in response to gonadotropin regulated different aspects of spermatogenesis. For example, oestrogens stimulated SSC self-renewal via Sertoli cell-derived platelet-derived endothelial cell growth factor, and androgens stimulated SSC differentiation and spermatogonial proliferation, which involved down-regulation of Sertoli cell-derived anti-Müllerian hormone (Amh). Using a tissue culture system for adult zebrafish testis, we found that recombinant Fsh not only stimulated steroid production via Fsh receptor-expressing Leydig cells but also modulated Sertoli cell growth factor expression and stimulated spermatogonial proliferation and differentiation in a steroid-independent manner. Hence, Fsh has effects independent of its steroidogenic activity; a similar conclusion was drawn based on large-scale gene expression studies in rainbow trout. In zebrafish, Fsh down-regulated *amh* expression and recombinant Amh inhibited steroidogenesis but also blocked the differentiation of type A_{und} spermatogonia, even in the presence of androgens. We then discovered that Fsh up-regulated in Sertoli cells the gonad-specific *igf* gene family member *igf3*, and that recombinant zebrafish Igf3 stimulated spermatogonial proliferation and differentiation in a steroid-independent manner. We propose that Fsh is the major regulator of testis functions and, supported by other endocrine systems (e.g. thyroid hormone), regulates Leydig cell steroidogenesis as well as Sertoli cell number and growth factor production to promote spermatogenesis.

KEYNOTE LECTURE

Chair: Yann Guiguen

Tuesday – 27 May 2014, 08h30-09h05

TRANSCRIPTIONAL REGULATION, REWIRING AND NEO-FUNCTIONALIZATION IN THE EVOLUTION OF A NOVEL SEX DETERMINING GENE: THE CASE OF *dmrt1bY* IN MEDAKA

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In medaka, *dmrt1bY*, the duplicated copy on the Y-chromosome of the autosomal *dmrt1a*, became the master regulator of male development after acquiring an upstream position in the sex-determining cascade. Remarkably, this evolutionary novelty requiring a rewiring of the gene regulatory network was brought about by co-optation of pre-existing *cis*-regulatory elements contributed by transposable elements. While the autosomal *dmrt1a* is essential for testis maintenance, in contrast, *dmrt1bY* was shown to be responsible for male-specific primordial germ cell (PGC) mitotic arrest in the developing gonad at the sex-determination stage. Accordingly, the onset of *dmrt1bY* expression was recorded exactly at this stage in the Sertoli cell precursors only. However, the mechanism for such a cell non-autonomous action and the relevance of an obvious PGC-specific expression regulation of *dmrt1bY* could not be resolved.

Strikingly, we observed a very early expression of *dmrt1bY* in the PGCs far before the somatic gonadal primordium is committed. This PGC-specific expression then progressively vanishes while the somatic expression of *dmrt1bY* rises only at hatching stage. Interestingly, also some other gonad development genes showed transient sex-specific expression in the pre-committed gonad primordium. Using *in vivo* whole tissue ChIP and *in vitro* gene regulation assays, we interestingly found clear evidences that the specific expression pattern of *dmrt1bY* is controlled by a differential cross-regulation of *dmrt1a, dmrt1bY* and another transcription factor. Temperature-induced sex reversal experiments and morpholino-knock down of *dmrt1bY* support the physiological relevance of this early germ cell expression.

Our data support a hypothesis that sex determination in Medaka occurs much earlier than previously thought and assigns a more important role to germ cell identity in the process of commitment towards testis or ovary development.

KEYNOTE LECTURE

Chair: Hanna Rosenfeld

Tuesday – 27 May 2014, 14h00-14h35

TRANSPLANTATION OF SALMON GERM CELLS INTO RAINBOW TROUT RECIPIENTS: CAN ITEROPAROUS TROUT REPEATEDLY PRODUCE GAMETES DERIVED FROM SEMELPAROUS SALMON?

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Introduction

Most Pacific salmon employ a reproductive strategy referred to as semelparity in which they only spawn once in their lifetime and then die shortly thereafter. However, the more primitive Pacific salmon, such as rainbow trout (*Oncorhynchus mykiss*) and cutthroat trout (*O. clarki*), employ a reproductive strategy referred to as iteroparity, which allows them to spawn multiple times throughout their life cycle. Since the ability to use iteroparous surrogate trout to produce semelparous salmon gametes would facilitate the production of semelparous salmon, we produced surrogate iteroparous rainbow trout with germ cells derived from semelparous masu salmon (*O. masou*) and examined the potential application of such a strategy to produce salmon more efficiently.

Methods

Most of the germ cells in testicular cell suspensions prepared from the immature testes of masu salmon by trypsin treatment were type A spermatogonia. Cell suspensions containing approximately 5,000 germ cells were transplanted into the peritoneal cavity of triploid rainbow trout hatchlings. During the following spawning season, the number of mature recipient rainbow trout was counted, and those with mature gametes were subjected to progeny tests using gametes obtained from wild-type masu salmon of the opposite sex. The timing of hatching of the resulting offspring was recorded and hatchlings were subjected to PCR-RFLP analysis to distinguish between haplotypes. These analyses were conducted for three consecutive spawning seasons.

Results

Although none of the female recipients reached reproductive maturity in the first spawning season, 5 out of 24 female recipients had matured by the second spawning season. Four of the five females that had spawned in the previous spawning season, spawned again in the third spawning season. Compared to same-aged control rainbow trout, these recipient rainbow trout produced similar numbers of eggs. In male recipients, 7 out of 25 recipients matured in the first spawning season, and two of them matured again in the second and the third spawning seasons. All of the offspring produced by the gametes obtained from the recipient trout hatched at the expected time for masu salmon. Further, PCR-RFLP analyses showed that the banding patterns obtained for the offspring were identical to those of masu salmon. Taken together, we concluded that both male and female rainbow trout recipients could repeatedly produce masu salmon gametes.

Conclusion

This transplantation procedure would facilitate the seed production of semelparous species. In particular, the ability to perform backcrosses, which is an important technique in selective breeding, becomes possible using this surrogate broodstock technology.

KEYNOTE LECTURE

Chair: Penny Swanson

Wednesday – 28 May 2014, 08h30-09h05

NOVEL FISH HYPOTHALAMIC NEUROPEPTIDES THAT REGULATE FISH REPRODUCTION

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It was recently established that the endocrine regulation of vertebrate reproduction is achieved by the coordinated actions of several peptide neurohormones. Fish have distinct characteristics of the hypothalamo-pituitary axis that make them an important model for studying these systems' molecular and physiological activities, as well as its evolution. In addition to the classical increase by GnRH and decrease by dopamine, kisspeptin, neurokinin B (NKB), neurokinin F (NKF), RFamide peptides, dynorphine, spexin and gonadotropin-inhibiting hormone (GnIH) are emerging as important regulators of the reproductive axis. These neuropeptides are regulated by environmental factors, social cues, day length, gonadal steroids and nutrient signals. Using the tilapia as a model fish we identified the involvement of the Neurokinin B/NKBR system in reproduction. Intraperitoneal injection of tiNKB increased both FSH and LH plasma levels, already after 2 h, while tiNKF was more effective in increasing LH levels. However, tiNKB was more effective than tiNKF in increasing both FSH and LH from tilapia pituitary dispersed cells. Using in situ hybridization and fluorescent immunohistochemistry, we have shown that LH cells possess *tac3*, *tac3ra* and *tac3rb* mRNAs, while FSH cells possess mainly tac3rb and tac3, and tac3ra in a much lesser extent. Administration of the tilapia pyroglutaminated-LPXRF-amide-2 peptide, the piscine ortholog of GnIH, to primary cell culture of tilapia pituitaries, or to reproductive female tilapia by intraperitoneal injection positively regulate both LH and FSH release *in vivo* and *in vitro*. LH pituicytes were found to co-express *tilpxrfa* and *tilpxrf-r* mRNA, while only some of the FSH positive pituicytes co-express the *tilpxrf-r* mRNA. No co-expression of *tilpxrf-r* was identified in GH positive cells. These findings indicate that the LPXRFa system is a potent positive regulator of the reproductive neuroendocrine axis of tilapia. Fish hypothalamic neuropeptides convey their direct effects on GnRH neurons, suggesting a complex regulatory neuronal network upstream of GnRH. Moreover, certain neuropeptides and their receptors have been identified in the pituitary of model fish species and localized in specific gonadotropin-producing pituitary cells. These neuropeptides may serve as paracrine and possibly autocrine regulators of both FSH and LH secretion. The recent development of transgenic lines of in which gonadtroph populations in the pituitary are labeled with fluorescent proteins open a whole new spectrum of possibilities to explore the effect of hypothalamic factors on gonaodtropin secretion in great detail within a physiologically relevant context.

KEYNOTE LECTURE

Sponsored Seminar from the Society for Endocrinology

Chair: Takeshi Miura

Wednesday – 28 May 2014, 11h50-12h25

MEMBRANE PROGESTERONE RECEPTORS: A CRITICAL REVIEW

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In addition to the classic genomic mechanism of steroid action mediated by activation of intracellular nuclear receptors, there is now extensive evidence that steroids also activate receptors on the cell surface to initiate rapid intracellular (i.e.nonclassical) signaling and biological responses that are often nongenomic. Two novel transmembrane proteins proposed to mediate nongenomic progestin actions, membrane progestin receptors (mPRs), and progesterone membrane receptor component 1 (PGRMC1), have been identified in fish and mammalian cells.

In this paper I shall review recent studies on the ligand binding, signaling and adaptor protein functions of mPRs and PGRMC1. The potential biological significance of the different tissue distributions, ligand binding specificities, and signaling characteristics of the five mPR subtypes will be briefly discussed. Comparative molecular field analysis (CoMFA) indicates the steroid binding characteristics of human mPRα significantly differ from those of human nuclear progesterone receptor. Preliminary mutational analysis of mPRα has identified several amino acid residues that are potentially important for ligand binding. A plausible model of the structure of the ligand binding site would satisfy one of the major remaining criteria for designation of mPRs as steroid membrane receptors. In addition, new evidence will be presented that PGRMC1 performs a central role in nongenomic steroid signaling as an adaptor protein regulating cell surface expression of mPRs and other steroid receptors. The current status of mPRs and PGRMC1 as progesterone receptors membrane regulating reproductive and nonreproductive functions in mammals and fish and some of the controversies over their purported functions will be critically reviewed. Although significant progress has been made in deciphering the roles of these proteins as intermediaries in nongenomic progesterone actions, significant gaps remain in our knowledge of the molecular mechanisms involved and the physiological functions they regulate. Remaining questions concerning the functions of mPRs and PGRMC1 and their physiological importance will be highlighted and some experimental approaches to address these questions will be proposed.

KEYNOTE LECTURE

Chair: Eva Andersson

Thursday – 29 May 2014, 08h30-09h05

OVARIAN YOLK FORMATION IN FISHES: MOLECULAR MECHANISMS UNDERLYING FORMATION OF LIPID DROPLETS AND VITELLOGENIN-DERIVED YOLK PROTEINS

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Fish egg yolk is largely derived from vitellogenins (Vtgs), which are synthesized in the liver, taken up from the maternal circulation by growing oocytes *via* receptor-mediated endocytosis and enzymatically processed into yolk proteins (YPs) that are stored in the ooplasm. Lipid droplets are another major component of fish egg yolk, and these are mainly composed of neutral lipids that may originate from maternal plasma lipoprotein particles. Our studies aimed to further elucidate the molecular mechanisms underlying yolk formation in fishes.

We employed biochemical and molecular biological analyses, as well as "-omics" techniques, to identify and characterize components of the molecular machinery of yolk formation. These components include gene transcripts and protein products of multiple Vtg subtypes and their YP derivatives, multiple receptors for Vtgs, and other proteins involved in endocytosis by growing oocytes. Components of vitellogenesis included the Vtg receptor (Vtgr), low-density-lipoprotein receptor (Ldlr), Ldlr-related protein 13 (Lrp13), clathrin heavy chain (CltC), and autosomal recessive hypercholesterolemia protein (Arh). Components of oocyte lipidation included lipid-transport precursors (plasma lipoproteins) as well as lipases and fatty acid transporters.

Recent findings indicate that vitellogenesis varies among fishes with regard to rates of production and deposition into oocytes of multiple forms of Vtg, as well as the course of proteolysis of the Vtg-derived YPs, which may especially tailor the resulting yolk to each particular species. In many species, the circulating Vtg ratios do not always correspond to their ratios of deposition into egg yolk. This selective deposition may be regulated by a system of multiple receptors (e.g. Vtgr and Lrp13) engaged in endocytosis of the different types of circulating Vtg. Patterns in ovarian expression and localization of these receptor gene transcripts and proteins were consistent with those of endocytosis-associated proteins (CltC and Arh), indicating possible involvement of CltC and Arh in fish vitellogenesis. In some teleosts, plasma very-low-density lipoprotein (Vldl) appears to be a major source of neutral lipids in the ooplasm lipid droplets. Both *in vivo* and *in vitro* experiments demonstrated that the fluorescently-labeled neutral lipid moiety of Vldl enters into oocytes, while the protein moiety remains extracellular. Gene expression patterns of lipoprotein lipases and some fatty acid transporters suggest their involvement in hydrolysis of Vldl-associated lipids followed by active transport of the resulting fatty acids into oocytes, respectively, prior to the *de novo* synthesis of neutral lipids by the oocyte.

KEYNOTE LECTURE

Chair: Silvia Zanuy

Thursday – 29 May 2014, 14h00-14h35

ORIGIN, FATE AND ROLES OF DUPLICATED GENES IN TELEOST NEUROENDOCRINE CONTROL OF REPRODUCTION: NEW INSIGHTS FROM THE EEL

Dufour, S. [(1)], Lafont, A.-G.[(1)], Maugars, G.[(1)], Pasquier, J.[(1)], Morini, M.[(1)], Kamech, N.[(1)], Rousseau, K.[(1)], Tomkiewicz, J.[(2)], Dirks, R.[(3)] and van den Thillart, G.[(3)]

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Introduction

Two rounds (1R and 2R) of whole genome duplication have occurred in early vertebrate history, followed by a third round (3R) at the basis of the teleost lineage, which may have contributed to the remarkable diversification of this group, the largest among vertebrates. In addition to these whole genome duplications, local events of gene duplications may also have increased the number of some gene paralogs. As representatives of a basal group of teleosts (Elopomorphs), *Anguilla* species provide a powerful model to investigate the number, origin and early fate in teleosts of paralogs involved in the neuroendocrine control of reproduction.

Methods

In silico prediction of endocrine genes were performed on the European Eel (*Anguilla anguilla*) and Japanese eel (*Anguilla japonica*) draft genomes. Gene structures and coding sequences were assessed by cloning of corresponding eel cDNAs. *In silico* search for homologous genes were performed in other teleosts, as well as in other phylogenetically relevant species, such as a basal sarcopterygian, the caelacanth and a non-teleost actinopterygian, the spotted gar. Phylogenetic and syntenic analyses were performed to infer the evolutionary scenarios of gene families across vertebrates and teleosts. This also allowed to propose phylogenetically-based new classifications of paralogs. Finally, in order to get some insight into the potential processes driving conservation or loss of multiple paralogs, we analyzed by real time qPCR their tissue distributions and regulations.

Results and Discussion

Data show that in most cases, the eel has conserved the largest number of paralogs amongst teleosts. This is illustrated for instance by the three kisspeptin receptors, the two luteinizing hormone receptors, or the two leptin receptors. Their differential tissue distribution and regulation during sexual maturation, likely represent selective forces that have contributed to the conservation of multiple paralogs in the eel. Evolutionary scenarios revealed various origins of the multiple paralogs, according to gene families. For instance, the two eel leptin receptors likely originated from the teleost-specific 3R, while the three eel kisspeptin receptors would come from the vertebrate 1R and 2R, with no impact of the 3R. The two eel LH receptors likely arose from a local gene duplication that would have occurred early in the actinopterygian lineage before the emergence of teleosts.

Conclusion

Likely related to its basal position and life traits, the eel exhibits many examples of the largest number of conserved paralogs amongst teleosts. This provides a potent tool to retrace the evolutionary history and roles of gene families involved in reproductive endocrinology in teleosts.

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KEYNOTE LECTURE

Chair: Abigail Elizur

Friday – 30 May 2014, 08h30-09h05

CATFISH OVARIAN VASOTOCIN SYSTEM: DISTRIBUTION, REGULATION AND FUNCTIONAL IMPLICATIONS

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The involvement of the neurohormone Vasotocin (VT) in spawning and oviposition/parturition in fishes, amphibians, reptiles and birds has been documented in the literature. In teleosts, VT is the basic neurohypophysial nonapeptide hormone along with the neutral peptide Isotocin (IT), secreted mainly in the preoptic nucleus, axonally transported to the neurohypophysis and stored/released into the circulation. The brain distribution apart, an ectopic distribution of VT in the ovary of the catfish *Heteropneustes fossilis* was reported from our laboratory. The ovarian expression of VT and IT genes, and VT and IT receptor genes were indicated previously in some teleosts. VT is implicated in the regulation of diverse functions including reproduction and behaviour. Brain, plasma and ovarian VT levels showed significant seasonal variations, which were positively correlated with the annual reproductive cycle. Ovarian VT was localized in the follicular layer of the vitellogenic and post vitellogenic follicles. VT and VT receptor (V1a1, V1a2 and V2) genes are expressed in the ovary. The VT receptor genes exhibited differential distribution; the V1a1 and V1a2 genes were expressed in the follicular layer while the V2 gene was expressed in the denuded oocytes. The transcript levels of the candidate genes showed seasonal patterns, correlated with the ovarian recrudescence. VT secretion is regulated by gonadotropin (hCG) and steroid hormones such as E_2, P_4 and 17, 20β-DP (MIS). Whereas E_2 produced a biphasic effect, hCG, P_4 and 17, 20β-DP stimulated VT levels. The expression of VT and VT receptor genes were up-regulated by hCG, E_2 and 17, 20β-DP. The commercial spawning agent Ovaprim induced ovulation and stimulated VT levels, and transcript levels of VT and VT receptor genes (a non gonadotropic role). VT, in turn, stimulated steroidogenesis, final oocyte maturation with induction of the shift in steroidogenesis, oocyte hydration and prostaglandin secretion. The evidence for VT as a potential regulator of ovarian function, acting in a paracrine/autocrine manner, through its cognate receptors is presented.

Oral Session:

Chemical Communication and Reproductive Behaviour

Chairs: Alexander Scott and João Saraiva

CHEMICAL COMMUNICATION IN TILAPIA

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Introduction

Fish use pheromones to coordinate reproduction, migration, aggregation and social interactions but few fish pheromones have been chemically identified. Cichlids provide an ideal model to study the role of chemical communication in reproduction and speciation. In the Mozambique tilapia (*Oreochromis mossambicus*), reproduction and male aggression are mediated through urinary cues tactically released by dominant males. Here we report on the chemical identity and biological function of the most potent odorants in the urine of dominant *O. mossambicus* males and compare these findings to its close but allopatric relative, the Nile tilapia **(***O. niloticus*).

Methods

Urine was collected from dominant, subordinate and intermediate males, extracted with C18-SPE cartridges and fractionated by HPLC. Compounds in the most active fraction were identified by mass- and NMR spectroscopy and synthesized. Electro-olfactogram (EOG) recordings were used to assess olfactory potency of urine and synthetic analogues. Female 17,20β-P release to the water in response to chemical stimulation was measured by radioimmunoassay.

Results and Discussion

Two steroid glucuronides (5β-pregnane-3α,17,20β-triol 3-glucuronide and its 20α-epimer) were identified as the most potent odorants in *O. mossambicus* male urine. The two steroids are potent olfactory stimuli; the underlying common olfactory receptor mechanism was found to be specific for 5β,3α-reduced 3-glucuronidated steroids. Another, distinct receptor mechanism was found for 17β-estradiol 3-glucuronide, a putative female chemical signal. Females exposed to dominant male urine or to a mix of the two pregnanetriol 3-glucuronides showed a rapid 10-fold increase in 17,20β-P release. Thus, the male urinary steroids stimulate the female endocrine system to accelerate final oocyte maturation. Interestingly, *O. niloticus* showed similar olfactory sensitivity to the pregnanetriol 3-glucuronides; both compounds were also present in male *O. niloticus* urine. This supports the idea that reproductive chemical cues have not been subjected to differing selective pressure in closely related, but allopatric species.

Conclusion

Dominant tilapia males release a steroid sex pheromone *via* their urine to prime females to spawn and thereby promote reproductive success.

PHEROMONAL AND HORMONAL CONTROL OF FINAL OOCYTE MATURATION IN GROUPERS

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Introduction

Groupers are one of the important fisheries resources in Asian countries and also occupy an important position in the ecosystem of coral reefs. In our previous studies, we have already demonstrated the biological characteristics of spawning, such as lunar-related spawning rhythm and spawning migration from the feeding habitat to the spawning ground in the species. Moreover, in the honeycomb grouper *Epinephelus merra*, it was observed that the pheromonal substance released from the mature male has a role as a trigger causing final oocyte maturation. However, there is little information regarding physiological changes during final oocyte maturation after being triggered by the sexual pheromone from the male. Therefore, we examined endocrine changes and the role of steroids after exposure to the pheromone in the final oocyte maturation and ovulation in groupers.

Methods

Exp. 1) The female honeycomb grouper were divided into 3 groups, which are the groups of female alone, female reared with a mature male, and female exposed to the rearing water of a mature male. Fish were kept in these conditions for 3 days. Thereafter, pituitary and ovary tissue were used to determine the mRNA transcription level of follicle stimulation hormone (FSH) and luteinizing hormone (LH), and the plasma steroid levels, respectively. Exp. 2) Testosterone (T) and 17β-estradiol (E2) were injected into the fish having ovary occupied with tertiary yolk stage oocytes. After treatment, the ovaries were cultured with human chorionic gonadotropin (HCG) and morphological changes of the ovaries were observed histologically.

Results and Discussion

Exp. 1) The final oocyte maturation was observed in females reared with a mature male and exposed to the rearing water of a mature male. Moreover, FHS and LH levels in pituitary and T and E2 levels in plasma in females exposed to the rearing water of the mature male were significantly higher than those in female alone group. Exp. 2) In the culture of ovaries with HCG, the migration of oil droplets and germinal vesicle were observed in the ovary of T treated fish. These results suggest that the pheromone from male to female induces T and E2 synthesis via the acceleration of GTHs transcription. Moreover, it appears that T has an important role on morphological changes in final oocyte maturation of grouper.

Conclusion

Final oocyte maturation of the grouper is regulated by sexual pheromone from the male. The pheromone stimulates T synthesis via the acceleration of GTHs transcription, which induces the morphological changes in final oocyte maturation.

HORMONES AND AGONISTIC BEHAVIOUR: WHAT THE SOUTHAMERICAN CICHLID FISH, *CICHLASOMA DIMERUS*, TELL US ABOUT THEM

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Introduction

The so called "chanchita", *Cichlasoma dimerus*, is a South American cichlid fish with a hierarchical social system, established and sustained through agonistic interactions. Individuals in the top ranked positions aggressively guard a territory which grants them access to reproduction. It is a monogamous species with biparental care of the eggs and larvae. In this framework, the aim of the study was to characterize the agonistic behaviour of territorial (Tr) pairs and non-territorial (NTr) male fish, and its relationship with plasma androgens, estradiol and cortisol.

Methods

Experimental aquaria housed 6 size-matched fish (random sex ratio). Once the linear hierarchy was established, top and bottom ranked individuals were identified. When the Tr pair began with the reproductive displays, agonistic interactions were recorded with a camera and quantified for the Tr male and female, and the lowest ranked individual. Aggressive displays included chasings, bites and assaults, while submissive behaviour included escapes and passive coping. Blood samples were obtained and plasma levels of 11-ketotestosterone (11-KT), testosterone (T), estradiol (E_2) and cortisol were measured by ELISA. Also, testes were weighed to calculate the gonadosomatic index (GSI). For every fish, 3 randomly chosen testes' slices were analyzed to assess its cellular composition (percentage of type A and B spermatogonia, spermatocytes, spermatids, spermatozoa and interstitial tissue).

Results and Discussion

Territorial males and females spent 50% of their time biting NTr individuals, while more than 80% of NTr males' displays were escapes. Even though Tr males and females did not differ in their overall frequency of aggressiveness, males performed almost twice more assaults and chases than females. The pair rarely attacked in synchrony, and the frequency of simultaneous attacks equalled the expected by chance. Tr males had 8 times more 11-KT than females and NTr males, and plasma levels positively correlated with male aggression. T was doubled in Tr males in contrast to NTr males, while females' levels did not differ from Tr or NTr males. E_2 negatively correlated with male aggression, was highest in females, and twice in NTr males than in Tr males. The relation E_2/T was 3 times lower in Tr males compared to NTr males, indicating a reduced conversion of T to E_2. Cortisol levels did not vary between social rank or sex. Tr males' GSI was 7% greater than NTr males'. Analyzes of the testes revealed that Tr males had 20% more spermatozoa than NTr males, while spermatids and spermatocytes were higher in NTr males.

Conclusion

In the highly social cichlid *C. dimerus*, social status is accompanied by changes in sex hormone profiles, behavioural repertoire and testicular physiology.

STUDY OF BEHAVIOURAL PROFILES IN SENEGALESE SOLE (*SOLEA SENEGALENSIS*) BROODSTOCK TO CHARACTERISE STRESS COPING STYLES IN RELATION TO REPRODUCTIVE SUCCESS

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Introduction

Senegalese sole (*S. senegalensis*) is a valuable aquaculture species. However, juvenile production depends on wild breeders as captivity bred G1 breeders present reproductive behavioural dysfunctions. The aim of the present study was to describe the stress coping styles behaviour, reactive (shy) or proactive (bold) of Senegalese sole breeders, in reared and wild organisms held in captivity, and establish a possible relation with reproductive success.

Methods

The study was conducted in two steps. In the first step, five different stress tests were applied to 59 Senegalese sole breeders in IRTA, test **1)** capture a fish with a net, **2)** reaction when transferred to a new environment, **3)** reaction when placed in confinement, **4)** time to recover position when the fish was flipped over and **5)** time to induce three anaesthesia levels and finally cortisol levels were determined. A PCA analysis was used to identify which tests characterised the stress coping styles of the breeders. In the second step, the stress coping styles of 198 breeders were determined (59 in IRTA, 139 in IEO, Santander) and comparisons were made when individuals were grouped by spawning success, origin or sex. Tests were repeated to determine the consistency over time of the behavioural responses of the fish.

Results and discussion

The range of behavioural responses of Senegalese sole breeders to the tests characterised a range of stress coping styles. The PCA analyses showed that test 1, 2 and 3 described 65% of total variance observed in all five tests and the cortisol levels. These three tests identified a wide range of coping style responses in the 198 Senegalese sole breeders and when repeated the fish exhibited consistency in behavioural responses over time (Pearson correlation coefficients R=0.571 to R=0.688). Extremes ranged from a "proactive" 15% of the group that presented high activity "fight or flight" in all tests (total activity times were 161±5.8s - test 1; 171.6±4.47.3s - test 2 and 163.9.3±5.2s – test 3) to a "reactive" 15%, that presented low activity "freezing or hiding" in the tests (activity = 31.9±5.6s - test 1; 27.9±6.6s - test 2; 25.8±6.4s - test 3). There was no significant difference (Chi-squared test) in the proportion of proactive (15%), intermediate (70%) or reactive (15%) breeders that spawned successfully compared to breeders that did not spawn, or wild compared to cultured breeders or male and female breeders. Senegalese sole, as others organisms, presented a consistent proactive-reactive behavioural axis with wide variation in responses and the full range of behavioural characteristics played a key role in factors such as origin, sex and reproductive success.

Conclusion

Sole breeders presented behavioural responses indicative of a wide range of stress coping styles including the response types "proactive" and "reactive" and breeders with these responses were evenly distributed in groups related to reproductive success, origin and sex.

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STERILE JUDAS CARP — SURGICAL STERILISATION DOES NOT IMPAIR, GROWTH, ENDOCRINE AND BEHAVIOURAL RESPONSES OF MALE CARP

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Introduction

Use of "Judas" fish is a powerful tool in management, control and eradication of invasive pest fish such as carp in Tasmania, Australia. This works on the premise that radio-tagged individuals readily integrate into wild populations and successfully betray the locations of conspecifics—assisting in their capture and removal. Nonetheless, mature Judas fish pose a risk of contributing to spawning and recruitment with disastrous ecological consequences. Our aim therefore was to generate surgically sterilised male common carp (*Cyprinus carpio*) and test whether they 1) readily assimilate into wild populations, 2) retain sexual behaviour and 3) successfully betray the locations of feral carp similar to control Judas carp, but in contrast mitigate the risks of contributing to spawning and recruitment.

Methods

Wild caught three-year old male common carp were surgically sterilised (n = 44) adopting a two-point nip technique, using either a haemoclip or suture to tie each of the testicular ducts about 2.5 cm cranial to urogenital sinus — retaining all of the glandular testis tissue. Fish were sampled pre- and post- surgery (8 times over 10 months): measured, assessed for sperm expression and blood sampled. Plasma steroids (11-keto testosterone and 17β-estradiol) levels were quantified by immunoassay. A subset of sterile and control male fish (n = 7 each) were implanted with radio-transmitters and released into Lake Sorell, a large lake (50 km^2) infested with feral carp. Their ability to assimilate and betray the location of feral carp was assessed using a battery of passive and active gears and concurrent radio tracking.

Results and Discussion

We have developed procedures for the surgical sterilization of male carp with high survival (95%) and success (77%) rates. Surgery wounds healed remarkably quickly (in about 10 days), with fish growing normally (not significantly different to controls at respective time points). There was a statistically significant difference in 11-keto testosterone and 17β-estradiol levels over time ($P < 0.05$), but not between the sterile and control groups within each sampling time ($P > 0.05$). The sterile radio-tagged male carp (n = 7) released into Lake Sorell, have integrated well into the population—behaving similarly to control Judas males and assisting in the capture of feral carp. There are no comparable studies in teleosts however; in other animals (e.g. murine and ovine) surgical vasectomy is known to cause regression of testicular tissue or loss of gonadal function over time. Long-term observations are therefore planned; consequences of which on gonadal function, behaviour and pest-fish management will also be discussed.

Conclusion

We have successfully developed surgical procedures for sterilisation of male carp that do not impair the growth and endocrine responses or their ability to assimilate and betray the locations of feral carp. The study marks a significant breakthrough in the management of this pest fish with potential adoption to the management of other pest fish globally.

Oral Session:

The Testis

Chairs: Luiz Renato França and Joan Cerdà

GERM LINE ACTIVATION OF THE LUTEINIZING HORMONE RECEPTOR DIRECTLY DRIVES SPERMIOGENESIS IN THE FLATFISH *SOLEA SENEGALENSIS*

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Introduction

In mammals and teleosts, the differentiation of post-meiotic spermatids to spermatozoa (spermiogenesis) is thought to be controlled by the luteinizing hormone (LH) acting through the LH/choriogonadotropin receptor (LHCGR) to stimulate androgen secretion in the interstitial Leydig cells. However, a non-steroidal role of LH mediating the spermiogenic pathway remains unclear, in part because in mammals or amphibians complete spermatogenesis *in vitro* has never been obtained from isolated germ cells. In the flatfish Senegalese sole (*Solea senegalensis*), spermatogenesis occurs in spermatocysts which develop within seminiferous lobules (SLs), but germ cell development is semi-cystic, i.e. round spermatids are released from the supporting Sertoli cells into the SL lumen (SLL) where they elongate and transform into spermatozoa. Using this model, here we test the hypothesis that Lh can directly control spermiogenesis through the activation of the Lhcgrba in germ cells.

Methods

Cells from the sole SLL were extracted and characterized by flow cytometry or isolated by fluorescence-activated cell sorting (FACS). Immunofluorescence staining and Western blot on males treated or not with intramuscular injection of homologous recombinant Lh (rLh) (6 µg/kg) were carried out using a sole Lhcgrba-specific antibody, a piscine Lhβ antiserum or a 6xHis antibody. SLL cell extracts and FACS-purified spermatids were incubated *in vitro* with rLh in the presence or absence of different inhibitors, and the effect on spermatid differentiation assessed by quantitative flow cytometry. Changes in gene expression were determined by real-time qPCR.

Results and Discussion

Sole spermatids released into the SLL express the Lhcgrba which correlated with the detection of both native Lh and intramuscularly injected His-tagged rLh specifically bound to their cell membrane, thus demonstrating that circulating Lh can reach the SLL. In vitro incubation of spermatids isolated from the SLL with rLh specifically promoted their differentiation into spermatozoa, while homologous recombinant follicle-stimulating hormone and steroid hormones did not. The Lh-Lhcgrba induction of spermiogenesis in vitro was mediated through a cAMP/PKA signaling pathway which initiated the transcription of genes potentially involved in the function of spermatozoa. We further found that Lhcgrba expression in late spermatocytes and/or spermatids also occurs in distantly related fishes with cystic spermatogenesis, such as zebrafish and gilthead seabream, suggesting that this feature is likely conserved in teleosts regardless of the type of germ cell development.

Conclusion

These data reveal a novel role of Lh in vertebrate germ cells, whereby a Lhcgrba-activated signaling cascade in haploid spermatids directs gene expression and the progression of spermiogenesis.

EVIDENCE FOR A LACK OF HIGH AFFINITY STEROID BINDING PROTEIN IN THE SEA LAMPREY

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Introduction

Most studies of lamprey species have found very low concentrations of sex steroids in the plasma, casting doubts on possible roles of sex steroids in lamprey reproduction. We hypothesized that the low circulatory steroid concentrations are due to a lack of steroid binding proteins (SBP's) that specifically transport and store these hormones. As a result, steroids produced in gonad are released into water shortly after synthesis. To test this hypothesis we examined concentration and the binding activity of steroids in lamprey plasma and steroid release in water.

Methods

A prespermiating male, spermiating male or preovulatory female was placed in a tank with 30 liter (l) water (approximately 6 g fish·l^{-1}). One l of water was taken at 0h, 2h, 6h and 24h after injection into lamprey of Gonadotropin Releasing Hormone III (GnRH III), progesterone, androstenedione, 11-deoxycortisol or saline (control) and replaced with 1l clean water at each sampling. Blood samples were taken at 24h. Water and plasma samples were extracted for steroid quantification by LC-MS. We measured 10 progestogens, 4 androgens and the estradiol. Plasma steroid binding was determined by incubation with tritiated steroids, separation by filtration and quantification of the bound fraction.

Results and Discussion

Binding assays using plasma showed a low affinity binding for 11-deoxycortisol and progesterone, and no specific binding for the rest of steroids. Hormonal treatments affected the release of several progestogens and androgens after 2 h, but not at 24h. Progesterone and 15α-hydroxyprogesterone were released at the level of 200-50μg/h/kg fish, showing that lamprey synthetize large amounts of steroids. Furthermore, we found a significant production of 11-deoxycortisol (600ng/h/kg fish) after treatment with androstenedione, suggesting a possibility that 11-deoxycortisol plays a role in lamprey sex maturation. Taken together, the lack of binding and the significant release of steroids can be linked to an inefficient plasma storage. The non-invasive measurement of water-borne steroids appears to be an adequate assay for studies of lamprey steroid hormones.

This lack of SBP's and consequentially low levels of circulatory represent the only example known to vertebrates. We speculate that the apparition of SBP in jawed vertebrates may have played a critical role in evolution of the link between central nervous system and peripheral glands, allowing specialization and centralization of brain control.

Conclusion

Sea lamprey produces significant amounts of a variety of sex steroids that are metabolized and released to water rapidly after hormonal treatments. These results are consistent with the hypothesis of lacking a high affinity sex steroid carrier.

MALE REPRODUCTIVE PHYSIOLOGY IN A CICHLID FISH WITH BIPARENTAL CARE: THE CHANCHITA *CICHLASOMA DIMERUS*

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Introduction

The Southamerican cichlid fish *Cichlasoma dimerus,* presents a hierarchical social system, high breeding activity and biparental care of the eggs and larvae. We divided its reproductive period in 4 different phases, according to the offspring degree of development: Male with prespawning activity (MP, day 0), male with eggs (ME, day 1), male with hatched larvae (MHL, day 3), and male with swimming larvae (MSL, day 8). In order to better understand the reproductive stage, the aim of this study was to characterize males' reproductive physiology, by measuring steroid plasma levels and analyzing their testes cellular composition.

Methods

We calculated the gonadosomatic index (GSI), and measured the steroid plasma levels 11-ketotestosterone (11-KT), testosterone, estradiol, and the glucocorticoid cortisol, using ELISA kits. We analyzed the cellular composition of the testes by estimating the average percentage of spermatogonia type A and B, spermatocytes, spermatids, spermatozoa and interstitial tissue; 180 random points per testis were evaluated (CPCe 4.1 software), and from 4 testes of each phase 30 Leydig cells were randomly selected for the measurement of its nuclear area (Image Pro-plus software).

Results and discussion

MP showed 40 times more testosterone ($p=0.0261$) and 20 times more 11-KT ($p=0.0355$) plasma levels than MHL. No significant differences were observed in estradiol and cortisol levels between the different phases. The cellular composition of the testes varied along the 4 phases of reproductive period and parental care. MP's testes were composed by a 50% of spermatozoa, showing the highest percentage ($p<0.05$), whereas spermatogonia type B and spermatocytes were predominant in the subsequent phases. The major percentage of spermatocytes was seen in ME ($p<0.05$), while in MHL had significant higher percentage of spermatogonia B, being the 35% of the testis's cell composition ($p<0.05$). The percentage of interstitial tissue remained constant trough the reproductive cycle. Leydig cells morphometric analysis revealed that MP had 1.35 bigger nuclear area than those from the last two phases ($p=0.004$), and positively correlated with circulating 11-KT ($p<0.05$) and estradiol ($p<0.001$). These results would implicate a higher steroideogenic activity during the pre-spawning stage, explaining the increased of testosterone synthesis available for its conversion to 11-KT and estradiol.

Conclusions

Chanchita males showed important changes in its hormonal profiles, and a particular testicular preponderance of cell types in each reproductive stage. In wildlife this reproductive cycle is repeated 7-9 times from October to March. As fish becomes ready to spawn, higher levels of 11-KT and testosterone, as well as predominance of sperm can be detected.

CHARACTERISATION OF PUTATIVE SPERMATOGONIAL STEM CELLS IN TROUT AND FSH EFFECTS ON POTENTIAL REGULATORY PATHWAYS

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Introduction

Continuous or cyclic production of spermatozoa throughout life in adult male vertebrates depends on a sub-population of the undifferentiated A spermatogonia (und A-Spg) acting as spermatogonial stem cells (SSCs). In *Oncorhynchus mykiss*, at the end of the spermatogenetic cycle, the seminiferous tubules contain only spermatozoa and scarce und A-Spg that remain on the tubular wall. These und A-Spg will be at the origin of the next annual round of spermatogenesis. Taking advantage of this model, we identified putative SSCs.

Methods

1) Morphological approach through photonic and electronic microscopy 2) the spatio-temporal expression of genes involved in SSC biology was studied by qPCR and by ISH, immunohistochemistry a- during the reproductive cycle and b- in populations of germ cells purified by Centrifugal Elutriation 3) "stemness" was assessed by transplantation of transgenic germ cells into embryos, then quantitative evaluation of gonad colonization and transgene transmission to offspring, 4) FSH action on SSC regulatory pathways was analyzed in vitro.

Results and Discussion

In all stages of maturation, large spermatogonia with ultrastructural characteristics of germinal stem cells were found isolated or in pairs. Trout homologs of SSCs/progenitor markers (*nanos2* and *nanos3*, *pou2*, *plzf* and *piwil2)* were preferentially expressed in the prepubertal testis (containing only und A-spg) compared to more advanced stages, and in an undifferentiated A spermatogonia population purified by centrifugal elutriation Moreover, transplantation of these purified und A-Spg into embryonic recipients demonstrated their high "stemness" property in terms of colonization of the gonads and ability to give functional gametes. Interestingly, we show that high levels of *nanos2* transcript were restricted to a small sub-population of und A-Spg present as single cells or in pairs in juvenile and maturing testes. In contrast, *nanos2* transcript was detected in all the und A-Spg remaining in the spawning testis. Plzf was also immuno-detected in und A-Spg from the spawning testis, which reinforces the idea that these cells are the most undifferentiated germ cells. Moreover, an homolog of *Gdnf* (the specific regulator of SSCs in mouse), was expressed in the somatic compartment of the trout testis, while *gfrα1, (Gdnf* specific co-receptor), is found by immunocytochemistry and flow-cytometer analysis in a sub-population of und A-Spg. Fsh was found to regulate *gdnf* and *gfrα1* expression *in vitro.*

Conclusion

From those results, we propose that the subset of undifferentiated A spermatogonia expressing *nanos2* transcript are putative SSCs and that Gdnf regulatory pathway of SSCs is possibly conserved in trout.

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IDENTIFICATION OF CELL SURFACE MARKER FOR ENRICHMENT OF TYPE A SPERMATOGONIA IN RAINBOW TROUT

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Introduction

Germ cell transplantation is a powerful tool in reproductive biotechnology. We recently established a surrogate broodstock technique in rainbow trout (*Oncorhynchus mykiss*), in which type A spermatogonia (A-SG) were transplanted into the body cavity of a recipient embryo. We previously revealed that only some of the transplantable A-SG migrated to, and be incorporated into the recipient gonad. Consequently, to increase the colonization efficiency of donor cells, we established a flow cytometric method to enrich A-SG. However, flow cytometry (FCM) techniques are time consuming and required expensive equipment. In this study, to employ the simpler method of magnet-activated cell sorting (MACS), we generated an antibody (Ab) capable of specifically recognizing a cell surface marker of A-SG.

Methods

To generate an Ab specific for A-SG, we inoculated four mice with living A-SG in the left hind footpad. After inoculation, the cells of the hind popliteal lymph nodes were fused to myeloma cells and seeded. To identify hybridoma cell colonies that produced the monoclonal Ab (mAb) that specifically recognized the A-SG, the supernatants from these cells were screened by the cell ELISA assay, cell immunostaining, and FCM analysis. The colonization efficiency of the cells isolated using the mAb obtained by this screening procedure was then analyzed by intraperitoneal transplantation.

Results and Discussion

By seeding the cells fused with myeloma cells on four 96-well plates, hybridoma colonies were apparent in all wells (576 wells). Among the 576 supernatants, a positive signal was obtained from 198 supernatants by cell ELISA for A-SG. Immunostaining of total testicular cells revealed that mAb in 60 of the 198 supernatants labeled A-SG. Of these mAbs, FCM analysis revealed that five preferentially recognized p*vasa-GFP*-positive A-SG in immature testis. Further FCM analysis revealed that a mAb preferentially labeled A-SG showing strong p*vasa-GFP* fluorescence in mature testis. To investigate the colonization efficiency of the cells isolated using the mAb, we performed an intraperitoneal transplantation assay. The results showed that, compared to total testicular cells, colonization efficiency was significantly increased in cells isolated using the antibody-mediated FCM. Thus, the A-SG of interest could be enriched using the mAb generated in this study.

Conclusion

We successfully identified a novel cell surface marker that could be used to preferentially label A-SG in both immature and mature testis. Using this mAb, colonizable A-SG could be enriched. This mAb will allow us to increase the efficiency of surrogate broodstock technology.

LENTIVIRAL TRANSDUCTION OF MALE GERMLINE STEM CELLS IN NILE TILAPIA (*OREOCHROMIS NILOTICUS*)

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Introduction

The spermatogonial stem cells (SSCs) are the foundation of the spermatogenic process and are unique stem cells in the adult organism due to their ability to transmit the genetic information to the subsequent generation. Importantly, the genetic modification of these cells provides a great opportunity to study the biology of their complex self-renewal and differentiation processes, as well as to enable the generation of transgenic animals in a wide range of species, including fish. In Nile tilapia, we have previously developed a competent SSC culture system and also an efficient methodology for SSCs transplantation into the testes of adult fish. Our aim was to investigate the ability of lentiviral vectors to transduce Nile tilapia SSCs *in vitro* by inserting the RFP (red fluorescent protein) marker transgene.

Methods

The DsRed2 sequence was cloned into pLenti6.3/V5-TOPO®; this recombinant vector was transformed into One Shot® Stbl3™ Competent *E. coli* and these bacterial cells were submitted to maxiprep. The recombinant vector was sequenced and used to transform 293 FT cells by Lipofectamine 2000. The viral particles were obtained after 72 hours into the supernatant of the complete medium (high glucose D-MEM). Nile tilapia (*Oreochromis niloticus*) SSCs were exposed to lentivirus for 12 hours under pre-established optimal culture condition, selected with Blasticidin, and formerly analysed for the expression of RFP under fluorescent microscopy.

Results and Discussion

Transduction of the reporter gene encoding the RFP was monitored in *in vitro* cultivated SSCs for 9 days with the first observation at 24h post-infection. Although in low intensity, at that time a substantial fraction of cells expressed the transgene. The percentage of RFP-positive cells increased during the *in vitro* cultivation, with gradual overgrowth of RFP-positive colonies until one week. We are currently developing new experiments to confirm the stable integration of the transgene into SSCs genome through long-term fluorescence analysis and DNA sequencing. In order to obtain transgenic offspring, such investigations are essential before transplanting transduced SSCs into the tilapia recipient testis.

Conclusion

We demonstrated here that *in vitro* lentiviral-mediated gene delivery into the Nile tilapia SSCs results in long-term integration and expression of RFP transgene. Our data indicate that, similar to mammals, viral transduction represents an efficient method to introduce genes into the fish male germline. Therefore, these findings provide the first step in establishing a system that will allow genetic manipulation of fish SSCs, representing an important progress towards the production of transgenic fish lines and new biotechnologies in aquaculture.

Financial Support: CNPq and FAPEMIG.

AQUAPORIN-1aa AND -8b ARE DIFFERENTIALLY LOCALIZED IN GILTHEAD SEA BREAM (*SPARUS AURATA*) SPERMATOZOA AND PLAY DISTINCT ROLES DURING THE ACTIVATION OF SPERM MOTILITY

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Introduction

In marine teleosts, such as the gilthead seabream, aquaporin water channels are implicated in sperm activation through mediation of water efflux from ejaculated spermatozoa exposed to hyperosmotic seawater. We have recently shown that seabream spermatozoa express up to five different aquaporin paralogs (Aqp1aa, -1ab, -7, -8b and -10b) with a segregated spatial distribution. However, the specific contribution of each paralog to the initiation and maintenance of sperm motility remains unknown. Here, we used paralog-specific antibodies as functional inhibitors to investigate the different roles of Aqp1aa and -8b during the hyperosmotic activation of sperm motility in the gilthead seabream.

Methods

Sperm was collected during the natural spawning season and used for immunofluorescence microscopy or activation assays. Aqp1aa and -8b mediated transport of water, urea or ammonia was determined from ectopic expression in *Xenopus laevis* oocytes, in the presence or absence of Aqp1aa and -8b affinity-purified antibodies. Motility parameters on seawater-diluted sperm were recorded using the Integrated Semen Analysis System software (ISAS version 1) in the presence of IgG (controls) or Aqp1aa or -8b antisera.

Results and Discussion

Expression of Aqp1aa and -8b in *X. laevis* oocytes revealed that Aqp1aa is water-specific, whereas Aqp8b is permeable to water, urea and ammonia. Double immunolocalization of Aqp1aa and -8b in ejaculated immotile spermatozoa confirmed that Aqp1aa is exclusively localized along the flagellum, whereas Aqp8b is dispersed in the head and the anterior flagellum. Upon activation, Aqp1aa localization is maintained along the tail while Aqp8b almost completely accumulates in the mitochondrion of the spermatozoa. Exposure of oocytes expressing Aqp1aa and -8b to their corresponding antibodies inhibited the osmotic water permeability of oocytes in a dose-dependent manner, and no cross-reaction of the antibodies was detected. Using these antibodies on sperm activation assays in seawater, we found that inhibition of Aqp1aa decreased most of the motion parameters (% of motile sperm, velocity, progressive motility), whereas Aqp8b inhibition only reduced the % of motile and progressive sperm.

Conclusion

These data suggest for the first time that Aqp1aa and -8b play distinct roles in the activation of teleost sperm. Aqp1aa possibly facilitates flagellar beating while Aqp8b may be required for the maintenance of spermatozoa progressivity. The function of Aqp8b-mediated mitochondrial transport of solutes (i.e. ammonia, urea) in this mechanism is under investigation.

THE ION PUZZLE WHICH MAKES THE EEL SPERM MOTILE

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Introduction

Sperm motility in marine fish starts due to the hyperosmotic shock, which causes a membrane depolarization, a water efflux from spermatozoa, and an increase in intracellular Ca^{2+} and K^+. Our goal was studying the role of Ca^{2+}, Na^+ and K^+ on European eel (*Anguilla anguilla*) sperm motility initiation, by removing selectively the ions from seminal plasma and/or activation media, as well as by using ion channel inhibitors.

Methods

Male eels were matured with hCGrec weekly injections. Sperm samples with motility >50% were selected for studies. Sperm was washed in several extenders (with or without Ca, K, Na) by diluting (1/25) and centrifuging (4 ºC, 500 g, 5 min). The effect of the different extenders on motility and intracellular ion changes was evaluated by CASA and flow cytometry (estimating relative fluorescence changes), respectively. Sperm was maintained in the different extenders for 30 min prior to activation or intracellular ion measurements.

Different sperm hyperosmotic activation media (with or without Ca, K, Na; pH 8.2; 1100 mOsm) were also evaluated. The effect of several Ca^{2+}, K^+ , and Na^+ inhibitors was studied, after 30 min of sperm incubation in the extender media containing inhibitors. Then, sperm was activated and sperm motility and intracellular ion changes were measured.

Results and Discussion

Effect of extenders: washing in K-free extender or Na-free extender caused a decrease in sperm motility of 92% and 44% respectively, but motility was not affected by washing in Ca-free extender.

Effect of activating media: sperm motility was not affected by activation in Ca-, K- or Na-free media. Intracellular sperm Ca^{2+} did not increase after activation in Ca-free media. Intracellular K^+ and Na^+ increased even after activation in K- or Na-free media, respectively.

Effect of inhibitors: Bepridil (a Na^+/Ca^{2+} exchanger inhibitor) reduced total motility (-75%); it not affected the rise in intracellular Ca^{2+}, but blocked the rise in Na^+ observed in controls. Valinomycin (a K-ionophore) highly reduced sperm motility (-75%) and caused a higher rise of intracellular K^+ after sperm activation than controls. Amiloride (a Na^+ channel blocker) partially reduced motility (-25%) and slightly reduced the rise of intracellular Na^+ saw in controls. A-23187 (Ca-ionophore) did not affect sperm motility, but induced a higher level of intracellular Na^+ than controls.

Conclusion

Our results indicates that the presence of Na^+ and K^+ (but no Ca^{2+}) in seminal plasma is important for keeping intact the sperm ability for motility. The increase of intracellular Ca^{2+} is not necessary for sperm motility acquisition, but a Na^+ increase (inhibited by bepridil) seems to be important for motility activation. Also, a Na^+/Ca^{2+} exchanger seems to be involved in sperm motility. A modified model for marine fish sperm motility acquisition is proposed.

Funded by the SPERMOT project (MINECO; AGL2010-16009).

THE SPEED OF PUBERTAL DEVELOPMENT IN ATLANTIC SALMON (SALMO SALAR) POST-SMOLTS IS MODULATED BY SALINITY AND PHOTOPERIOD

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Introduction

The Atlantic salmon shows substantial lifecycle plasticity, which also applies to the timing of puberty. While it is characterized by the activation of the brain–pituitary–gonad axis, many morpho-physiological aspects of puberty and the influence of environmental conditions are not well understood. Previous work showed that puberty can be induced in male postsmolts by certain photoperiod and temperature conditions. This model of postsmolt maturation was used to examine effects of salinity (and photoperiod) on the entry and completion of puberty in males.

Methods

An out-of-season smoltification regime was applied from October - December. At the beginning of January, the maturation regime started and fish were exposed to freshwater (FW) or seawater (SW) at 16°C to stimulate puberty, under 24h constant light (LL) or 12h light/12h dark (LD) photoperiod. These four treatment groups (FWLL, SWLL, FWLD and SWLD) were sampled four times every three weeks (Jan 26, Feb 16, March 9 and 30) after the start of the maturation regime, to quantify by qPCR pituitary *fshb*, *lhb*, and *gnrhr4* mRNA levels, 11-ketotestosterone plasma androgen levels by RIA, to determine the gonado-somatic index (GSI), and to evaluate spermatogenesis quantitatively (number of germ cells counted in the different phases of spermatogenesis).

Results and Discussion

When spermatogonial proliferation started, *fshb* mRNA levels increased steeply and began to decrease when spermatogonial mitosis approached completion and most germ cells had reached meiotic or post-meiotic stages. Conversely, *lhb* mRNA levels increased progressively during spermatogenesis. Most males in all treatment groups matured, but exposure to seawater resulted in the strongest stimulation of the onset of spermatogenesis and elevation of pituitary *gnrhr4* and *fshb* mRNA levels. Later on, the LD photoperiod accelerated, irrespective of the salinity, the completion of spermatogenesis, associated with higher *lhb* mRNA and 11-KT plasma levels than in the LL groups.

Conclusion

In general, a given developmental stage was associated with stage-specific GSI, plasma androgen, or pituitary gene expression levels, while the environmental conditions determined when a developmental stage was reached. Both salinity and photoperiod modulated the timing of puberty: seawater stimulated the onset and the shorter photoperiod the completion of puberty. To our knowledge, this is the first time that a stimulatory effect of seawater on pubertal development was demonstrated.

Oral Session:

Sex Determination and Differentiation

Chairs: Yann Guiguen and Minoru Tanaka

OVERVIEW OF EXTERNAL INFLUENCES ON THE PROCESS OF SEX DIFFERENTIATION IN ZEBRAFISH GONADS

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Introduction

Among vertebrates, fish exhibit all kinds of reproduction systems. Fish sex determining mechanisms and the process of sex differentiation can be modulated by the influence of the environment where they live. Thus, external factors such as temperature are known toinfluence the final sex ratios in fish. However, how these external factors interact with the gonad to modulate its plasticity and determine the final sex is still poorly known. In this presentation, we summarize the results of different types of experiments in our laboratory, showing how temperature, density and chemical insults (hormones and DNA methylation inhibitors) modulate the final sex ratio on the zebrafish populations. We also identify different transcriptomic profiles based on gene expression and molecular pathway analysis.

Methods

Zebrafish were subjected to different types of environments during the gonadal development period; heat treatments, high density confinement, hormonal treatments and DNA methylation inhibitors (5'-aza-C). Fish survival, biometry and sex ratios were recorded. Gonads were dissected for molecular biology studies. Transcriptomes were studied by microarrays and real time PCRs.

Results and Discussion

Sex ratio was altered in all cases. High temperature of either 34 or 36°C masculinized zebrafish and the period of highest thermosensitivity was identified at 7–32 dpf. Rearing zebrafish at high densities (> 30 fish/liter) also induced masculinization, indicating the existence of a upper threshold above which sex ratios are altered. Further results showed that treatment with 5-'aza-C, a DNA-methyltransferase inhibitor, increased the number of females, suggesting that methylation of key genes related to gonadal sex differentiation might be altered by this treatment. Gene expression analysis by microarray of fish subjected to heat treatment revealed different transcriptomic profiles in adult fish. Some altered pathways were conserved from juvenile to adulthood, indicating persistent effects of heat exposure. Overall, results show that *cyp19a1* (gonadal aromatase) is among the genes that are consistently affected by all these treatments, indicating its central role as a marker of females sex differentiation in fish.

Conclusion

Together, these studies contribute to our understanding on how changes in environmental conditions during early life influence sex ratios and thus fish population structure.

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CRISPR/CAS9, A VERSATILE TOOL TO STUDY SEX DETERMINATION AND FOR SEX CONTROL IN TELEOSTS

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Reverse genetics approaches have been important in demonstrating gene functions, genetic engineering and understanding complex biological processes. Recent advances using the CRISPR-associated (clustered regularly interspaced palindromic repeats/CRISPR associated) single guide RNA system (Cas9/gRNA) illustrate the potential of this simple technique for genome engineering in a number of model organisms. However, there is no report showing its application in non-model animals. The Nile tilapia, a gonochoristic teleost with a stable XX/XY sex determination system, has become one of the most important species in global aquaculture. The availability of monosex populations and the whole genome sequence made this species an excellent model for investigation of sex determination in teleost. However, studies to decipher genes involved in fish sex determination/differentiation have been limited by the approaches available for eliminating gene function. Here, we report that disruption of a selected gene or two genes simultaneously in tilapia through CRISPR/Cas9 system was achieved with high efficiency. Mutations induced by CRISPR/Cas9 were efficiently transmitted through the germline to F1. In addition, obvious phenotypes, partial or even complete sex reversal, were observed in G0 generation after mutation of genes related to sex determination and differentiation. For example, loss of Nanos2 and Nanos3 resulted in germ cell-deficient XY and XX gonads, respectively, and masculinization of somatic cells, as demonstrated by Dmrt1 and Cyp11b2 immunohistochemistry and also by up-regulation of serum androgen levels. Loss of Gsdf resulted in male to female sex reversal with up-regulation of Cyp19a1a expression and serum estradiol-17□, while loss of Foxl2 resulted in female to male sex reversal accompanying with down-regulation of serum estradiol-17□. Loss of Amh resulted in hyper-proliferation of oogonia and spermatogonia, and blockage of germ cell meiosis in both XY and XX gonads. Thereby, switch of gonadal fate can be easily achieved by loss of function of a single gene via a CRISPR/Cas9 system. Our study goes beyond model animals and shows the utility of the CRISPR/Cas9 as a genetic tool in generating genetically engineered tilapia and, potentially, other teleosts. Taken together, our data demonstrate that targeted, heritable gene editing can be achieved in tilapia. CRISPR/Cas9 provides a convenient and effective approach for generating loss-of-function mutants for deciphering genes involved in sex determination/differentiation and for sex control in fish.

PRIMORDIAL GERM CELLS EXHIBIT SEXUALLY DIFFERENT CHARACTERS BY SEX DETERMINATION GENE-INDEPENDENT MECHANISM IN MEDAKA

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Introduction

The sexual identity of germ cells in the vertebrates that have been examined to date is determined by the sex of gonadal somatic cells. In the teleost fish medaka, a sex determination gene on the Y chromosome, *DMY*/*dmrt1bY*, is expressed in gonadal somatic cells and regulates the sexual identity of germ cells. During the course of analysis of the gonadal sex differentiation, we found that several transcripts showed sexually different expression in germ cells before the gonad formation. Among them, a novel transcript, *olte54h09* was highly enriched in XY germ cells. Here, we addressed an issue of how germ cells could exhibit sexually different characters prior to gonad formation.

Methods

Q-PCR and *in situ* hybridizaiton of *olte54h09* was performed using st.30 embryos in which germ cells are migrating towards prospective the gonadal region. Chimeric analysis by transplantation of XY germ cells into XX hosts and vice versa was performed to reveal whether the sex difference occurred in a germ cell-autonomous manner or affected by somatic cells. We also investigated whether germ cells behaved differently between the two sexes using an *in vitro* culture system. Finally, knockdown and/or overexpression of *DMY/dmrt1bY* and *olte54h09* followed by q-PCR and the culture experiment were performed.

Results and Discussion

We found that *olte54h09* showed more than two-fold higher expression level in XY germ cells than that in XX germ cells prior to gonad formation. Chimeric analysis revealed that the sexually different expression of *olte54h09* is controlled in a germ cell-autonomous manner by the number of Y chromosomes. Interestingly, *DMY*/*dmrt1bY* was also expressed in germ cells prior to gonad formation, but knockdown and overexpression of *DMY*/*dmrt1bY* did not affect *olte54h09* expression. We also found that XX and XY germ cells isolated before the gonad formation behaved differently *in vitro*, which was affected by *olte54h09* but not by *DMY*/*dmrt1bY* expression. Since *olte54h09* maps close to the sex determination locus, insertion of *DMY*/*dmrt1bY* into the newly arising sex chromosome may have affected gene expression of nearby loci and thus cell behavior.

Conclusion

Primordial germ cells exhibit cell-autonomous sexual difference by sex determination gene-independent mechanism in a genetically sex-determined species, providing an insight into various processes of the acquisition of sexual difference at the cellular level.

TUG-OF-WAR OF SOMA FATE DECISION IN THE HERMAPHRODITIC BLACK PORGY

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Introduction

No ancient ancestry and no single sex-determining mechanism are involved in the hermaphroditic fishes. How the presence of more than one sex at a time existed during an evolutionary transition from gonochorism to hermaphroditism in fish remains unknown. Our aim was to understand the possible reason for the presence of more than one sex at a time during an evolutionary transition from gonochorism to hermaphroditism in fish. In this study, we generated the abnormal testicular part of the ovotestis by E2 treatment, in which newly regenerated testes contain ectopic oocytes. To test these ectopic oocytes adaptation we performed a detailed phenotypic and molecular analysis of this E2-induced ectopic location of the oocytes in the testicular part of the ovotestis.

Methods

We created an ectopic location of the oocytes in the testicular part of the ovotestis. Fish were fed a diet containing E2 (6 mg/kg of feed) at the undifferentiated gonad for 3 months (age, 2 mo). The short-term group was followed by the replacement of the E2 diet with control feed at the differentiated gonad (age, 5 mo) until the first spawning season. Fishes were collected for genetic analysis and histology. Anti-Dmrt1 (Sertoli cells marker) and anti-Cyp19a1a (follicle cells marker) were used to identify the types of cells surrounding the oocytes. Anti-Figla (oocyte-specific gene for folliculogenesis) and anti-Amh (gene for spermatogenesis) were used to understand the competition between male- and female microenvironment.

Results and Discussion

The ectopic oocytes in the regenerated testis do not undergo apoptosis; thus, a number of occytes are in the testis. Furthermore, the cells surrounding the oocytes are Dmrt1-positive cells (Sertoli cells) with high Figla expression in the oocytes at an early stage. Then, as the Dmrt1 expression diminishes, Cyp19aia-positive cells (follicle cells) with low Figla expression appear in the oocytes at a later stage. On the other hand, the number of oocytes in the regenerated testis is limited and the germ cells proliferation is arrested in the near region of ectopic oocytes. The candidate suppressor of self-renewing germ cells proliferation, Amh, was detected in the near region of oocytes in the regenerated testis. Furthermore, *in vivo* organ culture showed that recombinant Amh only to inhibit spermatogonia (or early germ cells) division in the testis and no affects to oogonia in the ovary. Thus, we demonstrated that oocytes are competent to create a microenvironment to protect against a testicular environment in black porgy. However, the number of ectopic oocytes in the testis is restricted through arrest the spermatogonia (or early germ cells) proliferation by Amh.

Conclusion

Even that Figla alter the fate of surrounding cells of ectopic oocytes. Amh was supressed the proliferating activity of the spermatogomia in the near region of ectopic oocytes. These results shed light on why the presence of more than one sex at a time existed during an evolutionary transition from gonochorism to hermaphroditism in fish.

SIGNIFICANCE OF TRANSCRIPTION FACTOR, Pax2 AND THE SIGNALING MOLECULES, Wnt4/5 IN THE GONADAL DIFFERENTIATION OF THE CATFISH, *CLARIAS BATRACHUS*

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Introduction

The transcription factor, *Pax2* belongs to the "paired box" family, is known to orchestrate the patterns of gene expression in specific cells during organ development, like its other family members. The studies thus far in mammalian models suggested it as one of the earliest markers expressed during kidney development and its functions may be multiplex, activating distinct panels of genes in different cell lineages at different stages. In this process, a prominent role for the secreted glycoproteins of Wingless MMTV (Mammalian Mammary Tumour Virus) integration site family was hypothesized where the regulation of *Wnt4/5* is known to be critical for successful nephrogenesis. Nevertheless, its precise gene targets or the specific developmental processes, leading to gonadal differentiation in lower vertebrates including teleosts were not well understood. Hence, our study aims to correlate the actions of these transcription factors/signaling molecules, if any, during the developmental events of urogenital system including gonadal differentiation using catfish *Clarias batrachus* as our animal model.

Methods

We cloned the full length cDNAs of *Pax2*, *Wnt4* and *5* using degenerate primer- and RACE-PCR strategies**.** The transcript levels of *Pax2*, *Wnt4* and *5* were measured using qPCR at different developmental stages of catfish. Localization of Pax2, Wnt4 and 5 in the gonads were done through immunocytochemistry and/or *in situ* hybridization. We are making efforts to use siRNA technology to assess the up/down regulation of these genes.

Results and Discussion

The full length cDNAs of *Pax2*, *Wnt4* and *5* were obtained from the catfish, *C. batrachus* which is a lower vertebrate model wherein the events of urogenital system formation are highly complex. The transcript levels of *Pax2*, *Wnt4* and *5* measured using qPCR found to be predominant in the ovary throughout the growth of the catfish. In addition, localization of Pax2, Wnt4 and 5 in the gonads through immunocytochemistry and/or *in situ* hybridization assumes importance. Our studies using siRNA technology define the significance of *Pax* and *Wnt* genes in the development of urogenital system including gonadal differentiation of catfish.

Conclusion

Based on our results, we suggest a major role for Pax2, Wnt4 and 5 in the development of urogenital system in teleosts.

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GONADAL SOMA-DERIVED GROWTH FACTOR SUPPRESSES THE EXPRESSION OF FOLLICLE-STIMULATING HORMONE RECEPTOR IN MEDAKA.

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Introduction

Medaka (*Oryzias latipes*) is a teleost fish that has a XX/XY sex determination system. We previously showed that the expression levels of follicle-stimulating hormone receptor (*fshr*) mRNA in XX medaka were higher than those in XY fishes at the hatching stage. Moreover, cortisol caused masculinization as well as suppression of *fshr* expression in XX embryos, suggesting that FSHR may be involved in the ovarian differentiation in medaka. However, the molecular mechanism of transcriptional regulation of *fshr* gene in medaka remains unclear. In this study, to analyze the effect of gonadal soma-derived growth factor (GSDF), a member of the TGFβ superfamily which is male-specifically expressed in the gonadal somatic cells, on *fshr* expression regulation in medaka, we established *fshr-GFP* transgenic (Tg) medaka and *gsdf*-overexpressed Tg medaka lines.

Methods

Fshr-GFP Tg medaka line was generated by injecting the EGFP vector fused to the regulatory region of the medaka *fshr* gene into fertilized eggs of the FLFII stock. All injected embryos were bred to adults and only F1 embryos possessing GFP fluorescence were selected and used to produce succeeding generations. *Gsdf*-overexpressed (*mis-gsdf*) Tg medaka line was generated by simultaneously injecting the *mis-gsdf* and *olvas-DsRed* vectors. F1 embryos possessing DsRed fluorescence were selected and used to produce succeeding generations. We then mated the *fshr-GFP* Tg medaka line with *mis-gsdf* Tg medaka line.

Results and Discussion

We first established *fshr-GFP* Tg medaka line and examined the expression patterns of GFP and FSHR in the Tg medaka by immunohistochemistry. The expression patterns of GFP were coincident with those of FSHR in Tg medaka, suggesting that GFP expression reflects FSHR expression in this line. On the other hand, we successfully generated the *mis-gsdf* Tg medaka line which expresses *gsdf* mRNA in the somatic cells of XX gonads, and the Tg XX fishes became typical males which are fertile. To investigate whether GSDF regulates *fshr* and *GFP* expression in *fshr-GFP* Tg medaka, we examined the expression pattern of *fshr* and *GFP* mRNAs in *fshr-GFP/mis-gsdf* Tg fishes by quantitative real-time PCR. As results, the expression levels of *fshr* and *GFP* mRNAs were significantly suppressed in the gonads of the Tg XX fishes.

Conclusion

In the present study, we showed that GSDF inhibited the expression of GFP and *fshr* mRNAs in *fshr-GFP/mis-gsdf* Tg medaka, suggesting that GSDF down-regulates the expression of *fshr* and induces male development in XY medaka.

A TRANSCRIPTOMIC STUDY ON TEMPERATURE AND ESTRADIOL EFFECTS ON EUROPEAN SEA BASS (*DICENTRARCHUS LABRAX*) GONADS

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Introduction

The European sea bass (*Dicentrarchus labrax*) is a gonochoristic species with a polygenic sex determination system with environmental influences. Elevated temperatures early in development alter sex ratios by masculinizing fish that otherwise would have developed as females. Furthermore, estradiol-17ß (E_2) treatment when applied at the hormone sensitive period is known to feminize fish. Our aim was to analyze the effects of E_2 at a whole transcriptomic level in fish exposed to high temperature.

Methods

European sea bass juveniles previously reared at 21°C from 20 to 90 dph, covering the early gonad formation period, were divided into two groups. One was fed with a diet containing E_2 at 10 mg/kg of food prepared with the alcohol evaporation method during the hormone sensitive period (90-160 dph), while the other was treated with a food treated with the solvent alone. At the end of the experiment, biometric data and tissues were collected for further analysis, and the sex ratio was calculated. We collected 170 days post hatch (dph) gonads from putative females based on *cyp19a1a* mRNA expression levels and determined the transcriptomic profile using a custom-made sea bass microarray (GPL13443). Microarray data was normalized and analyzed using Bioconductor in R. Microarray results were validated by qRT-PCR of selected genes of interest.

Results and Discussion

Because of the temperature regime used, 80% males were found in the control group at 337 dph. In contrast, the E_2-treated group had 100% females. Transcriptomic analysis of E_2-treated versus control gonads showed 420 differentially expressed (DE) genes. Elevated temperature was responsible of the up-regulation of the genes related to the male pathway in controls. However, E_2 treatment completely negated this masculinizing effect. Analysis of the transcriptomic results of the ovarian steroidogenesis-related genes showed a down-regulation of this pathway in the E_2-treated group. In addition, screening of several epigenetic mechanisms-related genes present in the microarray also showed a tendency to down-regulation in the E_2 group.

Conclusion

Together, these results corroborate the relationship between elevated temperature, the up-regulation of the male pathways and sex ratio bias. Moreover, the transcriptomic down-regulation of the ovarian steroid synthesis pathway after E_2 administration is in agreement with what has been previously reported for E_2 plasma levels in fish after E_2 treatment. Although some of the genes related with epigenetic regulatory mechanisms were DE in the E_2-treated group, it remains to be determined the possible causal relationship among these observations.

GENETIC VERSUS TEMPERATURE-DEPENDENT SEX DETERMINATION IN PEJERREY: EVIDENCE FROM THE TESTIS-DETERMINING GENE, *AMHY*

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Introduction

Sex differentiation in the pejerrey *Odontesthes bonariensis* is highly dependent on the water temperature experienced by an individual early in life. Thus, each individual can be reliably feminized or masculinized by early exposure to low and high temperatures, respectively. For this reason, genotypic determinants of sex have been considered virtually inexistent in this species. In this study, we examined the presence of a homologue of the congener *O. hatcheri* testis-determining gene *amhy* (Y-chromosome-linked, anti-Müllerian hormone) in pejerrey and its functionality at an intermediate, mixed sex-producing (MixPT) temperature.

Methods

To examine the presence of *amhy* in pejerrey, genomic DNA from laboratory-reared broodstock (n=24) was extracted, subjected to amplification by PCR using *amhy*-specific primers, and then sequenced. Larvae derived from a cross between an *amhy*-/-female and an *amhy*+/-male were reared at MixPT (25ºC) for up to 14 weeks after hatching (wah) and sampled weekly for the analyses of *amhy* and autosomal *amh* (*amha*) mRNA expression by Real-time PCR and *in situ* hybridization (ISH) in the gonads. The remaining larvae were sampled at the end of 14 wah for the determination of sex ratios by light microscopy.

Results and Discussion

Approximately 50% of broodstock fish tested in this study possessed *amhy* and 12 out of 14 were males. In contrast, the autosomal *amha* was present in all individuals. The sex ratio in the *amhy*+/-/*amhy*-/- full sibling progeny reared at 25ºC was 68.7% male: 31.3% female. Genotyping based on *amhy* revealed that all *amhy*+/- fish developed as males whereas about 2/3 and 1/3 of the *amhy*-/- were females and males, respectively. The temporal transcription pattern of *amhy* differed from that of *amha*: *amhy* levels increased transiently before the critical period of sex determination, whereas *amha* expression increased concomitantly with the *amhy* decrease and remained high during sex determination. In *amhy*-/- individuals, *amha* expression was detected in approximately 1/3 of the animals. All gonads of *amhy*-/- animals with *amha* ISH signals after histological differentiation were identified as testes and those without it as ovaries.

Conclusion

These results reinforce the importance of *amha* for testis formation in pejerrey regardless of genotypic sex trend. More importantly, they also suggest that *amhy*, alone or in combination with temperature, may direct animals to differentiate as males at intermediate temperatures whereas *amhy*-/- animals rely solely on temperature-activated *amha* expression. These findings represent the first clear evidence of a genotypic sex trend in pejerrey and suggest that genetic and environmental sex determinants can coexist even in species with marked temperature-dependent sex determination.

UP-REGULATION OF *DAX1* IN THE BRAIN OF PHENOTYPIC AND GENETIC MALES DURING SEX DIFFERENTIATION

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Introduction

Gonad development is governed by genetic factors and temperature in the Nile tilapia *Oreochromis niloticus*. When the gonads are still undifferentiated, the genetic sex can be inverted with elevated temperatures or 17α-methytestosterone (17MT) treatments resulting in functional phenotypic males. In contrast, 17α-ethinylestradiol (E2) treatment induces ovarian differentiation and female phenotypes. Characterisation of masculinising and feminising treatments on the expression of major ovarian genes (*cyp19a1a*, *foxl2*) and testicular genes (*dmrt1* and *amh)* has been performed in tilapia gonads during sex differentiation. However, information is scarce regarding the effect of these treatments in the brains of gonochoric fish during sex differentiation. Here we analysed the changes during the masculinisation and feminisation in the brain of tilapia studying notably the expression of the orphan nuclear receptor gene *dax1* (=nr0b1). *Dax1* expression was also analysed in the gonads and heads of genetic males and females during sex differentiation.

Methods

Gonads and heads were sampled throughout gonadal sex differentiation from progenies of all-male XY and all-female XX tilapia reared at either 27°C or 36°C masculinising-temperatures from 10 to 30 days post-fertilisation (dpf). In addition, heads were sampled from a mixed XX/XY progeny that was divided into 4 groups: the control reared at 27°C, a group treated from 9 to 30 dpf with 100 µg/g feed of E2, and two groups treated from 10 to 30 dpf respectively with 50 µg/g feed of 17MT or 36°C masculinising-temperatures (TM). Sex-ratios were studied at 90 dpf. *Dax1* expression was analysed by real-time PCR and relative values assessed using the housekeeping gene *ef1*. The aromatase *cyp19a1b* and *amh* were also analysed.

Results and Discussion

In contrast to previous studies, we found higher *dax1* expression in both the gonads and heads of genetic males compared to genetic females, at early stages of sex differentiation (10-15 dpf). Temperature-induced masculinisation stimulated *dax1* expression appearing around 11 dpf first in the gonads and subsequently (~17 dpf) in both gonads and heads. Induced masculinisation with 17MT or TM on the mixed progeny caused a rapid up-regulation of *dax1* in the heads at 14 dpf, with higher levels in TM males. Levels decreased in E2 treated fish. At 14dpf, *amh* was higher in genetic males in tilapia brain.

Conclusion

The *dax1* gene is affected in gonads and brain during the early stages of masculinisation of tilapia in both genetic and phenotypic males. Temperature-induced masculinisation exerts a higher stimulation of *dax1* and *cyp19a1b* expression than 17MT phenotypic males.

CHARACTERISATION OF MOLECULAR SEX DIFFERENTIATION IN THE SIBERIAN STURGEON, *ACIPENSER BAERII*

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Introduction

The factors that trigger the gonad sex differentiation in sturgeons are unknown. In teleost fish the male pathway is characterized by the expression of sex-determining gene followed by an early dimorphic expression of *amh*, *dmrt1*, *sox9* depending on species. In females estrogens (mediated by *cyp19a1a)* and *foxl2* seems to be essential for the ovarian differentiation. The participation of androgens in testis differentiation is controversial. Our hypothesis is that sturgeons share with teleosts the genes directing the gonad differentiation. We identified the period of sex differentiation at morphological level, and studied the expression of *dmrt1*, *sox9*, *amh*, *ar*, *cyp17*, *foxl2*, *cyp19* and *yy1* during early undifferentiated stages compared to differentiated gonads.

Methods

dmrt1, *sox9*, *amh*, *ar*, *cyp17*, *foxl2*, *cyp19a1a* and *yy1*were amplified from immature testis and vitellogenic ovaries of *A. baerii*, using primers designed to target conserved regions in fish. Sequences were validated in previous works. The histological analysis and gene expression was made in gonads sampled at different ages: 3 (n=8), 4 (n=14), 8 (n=7), 9 (n=9), 10 (n=7), 11 (n=10) and 14 (n=7) months old (mo). Fish were obtained at the farm 'Estuario del Plata' (Uruguay).

Results and Discussion

At 3 and 4 mo the fish were undifferentiated, while at 8 mo only 12,5% were differentiated. 9 months onwards all fish were differentiated.

The genes studied showed two different level of expression. The 'high expression genes' showed > 40 fold with respect to 'low expression genes' at 4 mo gonads (undifferentiated fish). The 'high expression group' was constituted by classic male markers as *amh*, *sox9* and *dmrt1* and the low expression group by female markers (*foxl2*, *cyp19*, *yy1*) and steroid production and receptivity genes (*ar*, *cyp17a1)*. The two groups showed a bi-modal expression with a first peak of expression during undifferentiated period and a second peak during post-differentiated time. The earliest and higher level of expression was reported in the *amh* and *sox9* (3 and 4 mo) while *dmrt1* started it increase later (4 mo). The *cyp19*, *foxl2*, *cyp17* and *yy1* showed relative high levels very early (3 and 4 mo), while *ar* showed an increase at 9 mo in differentiated fish.

Conclusion

The period of sex differentiation in Uruguay started at least at 8 mo. The early peak of expression of the genes studied suggests that the period between 3 and 4 months corresponds to the molecular sex differentiation period.

THE SENSITIVE PERIOD OF SEX DIFFERENTIATION BEGINS AT THE EMBRYONIC STAGE IN NILE TILAPIA: NEW INSIGHTS ON THE MECHANISMS OF EARLY FEMINIZATION

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Introduction

The sensitive period of sex differentiation is known to range from 10 to 28 days post-fertilization (dpf) in Nile tilapia. During this period, thermal or hormonal sex reversal treatments act on developing gonads to induce a phenotypic sexual differentiation which may oppose the sexual genotype. An increasing number of studies seem to indicate that not only gonads but also the brain could play a key role in fish sex differentiation. To test this hypothesis in tilapia (*Oreochromis niloticus*), we feminised embryos by hormonal immersion. We studied the efficiency and mechanisms of the sex reversal treatment, analysing sex differentiating gene expressions and sexual steroid levels during the embryonic development, when the brain is well differentiated but the gonadal anlage is not yet developed.

Methods

After artificial fertilization, 5 XY spawns were split into 3 batches: control, immersed in 2000 µg L^{-1} 17α-ethinylestradiol (EE_2) solution for 4 h at 24 hpf (T2000) and immersed in 20 µg EE_2 L^{-1} for 4 h at 10 dpf (T20). The first treatment was used to test the sex inversion efficiency of hormonal treatment on developing embryos. The second treatment was intended to verify that the first one really targeted the early development stages and did not act by a hormonal tissue accumulation and a delayed effect after 10 dpf. Sex ratio was assessed by gonadal squash at 90 dpf. EE_2 tissue concentration was measured by ELISA and testosterone (T), estradiol (E_2) and 11-ketotestosterone (11KT) by radioimmunoassay from 0 to 35 dpf in all batches. Expression levels of *cyp19a1b* (brain aromatase), *amh* (anti-müllerian), *foxl2* (forkhead box l2), *ER1* and *ER2* (estrogen receptors) were determined by QPCR in the head at 4 and 14 dpf in control and T2000 groups.

Results and Discussion

Survival rates (from fertilization to 35 dpf) ranged from 25.1 ± 8.3 (control) to 40.4 ± 10.1 % (T20). All control groups were 100 % male. Feminization rate in T2000 fish ranged from 0.9 to 60.6 % (mean 20.5 ± 23.8 %). In T20, from 0.0 to 8.6 % of females were observed (mean 2.5 ± 3.8 %). As EE_2 concentration in fish at 10 dpf was 1146 ± 342 ng g^{-1} in T2000 and 3975 ± 2225 ng g^{-1} in T20, it can be assumed that feminization observed in T2000 was induced by early treatment at 24 hpf, acting during the first 10 days of life. At 14 dpf, no difference in expression levels of studied genes was observed between control and EE_2-treated groups. In contrast, at 4 dpf, *cyp19a1b* was highly expressed in heads and EE_2 immersion increased the relative expression from 16.1 ± 2.6 % in controls to 39.1 ± 6.1 % in T2000. At this stage, no transcript differences were detected for *amh*, *foxl2*, *ER1* and *ER2* between the two groups. These results suggest a precocious role of brain aromatase in sex differentiation in tilapia. T, E2 and 11KT measurements are under progress.

Conclusion

This study confirms our work and shows that the sensitivity for sex inversion by external factors is much broader than initially thought, starting much earlier between 0 and 10 dpf. This period covers the development of the brain and the division of primordial germ cells (PGCs) that migrate to the gonadal anlagen around 9 dpf. EE_2 immersion of embryos could act on undifferentiated PGCs or on the brain as supported by the increase in brain aromatase expression at 4 dpf.

FURTHER CHARACTERIZATION OF RAINBOW TROUT SHBGB DURING SEXUAL DIFFERENTIATION

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Introduction

Sex hormone-binding globulin (SHBG) which specifically binds biologically active androgens and estrogens was found in the blood of several teleost species like the zebrafish and the goldfish. Interestingly, two paralogs of Shbg have been identified in the rainbow trout *Oncorhynchus mykiss*, Shbga and Shbgb. Shbg is a glycoprotein whose structure has been well characterized in Human [two laminine-G-like (LG) domains, disulfide bridges, glycosylation consensus sites and signal peptide]. These characteristics are well conserved between species. Shbgb is mostly expressed in rainbow trout granulosa cells. In the present work, Shbgb has been further characterized during rainbow trout sexual differentiation.

Methods

The protein domains structures were analyzed with the NCBI Conserved Domain Database (CDD) software. *shbgb* expression was followed in gonads during sex differentiation of all-male and all-female population using RT-qPCR and whole-mount hybridization. Finally, relative binding affinities to testosterone of recombinant Shbgb were determined for several steroids using competition experiments.

Results and Discussion

shbgb gene displays the same structure as *shbga*, but is nearly twice as long as the *shbga* gene. This variation seems to be due essentially to the intron sizes. As regards the protein structures, one LG-like domain was identified in the N-terminal part of both Shbga and Shbgb and a domain belonging to the LG-like family was identified in their C-terminal part. Thus, characteristics of SHBG have been conserved by Shbgb.

As expected, *shbgb* mRNA were only detected in female gonads along the full length of the embryonic female gonad. *In situ* hybridization showed a specific expression of *shbgb* in cells surrounding lamellae during ovarian differentiation. Gonadal aromatase (*cyp19a1a*) mRNA were also located in these cells during ovary differentiation. A first but not significant expression increase occurred at 61 dpf with the establishment of the ovarian lamellae. Then, a significant high increase was observed at the end of the ovarian sex differentiation when *cyp19a1a* expression tended to decrease.

Recombinant trout Shbga and Shbgb had different steroid binding affinities. Thus, Shbgb showed a greater affinity for E2 but a lower affinity for androstenedione. The whole data indicates that Shbgb is more discriminating against steroids than Shbga.

Conclusion

Trout Shbgb possesses some common characteristics to SHBG proteins but has its own properties such as its spatial and temporal expression as well as its steroid binding pattern. These observations should help to understand Shbgb functions in the rainbow trout ovary.

Oral Session:

Reproductive Technologies and Selective Breeding

Chairs: Hanna Rosenfeld and Oliana Carnevali

GONADOTROPIN ACTIONS IN EUROPEAN SEA BASS: ENDOCRINE ROLES AND BIOTECHNOLOGICAL APPLICATIONS

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The follicle stimulating hormone (Fsh) and the luteinizing hormone (Lh) are central endocrine regulators of gonad function in vertebrates. These gonadotropins act by binding and activating their specific receptors that are located in certain cell types of the gonads.
In fish, the differential roles of these hormones are being progressively elucidated with the development of different tools for their study. In the case of European sea bass *Dicentrarchus labrax*, the isolation of the genes coding for the gonadotropin subunits and receptors allowed in first instance for expression studies. Later, to overcome the limitation of native hormones, recombinant dimeric gonadotropins have been generated, which show different functional characteristics depending on the cell system and DNA construct. In addition, single gonadotropin beta-subunits have been produced and used as antigens for antibody production. This last approach has allowed the development of detection methods for native gonadotropins being the European sea bass one of the few species where both gonadotropins can be detected in their native form. The generation of antibodies has also allowed for their cellular localization and co-localization with other factors, helping to a better understanding of their function.
By administration of the recombinant gonadotropins to gonad tissues cultured *in vitro* we have studied their effects on steroidogenesis, the activated intracellular pathways and their target genes. Their administration *in vivo* has also been tested both for basic studies and as a biotechnological approach. In this frame, it is known from different studies in mammals that exogenous administration of native hormones in cases of deficiency, including assisted reproduction, can be unsatisfactory due to their rapid clearance from circulation or limited availability. Other than the production of recombinant hormones gene-based therapies by using somatic gene transfer are offered as an alternative. Such an approach has been tested in sea bass for gonadotropin delivery *in vivo*. The hormones produced by the injected genes were functional and have allowed for different studies on gonadotropin action in spermatogenesis.
All together, the use of gene therapy for hormone replacement in fish is a real alternative to the production of recombinant gonadotropins for *in vivo* use, due to the low cost of production and the high persistence of the injected DNA, and has a broad range of potential applications such as its use in out-of-season breeding programs or reproductive dysfunctions in fish species.

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PRODUCTION OF INFERTILE ZEBRAFISH BY DISRUPTING PRIMORDIAL GERM CELL DEVELOPMENT: TRANSGENIC AND NON-TRANSGENIC APPROACHES

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Introduction

Primordial germ cells (PGCs) are a population of cells in the embryo that give rise to the eggs and sperm of the adult. In fish, PGCs are specified during early development by the incorporation of maternally-derived germ plasm and then migrate to the developing gonad by following a gradient of the chemokine, stromal-derived growth factor 1 (Sdf1). Disruption of the Sdf1 signaling pathway prevents normal PGC migration in the fish embryo. Here we describe methods to produce infertile fish by disrupting the normal migration and development patterns of PGCs.

Methods

A transgenic line of zebrafish, *Tg(hsp70:sdf1a-nanos3, EGFP)*, was generated that expresses Sdf1a under the control of the heat-shock protein 70 (*hsp70*) promoter and *nanos3* 3'UTR. To better visualize the PGCs, the *Tg(hsp70:sdf1a-nanos3, EGFP)* fish were crossed with another transgenic line, *Tg(kop:DsRed-nanos3)*, that expresses DsRed driven by the PGC-specific *kop* promoter. Heat treatment of the transgenic embryos caused an induction of Sdf1a expression throughout the embryo resulting in the disruption of normal PGC migration. The *Tg(kop:DsRed-nanos3)* line was also used to identify compounds that are able to disrupt PGC migration and development, for application in non-transgenic approaches to induce sterility in farmed fish.

Results and Discussion

Optimal embryo survival and disruption of PGC migration was achieved when transgenic embryos at the 4- to 8-cell stage were incubated at 34.5°C for 18 hours. Under these conditions, disruption of PGC migration was observed in 100% of the embryos. Sixty-four adult fish were developed from three separate batches of heat-treated embryos and all were found to be infertile males. Histological examination revealed that each of the adult male fish possessed severely under-developed gonads that lacked gametes. The results demonstrate that disruption of the Sdf1a signal pathway is an efficient and reliable strategy to produce infertile fish. This approach has led to the use of *Tg(kop:DsRed-nanos3)* to identify compounds that are able to disrupt PGC migration and development and that eventually result in failure of gonadal development and sterility via non-transgenic approaches. A list of compounds and their potential for disrupting PGC development are discussed.

Conclusion

Using transgenic and non-transgenic approaches, PGC migration and development have been able to be disrupted in zebrafish embryos, which has led to the failure of gonadal development in adult fish and resulted in the successful generation of infertile fish.

IDENTIFYING SEX-ASSOCIATED MARKERS IN FARMED FISH USING RAD SEQUENCING

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Introduction

Many aquaculture species, like tilapias and flatfish, exhibit sex-related differences in desired traits like growth and age of maturation, making the production of all-male or all-female stocks appealing for the aquaculture industry. Sex determination is very variable in fish. Even in species with genetic sex determination, sex chromosomes are often at early stages of differentiation. Combined with the effect of autosomal loci in some cases, clarification of sex determination mechanisms and development of sex-associated markers can be challenging. Restriction-site associated DNA (RAD) sequencing has the potential for rapid discovery of sex–linked, or even sex-specific, SNPs. The current study is focused on using RAD markers to identify sex-associated SNPs in two important farmed species, the Atlantic halibut (*Hippoglossus hippoglossus*) and the Nile tilapia (*Oreochromis niloticus*).

Methods

For both species DNA from parents and offspring from mixed-sex families were used as a template for RAD sequencing using the Illumina HiSeq 2000 platform. Following quality control of sequencing output, good quality sequence reads were sorted in loci and genotyped using Stacks software. Genetic maps were then constructed using R/Onemap and TMAP and drawn using Genetic-Mapper and subsequent QTL analysis was then performed using R/qtl to identify sex associated markers. Once identified, allele specific endpoint-genotyping assays were established to then verify marker association with gender.

Results and Discussion

For Atlantic halibut, 5703 informative SNP markers were identified which were resolved into a linkage map consisting of 24 linkage groups. A major sex determining locus was mapped to linkage group 13 that explained 82% of the phenotypic variance. This QTL spanned a region of approximately 30cM and contained 38 putative sex associated SNPs which were then further resolved to a 4 marker based assay that had 97% accuracy in predicting gender. For tilapia, 3280 markers were used to identify a QTL in LG1 explaining nearly 96% of the phenotypic variance. The QTL was mapped to a 2cM interval in which 2 SNP markers could be used to predict phenotypic sex with >95% accuracy. Furthermore by alignment with the draft Tilapia genome, 10 annotated genes were identified in the region which are potential candidates for the sex-determining gene in this species.

Conclusion

This work showcases the speed and accuracy in which sex associated markers can be identified in commercially important finfish. The cost effectiveness of next generation sequencing methods is improving rapidly which the authors hope will see the expansion of trait associated marker generation to support commercial breeding programmes.

TOOLS FOR THE DEVELOPMENT OF A SELECTIVE BREEDING STRATEGY FOR ATLANTIC HALIBUT (*HIPPOGLOSSUS HIPPOGOSSUS* L.): GENETIC SEX MARKERS AND LARGE SCALE ALL FEMALE PRODUCTION

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Introduction

Commercial aquaculture of Atlantic halibut (*Hippoglossus hippoglossus* L.) has been hampered by the complicated biology of early life stages, as well as slow and uneven growth in sea cages. A pronounced sexual dimorphism in growth, mainly due to male maturation, makes production of all female populations particularly interesting. Identification of sex-specific markers and development of assays are essential steps in the establishment of breeding programmes based on use of sex-reversed females, neomales, as broodstock. At present, there are no such assays available that work across a wide number of populations.

Methods

For genome sequencing, twenty full siblings (ten males and ten females) were sampled and DNA extracted and pooled for each sex. The pools were subjected to sequencing by Illumina HiSeq 2000. The resulting sequences were assembled and differences between the female and male pools sequences data identified. Based on this a number of PCR assays were designed to differentiate between the sexes. Neomales were created by feeding weaned halibut postlarvae (30-50 mm TL) with a commercial feed that was supplied with either methyltestosterone (MT), dihydromethyltestosterone (DHMT) or fadrozole (Fad). A second generation of neomales was produced from all-female fry and conditions for, as well as timing of, sex reversal optimised. Gene transcript levels of key genes involved in sex differentiation were monitored in postlarvae.

Results and Discussion

Treatment with MT, DHMT or Fad resulted in dose-dependent sex reversal of halibut females. Identification of neomales was performed using either progeny testing or an assay based on a genetic sex marker, which was designed to work across different populations of halibut. The assay for a genetic sex marker can be an efficient way to identify neomales from different populations of halibut without progeny testing. This has great potential for use in selective breeding based on the production of neomales sired by male halibut with the desired genetic treats. Treatment with Fad or MT up-regulated key gene transcripts such as *cyp19a1a, er2, dmrt1 and ar* after two weeks, which is consistent with the general pattern observed during sex differentiation in other teleost species.

Conclusions

Large-scale production of all female populations is established and will be an important means to improve the results of commercial farming of halibut. Reliable assays for sex markers were developed that can be used to identify neomales at an early stage, and that work across different populations, which will be important for the development of selective breeding programmes.

DNA METHYLATION OF FISH GERM CELLS AND THE RISK OF ALTERATION AFTER CRYOPRESERVATION

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Introduction

DNA methylation of cytosine residues in CpG sites in the promoter region of some genes is one epigenetic modification associated with a repressed state of the gene expression. DNA methylation is a major factor in the regulation network during embryo development, and it was proposed that the methylation pattern of sperm DNA would still play a role after fertilization, during early embryo development. Because sperm cryopreservation in fish often relies on the use of permeating cryoprotectants bearing chemically reactive methyl group (methanol, dimethyl sulfoxide, dimethyl acetamide …), we wanted to decipher whether fish sperm DNA methylation pattern can be affected by cryopreservation.

Methods

Sperm from zebrafish (*Danio rerio*), goldfish (*Carassius auratus*), and rainbow trout (*Oncorhynchus mykiss*) were tested in this experiment. For each male, one sperm fraction was used for the fresh sperm control, whereas another fraction was cryopreserved. Cryoprotectants for cyprinids contained sucrose (60 mM) and methanol (10 %), those for rainbow trout contained dimethyl sulfoxide (7.5 %), sucrose (120 mM) and egg yolk (10 %). DNA from both fresh and cryopreserved samples was extracted with a phenol-chloroform method. Total DNA methylation level was estimated from differential DNA digestion with the restriction enzymes MspI and HpaII. MspI will cut all CCGG sites whereas HpaII will digest only the unmethylated CCGG sites. The extent of digestion was assessed by pyrosequencing according to the LUMA method.

Results and Discussion

The global sperm methylation of fish spermatozoa was different between species, but showed a general hypermethylation. This contrasts with the hypomethylation of some genes whose expression is activated during early development. After cryopreservation, sperm DNA will be randomly affected depending on the individuals (some showing hypomethylation, some hypermethylation, or no change compared to the fresh controls). Not all species will behave the same after cryopreservation, and the relationship with sperm nucleus organization will be discussed.

Conclusion

Sperm cryopreservation does affect global DNA methylation. It remains to be determined whether the genes involved in early embryo development are affected as well. The observed instability of the global methylation has now to be explored with regards to embryo development quality.

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TOOLS AND TECHNIQUES TO SUPPORT BALLAN WRASSE (*LABRUS BERGYLTA*) BROODSTOCK MANAGEMENT

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Introduction

There is increasing commercial pressure for a sustainable hatchery source of Ballan Wrasse (*Labrus bergylta*) for parasite control in the salmon farming industry across northern Europe. Due to the significant lack of understanding of basic reproductive physiology and behavior in the species, broodstock management has proven challenging in the initial phases. In addition there are few validated species specific physiological and genomic tools and methods which can help optimise management practices. The aim of this work was to develop such tools and techniques to help address basic management questions like, gender identification, gender manipulation, phylo-geographic structuring and parental contribution to spawning events. All of which are required to ensure that spawning harems are optimised to the best of our ability.

Methods

A dataset of > 400 Ballan wrasse sourced from across the UK and Norway where each individual was aged, morphometrics and external colour phenotype recorded, digital photograph taken, gender confirmed by histological examination of gonads, fin clipped for genomic analysis and blood plasma preserved for pigment analysis. As Ballan wrasse are protogynous hermaphrodites and in response to the output of the gender identification investigations, an additional series of experiments were performed to test IP injection of androgenic compounds to induce sex reversal in female wrasse.

Results and Discussion

The wild Ballan wrasse dataset was successfully used to develop gender identification methods; after screening potential techniques including ultrasound screening, hormonal profiling and colour morphotype, the most accurate method was a derivation of truss network analysis giving a gender prediction accuracy of *circa* 90%. A Ballan specific genomic marker library was also generated using the RAD-sequencing technique which identified >8000 SNP and >1000 putative microsatellite markers. This library was then interrogated to develop geographic specific assays, investigate genomic association with gender and colour phenotypes and develop pilot assays to determine parental contribution in spawning harems. This study also highlighted the lack of available males (≈10% of the population) thus a tank trial demonstration that female – male sex reversal is reliably inducible has helped assure male availability.

Conclusion

This body of work summarises the development and validation of a range of tools and methodologies that ultimately will help improve the productivity of captive Ballan wrasse broodstock populations by allowing informed management decisions to be made. The future application of the tools will be discussed in the context of the identified bottlenecks in hatchery production of this and other species.

PRODUCTION OF OFFSPRING DERIVED FROM FROZEN WHOLE FISH KEPT IN FREEZER

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Introduction

Although the long-term preservation of fish genetic resources is essential for the conservation of endangered fish species, cryopreservation techniques for fish eggs and embryos are still not developed. We recently established a method for harvesting functional eggs and sperm from cryopreserved trout spermatogonia (SG). However, derivation of viable gametes from whole fish that have been frozen without any cryoprotection remains a challenge. If the SG inside frozen whole fish were capable of differentiating into eggs and sperm in the recipients of different species, then it would be possible to produce fish from frozen extinct fish species by germ cell transplantation into closely related species. Therefore, in this study, we aimed to establish a germ cell transplantation system using the SG of frozen whole fish stored at -80°C.

Methods

Hemizygous p*vasa-Gfp* transgenic rainbow trout were frozen without cryoprotection in a standard deep freezer. After freezing for up to 3 years, testes isolated from the thawed fish were dissociated into cells and SG survival was assessed. Freeze-thawed SG were then transplanted into the peritoneal cavity of wild-type triploid masu salmon and the recipients were reared until maturity.

Results and Discussion

Relatively few of the SG from freeze-thawed fish remained viable (survival rate: 0.08%, approx. 1,200 SG per fish), and this proportion did not change significantly with an increase in freezing duration (up to 3 years). Freeze-thawed SG that were intraperitoneally transplanted into recipient fish migrated toward the recipient gonads where they were incorporated and proliferated rapidly. Although all triploid salmon that did not undergo transplantation were sterile, two out of 12 female recipients (16.7%) and 4 out of 13 male recipients (30.8%) reached sexual maturity at 2 years old. Eggs and sperm obtained from the recipients were capable of producing normal offspring, with approximately 75% of F1 individuals possessing the *Gfp* gene. These findings suggested that all of the haploid gametes produced by the recipients were derived from the frozen donor fish. Derivation of functional eggs and/or sperm from nonembryonic frozen animals has not yet been achieved in any animal species, as cells within frozen animals tend to lose their functional integrity. The protocol established in the present study is thus a breakthrough for complete regeneration of a frozen animal.

Conclusion

We derived functional eggs and sperm from SG recovered from the testes of a fish that was frozen without any cryoprotection. This methodology is considered to be applicable to the regeneration of extinct fish species stored in a deep freezer.

COMPARISON BETWEEN GnRHa INJECTIONS AND IMPLANTS ON SPAWNING KINETICS AND EGG/LARVAL PERFORMANCE PARAMETERS IN MEAGRE *ARGYROSOMUS REGIUS*

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Introduction

The meagre *Argyrosomus regius* (Sciaenidae) is a species with great potential for the diversification of aquaculture production in the Mediterranean region. Meagre aquaculture production has increased rapidly in the last decade, though reproduction in captivity still remains a problem. With few exceptions, females do not mature in captivity, and exogenous hormones need to be used to induce ovulation and spawning. The present study compares the spawning kinetics, and egg/larval performance characteristics of GnRHa injections vs implants. The information obtained is expected to be useful for better implementation of GnRHa induction therapies in meagre and optimization of egg production in commercial aquaculture operations.

Methods

Over the course of two consecutive years (2012-2013), females (mean body weight 7.3 – 8.3 Kg) were treated with a GnRHa (Des-Gly^{10},d-Ala^{6}-Pro-NEth^{9}-mGnRHa) injection of 15-25 μg kg^{-1} (n=2) or with a GnRHa implant for an effective dose of 40-100 μg Kg^{-1} (n=2). The GnRHa injections were given approximately every 10 days and the GnRHa implants every 20-30 days. All males were treated with a GnRHa implant at doses ranging between 30 and 160 μg kg^{-1} body weight, at the beginning of the experiment and as needed to increase sperm production thereafter. After treatment, females were placed individually with a single male in separate spawning tanks. Eggs were collected using passive over-flow egg collectors and were incubated in microtiter plates until absorption of the yolk sac (5 days after spawning).

Results and Discussion

The GnRHa implanted females spawned for 17-23 almost consecutive spawns after the first implant, but failed to repeat this spawning kinetics in subsequent treatments (Fig. 1). Batch fecundity was decreasing as time progressed after implantation. The GnRHa injected females spawned only 2 times after each GnRHa injection, with the first batch having the highest fecundity. Fertilization success was high in all treatments. Overall egg production was not significantly different between the females treated with GnRHa implants or injections, but there were significant increases in total relative fecundity in 2013 (Fig. 2). Similarly, there were no significant differences between the GnRHa treatments in any of the egg/larval performance parameters examined. Fertilization and hatching was significantly higher in 2013, whereas 5-d larval survival was lower in 2013, compared to 2012.

Conclusion

Both GnRHa treatments methods were effective in inducing spawning in meagre, without any differences in the overall production data, but with very different spawning kinetics. The results obtained indicate that a hatchery manager can manipulate the spawning kinetics of meagre and adapt them according to the egg production needs of the hatchery, by selecting GnRHa implants or multiple injections. Implants can be used to provide many consecutive spawns over a period of 2-3 weeks (though of decreasing fecundity), while GnRHa injections can be used to obtain 2 consecutive spawns of larger fecundity at required times over the course of 2-3 months.

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EFFECT OF ORAL ADMINISTRATION OF GnRHa+NANO PARTICLES OF CHITOSAN IN OOCYTE DEVELOPMENT OF GOLDFISH *CARASSIUS AURATUS*

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Introduction

Dysfunction in reproduction in aquaculture industry is characterized by improper oocyte development, final oocyte maturation and ovulation in female fishes. Also, gonadal development of some species like sturgeons confronted to lengthy period of vitellogenesis. So far several methods have been used to accelerate these stages, including oral or sustained-release delivery of hormones. But most of these routes make stress for fishes which leads to some dysfunction in reproduction or oocyte development, except the oral administration. In this study we proposed using of combination of GnRH analog with nano particles of chitosan (a biopolymer derived from chitin) may be capable of accelerating oocyte development and induce vitellogenesis in gold fish as a model through oral administration.

Methods

Nano particles of GnRH analog+chitosan were prepared and fed to adult female goldfishes in 6 experimental groups: Intact group, recipients of chitosan group, recipients of 50 µgr GnRHa/kg.BW, 100 µgr GnRHa/kg.BW, chitosan+50 µgr GnRHa/kg.BW and chitosan+100 µgr GnRHa/kg.BW. Fishes in each group were treated tertian by these doses.

From day 0 to day 40 of the experiment, 3 fishes were chosen randomly from each group once every 10 days and sampled from their ovary and photographed under optical microscope to evaluate its development through gonadal development indicators such as Gonadal Somatic Index (GSI), Oocyte Diameter (OD), Zona Radiata (ZR) appearance and Follicular Layer (FL) diameter.

Results and Discussion

The results demonstrated an increasing trend of oocyte development in most of the fishes in three groups of chitosan, 50 µgr GnRHa/kg.BW, 100 µgr GnRHa/kg.BW. This trend was different in the other two groups of 50 and 100 µgr GnRHa/kg.BW+nano particles of chitosan.

In these two groups we initially observed an increase in gonadal development indicators, then a decrease and at last another increase. This dip in the increasing trend was more prominent in the group treated with 100 µgr GnRHa/kg.BW. It seems the compound induced orally in this group was able to pass the acidic space of interior intestine and then be absorbed by the epithelial cells and finally affect the pituitary by entering the bloodstream**.**

Conclusion

The results showed that using the combination of chitosan+100 µgr GnRHa/kg.BW can accelerate oocyte development. It was also shown that oral administration of GnRHa without any additives does not stimulate oocyte development significantly.

Oral Session:

Brain and Pituitary

Chairs: Penny Swanson and Hamid Habibi

NEUROENDOCRINE CONTROL MECHANISMS OF IMPRINTING AND HOMING MIGRATION IN PACIFIC SALMON

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Introduction

Pacific salmon have an amazing ability to imprint their natal stream and to migrate thousands of kilometers from the open sea to their natal stream for reproduction after several years of oceanic feeding migration. In my laboratory, we tried to clarify neuroendocrine control mechanisms of the brain-pituitary-thyroid (BPT) and brain-pituitary-gonad (BPG) axes during juvenile downstream migration and adult homing migration, respectively. Changes in gene expression of thyrotropin-releasing hormone (TRH), gonadotropin-releasing hormone (GnRH), and kisspeptin (Kiss) in the brain as well as changes in hormone levels of thyroid hormones and gonadal steroid hormones were analyzed in Pacific salmon.

Methods

Under yearling chum salmon (*Oncorhynchus keta*) that were reared in the Chitose Hatchery in Hokkaido, Japan, and released into the Chitose River were collected during downstream migration to the Ishikari Bay. Homing adult chum salmon were collected from the Bering Sea to the Chitose Hatchery. One-year-old of sockeye salmon (*O. nerka*) were collected during parr-smolt transformation (PST) in the Shizunai Hatchery, and homing adults were collected from the Bering Sea to the Bibi River. Changes in TRH (TRHa and TRHb), salmon GnRH (sGnRH), and Kiss2 gene expression in the brain were analyzed by the real-time quantitative PCRs. Changes in thyroid hormones (T_4 and T_3) and gonadal steroid hormones (E_2, 11KT, and DHP) were analyzed by the time-resolved fluoroimmunoassay.

Results and Discussion

During downstream migration of chum salmon, the gene expressions of TRHa increased from the hatchery to the release point and decreased toward the Gulf of Ishikari, those of TRHb showed a drastic increase just after release, and T_4 and T_3 increased during downstream migration. During homing migration of chum salmon, the gene expressions of sGnRH in the olfactory bulb were higher in the Gulf of Ishikari, and decreased toward the hatchery. In contrast, those in the hypothalamus increased toward the hatchery. E_2 and 11KT increased during vitellogenesis and spermatogenesis, respectively, while DHP increased dramatically at the hatchery. During PST of sockeye salmon, the gene expressions of TRHa in the hypothalamus increased, but those of TRHb showed little changes. During homing migration of sockeye salmon, the gene expressions Kiss2 and sGnRH in the hypothalamus and telencephalon were high in the Bering Sea, and decreased in maturing and matured fish in the Bibi River. The gene expression levels of TRH, and sGnRH and Kiss2 showed brain region specific patterns, and these different patterns might control imprinting and homing migration in Pacific salmon.

Conclusion

In Pacific salmon, the BPT and BPG axes may control imprinting and homing migration during juvenile downstream migration and adult homing migration, respectively.

ESTROGENS MODULATE CELL PROLIFERATION IN THE BRAIN OF TELEOSTS

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Introduction

In teleosts, aromatase B (cyp19a1b) is expressed in radial glial cells, a unique type of brain cells, which act as progenitors in developing and adult vertebrates. In addition, aromatase B expression is strongly up-regulated by androgens and estrogens, so that aromatase activity is highest in sexually mature fish, when sex steroid levels are elevated. These properties represent unique features of teleosts compared to other vertebrates and raising the question of their functional significance. Using zebrafish, we tested the hypothesis that estrogens affect adult neurogenesis and brain regeneration by modulating the neurogenic activity of radial glial cells.

Methods

A combination of q-PCR, morphological and pharmacological techniques was used to assess the effects of manipulating estrogen levels on cell proliferation and newborn cells migration. This was examined in 6 months old adults, juveniles, larvae and embryos of wild or tg(*cyp19a1b*-GFP)-transgenic zebrafish. Animals were treated by estrogens, aromatase inhibitors or estrogen receptor blockers before being examined for cell proliferation and cell migration in different brain regions.

Results and Discussion

In both male and female adult zebrafish, estrogenic treatments, inhibition of aromatase activity or blockade of nuclear estrogen receptors lead to significantly decreased cell proliferation in most regions examined. This was particularly notable in the telencephalon, the preoptic area and the mediobasal or caudal hypothalamus, where aromatase B expression is elevated. Migration of newborn cells was also significantlyt reduced. In zebrafish embryos and larvae, exposure to estradiol or ethinylestradiol also consistently leads to increased expression of *cyp19a1b*, as expected, but this is also accompanied by decreased expression of PCNA. We also developed a model of telencephalic lesion to assess the role of aromatase and estrogens in brain repair. Proliferation increased rapidly immediately after the lesion in the ventricular surface of the injured telencephalon. Interestingly, aromatase B expression was significantly reduced 48 h and 7 days, after the lesion and estrogen treatment did not have any significant effects on cell proliferation. These data suggests that radial glial cell proliferation is inversely proportional to the estrogen environment, suggesting that in sexually maturing or mature fish, cell proliferation in the brain is reduced.

Conclusion

From the results obtained so far, estrogens appear to inhibit cell proliferation of radial glial cells in embryos, juveniles, larvae and adult zebrafish. It also seems that estradiol does not favor cell proliferation after brain lesion, in contrast with the situation in birds and mammals. All these data further point to a peculiar function of aromatase and estrogens in the brain of fish. The hypothesis, on which we are working on, stipulates that inhibitory effects of estrogens or aromatizable androgens would inhibit brain growth in maturing or mature fish, when energy demand for gonadal growth is high.

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FEMALE-SPECIFIC EXPRESSION OF NEUROPEPTIDE B IN THE MEDAKA BRAIN

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Introduction

Teleosts exhibit numerous sex differences in various traits. Most sex differences in behavioral traits likely originate from sex differences in the brain, about which only limited information is available. In this study, we searched for genes differently expressed in the male and female medaka brain and identified *npb* (neuropeptide B) as a gene that was female-specifically expressed in some neuronal populations. We then investigated the morphological and physiological features of the female-specific *npb*-expressing neurons to illuminate their biological significance.

Methods

Subtractive hybridization was performed to identify genes with sexually dimorphic expression in the medaka brain. One of the genes identified as having female-biased expression, *npb*, was selected for further analyses. The precise region of brain responsible for the female-biased expression of *npb* was determined by *in situ* hybridization. The fluctuation of *npb* expression during sexual maturation was also surveyed by real-time PCR. We then inspected the regulatory mechanisms underlying the sex difference in *npb* expression. First, possible genetic influences were examined using artificially sex-reversed medaka, whose genetic and phenotypic sex were discordant. Next, effects of sex steroids were assessed. We further analyzed the mode of action of sex steroids by examining the co-expression of *npb* and sex steroid receptors and by *in vitro* promoter assay.

Results and Discussion

Conspicuous sex difference of *npb* expression was found in ventral telencephalon and preoptic area, where *npb* was prominent in female but completely absent in male. These areas coincided with the areas implicated in the control of sexual behavior in teleosts. During sexual maturation, the expression level massively increased female specifically, with its peak at 3 month post fertilization, when medaka start spawning. In sex-reversed medaka, the levels of *npb* expression in the brain corresponded not with genetic sex but with phenotypic sex, indicating a regulatory role by sex steroids. Indeed, subsequent analyses revealed that the female-specific *npb* expression was totally dependent on estrogen secreted from the ovary. This estrogenic up-regulation is likely a direct transcriptional effect via estrogen receptors, since they were observed to be co-expressed in the female-specific *npb* neurons and be capable of activating *npb* transcription *in vitro*.

Conclusion

Here we report the female-specific expression of *npb* in the brain areas considered to control sexual behavior. This female-specific expression arises entirely by the stimulatory effect of estrogen. These findings suggest that *npb* may be involved in the feminization of sexual behavior in response to estrogen signaling.

UNDERSTANDING THE ROLES AND MODES OF ACTION OF KISSPEPTIN NEURONS IN REPRODUCTION

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Introduction

Kisspeptin is a key upstream regulator of the reproductive hypothalamic-pituitary-gonadal (HPG) axis in vertebrates, acting primarily on GnRH neurons. Many teleosts possess two kisspeptin systems: kiss1, kiss2 and two cognate receptors. The important roles of kisspeptin in regulating puberty and ovulation have been established in mammalian species. However, it is still unknown whether this scenario exists also in fish in terms of their exact roles and how these roles are exerted. The aim of this study was to establish an initial understanding of how (and if) kisspeptin1 and 2 regulate puberty and ovulation/spawning in the striped bass, *Morone saxatilis*.

Methods

1) To test the involvement of kisspeptin in puberty, pre-pubertal ('dummy-run') females were treated with 25 or 75 µg/Kg BW pGlu-Kiss1 or Kiss2 via EVAc sustained-release implants for 10 weeks from onset through mid-gonadal development. The effect on GnRHs, GnRHR, gonadotropins and gonadal development was then determined by histology, quantitative PCR and/or specific ELISA assays.
2) The involvement of kisspeptin in the regulation of ovulation and spawning was studied neuro-anatomically. Kisspeptin neurons were monitored in brain sections of male and female striped bass at different reproductive stages using immunohistochemistry and *in situ* hybridization. The appearance and location of neuronal soma, their projections and interactions with GnRH neurons and the gonadotrophs were studied.

Results and Discussion

1) None of the kisspeptin chronic administration treatments seem to trigger puberty. However, the lower dose of Kiss1 increased FSH transcript and advanced oocyte development. The observation that the stimulatory effect of Kiss1 was not correlated to GnRH raises the possibility that Kiss1 acts directly on the pituitary.
2) We detected a specific episodic appearance of both kiss1 and kiss2-expressing neurons in the hypothalamic nucleus lateralis tuberis (NLT) of males and females prior to spawning that presumably helps to execute the hormonally regulated events of spermiation and ovulation. In addition, we observed sexually dimorphic kiss2 neuronal innervations in the NLT and the neurohypophysis. We show that kiss1 and kiss2 neurons innervate the gonadotrophs, and like GnRH neurons, these neurons have the potency to affect gonadotrophs and perhaps also somatotrophs in the proximal pars distalis in the pituitary.
3) Initial results indicate that an episodic appearance of kisspeptin neurons occurs also at the onset of puberty.

Conclusion

These results underscore the NLT as important nuclei for kisspeptin action via kisspeptin – GnRH interaction and via the GnRH-independent effects of Kiss peptides on the pituitary. Kisspeptin neurons display a neuroanatomical plasticity in their neuronal location and projections that may intricately regulate specific reproductive events.

THE GONADOTROPIN-INHIBITORY HORMONE SYSTEM IN ZEBRAFISH: ONTOGENY, INTERACTIONS, AND FUNCTIONAL ROLES IN THE HYPOTHALAMUS-PITUITARY-GONAD AXIS

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Introduction

Gonadotropin-inhibitory hormone (GnIH) is a recently discovered hypothalamic neuropeptide that is capable of inhibiting the release of gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), from the anterior pituitary in birds and mammals. However, the functional role of GnIH is poorly understood in fish. Therefore, the purpose of this study is to characterize the ontogeny, interactions, and functional roles of GnIH in zebrafish (*Danio rerio*), a model for which multiple molecular tools are available.

Methods

To localize GnIH during ontogeny, a transgenic zebrafish line, expressing enhanced green fluorescent protein (eGFP) driven by the GnIH promoter, is now being generated. To corroborate eGFP localization, *in situ* hybridization (ISH) and immunohistochemistry (ICC) on wild-type embryos were conducted.

Using a dual luciferase reporter assay in COS7 cells, the activation of each of the three zebrafish GnIH receptors (GnIHR) by each of the three zebrafish GnIH peptides (encoded in the precursor polypeptide) was determined by monitoring the cAMP/cGMP pathways.

To determine the roles of GnIH, we employed loss-of-function methodology: (1) antisense morpholino (MO) oligonucleotide to knockdown GnIH and (2) transcription activator-like effector nucleases (TALENs) to introduce targeted and heritable GnIH mutations.

Results and Discussion

Transient expression of eGFP was observed in the hypothalamus of injected embryos at 48 hours post-fertilization (hpf), which was validated by *gnih* ISH. ICC also revealed GnIH expression in the hypothalamus of 72 hpf larvae with projections extending throughout the fore-, mid-, and hind-brain.

The receptor assay results show that GnIHR2 and GnIHR3 are activated with different potencies by all three GnIH peptides and that GnIHRs utilize the cAMP signal transduction pathway.

Transgenic *GnRH3:eGFP* embryos injected with a GnIH-targeting MO oligonucleotide had increased *gnrh3* expression at 24 hpf but no noteworthy changes in *gnrh2* expression. Transgene-based fluorescence at 48 hpf revealed a transient increase in *gnrh3* expression and advanced migration of GnRH3 neurons. Additionally, targeted and heritable GnIH mutations were obtained with GnIH-targeting TALENs administered to embryos. Founder fish carrying the mutation were outcrossed to produce F1 offspring, which are currently being raised to maturity to obtain $gnih^{-/-}$ fish.

Conclusion

A multi-level approach to studying and characterizing GnIH's role within the reproductive axis of the zebrafish has shown promising initial results to date. The addition of mature $gnih^{-/-}$ will enable future studies to elucidate the degree to which GnIH regulates neuroendocrine control of reproduction, as well as the mechanism(s) through which regulation takes place.

NEUROANATOMICAL AND GENETIC BASES FOR THE DOPAMINERGIC INHIBITION OF THE GONADOTROPE FUNCTION IN ZEBRAFISH

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Introduction

In many vertebrates, the stimulatory control by GnRH of the gonadotrope axis is opposed by an inhibitory control by dopamine (DA). This DA control has been found in various vertebrate species, but its morphological support has been studied in only three teleost species (goldfish, eel and trout). To get a deeper knowledge of the DA neurons involved in this control, we used another teleost, the zebrafish (*Danio rerio*), a popular vertebrate model, for which numerous genetic tools are available. We have recently shown that DA is able to inhibit its gonadotrope function and that the three zebrafish subtypes of DA D2 receptors are expressed by the gonadotrope cells and therefore likely involved in this control (Fontaine et al., 2013). In the present study, we localized the cell bodies of the hypophysiotropic DA neurons. We then characterized their tyrosine hydroxylase gene *(th)* expression, since in zebrafish two genes, *th1* and *th2*, code for TH protein, the key-enzyme in DA biosynthesis. We finally studied the ontogenetic development of these neuroendocrine DA neurons, as well as the genetic network at stake in their differentiation.

Methods

The DA neurons innervating the pituitary were identified by retrograde tracing experiments, in the adult brain. A microcrystal of DiI implanted in the pituitary was allowed to migrate and TH immunofluorescence (IF) was then performed. We then examined the expression of *th1* and *th2* genes in these neurons by double Fluorescent *In Situ* Hybridization (FISH) combined with a TH IF labelling. The development of these DA neurons was studied, firstly by examining the schedule of TH expression using TH IF, with the help of ZO-1 (ventricular epithelial marker) and DAPI (nuclear staining) which revealed the optic recess, an anatomical landmark. Secondly, we examined the expression patterns of several developmental genes (*foxg1*, *otpa/b*, *nurr1a/b*, *lhx5*, *dlx2a*, and *six3b*), by ISH combined with TH IF. For comparison, we studied in parallel another DA population, the suprachiasmatic DA (SCDA) neurons.

Results and Discussion

We determined that the DA cell bodies of the neurons projecting to the pituitary are localized in the most antero-ventral part of the preoptic area, within the anterior parvocellular nucleus (PPa). Two-color FISH revealed that all the PPa DA neurons express *th1*, and some of them co-express *th2*, with a proportion variable from one fish to another. We named this population the preoptico-hypophyseal DAergic neurons (POHDA).Thanks to early onset of the anatomical landmark, we could follow the development of this poorly studied population located just anterior to the optic recess. We observed that these neurons express TH relatively late (72hpf) as compared to other DA populations, and that their terminals reach the pituitary at around 84hpf. Gene expression analysis of POHDA and SCDA indicates that their developmental genetic networks are different. There are some genes (e.g. *six3b*) which are commonly expressed in both populations, and some are specific to POHDA (e.g. *foxg1*).

Conclusion

This is, to our knowledge, the first report characterizing the neurons that give rise to the neuroendocrine DA system, thus providing the morphological support for the inhibitory effect of DA on the gonadotropin secretion. It also provides some new insights about the development of POHDA neurons.

DEVELOPMENT OF APPROACHES TO INDUCE PUBERTY IN CULTURED FEMALE SABLEFISH

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Introduction

Efforts to establish sustainable and efficient aquaculture production of sablefish (*Anoplopoma fimbria*) have been constrained by delayed puberty in filial-1 generation (F1) females. This study integrates a series of basic and applied experiments aimed at gaining an understanding of the reproductive physiology and developing an approach for induction of puberty in sablefish, which would greatly facilitate selective breeding.

Methods

The reproductive endocrine axis (brain and pituitary dopamine D2 receptor (*drd2*), pituitary gonadotropin subunits (*fshb*, *lhb*, *cga*), ovarian gonadotropin receptors (*lhcgr*, *fshr*) and plasma sex steroids) of wild maturing female sablefish was compared to that of 7 year-old captive F1 females of a comparable body size. To determine whether puberty could be induced in F1 females, we tested the long-term *in vivo* effects (9 weeks) of implants containing dopamine receptor antagonist, metoclopramide (Met, 4 mg kg^{-1}), testosterone (T, 4 mg kg^{-1}) or gonadotropin-releasing hormone analog (GnRHa, 80 µg kg^{-1}) alone or in combination. In parallel, ovarian tissue incubation experiments tested the steroidogenic capability of primary follicles in response to gonadotropins, T, Met, and GnRHa using ultra-performance liquid chromatography with tandem mass spectrometry.

Results and Discussion

F1 female sablefish exhibited a less active reproductive endocrine axis and significantly elevated brain and pituitary *drd2* expression relative to wild maturing females. This suggested possible dopaminergic inhibition of reproductive function in F1 females. Using hormone implants in F1 females, we found that Met and T alone stimulated expression of pituitary *fshb* and *lhb*, respectively, whereas a combination of T and GnRHa had a strong synergistic effect on *lhb* expression (2000-fold higher than control). Although T induced a significant increase in follicle size, none of the treatments tested stimulated a transition into secondary oocyte growth. In vitro culture of follicles demonstrated that Met stimulates production of T and 11β-hydroxyandrostenedione, whereas gonadotropin preparations enhanced secretion of 17β-estradiol (E2), T and androstenedione. Testosterone was the most potent treatment in elevating production of E2, 11-ketotestosterone, androstenedione, 11β-hydroxyandrostenedione, 11β-hydroxytestosterone, and interestingly, 5α-dihydrotestosterone (DHT) production.

Conclusion

Our data suggest a possible dopamine inhibition of the reproductive axis in female sablefish. Treatments with Met and T elevated gonadotropin mRNAs in prepubertal, F1 females but failed to stimulate the transition into secondary oocyte growth, suggesting impairment in the pituitary gonadotropin synthesis/release. Primary ovarian follicles are equipped to synthesize steroids, including those required for the vitellogenic growth, and DHT, a sex steroid with a potential role during early ovarian growth.

THE ZEBRAFISH PINEAL GLAND, A CENTRAL CLOCK UNDER SPOTLIGHT

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Introduction

Seasonal reproduction is known to depend, in some cases, on the periodic melatonin signal originating from the pineal gland. While extensive research has greatly improved our understanding of the molecular mechanisms underlying night-time melatonin production, the molecular mechanism of the pineal gland clock, its entrainment by light and its links with downstream processes are not fully uncovered. Here, a systematic identification of rhythmic clock-controlled genes and light-induced genes in the zebrafish pineal gland was conducted using next generation transcriptome sequencing.

Methods

To identify rhythmic clock-controlled genes, pineal glands were collected from adult zebrafish kept under constant darkness, at 4-hr interval over two 24-hr cycles. To identify light-induced genes and miRNAs, pineal glands were also collected after 1-hr light pulse at six different circadian time points. RNA was extracted and subjected to RNA-Seq and miR-Seq. The data was subjected to stringent computational analysis. Specific genes and miRNAs were selected for functional analysis both *ex vivo* in zebrafish cell line and *in vivo* in the intact fish by gene knockdown and over expression manipulations.

Results and Discussion

In addition to a comprehensive view of the expression pattern of known clock components within this master clock tissue, this approach has revealed novel elements of the circadian timing system, which were functionally tested. These studies revealed new rhythmically expressed genes as players in the circadian clockwork, connecting the core molecular clock with downstream physiology of the pineal gland and linking the pineal master clock with the periphery. Likewise, newly identified light-induced genes were found to exert different effects on the clock. Both clock-controlled genes and light-induced genes exhibit a transient expression pattern, implicating mRNA stability as an important feature of the molecular clock. We show the involvement of specific miRNAs in this process.

Conclusion

The combination of cutting edge sequencing technology with stringent computational analysis and functional analysis in a genetically amenable model species, the zebrafish, enabled the detection of several new genes that affect the fish physiology by fine-tuning the circadian clock. In conclusion, we report a detailed functional characterization of the circadian clockwork in the zebrafish pineal gland and its entrainment by light. This knowledge will possibly lead to a better understanding of the timing of reproduction, as well as other time-dependent physiological processes.

Oral Session:

The Ovary

Chairs: Eva Andersson and Julien Bobe

MOLECULAR MECHANISM OF MATURATION-INDUCING HORMONE PRODUCTION DURING FINAL OOCYTE MATURATION IN THE JAPANESE EEL

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Introduction

17α,20β-dihydroxy-4-pregnen-3-one (DHP) has been identified as a maturation-inducing hormone of the Japanese eel, *Anguilla japonica*. In *A. japonica*, final oocyte maturation (FOM) and ovulation is routinely induced by injection of DHP as the eels do not produce DHP by standard methods using salmon pituitary extract (SPE). DHP production is regulated by production of its precursor, 17α-hydroxyprogesterone (17αOHP), and an enzyme which converts 17αOHP to DHP, termed 20β-hydroxysteroid dehydrogenase (20bHSD). Though carbonyl reductase-like 20bHSD (CR20bHSD) was isolated as an enzyme which converts 17αOHP to DHP in salmonid, eel CR20bHSD does not have 20bHSD activity. In a previous study, we demonstrated that a novel 20bHSD and not CR20bHSD is the 20bHSD responsible for DHP production during FOM in masu salmon. In this study, we isolated cDNAs encoding 20bHSD and cyp17a2 (17α-hydroxylase), and investigated changes in the expression of genes such as cyp17a1 (17α-hydroxylase/$C_{17–20}$ lyase), cyp17a2 and 20bHSD during FOM without DHP injection to understand the molecular mechanism of DHP production in *A. japonica*.

Methods

Eel 20bHSD and cyp17a2 cDNAs were isolated and their enzymatic activities were examined in HEK293T cells. Eels received weekly injections of SPE at 30 mg/kg body weight. After completion of vitellogenesis, the SPE dose was increased 10× to induce FOM and ovulation, instead of DHP. During vitellogenic growth and FOM up to ovulation, ovaries were biopsied and sera collected. Changes in cyp17a1, cyp17a2, and 20bHSD mRNA levels were measured by quantitative PCR and serum DHP levels were measured by time-resolved fluoroimmunoassay.

Results and Discussion

HEK293T cells transfected with eel 20bHSD or cyp17a2 efficiently converted exogenous 17αOHP to DHP or exogenous progesterone to 17αOHP and not androstenedione. mRNA expression of cyp17a1 increased rapidly after mid-vitellogenesis until late-vitellogenic stages. cyp17a2 levels increased gradually during vitellogenic growth. In contrast, 20bHSD mRNA levels were maintained at consistently high levels in ovaries at all stages. After high-dose SPE injection, cyp17a1 expression decreased to almost undetectable levels. However, levels of cyp17a2 and 20bHSD increased gradually as FOM proceeded until completion of ovulation. Serum DHP levels were significantly increased after FOM.

Conclusion

FOM and ovulation can be induced by injection of high-dose SPE instead of DHP in eels. This study demonstrated that eels can produce DHP and suggested that DHP production is controlled mainly by 17αOHP production, due to a rapid drop in cyp17a1 expression and not upregulation of cyp17a2 and 20bHSD mRNA expression.

ESTROGEN RECEPTOR β2 IS REQUIRED FOR GERM CELL MIGRATION AND OVARIAN DIFFERENTIATION IN GENETICALLY FEMALE MEDAKA

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Introduction

In vertebrates, estrogen receptors (ERα and ERβ) are essential for estrogen-induced gonadal development. Although it has been reported that ERβ is involved in early stages of gonadal sexual differentiation, there are no studies describing the exact roles of ERβ in the migration and maintenance of germ cells and gonadal sexual differentiation. Majority of teleosts have two *ERβ* isoforms, i.e. *ERβ1* and *ERβ2*. Previously, we reported that only *ERβ2*, not *ER*α or *ERβ1*, expression was markedly increased during sex differentiation of genetically female embryos. The present study aimed to determine the roles of *ERβ2* in the migration and maintenance of germ cells, expression of female- and male-associated genes and ovarian differentiation in medaka.

Methods

In this study, we generated *ERβ2*-knockdown transgenic medaka lines (*ERβ2*-KD) and used F4 generation of these transgenics to determine the roles of *ERβ2* in different stages of gonadal development. PGC migration rescue experiments were conducted using utr (untranslated regions) tagged and reporter fused mRNA over expression. *Dmy* and *rspo1* knockdown transgenics were used to assess the meiotic division patterns. We also used *cyp19a2-gfp* transgenics for the study of steroidogenic activity.

Results and Discussion

Analysis of *ER*β2-KD XX larvae revealed that *ERβ2* knockdown up-regulated the expression of male associated genes (*gsdf1, sox9a2,* etc) and down-regulated the expression of female associated genes (*spo11, foxl2, cyp19a1*). This shift in the gene expression pattern resulted in a complete male-to-female sex-reversal, producing fertile sperm. We also found that the mitotic and meiotic activities of *ERβ2*-KD XX larvae at 0 day after hatching were identical to those of the normal XY larvae. Whole mount *in situ* hybridization of *ERβ2*-KD XX embryos showed abnormal germ cell migration and further loss of germ cells. In these embryos, expression of stromal cell-derived factor 1 (*sdf1a*) and its G-protein-coupled receptor (*cxcr4b*), known to control cell migration, was markedly reduced. Co-injection of *sdf1a* and *cxcr4b* into fertilized eggs of these F4 fish re-established the ovary development pathway. These findings indicate that medaka germ cell migration and maintenance requires the ERβ2 responsive SDF1/CXCR4 interaction. We also will discuss the effect of *ERβ2* knockdown on the *Rspo1* pathway.

Conclusion

These findings indicate that *ERβ2* is required for normal ovarian differentiation in medaka. We also demonstrated that ERβ2 responsive SDF1-CXCR4 interaction is crucial for migration and maintenance of germ cells. ERβ2-knockdown transgenic medaka lines will be useful for the study of germ cell migration and early stages of ovarian differentiation.

MIS RECEPTOR VARIATIONS AND EXPRESSION IN THE REPRODUCTIVE CYCLE OF *LABEO ROHITA* WITH A SPECIA REFERENCE TO *Cyp19* GENE

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Introduction

Membrane progestin receptors (mPRs) are involved in oocyte maturation in teleost. 17α,20β-dihydroxy-4-pregnen-3-one (17α,20β-DHP) has been identified as maturation inducing steroid (MIS) in many teleosts. It induces the oocyte to enter into final meiotic maturation and leads to ovulation. The MIS receptors, mPRs are responsible to mediate rapid non-genomic progestin action. The lack of comprehensive information on the identifying the molecular structure of membrane steroid receptors in Indian major carps is a block to know the critical molecular aspects of steroid actions. Present study reveals the types of mPRs at different seasons in both sexes of an Indian major carp, *Labeo rohita* along with cyp19 expression.

Methods

Sigma RNAlater preserved ovary, testis, brain, liver, kidneys, gills and muscle of *L. rohita* were processed to isolate the total RNA and reverse transcribed to cDNA using the RT-PCR method. Gene specific primers were constructed for mPRα, mPRβ, mPRγ and Cyp19. Synthesized cDNA subjected to PCR amplification along with specific primers in suitable thermal cyclic programme. Gene specific products were sequenced for further analysis.

Results and Discussion

Present results showed that the presence of diverse expression of mPRα, mPRβ and mPRγ in the different tissues of *L. rohita* and its mRNA expression levels were higher in the gonadal tissues compared to other tissues. The mPRγ was not found in the gravid ovary whereas the mPRα identified in all the stages of reproduction. mPRβ expressed very high level in the late vitellogenic period documents the mPRβ playing an important role in the meiotic maturation. This study compares with another Indian carp, *Cirrhinus mrigala*. Cyp19 mRNA was highly expressed in the female brain and ovarian tissues compared to male brain, testis, gills, liver, kidney and muscle of the *L. rohita*. The sequence comparison analyses with other species of fishes have shown the high similarity with *Danio rerio* and *Carassius auratus*.

Conclusion

The study suggests that the expression of mPRs is tissue specific and cyp19 are sex specific along with reproductive season. Further comparative gene analysis proved that cyprinidae has common conserved regions in compare with other teleosts.

A COMPARISON BETWEEN EGG TRANSCRIPTOMES OF COD AND SALMON-REVEALS SPECIES SPECIFIC TRAITS IN EGGS FOR EACH SPECIES

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Introduction

Fish in use in aquaculture display large variation in gamete biology. This study tries to find if "egg life history traits" can be mirrored or hidden in the egg transcriptomes. To pursue this, salmon and cod eggs were selected due to their largely differencing phenotypes (size, robustness for handling, fresh/marine environment) with the main aim to find conserved species specific patterns in the transcriptome.

Methods

An Agilent oligo microarray was performed on ovulated eggs from eight and seven pools of eggs obtained from cod (~23 000 genes) and salmon (~44 000 genes) respectively. The arrays were normalized to a similar spectrum for both arrays. Both arrays were re-annotated based on official gene symbol to retrieve an orthologous KEGG annotation (**http://www.genome.jp/kegg/**). The KEGG annotation was obtained to link species unique genes to pathways.

Results and Discusson

In the whole transcriptome analysis the KEGG annotation in salmon and cod represented 14009 and 7437 genes respectively. As the salmon genome has undergone a recent whole-genome duplication (WGD) event, this species displays many more duplicated copies of the same ancestral gene compared to cod. This represented a major problem in the data set, as one KEGG annotation could have 10 probes associated to it in salmon, while the same gene symbol had only 2 probes associated with this symbol in cod. In addition, due to the incompleteness of the genome data of both species, the same gene could also have several probes associated with it. To solve this problem, the probe linked to the highest gene expression for that particular KEGG annotation was used to compare the expression profile between species. Differential expression was calculated for genes that had an annotation with score > 300, resulting in a total of 2974 KEGG annotations (genes) being differently expressed between the species. The most differentially expressed genes in salmon and cod ($FD \geq 2$), were used to reveal pathways that were overrepresented in the eggs of each species. This analysis revealed that different immune related pathways were upregulated in each species. Moreover, calcium balance and signal transduction pathways were upregulated in salmon compared to cod. The most differentially expressed pathways were related to transcription and metabolism in cod, which may be related to the energy requirement to fulfill the high requirement for the coming fast numerous cell divisions as also indicated by the cod egg transcriptome containing around 50% mitochondrial transcripts.

Conclusion

Comparative transcriptomics between cod and salmon eggs reveals upregulated pathways associated immune system, calcium balance and signal transduction in salmon, and transcription and metabolism in cod.

MASS PRODUCTION OF JAPANESE EEL RECOMBINANT FOLLICLE-STIMULATING HORMONE AND LUTEINIZING HORMONE BY A STABLE EXPRESSION SYSTEM: THEY FULLY INDUCED OVARIAN DEVELOPMENT AT A DIFFERENTIAL MODE

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Introduction

Gonadotropins (GTH), follicle-stimulating hormone (FSH) and luteinizing hormone (LH), play central roles on gametogenesis through vertebrate. However available information on their differential actions, especially *in vivo*, is far insufficient in fish. In this study, we have established cell lines stably expressing long-lasting recombinant FSH (reFSH) and LH (reLH) of Japanese eel and the reFSH and the reLH were produced in abundant. Furthermore, ovarian development induced by administration of reFSH and reLH was thoroughly investigated.

Methods

Expression vectors encoding single-chain reFSH and reLH, composed of the β subunit, a C-terminal peptide of human chorionic gonadotropin, a GS spacer, the α subunit and a 6xHis-tag, were constructed. Chinese hamster ovarian cells were transfected with the vectors and cells constantly expressing the reFSH and the reLH were selected and expanded. Recombinant GTHs (reGTHs) were purified from the culture media by an immobilized metal affinity chromatography. Immature female eels received weekly i.p. injections of reGTHs (500μg/kg-BW) or PBS. Eels were periodically killed, the ovaries were removed and the gonad-somatic index (GSI) was calculated. The diameters of 100 ovarian follicles were randomly measured and the frequency distribution was examined. The developmental stages were determined by the existence of yolk globules, the size of most advanced group of follicles. Ovarian transcript abundance of genes related to reproduction was measured by quantitative real-time RT-PCRs.

Results and Discussion

The yield of reGTHs ranged from 10 to 20mg/L-medium and sufficient amount (approximately 500mg) of reFSH and reLH were prepared. All the pretreated and saline-injected eels were at the pre-vitellogenic stage. reFSH induced the entire ovarian development by 8 weeks from the beginning of injections, thus most fishes were at the migratory nucleus stage while eels at the same stages were observed after only 4 weeks in reLH group. GSI in reFSH group was significantly higher (~1.5 times) than that in reLH group at migratory nucleus stage, due to significant higher frequency of the advanced follicles. All the genes except for HSD3β were induced by both reGTH with a peak either at the mid- or late vitellogenic stages. Transcript abundance of CYP19A and FSH receptor in reLH group was significantly higher than those in reFSH group although no difference in expression of other genes was seen between the groups.

Conclusion

FSH and LH regulated ovarian development and the expression of genes related to reproduction with distinct differences in the Japanese eel.

SEASONAL DYNAMICS OF RIBOSOMAL RNA COMPONENTS PRODUCTION AND ACCUMULATION DURING OOGENESIS IN FISH; CAUTIOUSLY INVESTING IN REPRODUCTION

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Introduction

Oogenesis in fish is characterized by a massive growth of the oocytes that will be spawned each season. Production and stockpiling of certain molecules is essential to ensure proper development of the future embryo. Ribosomal RNAs are accumulated in the oocyte during oogenesis to assist the quick assembly of ribosomes for new protein synthesis into the newly formed embryo. The massive expression of 5S rRNA in oocytes, above that in any other cell type, allows using 5S as a molecular marker of sex in fish. This production of 5S rRNA is allowed by transcription factor IIIA (TFIIIA) that regulates RNA polymerase III (Pol III) activity. Instead, production of the other rRNAs requires Pol I that is regulated by the RNA polymerase I upstream binding transcription factor (UBTF). Here we wanted to study rRNA production dynamics during oocyte development in female fish.

Methods

Thicklip grey mullets *Chelon labrosus* were sampled in Pasaia (43º19'18"N 1º55'53"W), a polluted harbour with high prevalence of intersex males, during 1 year. All mullets were histologically sexed and gonad RNA extracted. 5S and 18S rRNA concentrations were quantified by capillary electrophoresis to obtain a 5S/18S rRNA index. cDNA was produced, concentrations quantified by OliGreen stain and TFIIIA and UBTF (sequences obtained by 454 pyrosequencing of mullet transcriptome) mRNA levels were quantified by qPCR in previtellogenic/vitellogenic females.

Results & Discussion

5S rRNA is highly expressed in oocytes and identifies females and intersex males exposed to xenoestrogens. 5S rRNA massive expression occurs during the first stages of oogenesis while 18S/28S rRNA production begins with the onset of vitellogenesis. 5S/18S rRNA index also allows identification of sex, the lowest 5S/18S rRNA values, closest to those in testes, being recorded in ovaries with mature oocytes. Highest index values appear in ovaries with previtellogenic oocytes. In agreement, TFIIIA expression is highest during previtellogenesis decreasing during vitellogenesis, while UBTF shows an opposite pattern of expression with highest transcription levels once vitellogenesis initiates.

Conclusion

5S/18S rRNA index allows quantitative identification of sex and ovarian developmental stage in mullets. TFIIIA and UBTF mRNA levels demonstrate sequential activation of Pol III and I during oocyte development which indicates that 5S rRNA is accumulated from the beginning of oogenesis while 18S rRNA, whose production is more energetically demanding, only begins to accumulate during vitellogenesis, once successful reproduction can be envisaged.

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ISOLATION, *IN VITRO* CULTURE AND NUCLEIC ACID TRANSFECTION OF RAINBOW TROUT GRANULOSA CELLS

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Introduction

A large number of *in vitro* studies have been used whole ovarian follicles to study fish ovarian functions and regulations but very few works aimed to use isolated granulosa cell (GC). However, such methods may be of great interest to specifically analyze GC biology. In the present work, we isolated rainbow trout GC and analyzed their steroidogenic capacities after several days of *in vitro* culture. Furthermore, various methods for plasmid and small nucleic acid transfection have been assessed.

Methods

Vitellogenic and pre-ovulatory ovaries were dissected to isolate ovarian follicles in a complete trout culture medium (CTCM, pH= 7.7, Po=280 mosmol/L). Follicles were digested with collagenase H, then oocytes were removed and follicular layers were transferred in Hank's medium without Ca^{++} to facilitate cells dissociation but with bovine serum albumin (BSA, 1%) to stop collagenase action. GC were kept overnight at 12°C in CTCM with BSA then transferred in CTCM with 5-10% steroid free Ultroser or calf serum, and distributed in 24-well gelatinised plastic plates. The use of Percoll^{TM} for cell separation was also tested. After 24h to several days of culture, 17,20β-dihydroxy-4-pregnen-3-one, testosterone and estradiol levels were measured in medium by RIA. For transfection, a fluorescent morpholino-RNA or a pCMV-GFP was added to GC culture along with various chemical transfectants or electroporated (pipet type electroporator MP100).

Results and Discussion

On average, 5-10 10^6 GC could be obtained from 3000 trout ovarian follicles depending on the ovarian development stage. GC could be maintained *in vitro* during at least three weeks. After 2-3 days of culture GC actively agglomerated to form spherical clusters especially when non-gelatinized plates were used. A high number of red cells could also be collected with GC but they degenerated after 3 days of culture. When necessary they could efficiently be eliminated by a Percoll^{TM} filtration. GC collected during vitellogenesis or just before ovulation showed steroidogenic activities *in vitro*. Furthermore, GC response to luteinizing hormone was maintained. However, steroid production in the only TCTM decreased with time even when precursors were added. Transfection was more efficient using electroporation than chemical transfectants but electroporation by itself was deleterious with a decrease of steroidogenic capacity.

Conclusion

Granulosa cells from vitellogenic and preovulatory rainbow trout ovary can be isolated and cultured *in vitro*. They show steroidogenic capacities in both cases. Electroporation is an efficient method for short acid nucleic and plasmide transfection. However, the cell biology disturbance induced by the microporation itself should be taken into account for data interpretation when looking for the effects of under- or over-expression of candidate genes.

VERY LOW-DENSITY LIPOPROTEIN IS PRIMARY CARRIER OF NEUTRAL LIPIDS TO OOPLASM LIPID DROPLETS IN TELEOSTS

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Introduction

In many teleosts, neutral lipids (NLs) accumulate in ooplasm lipid droplets during oocyte growth, for later use as an energy resource by developing offspring. Little is known about origin of the NLs or mechanisms underlying their accumulation. Triacylglyceride-rich lipoproteins, such as very low-density lipoprotein (Vldl), are known to be primary carriers of NLs to the cell. We labeled Vldl and other plasma lipoproteins in their lipid moiety with fluorescent fatty acids, or in their protein moiety with fluorescent dye, and traced their uptake, distribution and fate in ovarian follicles of several species of teleosts both *in vivo* and *in vitro*.

Methods

Three classes of lipoproteins, Vldl, low-density lipoprotein (Ldl) and high-density lipoprotein (Hdl), were isolated from plasma of cutthroat trout, *Oncorhynchus clarkii*, by sequential ultracentrifugation, labeled with fluorescent fatty acid analogue, and added to cultures of trout ovarian follicles. The Vldl was also dually labeled in its lipid and protein moieties with fluorescent fatty acids and fluorescent dye, respectively, and then cultured with ovarian follicles isolated from trout and from black skipjack tuna, *Euthynnus lineatus*. Double-labeled Vldl was also injected to female medaka, *Oryzias latipes*. Selective, time-dependent uptake into oocytes of labeled lipoprotein products was analyzed by histological observation and quantification of incorporated fluorescence.

Results and Discussion

Among the three classes of lipoproteins, Vldl showed the significantly fastest and highest level of uptake of fluorescent fatty acids into trout follicles, with numerous intensely fluorescent lipid droplets evident in the ooplasm. The Hdl-treated follicles showed fewer intensely labeled lipid droplets, and Ldl treatment resulted in just a faint fluorescent signal. These findings indicate that Vldl is a major contributor to NLs in ooplasm lipid droplets. Treatments of ovarian follicles with double-labeled Vldl *in vivo* (medaka) and *in vitro* (trout and tuna) gave almost identical results. Faint to moderate fluorescence was evident across the entire follicular area, with intense fluorescent signals appearing only in ooplasm lipid droplets. Labeled Vldl protein moieties were restricted from the ooplasm, appearing only in the follicle layers and vitelline envelope. These results indicate that Vldl is processed into apoproteins excluded from oocyte entry, and into free fatty acids that are incorporated into ooplasm NL droplets.

Conclusion

This study provides, for the first time, direct evidence that Vldl is a primary career of NLs to the ooplasm lipid droplets. This discovery sets the stage for elucidation of molecular mechanisms by which NLs accumulate in teleost oocytes.

HOW DO EGGS GET FAT? INSIGHTS INTO TRIACYLGLYCERIDE UPTAKE IN THE OOCYTES OF THE SHORTFINNED EEL, *ANGUILLA AUSTRALIS*

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Introduction

Lipids play an essential role in reproduction, especially in oviparous species such as some fish, in which the developing embryo does not receive nutrients from the mother after fertilisation. Triacylglycerides (tags), the main component of the oil droplets in the oocytes of multiple fish species, can be taken up via a number of different pathways; thus, lipids are likely to make their way into the oocyte either by receptor-mediated endocytosis or as free fatty acids after hydrolysis by lipoprotein lipase (lpl) via free fatty acid receptors, scavenger receptors or fatty acid transport proteins (fatps). Following uptake, free fatty acids are re-esterified into tags to end up as oil droplets in the ooplasm. To date, it is unknown which of these pathways is the main one in fish, but the need to incorporate large amounts of tags into the gamete suggests the involvement of receptor-mediated endocytosis. Previous research has shown that 11-ketotestosterone (11-KT) can induce ovarian tag uptake both *in vivo* and *in vitro*, and that *in vitro* co-incubation of 11-KT and very low density lipoprotein (vldl) leads to even more enhanced uptake. This study aimed to determine the relative importance of receptor-mediated endocytosis versus LPL-mediated hydrolysis during 11-KT induced tag uptake in the oocytes of the shortfinned eel, *Anguilla australis*.

Methods

In vitro incubations of ovarian tissue using 11-KT and vldl were carried out together with specific antibodies intended to block the vitellogenin receptor (vtgr) and/or the low density lipoprotein receptor (ldlr). After 21 days, histological analysis of resin embedded ovarian tissue was used to identify the amount of oil accumulated within the cytoplasm of the oocytes.

Results and Discussion

Incubation with 11-KT and vldl significantly increased both oocyte surface area and oil droplet accumulation, confirming the results of previous studies. Interestingly, blocking the ldlr appeared to reduce the oocyte surface area and the amount of oil present within the oocyte. In contrast, blocking the vtgr seemed to have little effect on either oocyte surface area or the abundance of oil droplets present.

Conclusion

We conclude that endocytosis by the ldlr is a major, if not the main, pathway driving ovarian tag uptake in the eel. This is in keeping with findings on chicken, but contrasts the strategy used in mammals in which hydrolysis is the main pathway.

MULTIPLE VITELLOGENIN YOLK PRECURSORS IN EUROPEAN SEA BASS (*DICENTRARCHUS LABRAX)*

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Introduction

We characterized three deduced sea bass vitellogenin (Vtg) polypeptides with regard to linear and 3-dimensional (3-D) structural and functional features, verified the presence and relative contribution to the yolk of each form of Vtg, and assessed maturational degradation and potential contribution of each product lipovitellin (Lv) to the free amino acid (FAA) pool driving oocyte hydration and supporting early embryonic nutrition.

Methods

Three complete *vtg* cDNAs were assembled from contiguous partial sequences obtained by RT-PCR. Deduced Vtg proteins were classified using BLAST, revealing homologies across a broad array of taxa. ClustalW alignments of Vtg sequences revealed conservation of cysteine (C), proline (P) and glycine (G) residues by Vtg-type. Residue positions were localized in the 3-D structure of a lamprey Lv template using Cn3D and mapped to phylogenetically-conserved functional surfaces using EvoTrace. Relative concentrations of each Vtg or their yolk protein (YP) products in postvitellogenic female liver, plasma and ovary were measured by nanoLC-MS/MS as ProteoIQ-normalized spectral counts. Maturational degradation of each type of Lv was detected by Western blotting using antisera raised against purified grey mullet (*Mugil cephalus*) Lvs.

Results and Discussion

Homology analyses definitively identified sea bass VtgAa, VtgAb and VtgC. The VtgC lacks two C-terminal domains ($\mathcal{B}$'-component, $\mathcal{B}$'c; C-terminal component, Ct) and its N-sheet, which bears the functional surface of the classical Vtg receptor (Vtgr), has undergone extensive substitutions at C, P and G residues likely to result in massive alteration of its structure and Vtgr-binding properties. VtgAb tryptic peptide spectra were generally several fold more abundant than for the other Vtgs, and VtgC spectra were very limited, except in ovary. Comparison of contiguous tryptic peptides detected in plasma versus ovary indicate that most Ct is degraded just after VtgA uptake by oocytes, whereas Lv degradation is minor and $\mathcal{B}$'cs are not degraded. Western blotting revealed limited degradation of all three forms of Lv during oocyte maturation, unlike the case in other marine pelagic spawners. Virtually identical Vtgs and patterns of YP degradation during oocyte growth and maturation in sea bass and *Moronidae* that spawn in freshwater (striped bass, pelagic; white perch, demersal) indicate that Vtg system structure and function cannot be inferred solely from reproductive life history.

Conclusions

The multiple Vtg system of D. labrax was characterized in detail using some novel technical approaches, setting the stage for studies of Vtg involvement in determining egg quality in this premier aquaculture species.

MATERNALLY-INHERITED NPM2 mRNA IS CRUCIAL FOR EARLY DEVELOPMENTAL SUCCESS IN ZEBRAFISH

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Introduction

The molecular mechanisms controlling the ability of the egg to successfully support early embryonic development remain poorly understood. Previous studies have highlighted the link between egg developmental potential and egg transcriptome. Among candidate genes analyzed in fish, nucleoplasmin 2 (*npm2)* is of specific interest. This gene was shown to be a maternal-effect gene in mouse, and *Npm2* KO led to major developmental failures in this species. The aim of the present work was to characterize the maternal contribution of the *npm2* mRNA to embryonic developmental success in zebrafish *Danio rerio*.

Methods

npm2 expression profiles were analyzed by QPCR analysis during oogenesis and early embryonic development. Morpholino-oligonucleotide (MO) injection was performed in 1-cell zebrafish embryos. An ATG-MO directed against the translation initiation site was used in order to knock down Npm2 protein expression. A Mismatch MO was used as negative control. Effective Npm2 protein knock-down was checked by Western Blot analysis. In order to validate phenotypic observations, a rescue experiment was performed by co-injecting the ATG-MO with a mutated flagged RNA, not recognized by the ATG-MO, but coding for a recombinant Npm2 protein.

Results and Discussion

We show that *npm2* mRNA is mainly expressed in the ovary, and more specifically within the oocytes. While *npm2* mRNA decreased throughout oogenesis, Npm2 protein was detected at high levels at all stages of oocyte development. During early embryogenesis, we observed a rapid and dramatic decrease of *npm2* mRNA levels after fertilization. In contrast, the decrease in Npm2 protein was not observed until 24hpf. A knock-down of Npm2 protein resulted in major and early embryonic development failure as knocked-down embryos did not progress beyond mid-blastula transition (*i.e.* embryonic development stopped around 4 hours post-fertilization (hpf) and embryonic survival rate at 24hpf was null).

A Western blot analysis confirmed that, following ATG-MO injection, Npm2 protein levels were significantly reduced ($p=0.023$) and that development beyond mid-blastula transition was rescued by the translation of the recombinant Npm2 protein. Our results demonstrate, for the first time in any teleost species, that maternally-inherited *npm2* mRNA is required for early embryonic success, and more specifically for progress beyond mid-blastula transition.

Conclusion

Here we show that maternally-inherited *npm2* mRNA is crucial for early developmental success in zebrafish and therefore contributes to egg developmental potential (*i.e.* egg ability to support early embryo development, once fertilized). Together with the previously established link between *npm2* mRNA levels in the rainbow trout egg and embryonic success, the present results suggest an important role of *npm2* in the molecular mechanisms controlling egg quality in teleost fish.

Oral Session:

Reproduction and Environment

Chairs: Silvia Zanuy and Hiroshi Ueda

STUDY OF REPRODUCTIVE IMPAIRMENT BY ENVIRONMENTAL CONTAMINANTS USING GENOMIC AND METABOLOMICS APPROACH

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Introduction

Environmental contaminants are known to exert adverse health effects in fish and other vertebrates. Many of these contaminants of emerging concern have hormone-like activities and can disrupt normal reproduction by affecting components of brain-pituitary and gonadal axis. Previous field studies demonstrated the presence of a number of pollutants in the Oldman River, Canada with hormone-like activity causing significant female bias (85-90%). Significant changes were observed in fish caught down stream of certain municipalities along the Oldman River correlating with high levels of vitellogenin expression in male fish. These observations suggested severe endocrine disruption of gonadal development likely due to presence of compounds with estrogen-like activity.

Methods

To investigate the effects of chemicals, we performed controlled laboratory experiments in which fish in aquaria were exposed to the same concentrations of a selected number of chemicals detected in the river system, individually and as mixtures. The main focus of the present study was to investigate the mechanisms by which these compounds disrupt reproduction, using transcriptomics and metabolomics approach. We studied the expression of various genes involved in gonadal development and differentiation as well as performing microarray and 1H-NMR metabolomics.

Results and Discussion

Concentrations of multiple metabolites were measured in the liver, gonad and brain tissue to evaluated disregulation of pathways involved in reproduction. The results suggest significant changes in the levels of amino acid, lipid, energy, carbohydrate, nucleotide and cofactor/vitamin metabolism. The effect of mixture of contaminants was found to be significantly different from the individual treatments. In the present study, we also exposed fathead minnows to environmental contaminants individually and in mixture and performed microarray analysis, using well-characterized 15K gene array. The "Omics" data identified new cellular response and biological endpoints, and provide information on mechanism-based cell and tissue response affecting energy cycle and reproduction. The observed information on metabolic shift is particularly important because of significant energy investment at the time of reproduction in fish. The results demonstrate that contaminants exert significantly different effects as a mixture, compared to individual compounds in the liver, ovary, testis and brain.

Conclusion

Together with our previous field data, the present results provide a framework for better understanding of ecological consequences of exposure to contaminants, and resulting reproductive abnormalities seen in fish and other vertebrates.

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THE IMPACT OF ENVIRONMENTAL FACTORS ON THE GONADOTROPIC SYSTEM IN ATLANTIC SALMON (*SALMO SALAR*)

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Atlantic salmon experience seasonal changes in temperature and photoperiod in fresh- and seawater and display an impressive lifecycle plasticity. Photoperiod is the key environmental factor adjusting the seasonal timing of initiation of puberty and spawning in Atlantic salmon, while water temperature can modulate this by allowing or inhibiting ovulation/spermiation, possibly also affecting the period of rapid gonadal growth prior to spawning. Puberty is characterized by activation of the brain–pituitary–gonad axis. Environmental cues are integrated in the brain with other signals relevant for reproduction, leading to a modulation of the neuroendocrine input to the pituitary gonadotroph cells that changes the release of gonadotropins, and in turn gonadal hormone production and germ cell development.
Atlantic salmon of both sexes were exposed to different photoperiod, temperature and/or salinity condition during the start of puberty, during rapid gonadal growth and also during spawning. Blood and tissue samples were analysed for mRNA levels of pituitary gonadotropin subunits (*fshb* and *lhb*) and gonadotropin releasing receptor 4 (*gnrhr4),* gonadal histology and gonadotropin receptor (*fshr* and *lhcgr*) expression, and changes in plasma sex steroid levels.
The first sign of pubertal initiation is an up-regulation of pituitary *fshb* mRNA levels, followed by increase in plasma sex steroid levels and a gradual increase in pituitary *lhb*. The *fshr* is highly and relatively constantly expressed over the period of puberty entry, rapid gonadal growth and spawning in both sexes with some fluctuations around spawning, while *lhcgr* shows a gradual increase during the same period, with a peak around spawning. However, the seasonal timing of these events varies with age at puberty and environmental conditions. Combined photothermal regimes had strong effects on pituitary *fshb* and *lhb* during both gametogenesis and spawning. Interestingly, both salinity and photoperiod were found to modulate pituitary mRNA levels of *gnrhr4*, *fshb*, and *lhb* during the initial phases of pubertal initiation in male salmon post-smolts.
Our observations support the notion that Fsh initiates and sustains pubertal development until spawning. Furthermore, in our studies of induced puberty in post-smolt males, salinity was for the first time identified as triggering recruitment into puberty, while photoperiod had a stronger effect on the final stages of maturation.

RECENT FINDINGS ILLUSTRATING ANTI-ANDROGENIC ACTIVITY OF VINCLOZOLIN THAT CAUSE IMPAIRMENT IN MALE FERTILITY IN GOLDFISH

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Introduction

It has been demonstrated that Vinclozolin (VZ), a pesticide widely used in agriculture for control of disease in fruits and vegetables, possess the ability to interfere with androgen functions via binding to androgen receptor (AR) which cause male infertility in mammals. In fish, VZ impacts on reproduction and its mode of action are largely unknown. In the present study, VZ effects on male fertility were studied by evaluating sperm quality. Sex steroids and their upstream neuroendocrine regulators were measured. To understand VZ mode of action, expression of neuroendocrine peptides/hormones and their receptors, sex steroid receptors and genes encoding enzymes in steroidogenesis were screened.

Methods

In experiment 1, sperm motility were evaluated in the mature goldfish (*Carassius auratus*) exposed to 100, 400, and 800 $\mu g.L^{-1}$ VZ and an estrogen (5 $\mu g.L^{-1}$, 17β-estradiol (E_2)) for 30 days. Alternations in luteinizing hormone (LH), E_2 and 11-ketotestosterone (11-KT) and cholesterol were investigated in blood plasma. Expression of sGnRH, LH receptor (LHr), Vitellogenin (VTG), and AR, and genes encoding enzymes in steroidogenesis (StAR, CYP11a, 3β-HSD, CYP17) were evaluated using a qRT-PCR in the brain, testis or liver. In experiment 2, mature goldfish were exposed to VZ (30 $\mu g.L^{-1}$), an anti-androgen (flutamide (Flu) 300 $\mu g.L^{-1}$), and an androgen (testosterone (T) 1 $\mu g.L^{-1}$) for 10 days. Expression of Kiss1-b, GPR54, sGnRH, LHr and AR, and StAR were evaluated using a qRT-PCR in the brain or testis.

Results and Discussion

In experiment 1, sperm motility was decreased in goldfish exposed to VZ and E_2. LH, 11-KT and cholesterol were increased in goldfish exposed to VZ. These were associated with upregulation of sGnRH mRNA in the brain and LHr, StAR, CYP11a, 3β-HSD and CYP17 mRNA in the testis. AR was unchanged in goldfish exposed to VZ. In goldfish exposed to E_2, LH and 11-KT were decreased, while E_2 and VTG mRNA were increased. These results suggests that VZ does not exhibit estrogenic activity, but alters steroid biosynthesis via disruption in hypothalamus-pituitary-testis functions. In experiment 2, expressions of Kiss1-b, GPR54 and sGnRH mRNA in the brain and expression of LHr and StAR in the testis of goldfish exposed to VZ and Flu were increased, while they remained unchanged in goldfish exposed to T. In all groups, AR was unchanged. This suggests that neuroendocrine and endocrine dysfunctions are very similar in goldfish exposed to VZ and Flu and differ from those exposed to T.

Conclusion

This study suggests an anti-androgen activity for VZ which cause male infertility in fish through disruption in neuroendocrine and endocrine functions involved in testicular steroidogenesis.

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IS THE CONTRACEPTIVE PILL AS OESTROGENIC TO FISH AS WE THINK?

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Introduction

Assessing the risks posed by complex mixtures of environmental contaminants is a major challenge for aquatic toxicology. As *in vivo* testing of all possible mixtures is clearly impractical, computational modelling will be required in future. Chemicals integrate their effects within the underlying molecular networks governing organism physiology, resulting in complex and often unpredictable outcomes. Systems toxicology offers a powerful approach to develop the evidence base to understand chemical mixture interactions and identify their mechanisms.

Methods

Three-spined stickleback (*Gasterosteus aculeatus*) were exposed to ten individual priority and emerging pollutants (B(a)P, CdCl2, dibutyl-phthalate, ethinyl-oestradiol, fluoxetine, gemfibrozil, ibuprofen, levonorgestrel, PCB-118 and triclosan) in flow-through systems for 4 days. In addition, sticklebacks were exposed to twenty-six different mixtures of these compounds. Hepatic transcriptomics and metabolomics were employed to characterize the molecular responses of the fish to these acute sub-toxic exposures

Results and Discussion

We found that although many molecular alterations can be explained by additive response models, some genes were activated in chemical mixtures alone whilst others were silenced by the presence of certain chemicals in the mixture. The latter was particularly evident when two specific chemicals, namely ethinyl-oestradiol (EE2) and levonorgestrel (LV), a widely used synthetic progestin, were present in the mixtures. LV antagonised the action of EE2 in terms of key genes (vitellogenin, choriogenin) that lead to oestrogenisation. Ironically EE2 and a progestin are always used together as the active ingredients of the contraceptive pill, suggesting that the overall oestrogenising potential of the pill may be overestimated when fish are exposed to EE2 alone. Preliminary analysis suggests a mechanism for this antagonistic behaviour between the two chemicals; LV appears to down regulate transcription of oestrogen receptor (ERα), potentially reducing or preventing the ER-dependent effects of its ligand, oestrogen (EE2). To our knowledge this is the first report of this antagonism between two key components of the contraceptive pill. Chronic exposures are underway to determine whether these acute molecular responses are maintained over a longer time period and to assess the physiological effects. These will be completed in April 2014.

Conclusion

The synthetic progestin levonorgestrel appears to be very effective at antagonising the oestrogenic potential of EE2, questioning the assumption that the contraceptive pill is the main inducer of the widespread oestrogenisation of wild fish observed in many aquatic environments.

METHYLATION LEVELS OF KEY GENES IN EUROPEAN SEA BASS (*DICENTRARCHUS LABRAX)* EXPOSED TO DIFFERENT THERMAL CONDITIONS

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Introduction

An organism integrates the environmental information received during early development through epigenetic mechanisms, such as DNA methylation. This process may confer alterations in gene expression or changes in the ability to respond to environmental factors when experienced later in life. Temperature is such an environmental factor related to changes in DNA methylation levels. Previous results from our group showed higher levels of DNA methylation of the aromatase promoter in animals subjected to higher temperature during the thermosensitive period. Here, we aimed to survey the DNA methylation patterns of several key genes in fish subjected to different thermal conditions using next generation sequencing (NGS) technology.

Methods

Adult 3-year-old sea bass raised at natural (17ºC) or at high (21ºC) temperature when larvae were used for this study. Four tissues from these two groups were initially considered in this experiment: liver, spleen, testis and muscle. DNA was extracted and quality checks were performed. DNA methylation levels were measured using Reduced Representation Bisulfite Sequencing (RRBS). Briefly, libraries were constructed using a modified RRBS protocol: *Msp*I digestion, end repair, A-tailing, adapter ligation and size selection were performed in individual samples, followed by pooling and bisulfite conversion. The quality of the libraries was checked by Qubit measurements and the Experion automated electrophoresis system prior to sequencing performed by Illumina HiSeq2000. All bioinformatic analysis from raw sequencing data to methylation profiling are performed using R (**http://www.r-project.org/**) and RRBS-dedicated packages, specifically BSMAP and RnBeads, modified for the sea bass genome.

Results and Discussion

The analysis of DNA methylation levels is focused on a subset of the temperature x tissue combinations and concerns key genes related to the reproductive physiology of the fish including sex differentiation and gonad maturation (e.g., forkhead box L2, androgen receptor, doublesex and mab-3 related transcription factor I, HMG-box transcription factor SOX17). For comparison, genes related to other important functions such as muscle growth, liver metabolism, immune response, etc. are also surveyed (e.g., insulin-like growth factor I, growth hormone, tumor necrosis factor-alpha, interleukin 1-beta).

Conclusion

Fish from groups submitted to natural or high temperature are compared in terms of DNA methylation levels of specific gene promoters and gene bodies related to key biological pathways using genome-wide NGS data.

IN- AND OUTDOOR REPRODUCTION OF FIRST GENERATION COMMON SOLE UNDER A NATURAL PHOTOTHERMAL REGIME: TEMPORAL PROGRESSION OF SEXUAL MATURATION DETERMINED BY PLASMA STEROIDS, GENOME SEQUENCING AND PITUITARY GONADOTROPIN EXPRESSION

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Introduction

Reproduction of many temperate fishes is seasonal and the maturation and spawning of their gametes is under photothermal control. Reproductive success of first generation (G1) common sole *Solea solea* has been low. This restricts domestication and consequently economic viability of commercial culture. In this study, we have investigated the photothermal effects on the sexual maturation during the prespawning months in G1 sole that were a) outdoor housed under the natural photothermal regime, or b) indoor housed under artificial photothermal induction.

Methods

Maturation status was assessed in male and female G1 broodstock in November as controls, after which the remaining population was divided over two outdoor tanks placed in a pond and two indoor recirculating aquaculture system (RAS) tanks. Repeatedly maturation status was assessed for male and female fish in one tank for each condition in January, February and during spawning in early April, while the other tank was untouched not to disturb the fish in achieving reproductive success. Maturation status was assessed by determining the gonadosomatic index (GSI) and blood plasma steroid levels. The sole genome was sequenced in order to retrieve gonadotropin sequences and perform quantitative real-time PCR in females.

Results and Discussion

Successful mass spawning and egg fertilisation occurred in all experimental outdoor and indoor tanks and gonadal development was similar for males and females under both conditions. Higher E_2 and T levels were found in indoor housed females which may have caused differences in vitellogenesis as also indicated by lower hepatosomatic indices. The expression of *common glycoprotein unit α* (*gpα*), *fshβ* and *lhβ* revealed similar temporal expression profiles between outdoor and indoor housed females but the latter showed less individual variation.

Conclusion

G1 sole was reproduced successfully at large scale in captivity, both in outdoor pond tanks as in indoor RAS tanks. The results indicate the importance of the natural photothermal regime in inducing sexual maturation in sole, as in temperate fish species in general. Specifically, a significant cold period (2-3 months at 5-7 °C) may be permissive for the action of FSH which levels will subsequently decrease and LH levels increase during rising temperatures towards spawning. The sole genome will be instrumental in unraveling its maturation and reproduction impairment under captive conditions in future studies.

TEMPERATURE AS MODULATOR OF THE STEROIDOGENIC PROCESS IN EUROPEAN EEL: MIGRATORY IMPLICATIONS

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Introduction

Little is known about the role of temperature in fish spermatogenesis and sperm production, but its influence on the European eel (*A. anguilla*) maturation process has been evidenced recently. This study tried to evaluate the effect of temperature on the dynamics of sperm production and testis steroidogenic enzymes gene expression.

Methods

Three hundred males were hormonally-induced with weekly doses of hCG under three thermal regimes (T10: 10 °C first 6 weeks, 15 °C next 3 weeks and 20 °C last 6 weeks; T15: 15 °C first 6 weeks and 20 °C last 9 weeks; and T20: constant 20 ºC). Eight fish per treatment were weekly sacrificed. Sperm was collected and volume, density and motility parameters were estimated. Blood samples were extracted to determine T, E2, 11KT and 17,20βP plasma levels. Total RNA was extracted from testicular fragments and retro-transcripted in cDNA. Primers were designed and used to develop specific qPCR to quantify the expression level of some genes encoding some key steroidogenic enzymes (*P450ssc, P450c17, cyp19a1, 11β-HSD, cyp21, 20β-HSD*).

Results and Discussion

The process of testis maturation, as well as the sperm quantity and quality, were affected by the water temperature. T20 induced the best sperm results. Gene expression evaluation probed that this temperature effect was caused through the modulation of the expression of steroidogenic enzymes. T20 and T15-treated males showed earlier and narrow peaks of steroidogenic enzymes gene expression, while T10 males showed delayed peaks with high values during several weeks, which was translated on different timings of steroids production. I.e.: *P450scc* and *P450c17*, involved in the path of T synthesis, maintained high expression at low temperatures coinciding with high T levels. However, the gene expression of steroidogenic enzymes involved into the T conversion to E2 and progestagens synthesis were delayed at lower temperatures. A relative good correlation was found between 11KT plasma levels and *11β-HSD* gene expression, but the E_2 plasma level increased before than the *cyp19a1* gene expression peaked. The sea water acclimation was enough to increase E_2 plasma level, independently of water temperature.

Conclusion

These results suggest that could be a differential activation of genes at the testis level depending on the environment temperature, and that could be the regulatory system used by the male European eels to regulate their sexual maturation process during their travel to the spawning areas in the West Atlantic.

Funded by the PRO-EEL Project. M.C.V. and M.M. have predoctoral grants from UPV (2011-S2-02-6521) and Generalitat Valenciana (Programa Grisolía), respectively. D.S.P. was supported by MICINN and UPV (PTA2011-4948-I).

CHARACTERISATION AND REGULATION DURING REPRODUCTION OF FOUR NUCLEAR CORTICOSTEROID RECEPTORS IN THE EUROPEAN EEL, *ANGUILLA ANGUILLA*

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Introduction:

Cortisol, the major corticosteroid in vertebrates, is often considered as a negative actor in the control of reproduction. However, as a key-hormone in metabolism, it is also necessary for the mobilization of energy and metabolite stores crucial for migration and gonadal growth. This is particularly important in species, like the eel, which are fasting during their reproductive migration. In teleosts, where there is no aldosterone, cortisol itself may act through two types of nuclear receptors, glucocorticoid receptor (GR) and mineralocorticoid receptor (MR).

Methods

Corticosteroid receptors were characterized in the European eel, *Anguilla anguilla*, draft genome, and subsequently cloned. *In silico* analyses were performed and phylogeny was produced. Specific qPCR were developed for each receptor and applied to tissue distribution. The corticosteroid receptor regulations were also measured by qPCR during experimentally induced maturation in female eels. Finally, *in vitro* regulation of the receptors was measured in primary hepatocyte culture treated with estradiol and cortisol, alone and in combination.

Results and Discussion

Two GR and two MR were characterized in the European eel, probably resulting from the whole genome duplication event that occurred specifically in teleosts. Both tissue distribution and expression during sexual maturation showed that the receptors are differentially regulated in the eel. These receptors are expressed in the organs of the hypothalamic-pituitary-gonad-liver (HPG-liver) axis, suggesting a role in reproduction. Hepatocyte cell culture revealed that cortisol and estradiol might have a synergic effect on vitellogenin mRNA synthesis, through an up regulation of the nuclear estradiol receptor ERa.

Conclusion

This represents the first evidence of the presence of four corticosteroid receptors in a basal teleost. In contrast to most of the teleost species, the eel would have retained the two duplicated copies of GR and MR, resulting from the specific whole genome teleost duplication event. Cortisol appears to be involved in the eel reproduction, probably through an interaction with sex steroid receptors. Further studies should aim at investigating the specific role of each corticosteroid receptor in these interactions.

This work was supported by European Community 7FP No. 245257 "PRO-EEL".

EFFECTS OF ESTROGENIC CHEMICALS ON TRANSCRIPTS FOR GONADOTROPIN-RELEASING HORMONE SIGNALING AND CIRCARDIAN RHYTHM PATHWAYS IN FEMALE COHO SALMON, *ONCORHYNCHUS KISUTCH*

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Introduction

Considerable research has been done on the effects of natural and synthetic estrogens on reproduction and gene expression in the brain, liver and gonads of teleost fish, but information on impacts to the pituitary gland are still limited despite its central role in regulating reproduction. Using high-throughput Illumina® sequencing, RNA-Seq and pathway analysis we previously found that waterborne exposure of previtellogenic female coho salmon to 17β-ethynylestradiol (EE2) significantly altered transcripts for gonadotropin subunits and genes in the gonadotropin-releasing hormone (GnRH) and circadian rhythm signaling pathways. The aim of this study was to further our understanding of the effects of 17β-estradiol (E2) and EE2 on pituitary luteinizing hormone (LH) and follicle-stimulating hormone (FSH) subunit mRNAs, and expression of genes in GnRH and circadian rhythm signaling pathways in female coho salmon in late stages of primary oocyte growth

Methods

The sensitivity of 1+ age coho salmon to the effects of EE2 on pituitary gene expression *in vivo* was examined by a 2-week waterborne exposure to either 0, 0.25, 2.5 or 25 ng/L. Primary pituitary cell cultures were used to assess potential direct effects of estrogens on pituitary gonadotropin subunit mRNA levels as well as transcripts for GnRH receptor 1 (*gnrhr1*) and period 1 (*per1*). Cells were exposed to 0, 1, 10, or 100 ng/mL E2 for 72 hours. Levels of mRNAs for target genes were measured by quantitative real time RT-PCR.

Results and Discussion

In vivo exposure of coho salmon to at least 2.5 ng/L EE2 for 2 weeks significantly increased mRNAs for LH beta subunit (*lhb*; 100-fold) and *gnrhr1 (*3-fold*)* while FSH beta subunit (*fshb*) and *per1* mRNAs decreased by 40%. These results are consistent with our previous observations and suggest exposure to low concentrations of EE2 *in vivo* differentially affects FSH and LH production, and potentially alters circadian rhythms in pituitary function. In primary pituitary cell cultures, E2 increased *lhb* (up to 15-fold) at all concentrations tested, but had no significant effect on *fshb, gnrhr1,* or *per1* levels. These data suggest that positive effects of estrogen on *lhb* are in part due to direct effects on the pituitary, but do not support a direct action of estrogen on *fshb, gnrhr1,* or *per1* expression.

Conclusions:

In previtellogenic salmon, low levels of estrogen differentially regulate LH and FSH by positive and negative effects on *lhb* and *fshb* gene expression, respectively. Estrogen alters pituitary expression of genes in circadian rhythm signaling pathways, but further studies are needed to determine if estrogen alters basal or GnRH-induced gonadotropin secretion and rhythms in pituitary gene expression.

Oral Session:

Reproduction and Growth

Chairs: Abigail Elizur and Mark Lokman

THE ROLE OF GONADS FOR GROWTH IN TELEOSTS

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In general, there is a relationship between growth and reproduction, and gonads are known to be important organs for growth, but direct evidence for their role is lacking. In this report, we show the direct evidence that gonads are endocrine organs equal to the pituitary in controlling body growth. To understand the expression of growth hormone (Gh) in testis and ovary, RT-PCR, Western blot analysis and histochemistry were performed using male and female Mozambique tilapia (*Oreochromis mossambicus*). For the investigation of the relationship between body growth and the presence of gonads, we performed the experiments for gonadal loss-of-function experiment by surgically removing gonads and gain-of-function and growth rescue by autotransplantation of their own gonad to gonadoectomized fish. For control experiment, Sham-operation was performed. These surgeries were carried out using juvenile 40 days male and female tilapia after hatching. On 50 days after surgeries, fish were sacrificed and investigated. In RT-PCR and western blot analysis, mRNA and protein of Gh are expressed not only in pituitary but also in both testes and ovaries. In histochemical observation, the expression sites of Gh in gonads were in Sertoli cells surrounding spermatogonia in male and follicle cells layer surrounding oocyte in female. In ectopic transplanted fish, implanted gonads has progressed their gametogenesis in ectopic site in both male and female. This finding indicates that transplanted gonads are functional. Gonadectomy significantly delay growth of these fish compared to sham operated fish. However, this delay was rescued by implantation of surgically removed gonads into the gap between skin and muscle. Serum Gh level of gonadectomised fish were lower than those of sham operated fish and gonad transplanted fish in both male and female, significantly. These finding suggest that gonads control body growth through the secretion of growth hormone and/or other endocrine factors. The gonads are necessary for normal growth and thus, from the perspective of regulation of body growth, can be considered a “secondary pituitary.”

OVARIAN TRANSCRIPTOME RELIABLY PREDICTS EGG QUALITY IN WILD AND DOMESTICATED STRIPED BASS (*MORONE SAXATILIS)*

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Abstract
Poor egg quality, the inability of developmentally incompetent eggs to produce viable embryos, is a major problem in finfish aquaculture. Maternal RNAs deposited in the oocyte direct embryogenesis from fertilization until transcriptional activation of the embryonic genome at mid-blastula transition (MBT). This study evaluated whether ovary transcriptome profiling via application of artificial intelligence could predict egg quality in striped bass. Females with uniformly fully-grown oocytes showing no signs of atresia were biopsied for ovary tissue, induced to spawn, and assessed for percentage of eggs producing viable 4-h-old blastulas at MBT (range 0-92%). Transcripts in the biopsies were initially measured using an Agilent Technologies microarray including ~12,000 UniGene probes targeting a published ovary transcriptome (eArray Striper Group, Design 029034). Ovary and egg transcripts (~39,000 contigs) were subsequently measured by Illumina HiSeq™. Sequences were pooled for *de novo* assembly using Trinity, mapped onto the assembly using the Bowtie short-read aligner, and then quantified as FPKM values via the RNA-Seq Tuxedo pipeline. We used artificial neural networks and supervised machine learning to model profiles of gene expression and their relationship to egg quality. In the microarray study, collective changes in expression of a limited suite of genes (233) representing <2% of the queried ovary transcriptome explained >90% of the variance in surviving embryos. Egg quality related to minor changes in gene expression (<0.2-fold), with most transcripts making a negligible individual contribution (<1%) to predictions of egg quality. Correlation analyses of candidate genes indicated that dysfunction of the ubiquitin-26S proteasome, COP9 signalosome and cell cycle engenders developmental incompetence. The RNA-Seq studies replicated these findings for both farmed fish and wild broodstock with remarkable precision. Models for ovary tissue explained slightly more variation in egg quality than those for eggs. These models predicted an average of 78% to 91% of the variation in egg quality of new fish whose gene expression data was entered into the models. Thus, subtle changes in ovarian transcriptome profiles predict most variation in egg quality not due to incomplete vitellogenesis, atresia or maturational incompetency. These changes involve a discrete spectrum of maternal RNAs forming a 'transcriptomic fingerprint' that is highly predictive of and likely to regulate egg quality and reproductive fitness. The extension of these findings to wild stocks has important implications for fishery management as they indicate clear and predictable differences in the fertility of individuals whose eggs are morphologically indistinguishable.

LINKS BETWEEN SOMATIC GROWTH, GROWTH RELATED HORMONES AND ONSET OF PUBERTY IN ATLANTIC SALMON REARED UNDER DIFFERENT FEEDING AND PHOTOPERIOD CONDITIONS IN SEA CAGES

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Introduction

A positive correlation between rapid growth, adiposity and early puberty has been noted in fish. GH, IGF11, leptin and ghrelin are endocrine factors that may mediate growth effects on puberty by affecting the Gnrh/Gnrhr/Fsh system. This study aims to characterize these factors in Atlantic salmon under different feeding and photoperiod conditions that modulate entry into puberty.

Methods

Salmon postsmolts (n = 2400) were subjected either to full feeding (HF) or restricted feeding (40% of satiation; LF) from September in triplicate sea cages. HF and LF fish were subjected to continuous light (LL) or natural light (NL) from January creating 4 experimental groups; HFNL, HFLL, LFNL and LFLL. Individually tagged fish (n = 200 pr group) were monitored for growth, and either terminally sampled at 6 time points for pituitary gene expression (qPCR), endocrine parameters, adiposity and gonadal development, or repeatedly blood sampled until the final sampling in June.

Results and Discussion

Both feeding and photoperiod affected puberty; in males, the incidence of maturation was reduced from 100% under HFNL to 31, 76 and 14% in the HFLL, LFNL and LFLL groups, respectively. In females, the incidence was reduced from 65% under HFNL to 17, 6 and 5%, in the HFLL, LFNL and LFLL groups, respectively. Puberty initiation was indicated from January onwards by elevated pituitary *fshb* and *gnrhr4* mRNA levels, plasma levels of 11KT (males) or E2 (females), as well as testicular or ovarian development. The timing of this initiation varied among individuals and groups, and while some individuals did not start at all, others started but then aborted the development. LF individuals tended to start later in the season. There was a positive correlation with growth parameters and incidence of puberty, except in fully fed males and in LFNL females. Despite strong negative effects of restricted feeding on growth, condition factor and adiposity in viscera and fillet, only a few effects were noted on the mean plasma levels of GH, IGF1, ghrelin and leptin. IGF1 seemed to correlate best to the observed differences in growth and adiposity at the level of feed treatment.

Conclusion

The study demonstrates strong effects of both restricted feeding and continuous light on pubertal initiation in Atlantic salmon, and links between of somatic growth parameters and puberty, but with limited correlation to plasma levels of growth, metabolic and appetite-related hormones. Current work on individual profiles may provide additional information.

TRANSCRIPTOME PROFILING OF EUROPEAN SEA BASS BRAIN AND ADIPOSE TISSUE RESPONSES TO PHOTOPERIOD TO IDENTIFY PUBERTY ONSET RELATED GENES

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Introduction

Puberty onset is initiated by the activation of the hypothalamus-pituitary-gonadal (HPG) axis to produce gonadotropins and its timing is influenced by internal and external factors, including photoperiod. However, how these external signals are integrated and which gene networks are triggered to initiate this process is still unknown. Thus, the aim of this study was to identify the gene networks that respond to either puberty inhibiting (continuous light-CC) or accelerating (short days- SD) photoperiod regimes at the early events of puberty onset in sea bass, as a step to understand the high incidence of precocious puberty of male sea bass in culture. To achieve this aim, the global transcriptomic changes occurring in pre-pubertal sea bass brain and adipose tissue were characterized throughout the first month after changing photoperiod regimens known to influence puberty onset in sea bass.

Methods

Immature sea bass of two sizes – large fish, the majority expected to go into puberty, and small fish, not expected to go into puberty - were either maintained in natural photoperiod or changed from natural photoperiod to photoperiod regimens known to delay (continuous light) or accelerate (long to short days) gametogenesis progression. SuperSAGE (Serial Analysis of Gene Expression) libraries (72) were constructed from brain or adipose tissue of fish exposed to the three photoperiod regimens for one to 38 days and sequenced by next generation SOLiD4 sequencing. Transcript tags were extracted, counted and their expression profiles statistically analyzed by pairwise comparisons between photoperiod regimens with false discovery rates < 0.05. The differentially expressed transcripts were mapped to the sea bass genome and available cDNAs using a multi-step Blast approach and mapped to cell pathways for enrichment analysis.

Results and Discussion

Gene annotation allowed matching 35-54% of the differentially expressed tags to known proteins and 20-24% to anonymous DNAs, with a global annotation rate up to 78%. Genes related to the brain circadian clock, to reproduction, to metabolism and neurotransmitter signaling were identified as differentially expressed between photoperiods in large or small fish in brain and/or adipose tissue.

Conclusion

In this study we successfully identified key genes and signaling pathways that are changed centrally and peripherally during the early events in response to photoperiod changes highlighting candidate genes and pathways that may be involved in triggering the onset of puberty in sea bass.

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EFFECTS OF GROWTH HORMONE OVER-EXPRESSION ON REPRODUCTION IN THE COMMON CARP *CYPRINUS CARPIO* L.

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Introduction

There is considerable 'cross-talk' between the neuroendocrine axes controlling growth and reproduction. GH-transgenic fish have enhanced growth rate but with reproductive abnormality traits compared with their non-transgenic counterparts. Here, we investigated the cause of reduced reproductive performance using the GH-transgenic common carp to provide data for the complex interactions between the growth and reproduction in fish.

Methods

The GH-transgenic common carp used in the study carried the grass carp growth hormone gene. 15 individuals each were randomly selected from the transgenic and non-transgenic groups once every month. The body weights and gonad weights of the sampled carp were measured to calculate the GSI. The gonads were dissected and stained with H&E to observe the development. Real-time PCR, ELISA and Western blot were performed to evaluate GH and LH levels in the hypothalamus, pituitary, liver, gonad, muscle and serum in samples. *In situ* hybridization and immunofluorescence were performed to identify the colocalization of *ghr* and LH, to compare the distribution of LHβ subunit in GH-transgenic and non-transgenic carp. Primary cell of the pituitary glands were cultured and incubated with GH, a GnRH agonist and a dopamine antagonist to determine the effects of GH on pituitary *gthα*, *fshβ*, and *lhβ* gene expression.

Results and Discussion

The GH-transgenic fish showed delayed gonadal development compared with non-transgenic common carp. To gain a better understanding of the phenomenon, we studied body growth, gonad development, changes of reproduction related genes and hormones of GH-transgenic common carp for 2 years. Over-expression of GH elevated peripheral *gh* transcription, serum GH levels, and inhibited endogenous GH expression in the pituitary. Hormone analyses indicated that GH-transgenic common carp had reduced pituitary and serum level of luteinizing hormone (LH). Among the tested genes, pituitary *lhβ* was inhibited in GH-transgenic fish. Further analyses *in vitro* showed that GH inhibited *lhβ* expression. Localization of *ghr* with LH indicates the possibility of direct regulation of GH on gonadotrophs. We also found that GH-transgenic common carp had reduced pituitary sensitivity to stimulation by co-treatments with a GnRH agonist and a dopamine antagonist.

Conclusion

We demonstrate that pituitary gonadotroph function is suppressed in GH-transgenic common carp. We propose that reduced reproductive performance of GH-transgenic common carp may be partially due to directly action of GH on the pituitary mediated by GH receptors in gonadotrophs. Our results further support the central importance of GH for the coordinated regulation of growth and reproduction.

PLASMA NESFATIN-1 IS NOT AFFECTED BY LONG-TERM FOOD RESTRICTION AND DOES NOT PREDICT REMATURATION AMONG ITEROPAROUS FEMALE RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

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Introduction

The metabolic peptide hormone nesfatin-1 has been linked to the reproductive axis in fishes. The purpose of this study was to determine how energy availability after spawning affects plasma levels of nesfatin-1, the metabolic peptide hormone ghrelin, and sex steroid hormones in rematuring female rainbow trout (*Oncorhynchus mykiss*).

Methods

To limit reproductive maturation, a group of female trout was food-restricted after spawning and compared with a control group that was fed a standard broodstock ration. The experiment was conducted twice, once using two-year-old trout (second-time spawners) and once using three-year-old trout (third-time spawners). During monthly sampling, blood was collected from all fish, and a subset of fish from each treatment was sacrificed for pituitaries. Pituitary follicle-stimulating hormone-beta (fsh-β) mRNA expression was analyzed with q-RT-PCR; plasma hormone levels were quantified by radioimmunoassay (17β-estradiol and ghrelin) and enzyme-linked immunosorbent assay (11-keto-testosterone and nesfatin-1).

Results and Discussion

Although plasma nesfatin-1 levels increased significantly in the months immediately after spawning within both feeding treatments, plasma nesfatin-1 did not differ significantly between the two treatments at any point. Similarly, plasma ghrelin levels did not differ significantly between the two treatments at any point. Food restriction arrested ovarian development by 15-20 weeks after spawning, shown by significantly lower plasma E2 levels among restricted-ration fish. Pituitary fsh-β mRNA levels were higher among control-ration fish than restricted-ration fish starting at 20 weeks, but did not differ significantly between treatment groups until 30 weeks after spawning. Within both treatment groups, plasma 11-KT was elevated immediately after spawning and rapidly decreased to and persisted at low levels; starting between 20 and 25 weeks after spawning, plasma 11-KT was higher among control-ration fish than restricted-ration fish.

Conclusion

The results from these experiments do not provide support for plasma nesfatin-1 as a signal for the initiation of reproductive development in rematuring female rainbow trout.

SEX STEROIDS MODULATE THE LEPTIN SYSTEM IN VITRO AND IN VIVO IN MALE ATLANTIC SALMON (*SALAR SALAR* L.)

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Introduction

The adiposity signaling hormone leptin plays an important role for reproductive function. In mammals, leptin is known to affect all levels of the endocrine reproductive axis and leptin expression is in turn modulated by sex steroids. The main site of expression of leptin in fish is, unlike mammals, the liver. In Atlantic salmon (*Salmo salar* L.), hepatic leptin expression levels are elevated in early maturing males, a stage associated with high androgen levels, and it is likely that sex steroids can alter leptin expression directly also in fish. In the present study we investigated the effects of sex steroids on the expression of the leptin and leptin receptor genes both in vitro and in vivo in Atlantic salmon male parr.

Methods

For *in vitro* experiments, primary hepatocytes cultures were obtained from male parr livers at different developmental stages and incubated with testosterone (T), 11-ketotestosterone (11KT), 17 β-estradiol (E2) and T in combination with the aromatase inhibitor fadrozol at different doses and time points. Relative gene expression of the two leptin genes, *leptin-a1* (*lepa1*) and *leptin-a2* (*lepa2*) were measured using qPCR.

For the in vivo experiment, immature 1+ Atlantic salmon parr were implanted with silicone capsules containing either T, 11-ketoandrostenedione (11KA) or empty capsules (control). Treatment lasted for one month after which the experiment was stopped and tissues were collected for gene expression analysis. Relative gene expression of *lepa1* and *lepa2* was measured in the liver and *lepa1* and the leptin receptor (*lepr)* were measured in the pituitary.

Results and Discussion

In vitro, T, 11KT and E2 all increased *lepa1* mRNA levels in a dose dependent manner, with different potency depending on hormone and developmental stage. Treatment with T in combination with the aromatase inhibitor indicated that the stimulatory effect on *lepa1* expression was androgen specific. The hepatic *lepa2* gene response to sex steroid treatments in vitro was generally weaker than that of *lepa1*.

In vivo treatment with T or 11KA did not affect gene expression of *lepa1* or *lepa2* in the liver, but T significantly up-regulated *lepa1* and leptin receptor expression in the pituitary.

Conclusion

These results show that the leptin system is regulated by sex steroids both in vitro and in vivo, suggesting that leptin plays a role in the process of sexual maturation in fishes.

BENEFICIAL BACTERIA AFFECT EGG QUALITY BY THE MODULATION OF MATERNAL FACTORS INVOLVED IN AUTOPHAGIC AND APOPTOTIC PROCESSES DURING *DANIO RERIO* DEVELOPMENT

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Introduction

It is well known that both autophagic and apoptotic processes are fundamental for a proper embryonic and larval development. In addition, probiotic strains' have the capability to exert important roles in many biological systems including immune response, growth, development and reproduction. However, to date, no studies have focused either on the relation among probiotics, autophagy and apoptosis or on probiotics' ability to qualitatively and/or quantitatively modulate maternal transcripts. In this study the effects of *Lactobacillus rhamnosus* on the control of maternal factors involved in the above-mentioned processes during *Danio rerio* embryo development were investigated.

Methods

Embryos and larvae deriving from parental zebrafish fed with commercial food supplemented with 10^6 CFU/ml water of *L. rhamnosus* IMC 501® (Sinbyotec, Italy) were collected at 0, 2, 4, 8, 12, 24, 48, 72, 96 hours (hpf) and 6 days post fertilization (dpf). Fish tanks parameters of temperature and photoperiod for embryos/larvae were kept at 28°C and 10/14 L/D, respectively. The gene expression patterns of known autophagic and apoptotic biomarkers were assessed by mean of q-PCR and whole mount *in situ* hybridization.

Results and discussion

Probiotic administration induced significant changes in maternal and zygotic mRNA levels involved in embryo development.

With regards to the three maternal autophagy-regulating genes *ambra1a1*, *ambra1b* and *beclin*, lower levels of maternal mRNAs were observed in embryos from treated parents with respect to control ones. These results suggest that the formers hereditarily received a minor quantity of the autophagic maternal messengers.

Noteworthy, both *ambra1a1* and *beclin1* expressions at 8 hpf in the treated group were significantly higher than in controls. Keeping in mind the progressive exploitation of maternal mRNAs from 0 to 8 hpf typical of zebrafish, these data suggest that *L. rhamnosus* may interfere with the zygotic control of embryonic transcription. It is also possible that embryos deriving from treated zebrafish may experience a decrease in maternal mRNAs' degradation. However, both scenarios would lead to the same result in terms of transcripts' levels.

As far as apoptosis is concerned, we analyzed two well-known apoptotic biomarkers, *caspase3* and *p53*. During the first 8 hpf both transcripts appeared higher in the treated experimental group than in controls. Likely, this finding suggests the ability of *L. rhamnosus* to prompt *Danio rerio*'s embryonic development. This hypothesis was confirmed by the earlier expression of genes *goosecoid* and *chordin*, which are both involved in the dorsalization process.

Conclusion

The beneficial *Lactobacillus rhamnosus* showed the ability to modulate important physiological processes involved in embryo development. Its ability of influencing the storage of maternal factors inheritable from parents is a fascinating subject, and a deeper knowledge of the matter would be beneficial for further applications.

NEUROPEPTIDE Y (NPY) INCREASES GONADOTROPINS (GTH) AND GROWTH HORMONE (GH) RELEASE AND SYNTHESIS IN THE CICHLID FISH *CICHLASOMA DIMERUS*

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Introduction

Reproduction and growth depend on feeding status. NPY is considered the most important orexigenic peptide, increasing before meal time and during fasting. In mammals, NPY modulates reproduction and growth acting through the hypothalamic-pituitary axis; however in teleosts few studies have been conducted. The aim of this study was to evaluate whether NPY modulates the expression and release of GtHs and GH in *C. dimerus*. First, we studied possible morphological relationships between NPY fibers and GtHs and GH cells. Then we performed *in vitro* studies to evaluate the effect of NPY on GH, GtHs synthesis/release. Finally, we analyzed variations of GH, GtHs and NPY expression in brain and pituitaries of animals exposed to 1 week of fasting.

Methods

Morphological relationships: double label confocal inmunofluorescences were performed in hypothalamic-pituitary complex. *In vitro* studies: male's and female's pituitaries cultures were incubated with different concentrations of NPY (0, 2, 20, 200nM). We evaluated the expression of GtHs and GH by Real time PCR (qPCR) in 4h culture, and the amount of protein released into the culture medium by western blot, in 24h culture. *In vivo* studies: animals of the same size were isolated and maintained in aquaria for two weeks (adaptation period) with a fix schedule of daily feeding time with commercial pellets at 1.5% of their body weight. Then fish were randomly assigned to feeding or fasting groups. After 1 week, variations in mRNA expression of GH, GtHs and NPY were determined by qPCR in brains (telencephalon-POA; hypothalamus; and medium-posterior brain) and pituitaries. A two way ANOVA design was used to evaluate differences between treatments and sexes.

Results and Discussion

We detected contacts between NPY fibers and somatotropes and gonadotropes, indicating possible functional relationships. We observed an increase in GH release of male's (2nM vs control (C),$p<0.05$) and female's (20nM vs C,$p<0.05$) pituitaries, with no changes in mRNA levels. NPY increased LH release in male's culture (2 and 200nM vs C,$p<0.05$), and LH mRNA levels in female's (20nM vs C,$p<0.05$). In females' pituitaries, there was a tendency of increasing FSH mRNA levels at 20nM of NPY. These results strongly suggested that NPY modulates the release and/or expression of GtHs and GH *in vitro*. In fasting conditions, there was a significant increase in GH and NPY mRNA levels in pituitaries ($p<0.05$) and a tendency of increasing in GtHs expression. The increase of NPY expression in different parts of the brain was area and sex dependent.

Conclusion

NPY is involved in the regulation of GH and GtHs. This is support by morphological and *in vitro* studies. Furthermore, NPY could be a link between feeding status, reproduction and growth in *C. dimerus*.

THE NEUROENDOCRINE CONTROL LINKING ENERGY BALANCE AND REPRODUCTION IN CAPTIVE ATLANTIC BLUEFIN TUNA (*THUNNUS THYNNUS*)

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Introduction

To understand the regulation of reproduction in Atlantic bluefin tuna (BFT), which is notable among fishes for its pseudo- endothermic metabolism and its capacity for rapid lipid mobilization, our study focused on the characterization of the Kiss1& 2–leptin interplay. Our goal was to follow the temporal expression profiles of the related genes during BFT natural spawning season within the Mediterranean Sea.

Methods

Mature BFT captured at the Balearic Islands and held in floating cages were sampled temporally (April, May, July, 2008) during the natural spawning season within the Mediterranean Sea. Biometric parameters were recorded and organ (gonad, liver, fat) indices (GSI, HIS, FSI, respectively) were calculated for individual fish. Tissue samples (pituitary, brain, gonad and liver) were collected for hormone (FSH and LH) and/or gene expression (FSHβ & LHβ, Kiss1 & 2, leptin and cognate receptors) profiling.

Results and Discussion

The GSI values were maximal during May (peak of gametogenesis) and minimal during July (end of the spawning season). HIS and FSI declined with the progression of the reproductive season, highlighting clear sex-dependent energy resource, with males and females utilizing hepatic lipids and perigonadal fat, respectively.

The pituitary FSH content was highest at the onset of the reproductive season (April) and decreased towards maturation, exemplifying the classic role of this hormone during gametogenesis. The pituitary LH levels increased concomitant with gonadal growth, yet, were high following gonadal regression. The latter suggests a pituitary disorder halting LH release, a common reproductive dysfunction of cultured fish.

The target gene expression patterns revealed seasonal and gender-dependent interplay along the brain-pituitary-gonad/liver axes. Our key findings denote: (i) an association between regressed gonads, depleted energy resources and increased transcription of leptin in the hypothalamus, (ii) comparable profiles of Kiss1 and leptin in the hypothalamus, (iii) reversed profiles of the hypothalamic leptin and its cognate receptor (Lep-R), (iv) comparable Kiss2 and Lep-R profiles in the pituitary, (v) sexual dimorphic patterns of the hypothalamic and pituitary Kiss2 during the onset and termination of the reproductive season, respectively.

Conclusion

Our study demonstrates sex-dependent energy resource expenditure in captive BFT undergoing a reproductive cycle, and emphasizes the differential physiological needs between the two genders. The results show associations between the profiles of Kiss members and leptin, at both hypothalamic and pituitary levels, and highlight their interplay coinciding with two critical stages: the onset and termination of reproduction. Further understanding of these factors, will enable us to take a step closer to reduce impaired fertility in BFT due to environmental and metabolic factors.

GROWTH AND GONADAL DEVELOPMENT OF F1 HAPUKU *(POLYPRION OXYGENEIOS)* UNDER TWO DIFFERENT TEMPERATURE REGIMES

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Introduction

In recent years, the National Institute of Water and Atmospheric Research (NIWA) has established a captive breeding programme in New Zealand for *Polyprion oxygeneios*, a wreck fish locally known as hapuku or groper. This species is considered a candidate for aquaculture, but a number of bottlenecks remain a challenge, including highly variable egg quality. To increase our understanding of hapuku reproductive physiology and the conditions required for spawning and for improving broodstock management, gamete quality and collection practices, we examined growth, gonadal development and plasma levels of sex steroids in F1 broodfish during their transition from immature to spawning adults. Ultimately, we aimed to produce F2 offspring and to close the life-cycle of this species.

Methods

Pre-pubertal 4-year-old F1 were divided between two cohorts maintained under the same simulated natural photoperiod but under different water temperature regimes in semi-recirculating systems. One cohort of fish (n = 68, 3 replicate tanks) was maintained at constant 17ºC while the other (n = 85, 4 replicate tanks) was held at temperatures that changed in a circannual fashion between 10ºC and 17 ºC during two breeding seasons (~ 20 months). Gonad biopsies, morphometrics and blood samples were collected every two months from all fish in one tank under each temperature regime until females reached late stages of vitellogenesis. Remaining tanks were only sampled four times during the study.

Results and Discussion

Spawning competency was acquired in F1 hapuku at five years of age. Average weight gain was higher in fish maintained under an annually cycling temperature regime (~ 800 g) than in fish maintained at a constant 17ºC (< 200 g) after two seasons. Changes in oocyte growth from the immature stage (oocyte diameter ~0.1 mm) were observed as early as 15 months prior to spawning. The proportion of females (~60%) to reach late vitellogenesis was higher under the annually cycling temperature regime than in the constant 17ºC group (~ 25%). Fertilised eggs averaged 2 mm in diameter. Plasma levels of sex steroids are currently being quantified by radioimmunoassay.

Conclusion

Average weight gain was higher in fish maintained under an annually cycling temperature regime and a higher proportion of females reached late vitellogenesis in this cohort compared to those maintained at a constant 17ºC. Results provide a foundation for controlling broodstock reproduction and insight into the basic reproductive physiology of hapuku.

Poster Presentations

Abstracts

Posters with odd numbers are presented in **poster session I** on Monday - 26 May, 17h20.

Posters with even numbers are presented in **poster session II** on Thursday- 29 May, 17h30.

Please stand by your poster during your session.

OBSERVATIONS OF THE COURTSHIP OF MIXED WILD AND CAPTIVITY BREED SENEGALESE SOLE (*SOLEA SENEGALENSIS*) BROODSTOCK

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Introduction

The culture of Senegalese sole (*Solea senegalensis*) has attracted investment during the economic crisis (2008-2013). However, expansion has been limited as broodstock reared in captivity, G1 generation, present a complete reproductive failure and absence of successful spawning. The failure has been attributed to G1 males, which were observed to not participate in the courtship to fertilise eggs. The hypothesis was proposed that G1 males must learn the complicated courtship behaviour. The aim of the present study was to observe reproductive behaviour in a broodstock that contained males and females of both wild and G1 origin.

Methods

A total of 17 Senegalese sole broodstock (mean weight 1,277 ± 422 g) of mixed origin (5 males, 5 females G1 and 2 males, 5 females wild) were held in a 10 m^3 tank with half the bottom covered with sand. The behaviour of the broodstock was video recorded (17:00-07:00) during the spawning season in April and May. All spawns were collected and microsatellite analysis used to identify paternity of the offspring. The activity was measured on five randomly selected nights that presented spawning. Behaviour of the broodstock was identified using an ethogram during the hour of peak activity 18:30-19:30 on 12 nights that presented spawns. The following behaviours were recorded: 1) rest the head, a social behaviour when one sole rests the head on another, 2) guardian, where one sole protects another from advances from a third, 3) persecutions, where sole swim in sequence following exactly the swimming of the lead sole, 4) coupled swimming when two sole swim in synchrony to the surface to spawn and 5) gamete liberation. Particular attention was given to the behaviour persecutions that in previous studies were only associated with spawning. Three researchers independently identified the origin of sole involved in persecutions.

Results and Discussion

A total of 20 spawns were collected (over a period of 59 days) and all larvae analysed were attributed to five parents, 1 wild male and 4 wild females. All spawns that were observed were between the hours 18:00-20:00, which coincided with the peak of activity. In general the courtship was similar to that previously described for the species and included the behaviours rest the head, guardian, persecutions, coupled swimming and gamete liberation. Males reared in captivity (G1) were observed to participate in persecutions. This is the first report that indicates that G1 males have participated in aspects of the courtship. Other aspects that differed from previous descriptions were that 1) the sole appeared to aggregate prior to spawning, 2) the sand allowed the male to enter under the female and initiate the swim towards the surface to liberate gametes and 3) the same couple was observed to spawn more than one time in a single night.

Conclusion

These are the first preliminary observations that indicated that G1 males can participate in aspects of the courtship. Further studies are in progress to determine if G1 males can learn or be put in a situation to participate in the full courtship including gamete release.

Acknowledgement: The study was funded by project INIA-FEDER RTA2011-00050.

EFFECTS OF INSL3 (INSULIN LIKE PEPTIDE 3) ON SPERMATOGONIAL DYNAMICS IN ADULT ZEBRAFISH

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Introduction

INSL3 is a member of the relaxin peptide family that is specifically expressed by Leydig cells in the vertebrate testis. In mammals, INSL3 mediates the testicular descent during embryogenesis while information about its functions in the adult testes is limited. In most submammalian vertebrates including teleost fish, there is no testicular descent and the testes remain in the body cavity. INSL3 expression in Leydig cells in fish may therefore be indicative of evolutionary older functions of INSL3. We aimed at studying the biological activities of INSL3 in adult zebrafish by quantifying the testicular expression of selected genes, germ cell proliferation/differentiation, and androgen release.

Methods

Adult zebrafish testes were collected and incubated in a previously established tissue culture (TC) system with or without human recombinant INSL3 (hrINSL3) at a concentration of 100ng/mL. To study if basal or Fsh-stimulated androgen release is modulated by INSL3, 11-ketotestosterone levels were quantified in the medium by RIA, after an overnight TC. After 7 days of TC, testis tissue was processed for qPCR analysis to quantify the mRNA levels of selected growth factors genes in response to hrINSL3. To analyse the effects of hrINSL3 on germ cell proliferation, we determined the BrdU-index of different types of spermatogonia by immunocytochemistry (ICC); BrdU was present during the last 6 hours of the 7 days long TC period. Finally, we pre-labelled undifferentiated type A spermatogonia (A_{und}) with BrdU *in vivo* during 3 days pulse/4 days chase, and then submitted testis tissue to 4 days of TC with or without hrINSL3, before quantifying the BrdU-index of spermatogonia by ICC.

Results and Discussion

hrINSL3 did not modulate basal or Fsh-stimulated androgen release, suggesting that INSL3 effects are not mediated by androgens. TC studies showed that hrINSL3 increased the proliferation activity of type A_{und} spermatogonia while no effects were observed for later germ cells generations (type A differentiated and type B spermatogonia). To elucidate if this increased BrdU-index reflects self-renewal or differentiating proliferation, we used testis tissue with type A_{und} spermatogonia that were pulse/chase labelled with BrdU *in vivo* for subsequent TC in the present of hrINSL3. Under these conditions, hrINSL3 clearly decreased the BrdU-index of A_{und} spermatogonia, suggesting that the loss of BrdU label from this early germ cell generation reflected hrINSL3-stimulated cell cycling in the absence of BrdU in TC. Monitoring the expression of selected growth factor genes showed that hrINSL3 decreased anti-müllerian hormone (*amh*) mRNA levels. Interestingly, previous work showed that Amh blocked germ cell proliferation and differentiation.

Conclusion

Our results indicate a possible stimulatory effect of INSL3 on spermatogonial differentiation in adult zebrafish, potentially involving down-regulation of Amh while not modulating androgen release.

WATER TEMPERATURE EFFECT ON FATTY ACID MOBILIZATION IN MALE EUROPEAN EELS DURING SEXUAL MATURATION AND RELATIONSHIP BETWEEN FATTY ACIDS, STEROIDS AND SPERM QUALITY PARAMETERS

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Introduction

We studied the changes in fat content and analysed quantitatively the fatty acid composition of different tissues from males hormonally-induced under three thermal regimes, looking for the relationships between fatty acids, steroids plasma levels and sperm quality parameters.

Methods

Males were subjected to three thermal regimes (T10: 10 °C first 6 weeks, 15 °C next 3 weeks and 20 °C last 6 weeks; T15: 15 °C first 6 weeks and 20 °C last 9 weeks; T20: constant 20 ºC). Fish were weekly treated with hCG. The testis development stages were established by histology. Fatty acids of muscle, liver and testis were analyzed by gas chromatography. Sperm motility parameters were evaluated using a CASA software. Samples from blood plasma were collected to analyse the plasma concentrations of T and 11KT.

Results and discussion

The eels reached spermatogenesis earlier and showed the best sperm production parameters under treatment T20, suggesting that eel spermatogenesis is closely regulated by water temperature. Males did not show variations of fatty acid content in the muscle. Regarding the liver, the levels of palmitic, eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids increased when eels began to produce sperm, suggesting *de novo* biosynthesis and a probable subsequent mobilization to the testis, due to their important function during gametogenesis. In the testis, EPA, araquidonic acid (ARA) and DHA remained constant during the maturation process, while the levels of the rest of fatty acids decreased significantly. Maintenance of ARA and EPA levels may have a physiological meaning (as prostaglandins precursors, i.e.), while the maintenance of DHA levels may have a structural one (spermatozoa membranes formation). In the liver, the levels of PUFA precursors (α-linolenic and linoleic) decreased when the sperm motility increased. In the testis, ARA levels were negatively correlated with different sperm velocity parameters. The consumption of ARA coincided with an increase in the speed of spermatozoa, supporting not only the importance of the role of this fatty acid for male testis maturation, but especially conditioning sperm quality. The levels of T and 11KT correlated positively with 16:0 and 16:1 (the main products of fatty acid *de novo* biosynthesis), especially when the cell meiosis happened, suggesting the role of these fatty acids as precursors in cell membrane formation. Later, when the eels were producing sperm, EPA and DHA were then positively correlated with T and 11KT. It is known that EPA and DHA act as modulators of T production, and this regulatory function could become more important during the sperm final maturation process.

Conclusion

Achieved results are coherent with the importance of PUFA in teleosts reproduction due to their regulatory role in male sexual maturation and sperm composition. All this information could be useful to improve the sperm quality throughout broodstock diet.

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ENDOCRINE CONTROL OF AQUAPORIN EXPRESSION DURING GILTHEAD SEABREAM (*SPARUS AURATA*) SPERMATOGENESIS

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Introduction

Recent studies in mammals have inferred that multiple classes of aquaporins may play different roles for water and solute transport during spermatogenesis and spermiogenesis. In teleosts, immunolocalization experiments in gilthead seabream have demonstrated that seven distinct aquaporins, Aqp0a, -1aa, -1ab, -7, -8b, -9b and -10b, are differentially expressed in the somatic and germ cell lineages of the mature (spermiating) testis. In this work, we used recombinant piscine gonadotropins and steroid hormones to investigate the *in vivo* and *in vitro* endocrine regulation of aquaporin expression during gilthead seabream spermatogenesis.

Methods

Males were sampled during the resting phase, at the onset of spermatogenesis and during the spermiating period. Plasma levels of testosterone (T) and 11-ketotestosterone (11-KT) were measured by EIA. Testicular subsamples were used for immunofluorescence microscopy using paralog-specific antibodies, or incubated *in vitro* in the presence of European seabass recombinant follicle-stimulating hormone (rFsh) or luteinizing hormone (rLh), T, estradiol (E2), 11-KT or 17α,20β-dihydroxy-4-pregnen-3-one (17,20β-P). Changes in aquaporin gene expression were determined by real-time qPCR.

Results and Discussion

During the resting phase, when plasma levels of T and 11-KT were low and the testis exclusively contained spermatogonia, only Aqp1ab was detected in the cytoplasm of spermatogonia, whereas Aqp9b and -10b were expressed in Leydig and Sertoli cells, respectively. At the onset of spermatogenesis and in the spermiating testis, androgen plasma levels progressively increased which correlated with the appearance of spermatocytes and spermatids, and few spermatozoa, in the seminiferous tubules. At both stages, Aqp0a and -9b were localized in Sertoli and Leydig cells, respectively; Aqp1ab, -7, and -10b from spermatogonia to spermatozoa; and Aqp1aa and -8b in spermatids and spermatozoa. *In vitro* incubations of testis explants with rFsh and rLh indicated that both gonadotropins induced elevated expression levels of *aqp0a*, *-9b* and *-10b* at the onset of spermatogenesis, whereas *aqp1aa* and *-7* only increased in the presence of rLh. At the spermiating stage, gonadotropins only promoted the expression of *aqp7* and *-8b*. The response to the steroids was also different depending on the spermatogenic stage. Thus, at the onset of spermatogenesis *aqp0a*, *-1aa*, *-7*, *-9b* and *-10b* mRNA levels increased in the presence of 11-KT, and in the case of *aqp9b* also with E2 and 17,20β-P. In contrast, during spermiogenesis the mRNA levels of *aqp0a*, *-8b*, *-9b* and *-10b* were only stimulated with T, and 17,20β-P for *aqp9b*, whereas those of *aqp7* were still responsive to 11-KT. Interestingly, *aqp1ab* expression was not regulated by gonadotropins or steroids at the onset of spermatogenesis or during spermiation, suggesting an earlier control of this channel.

Conclusion

These observations suggest a differential and complex gonadotropic and steroid regulation of aquaporin expression during gilthead seabream germ cell development.

PARACRINE MEDIATORS OF FSH EFFECTS IN THE ADULT ZEBRAFISH TESTIS: SEARCHING FOR CANDIDATES

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Introduction

Recent work has identified a small number of paracrine factors that participate in mediating the biological activity of Fsh in stimulating spermatogenesis in fish. In this study, we aimed at identifying more such candidate factors, using the zebrafish (*Danio rerio*) model, including members of Tgfβ, Egf and Igf signaling systems, as well as signaling molecules from the immune system. Candidates were selected from large-scale gene expression profiling data sets, and experimental data were obtained by quantifying the respective mRNA levels after challenging adult zebrafish testis tissue with recombinant zebrafish follicle-stimulating hormone (rzfFsh). We also studied in a global manner the potential involvement of sex steroids in candidate gene expression.

Methods

Using a previously established testis tissue culture system, we compared candidate gene expression by qPCR in adult zebrafish testis tissue incubated for 48 hours in the absence or presence of rzfFsh (100 ng/mL).

To differentiate effects of Fsh that are mediated by its steroidogenic activity from direct Fsh effects on Sertoli cells, these incubations were carried out in the absence or presence of trilostane, an inhibitor of the 3β-hydroxysteroid dehydrogenase enzyme, which prevents the production of biologically active steroids. Moreover, a parallel set of tissue culture experiments was carried out following exposure of adult males to 10 nM estradiol (E2) for 3 weeks, which interrupts spermatogenesis and induces an androgen insufficiency, resulting in a testis enriched in type A spermatogonia.

Results and Discussion

rzfFsh treatment significantly increased the testicular expression of a Tgfβ family member and an Egf family member, and this stimulatory effect was blocked when trilostane was added to the incubation medium. The mRNA abundance of a member of the Igf signaling system was strongly stimulated by rzfFsh, an effect that was only slightly attenuated by trilostane. Only one of the immune factors tested, a protease inhibitor, was differentially expressed in the presence of rzfFsh.

In order to study the effect of rzfFsh on testis tissue in a different physiological state, candidate gene expression was examined using testis tissue from males pre-exposed to E2 in vivo. In general, we observed higher amplitudes of changes in gene expression. Furthermore, a significant effect was observed for an immune factor that did not respond in males not exposed to E2.

Conclusion

The present study provides a number of interesting leads in the context of improving our understanding of the local, intratesticular mediation of Fsh effects with its complex range of bioactivities that include stimulation of steroidogenesis in Leydig cells and modulation of the expression of paracrine factors from Sertoli cells.

ENRICHMENT OF TRANSPLANTABLE GERM CELLS USING MAGNET-ACTIVATED CELL SORTING IN RAINBOW TROUT

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Introduction

We established surrogate broodstock in which donor germ cells transplanted into the peritoneal cavity of recipients were capable of developing into functional eggs and sperm in rainbow trout. However, when we transplanted a whole testicular cell suspension into recipient body cavities, only the type A spermatogonia (A-SG) and oogonia were incorporated into the genital ridges of the recipients and underwent gametogenesis. We previously employed flow cytometry (FCM) to enrich undifferentiated germ cells and demonstrated how the method could be applied to increase colonization efficiency. However, since FCM techniques are both costly and time consuming, we assessed the potential application of magnet-activated cell sorting (MACS) to enrich undifferentiated germ cells in rainbow trout. MACS is an inexpensive and intuitive method by which specific cells labeled with antibody can be isolated using a magnet. In this study, we developed a simple and rapid technique for enriching undifferentiated rainbow trout germ cells using a monoclonal antibody and MACS.

Methods

All experiments were conducted using transgenic rainbow trout carrying the *vasa-GFP* construct, which expressed green fluorescence in undifferentiated germ cells. Monoclonal antibodies were generated by inoculating mice with freshly harvested A-SGs, followed by cell ELISA screening, cell immunostaining, and FCM analysis. To identify which monoclonal antibodies could be applied to MACS, we first conducted immunohistochemistry assays in testes and ovaries. After screening, undifferentiated testicular germ cells were isolated using a MACS separator and an enrichment rate was evaluated by FCM analysis using GFP as an indicator. We examined the transplantability of the isolated cells by intraperitoneal transplantation of sorted and unsorted testicular germ cells. We also applied the same procedures to ovarian germ cells.

Results and Discussion

Screening results identified an antibody (No. 172) that produced a strong signal for ASG in testes. In addition, an immunoreaction was also detected in oogonia and primary oocytes. When MACS cell sorting was performed using antibody No. 172, antibody-positive cells could successfully be isolated from both testies and ovaries. Further, FCM analysis revealed that antibody-positive cells were also GFP positive germ cells. Transplantation assays revealed that the colonization efficiencies of the cells isolated using MACS were significantly higher than those of unsorted testicular and ovarian cells. Finally, we confirmed that MACS-mediated enrichment of undifferentiated germ cells could be applied to wild-type rainbow trout.

Conclusion

We successfully enriched undifferentiated germ cells from both male and female rainbow trout using MACS. The method does not require the use of expensive equipment, such as FCM, or transgenic fish. Using this approach will facilitate an increase in the efficiency of surrogate broodstock technologies in practical applications, such as in aquaculture and conservation.

EFFECTS OF DOSE AND ROUTE OF ADMINISTRATION OF HUMAN CHORIONIC GONADOTROPIN ON TESTICULAR DEVELOPMENT IN IMMATURE MALE SHORTFINNED EEL, *ANGUILLA AUSTRALIS*

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Introduction

Human chorionic gonadotropin (hCG) is routinely used to induce spermatogenesis in captive male anguillid eels. Notable differences exist in terms of injection dose, frequency and route of administration between experiments and the rationale for choice of these parameters is often obscure.To date, a systematic study aimed at identification of the optimal dose and route of administration of human chorionic gonadotropin hCG in shortfinned eels has not been performed; we therefore set out to compare the effects of several doses of hCG, administered as a single intramuscular (im) or intraperitoneal (ip) dose, on testicular development in this species.

Methods

Male silver shortfinned eels (~ 150 g) were recruited into a dose-response study and injected with 0-4000 U hCG, either im or ip. Fish were euthanized after 4 weeks and effects of treatment on gonadosomatic index (GSI), testicular histology and plasma levels of hCG and 11-ketotestosterone (radioimmunoassay) were determined.

Results and Discussion

Plasma hCG levels were detectable in most hCG-treated eels, even at 4 weeks after administration, and showed a clear dose response, peaking at *ca* 4 U/mL in fish that received a single 4000 U im injection. Testicular growth could be discerned histologically when eels were exposed to as little as 20 U of hCG, judged from limited spermatogonial proliferation. Testicular weight, however, remained unchanged. A 10-fold increase of dose (200 U) resulted in dramatically elevated, albeit variable GSI values, that were notably higher in fish injected im (~3.6%) than in those injected ip (~2.0%). This trend, an approximate doubling of GSI in im- compared to ip-treated eels, was also seen in response to 2000 U of hCG. Doses of hCG that yielded half-maximum responses (EC50) in terms of GSI and plasma 11-ketotestosterone levels were estimated at 102 and 114 U/fish, respectively.

Conclusion

In keeping with previous studies, hCG proved highly effective at increasing testicular mass and advancing testicular development in male eels, even in response to a single injection. Intramuscular administration is preferable over i.p treatment, and a dose of ~100 U/fish will give a half-maximal response by 4 weeks of treatment.

CHARACTERIZATION OF GERM CELLS AND ISOLATION OF TYPE A SPERMATOGONIA FROM THE TESTES OF *PROCHILODUS LINEATUS* (CURIMBATÁ)

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Introduction

Morphometric and stereological analysis allied techniques of cell isolation should clarify some aspects of the testes biology. Our aim was to identify testicular germ cells during spermatogenesis, to evaluate the individual volume of type A spermatogonia (Agonia) to estimate the mean number of these cells per testes, and to standardize techniques of testicular digestion and isolation of Agonia.

Methods

The mean diameter (μm) of thirty nuclei of germ cells of testes per animal was obtained. The individual volume of Agonia was obtained from the nuclear volume and the proportion between the nucleus and cytoplasm. Then, the number of Agonia of testes was calculated. For isolation of Agonia, germ cells were obtained by enzymatic digestion (collagenase, trypsin and DNAse) of the testes and were fractionated by density discontinuous gradient with Percoll (10%, 20%, 30 % and 40 %). The efficiency of cell separation (n=5) from the pellet in different bands of Percoll was histologically evaluated.

Results and Discussion

The nuclear diameters of all germ cells varied significantly among themselves (=3618.508 F / p <0.001). Therefore, the mean value of each class can be used as a parameter for the determination of these cell types, since comparing subsequent classes, the difference between the variances remains to a greater or lesser degree (SG2 - tSG1 = 18,389; tSG2 -PL = 50.110; TPL- LZ = -41.316; TLZ - P = 8.463; tP - D = 8,388; tD - SS = 27,540; TSS- S1 = 28.519; tS1 - S2 = 32.199; TS2 - S3 = 23.114; TS3 - SPZ = 11,857, [p < 0.001]; Abbreviations: SG1, Agonia; SG2, type B spermatogonia; PL, primary spermatocyte in pre - leptotene; LZ, primary spermatocyte in leptotene/zigotene; P, primary spermatocyte in pachytene; D, primary spermatocyte in diplotene; SS, secondary spermatocyte; S1, initial spermatid; S2, intermediate spermatid; S3, late spermatid; SPZ, spermatozoa The estimated cellular volume of Agonia were 1,252.00 μm^3, while the mean number of Agonia per testes was approximately 2.40 x 10^6. Results obtained in the standardization of the method of Agonia isolation showed the presence of four different cell bands of the Percoll column and the two upper bands had high concentration of Agonia. Morphological analysis and the use of trypan blue showed large number of viable cells.

Conclusion

The spermatogenic process in *P. lineatus* shows a decrease in nuclear diameter in germ cells, from the Agonia to sperm. Because of the morphological characteristics of stem spermatogonia are very similar to the Agonia present in large number in the two top layers of Percoll column, one can consider that the Agonia were in the pool of these layers.

AMH INHIBITS AND IGF3 PROMOTES SPERMATOGONIAL DIFFERENTIATION – DOES AMH MODULATE IGF3 EFFECTS?

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Introduction

Growth factors participate in the regulation of spermatogonial stem cell (SSC) activity, for example by modulating the balance between self-renewal and differentiating proliferation of SSCs. In zebrafish, two growth factors seem to play important functions in this regard; insulin-like growth factor 3 (Igf3) and anti-müllerian hormone (Amh). Unpublished results from our group show that Igf3 promotes proliferation/differentiation of type A undifferentiated spermatogonia (A_{und}) while Skaar, Nóbrega and colleagues (2011) demonstrated that Amh inhibited SSC differentiation. In the present study, we investigated if (i) Amh can modulate Igf3 effects, and (ii) how Fsh regulates the testicular mRNA levels of these two growth factors.

Methods

Using previously established tissue culture system, adult zebrafish testis was incubated with recombinant zebrafish (rzf) Igf3 (100 ng/mL), in the presence or absence of rzf Amh (10 µg/mL) for 7 days. BrdU (50 µg/mL) was added to the culture medium for the last 6 hours of incubation. Testis tissue was processed for BrdU immunodetection to determine the BrdU-labelling index of A_{und}. Additional cultures served to quantify *igf3* and *amh* mRNA levels in response to rzf Fsh at different doses (25, 75, 100 and 1000 ng/mL), or to 75 ng/mL rzf Fsh at different times (1, 3, 5 and 7 days).

Results and Discussion

The BrdU-labelling index of A_{und} recorded after exposure to Igf3 was significantly reduced in the presence of Amh, suggesting that Amh exerts a dominant negative effect on the stimulatory role of Igf3, as has previously been observed for the stimulatory role of 11-ketotestosterone (Skaar, Nóbrega et al., 2011). *igf3* mRNA levels increased already at 25 ng/mL rzf Fsh and increased further at higher rzf Fsh doses but showed no effect over time. Higher rzf Fsh doses (100 and 1000 ng/mL) were required to reduce *amh* mRNA levels and incubation periods above 3 days were required for significant effects.

Conclusion

These results suggest that at intermediate Fsh levels, elevated but not yet maximal Igf3 production and still high Amh levels would allow an intermediate stimulation of germ cell production which is strongly stimulated when Igf3 production is maximal in combination with reduced levels of the inhibitory Amh.

SPERM PROTEIN FOR EGG ACTIVATION: PLCZ1 EXPRESSION IN EUROPEAN EEL TESTIS THROUGH SPERMATOGENESIS

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Introduction

Egg activation at fertilization is characterized by a rise in intracellular egg calcium. In mammals, a sperm specific phospholipase C, the PLCZ1 (phospholipase C zeta 1) enters into the egg and triggers the egg activation through IP3 signaling, which causes the Ca^{2+} release from intracellular stores. Some evidences suggest that fish could have a similar mechanism, as sperm extracts from tilapia induced release or oscillations in Ca^{2+} in mouse and sea urchin eggs, and also microinjection of cRNA of tilapia *plcz1* induced calcium oscillations in mouse eggs. In contrast, in two fugu species, *plcz1* expressed in ovary, but not in testis. Our goal was studying *plcz1* gene tissue distribution and expression through spermatogenesis in European eel (*Anguilla anguilla*).

Methods

Male eels were induced to mature with weekly injections of hCG. Between 5-8 males were sacrifized weekly, and after testis histological classification, 8 samples from each spermatogenic stage (S1: spermatogonia; S2: spermatocytes; S3+4: spermatids, first sperm cells; S5: spermatozoa represented 50-70% of the cells; S6: spermatozoa represented 75-95% of the cells) were selected for gene expression analyses. Also, samples from 3 immature males and females were collected for *plcz1* screening study. mRNA sequence of *A. anguilla* PLCZ1 (*plcz1*) was obtained by a combination between *in silico* genome analysis and gene cloning (Genbank accession JX868847.1). Specific qPCR assay was developed.

Results and Discussion

A. anguilla plcz1 gene showed 73-76% sequence conservation with *plcz1* from other fish species (medaka, tilapia, 2 fugu species). Tissue distribution of *plcz1* gene revealed high *plcz1* expression in testis, and very low in ovary. Also, both in males and females a high brain *plcz1* expression was found. Through spermatogenesis, testis *plcz1* gene expression was very low in stages S1 and S2, increasing from S3+4 (spermatids) to S6 (full spermiation), when it was 75-fold higher than at S1. That suggests that testis *plcz1* mRNA synthesis starts after the onset of spermiogenesis, like it was shown in some bird and mammal species. In medaka fish *plcz1* express also in testis, and there are evidences of PLCZ activity in tilapia sperm. Our results support the hypothesis for a sperm-specific PLCZ1 egg activation in some teleost fish species, similar to mammals, nemertins, ascidians and the newt *Cynops*, but different to other fish species, which express this protein in ovaries, but not in testes.

Conclusion

By first time, *plcz1* gene expression has been analyzed in testis through fish spermatogenesis, showing the highest levels in the spermiation stages. That supports the existence in teleost fish of a sperm-oocyte activation mechanism similar to mammals.

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PROGESTIN RECEPTORS EXPRESSION IN EUROPEAN EEL THROUGH SPERMATOGENESIS

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Introduction

The progestin DHP (17α,20β-dihydroxy-4-pregnen-3-one) regulates the final sperm maturation in several teleosts species, including Japanese eel. That final sperm maturation is the phase during which not yet functional gametes develop into mature spermatozoa. This process involves physiological changes, which are regulated by the endocrine system. It seems that DHP action is mediated through an increase in the seminal plasma pH, which in turn increases the cAMP content in sperm, thereby allowing the acquisition of sperm motility. However, DHP has been also proposed as an essential factor for the initiation of testicular meiosis in Japanese eel. We studied the expression of two nuclear progestin receptors (nPR; *pgr1*, *pgr2*) involved in sperm maturation, evaluating their expression in testis, brain and pituitary through the spermatogenesis.

Methods

Hundred males were matured with weekly injections of hCG at 20 ºC. 5-8 males/week were sacrifized to collect testis, brain and pituitary samples. After histological classification, 8 selected samples per spermatogenic stage (S1: spermatogonia; S2: spermatocytes; S3+4: spermatids, first spermatozoa; S5: spermatozoa represented 50-70% of the cells; S6: spermatozoa representing 75-95% of the cells) were selected for gene expression analyses. Samples from 3 immature males were collected for screening study. mRNA sequence of *A. anguilla* nPR (*pgr1, pgr2*) were obtained by a combination between *in silico* genome analysis and gene cloning (Genbank accession JX494229, JX494230). Specific qPCR assays were developed to quantify expression.

Results and Discussion

pgr2 was more highly expressed in pituitary than in brain, while *pgr1* was more highly expressed in the brain compared to the pituitary. Other tissues outside the BPG axis showed high expression of *pgr1* (kidney, muscle) or *pgr2* (gill). In the testis, different expression profiles were observed: while *pgr2* expression was the highest at S1 (when only spermatogonia were present), *pgr1* expression was stable during testis development. That suggest that *pgr2* can mediate the reported effect of DHP on meiosis initiation. Pituitary *pgr1* expression increased by 480-fold from S1 to S2, and pituitary *pgr2* expression also increased by 9-fold from S1 to S2, maintaining high levels until S6, in an opposite profile than in testis. Low variation in brain *pgr2* expression through development was observed, while brain *pgr1* increased between 2 and 13-fold at the end of testis development.

Conclusion

Our results suggest that brain, pituitary and testis show different regulation by progestins, with *pgr1* being the most important in brain, and *pgr2* being the most important in testis and pituitary during eel spermatogenesis. Moreover, these results suggest the possible role of membrane PR on the final sperm maturation.

Funded by the SPERMOT project (MINECO; AGL2010-16009).

EVALUATION OF SPERMATOGENESIS AND MILT QUALITY IN THE JAPANESE EEL MATURED BY RECOMBINANT EEL GONADOTROPIN INJECTIONS

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Introduction

Pituitary gonadotropins, follicle-stimulating hormone (Fsh) and luteinizing hormone (Lh), control fish spermatogenesis as in other vertebrates. However, the respective roles of Fsh and Lh on spermatogenesis remain unclear in fish. Japanese eels (*Anguilla japonica*) do not mature under aquaculture conditions, requiring techniques to obtain fertile gametes. Recently we have established mammalian cell lines, which consistently produce recombinant eel Fsh and Lh connected with C-terminus of human chorionic gonadotropin (hCG) including glycosilation sites (reFsh and reLh). We aimed to evaluate spermatogenesis induced by reFsh and reLh injections and quality of milt from those matured eels.

Methods

Male eels received weekly injections of reFsh, reLh, hCG (500 μg/kg-body weight), or a mixture of reFsh and reLh (reFsh+reLh, 500 μg/kg-body weight respectively). Five eels from each group were killed after 0, 1, 2, 4, 6, 8 weeks from the start of injections and blood was collected to measure serum 11-ketotestosterone (11-KT) levels by time-resolved fluoloimmunoassay. Testes were dissected to measure gonadal-somatic index (GSI) and to use for histological observations. Milt was hand-stripped on the day following the injection at Week 8, 10 and 12. Milt quality was evaluated by volume, spermatoclit and sperm motility.

Results and Discussion

In eels injected with reLh, hCG and reFsh+reLh, GSI dramatically increased and reached to 10 % by 6 weeks. GSI in reFsh-injected eels was approximately 5.5 % even after 8 weeks. Serum 11-KT levels in eels injected with reLh, hCG and reFsh+reLh immediately increased and show the peak after 1 week, while those in reFsh-injected eels gradually increased, peaking after 4 weeks. As results of histological observations, seminiferous tubules in reFsh-injected eels were apparently smaller than those in other groups throughout the experimental period. Volume of milt from eels injected with reLh and reFsh+reLh was more than 5 fold to that from eels injected with reFsh and hCG. Spermatoclit in eels injected with reLh and reFsh+reLh was lower than that in other groups, and sperm motility in reLh-injected eels was highest (more than 70 %) throughout the experimental period.

Conclusion

In Japanese eels, reFsh injections were less effective on the progress of spermatogenesis and milt production than reLh injections. The quality of milt from eels injected with reLh was better than that from other groups, indicating reLh injections are the most suitable technique to obtain milt in eel aquaculture.

DEVELOPMENT OF WISTAR AND GFP RAT GERM CELLS TRANSPLANTATED TO TILAPIA (*OREOCHROMIS NILOTICUS*) TESTES

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Introduction

Germ cell transplantation consists in the removal of spermatogonial stem cells (SSCs) from the testis of a donor species and the transferring of them into the testis of a recipient animal with depleted endogenous spermatogenesis, where these cells will develop to form mature functional spermatozoa presenting donor genetic characteristics. Using the Nile tilapia (*Oreochromis niloticus*) as an experimental model, we demonstrated in our laboratory the production of donor fertile spermatozoa in the recipient testis and these spermatozoa were able to generate healthy progeny. In order to provide a technique to investigate the plasticity of the tilapia testis environment, our aim in the present study was to study the development of rat germ cell in the tilapia testes.

Methods

To deplete endogenous spermatogenesis, young sexually mature male recipient tilapias, maintained at a temperature of 35° C, were treated with two intracelomic injections (18 and 15mg/kg/BW) of the chemotherapeutic drug busulfan (Sigma, MO, USA), with an interval of two weeks between each injection. The donor germ cells were harvest from the testes of Wistar and eGFP rat (8-10 days of age) through enzymatic digestion according to Zhang (2003) with some modifications; and these cells were labeled with membrane lipophilic dye PKH26. The recipient fish received the donor germ cells through the common spermatic duct using a glass micropipette under a stereomicroscope (Olympus SZX-ILLB2-100). Four tilapias that received Wistar rat germ cells were sacrificed two and five weeks after transplantation, whereas two tilapias that were subsequently sacrificed at seven weeks post-transplantation received eGFP rat germ cells.

Results and Discussion

Two weeks post-transplantation, donor spermatogonia were seen in contact with the recipient tilapia Sertoli cells. Remarkably, spermatocytes and round and elongated rat spermatids were observed in some areas of the seminiferous tubules of fish sacrificed five weeks after transplantation. As only spermatogonia were present in the 8-10 days old donor rat testes, the meiotic and spermiogenic rat cells observed in the tilapia testes were undoubtedly originated from the transplanted rat spermatogonia. The histological analyses suggested that rat GFP germ cells were also present in the recipient tilapia testes evaluated seven weeks post-transplantation.

Conclusion

The development of rat germ cell within the tilapia testes strongly suggests the plasticity of the seminiferous tubules environment of this species, providing a new and important tool to investigate SSCs biology and spermatogenesis in vertebrates. In order to verify if fertile rat sperm is eventually formed in the tilapia testes we are currently developing new experiments.

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FSH MODULATES THE INSULIN GROWTH FACTOR BINDING PROTEINS (IGFBPS) EXPRESSION IN ZEBRAFISH TESTIS

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Introduction

The insulin-like growth factors (Igfs) promote proliferation and differentiation of different cell types. In most vertebrates, the Igf signaling system is composed of Igf1 and Igf2, its respective receptors (Igfrs) and the Igf binding proteins (Igfbps). Recently, a new member of this system has been identified in fish that is expressed in gonads: Igf1b (a.k.a. Igf3). However, prior to Igf/Igfr interaction, Igfbps bind Igf with equal or higher affinity than Igfrs. Igfbps can modulate the bioavailability of Igfs, thereby inhibiting or potentiating Igf actions. Our previous work showed that zebrafish testis tissue expressed Igf1, Igf2a and b, Igf3 and both known Igfr variants. Here, we examine which Igfbps are expressed in the testis and in how far Fsh (promoting spermatogenesis and Igf3 expression) changes Igfbp expression.

Methods

Ten zebrafish Igfbp cDNAs were cloned from testis and/or liver and sequenced. Primers for Igfbps were tested using plasmid DNA as a template. To study the effect of FSH on Igfbp expression, adult zebrafish testes were incubated for 5 days in a previously established tissue culture system in presence of 25, 50, 100 or 1000 (ng/mL) recombinant zebrafish (rzf) Fsh; trilostane (25 µg/mL) was present to block the production of biologically active sex steroids. Tissue samples were then processed to quantify the Igfbp expression by qPCR; specific assays could be developed for 7 of the 10 proteins so far.

Results and Discussion

We found that zebrafish testis tissue expressed all 10 Igfbps (Igfbp1a, 1b, 2a, 2b, 3, 5a, 5b, 6a, 6b and 7), although some (Igfbp1a, 1b and 2a) were reported to be exclusively expressed in the zebrafish liver previously. Fsh selectively modulated the expression of 4 of 7 Igfbps analyzed by qPCR. Both down-regulatory (Igfbp1a and Igfbp7) and up-regulatory (Igfbp2a and Igfbp5b) responses were recorded, which occurred at different Fsh concentrations, suggesting a complex response pattern of the Igf signaling system. Igfbp1b, 2b and 5a were not significantly regulated by rzfFSH at any of the concentrations used here.

Conclusion

All Igfbps evaluated are expressed in zebrafish testis, and 4 of 7 binding proteins were up- or down-regulated. Similar to studies in other models, our data seem compatible with the concept that some Igfbps enhance, while others reduce Igf bioactivity, suggesting overall that Igfbps are relevant for regulating testis functions. To obtain further information on Igfbp function, the cellular localization of Igfbp expression should be studied, and Igfbp expression should not only be investigated under conditions stimulating germ cell differentiation (e.g. in response to Fsh; see above), but also under inhibitory conditions.

OXIDATIVE STATUS OF SEMINAL PLASMA AND SPERMATOZOA IN DIFFERENT FISHES

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Introduction

Reactive oxygen species (ROS) are chemically reactive molecules containing oxygen. The ROS by the sperm mitochondria is excessive, the gamete's endogenous antioxidant defenses are rapidly overwhelmed and oxidative damage induces lipid peroxidation in the spermatozoa with a resultant loss of fertilizing potential. Therefore the aim of our study was to investigate oxidant status of the seminal plasma with that of spermatozoa in Russian sturgeon (*Acipenser gueldenstaedtii*), Siberian sturgeon (*Acipenser baerii*), sterlet (*Acipenser ruthenus*), common carp (*Cyprinus carpio*), and brook trout (*Salvelinus fontinalis*).

Methods

Sperm samples from each species were centrifuged at 5000g for 10min. The seminal plasma and sperm pellet were suspended in 50 mM KPi buffer, pH 7.0, and homogenized in an ice bath. The homogenate was divided into two portions: one in which thiobarbituric-acid-reactive substances (TBARS) and carbonyl derivatives of proteins (CP) were measured and a second that was centrifuged at 12000g for 30min to obtain post-mitochondrial supernatant for the antioxidant enzyme activity assay. The TBARS method was adapted to evaluate lipid peroxidation (LPO) in fish seminal plasma and spermatozoa. The TBARS concentration was calculated by light absorption at 535nm on a spectrophotometer. Carbonyl derivatives of proteins were detected by reaction with 2,4-dinitrophenylhydrazine (DNPH). The amount of CP was measured spectrophotometrically at 370nm.

Results and Discussion

The relatively high polyunsaturated fatty acid content of the spermatozoon membrane makes it susceptible to oxidative damage. Lipid peroxidation can lead to a loss of membrane integrity, causing increased cell permeability, enzyme inactivation, resistance to osmotic shock, and decrease in fertilization potential. In our study non-significant differences in levels of TBARS were observed between seminal plasma and spermatozoa of *A. gueldenstaedtii*, *A. baerii*, and *A. ruthenus*. In carp and brook trout, TBARS levels were significantly higher in spermatozoa (0.88 ± 0.14 and 0.57 ± 0.1nmol/mg protein, respectively) than in seminal plasma (0.49 ± 0.09 and 0.34 ± 0.06nmol/mg protein, respectively). Significant differences in CP concentration were detected between spermatozoa and seminal plasma in all experimental species.

Conclusion

Based on our results, we conclude that the low level of oxidative stress indices in seminal plasma seem to play no role in cellular metabolism (e.g. oxidative phosphorylation) of sperm, which could lead to subsequent decline of motility variables.

MORPHOLOGY AND ULTRASTRUCTURE OF ASIAN SEA BASS (*LATES CALCARIFER*) SPERMATOZOA

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Introduction

In the present study the spermatozoa of the catadromous and protandrous hermaphroditic Asian sea bass (*Lates calcarifer*) was investigated using scanning and transmission electron microscopy.

Methods

The sperm of five males was used as an experimental material. Individuals aged 31 and 36 months were cultured at a warm-water recirculating aquaculture system in Jászkisér, Hungary. The samples were prepared according to Pšenička et. al. 2006. Sperm was fixed with 2.5% glutaraldehyde in phosphate buffered saline and stored at 4°C till the preparation for the examination. Sperm samples were prepared for high vacuum SEM and examined with SEM JEOL 6300. Images were digitally recorded for the determination of morphological parameters. Samples for TEM were fixed in the same way, after the preparation a series of ultrathin sections were cut using a Leica UCT ultramicrotome, counterstained then examined in a TEM JEOL 1010.

Results

The spermatozoon consisted of a round head with a distinct acrosome and nucleus region, a midpiece and a flagellum. Total length of the barramundi spermatozoa is 32.31±2.91 μm, width of head is 1.58±0.16 μm, length of head is 1.72±0.21 μm and length of flagellum is 28.86±2.87 μm.

Conclusion

According to our findings the spermatozoa of *Lates calcarifer* belong to type II. among percoidei consistent with Mattei (1991). Nevertheless comparison between the present study on Lates calcarifer spermatozoa morphology and previous studies on other percoidei confirmed large inter-specific differences that could be of substantial taxonomic value.

Acknowledgements
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BLOCKING OF PROGESTIN ACTION DISRUPTS SPERMATOGENESIS IN NILE TILAPIA (*OREOCHROMIS NILOTICUS)*

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Introduction

Maturation inducing hormone,17α,20β–Dihydroxy-4-pregnen-3-one (DHP),a typical piscine progestin, plays essential roles during sperm maturation, spermiation, as well as early stage of spermatogenesis in male fish. However, lack of *in vivo* evidences restricts the probability of DHP action through nuclear progestin receptor (Pgr) in teleosts.

Methods

The tilapias for this study were reared in large tanks with a re-circulating aerated freshwater system (26 ºC).Tilapia *pgr* was cloned from the testis, and the expression during ontogeny was analyzed by real-time PCR. Furthermore, Pgr antagonist (RU486) treatment was carried out in XY fry from 5 day after hatching (dah) for two or three months. Finally, DHP and 11-KT was used to rescue the RU486-disrupted spermatogenesis.

Results and discussions

Tilapia Pgr possessed conserved DNA-binding domain and ligand-binding domain of nuclear receptor. Phylogenetically,tilapia Pgr clustered with other teleostean pgrs, while tetrapods Pgrs formed a separate clade. Real-time PCR analysis demonstrated that tilapia *pgr* expression was significantly up-regulated from 10 day after hatching (dah),peaked at 50dah and persisted till adulthood in XY gonads. Surprisingly, continuous treatment exceeding 3 mah severely disrupted spermatogenesis and resulted in loss of spermatocytes and spermatids. Transcript level of germ cell marker gene (*vasa*),meiosis marker gene (*sycp3*) and spermatogonial markers (*piwil1*and *dazl*) were substantially reduced in RU486 treated testis. However, the expression level of Sertoli cell marker genes,i.e.*dmrt1*,*amh* and *gsdf* remained unchanged. Moreover, RU486 treatment didn't alter the level of 11-KT production and expression profiles of steroidogenc genes including *star*, *cyp11a1*, *cyp17a1*, *hsd11b2* and *cyp11b2* in the Leydig cells. Simultaneous excessive DHP, but not 11-KT, supplementation could override the RU486-distrupted spermatogenesis, and restore spermatogenesis. Consistently, combination of DHP and RU486 treatment could re-activate expression of Vasa in spermatogonial cells and spermatocytes, while simultaneous supplementation of 11-KT couldn't.

Conclusion

Our data suggested that DHP, mediating by Pgr, plays a critical role in both the proliferation of spermatogonial cells and later phase spermatogenesis in tilapia.

THE RELATIVE ROLE OF LH AND FSH DURING SEX CHANGE IN A PROTANDROUS HERMAPHRODITE MODEL SPECIES, THE GILTHEAD SEA BREAM (*SPARUS AURATA*)

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Introduction

Many marine teleosts reproduce as sequential hermaphrodites. During this process the gonad changes from ovary to testis (protogynous), or vice versa (protandrous). Endocrine studies aimed to reveal the physiological mechanisms underlying sex change pointed to sex steroids (i.e., 11KT and E_2) as major effectors of this process. Nevertheless, the upstream mechanism of sex change is not yet entirely clear. The current study, therefore, focuses on the pituitary gonadotropins (LH and FSH), the key regulators of steroidogenesis, and evaluate their relative role during sex change in a protandrous hermaphrodite model species, the gilthead seabream (*Sparus aurata*).

Methods

One year old seabream males were divided into 3 groups (in triplicates) and treated with either: (i) rsbLH, (ii) rsbFSH or (iii) saline only (Control). The experiment proceeded from January to April, spanning seabream natural spawning season. Temporal samplings were conducted throughout the experiment prior to each injection. Biometric parameters were recorded and the respective GSI values were calculated. Plasma and tissues (i.e., pituitary and gonads) were collected and processed for further analyses. The latter included: (i) sperm quantity/ quality evaluation, (ii) examination of histological sections of the gonads, (iii) sex steroid (11KT and E_2) measurements, and (iv) transcript quantification of βLH and βFSH.

Results and Discussion

The GSI values in all experimental groups gradually declined reaching their minimum at the end of the natural spawning season. Nonetheless, higher GSI values and higher abundance of spermiating males were maintained over an extended period in the rsbLH treated group. Furthermore, the rsbLH treatment was found to promote dual affects: it enhances sex change and oocyte development in the segregating females vs. spermiation in males. At the hormonal level both the rsbLH and rsbFSH treatments induced the synthesis and secretion of pituitary LH. Nevertheless, the rsbFSH treatment effect on LH synthesis and secretion was prominent only at the end of the reproductive season. Furthermore, while the rsbLH treatment had no effect on pituitary FSH synthesis, the rsbFSH treatment negatively affected it. One of the striking results of this study is the evident positive correlation between intra pituitary βFSH /βLH mRNA ratio and GSI values. Steroid profiles correlated with gonad morphology revealed that a precipitate drop in 11KT levels rather than a change in E_2 levels promotes sex change towards segregating females.

Conclusion

Both rsbLH and rsbFSH were physiologically active and appeared to have differential role in the one-year old seabream undergoing sex change. Phenotypically, the effect of the rsbLH treatment was more discernible as compared to the rsbFSH treatment: it enhanced sex change and oocyte development in the segregating females, and spermiation in specimens that will maintain their sex in the consecutive year ("true males"). The latter dual effect suggests that LH is a down rather than an up regulator facilitating the natural path of the sex change process. The positive correlation between the GSI values and the ratio of the pituitary gonadotropins (βFSH / βLH) suggests that the ratio, rather than their absolute amount, better reflects the reproductive status of the fish.

DIFFERENTIAL EXPRESSION PATTERNS OF TWO *sox9* GENES IN THE TELEOST FISH *DICENTRARCHUS LABRAX*

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Introduction

The *Sox* (SRY-related genes containing a HMG box) genes code for a family of transcription factors that are involved in certain developmental processes. Several *Sox* genes are present in the genomes of vertebrates and particularly, *Sox9* has been demonstrated to be responsible for testis development in mice. Previous analysis of the *sox* gene family in the European sea bass (*Dicentrarchus labrax* L.) showed that this teleost species has two distinct *sox9* genes, namely *sox9a* and *sox9b*. Here, we report the isolation and characterization of the full length cDNAs of both genes, together with the identification of these two genes in non-teleost genomes. Their tissue expression patterns and the role of gene duplication for the evolution of developmental gene function is investigated.

Methods

Medaka and zebrafish sox9a and sox9b amino acid sequences were used as queries in TBLASTN searches against the sea bass genome database. Two sequences were identifed and the complete coding sequences of both genes were amplified from sea bass cDNAs using specific primers. The amplified products were cloned into the pGEM-T Easy vector and plasmids were verified by sequencing. The 3´untranslated regions (UTRs) of *sox9a* and *sox9b* were amplified to analyze the expression of both genes by qPCR of the cDNA samples. The expression of the elongation factor alpha (*ef-1*□) and the ribosomal protein L13a (*rpl13a*) genes were used as reference for ovary and testis samples, respectively. Tissue distribution and female and male gonad samples throughout their first reproductive annual cycle were analysed. Phylogeny and synteny analyses were performed to understand the evolutionary relationship between *sox9a* and *sox9b* in vertebrates.

Results and Discussion

Both *sox9a* and *sox9b* mRNAs are present in the brain and gonads of sea bass, similarly to those observations found in other teleosts. Both genes were also found to be expressed in other tissues. Gonad expression patterns of *sox9* genes in the sea bass showed a strong sexual dimorphism, with high mRNA levels of *sox9a i*n ovary and *sox9b* in testis of adult fish. The mRNA levels of both genes in gonads also significantly varied during the reproductive cycle of female and male sea bass. The *sox9* gene sequences are highly conserved among vertebrate species, including the sea bass. Phylogenetic and synteny analyses supports that these two *sox9* genes might originate from the teleost-specific whole genome duplication.

Conclusion

Our observations provide evidence that the two *sox9* genes are expressed in both male and female adult gonads. Further studies are focused in their expression during the first year of life and their functionality as transcription factors.

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N-MYC DOWNSTREAM REGULATED 3 (NDRG3): CHARACTERIZATION DURING PRIMORDIAL GERM CELL PROLIFERATION AND REGULATION BY ANDROGEN IN MEDAKA

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Introduction

In medaka primordial germ cells (PGCs) develop through various stages of mihotic proliferation, a process highly regulated in a sexually dimorphic manner. In females, the number of PGCs is increased dramatically after stage 37, previous to hatching, meanwhile in males the number of PGCs can be observed from the second weed after hatching. The desregulation of the PGCs proliferation can produce a male-to-female sex reversal, e.g. *hotei* mutant.

Methods

To evaluate if *ndrg3* could be involved on the gonadogenesis was analyzed its expression during different stages of gonadal development by RT-qPCR using medaka (*Oryzias latipes*, himedaka stain). Furthermore, the regulation of *ndrg3* expression by androgen (11-ketotestosterone, 11-KT) was analyzed using an *ex-vivo* approach.

Results and Discussion

A pattern of differential expression between sexes, with significant differences in stage 37 in females and 14 and 30 days post hatching in male was obtained. For both sexes, the increased of *ndrg3* expression was correlated with the time of proliferation PGCs characterized for this species. Moreover, the expression of *ndrg3* was up-regulate with 11-KT exposition.

Conclusion

The *ndrg3* expression at different stages of medaka showed significant temporary differences between sexes, with an increase in expression upon proliferation of PGCs of both sexes, so that might be involved in this process. In turn, *ndrg3* expression was positively regulated by androgen, suggesting a regulation by sex steroids.

VISUALIZATION AND MIGRATION OF PRIMORDIAL GERM CELLS IN A MARINE FISH, THE JAPANESE ANCHOVY, *ENGRAULIS JAPONICAS*

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Introduction

Primordial germ cells (PGC) are the origin of germ cells and each one differentiates into eggs or sperm through gametogenesis. In teleosts, PGCs specified as single cells that contain maternally inherited germ plasm at the blastula stage, and migrate toward the gonadal region under the guidance of chemokine signals. The inherent nature of PGCs offers important advantages of applications in reproductive biotechnology such as the control of fertility. However, in teleosts, visualization of PGCs in live embryos has been limited to some model species and currently very few studies are available for marine fishes. In the present study, we labeled PGCs of Japanese anchovy, a commercially important marine fish, with a green fluorescence protein (GFP). The resulting labeled PGCs were traced to determine the route of PGC migration.

Methods

Adult anchovy were captured in the wild and kept at 22-26˚C in a flow-through seawater 1.5 ton tank. Fertilized eggs were obtained by natural spawning. PGCs were labeled with GFP by injecting *gfp*-zebrafish *nos*3 3'UTR mRNA (Köprunner et al., 2001) at 1 to 2-cell stage of embryos in 50% seawater. Embryos were then incubated at 26˚C with filtered 100% seawater supplemented with antibiotics. Observations of embryos were performed in an agar coated glass dish with 50% seawater using a fluorescence microscope.

Results and Discussion

Fourty-five per cent of the embryos that received an injection possessed GFP-labeled PGCs on Day 1. The GFP-labeled PGCs were initially observed in the embryo at 50% epiboly, 8 hours post fertilization (hpf). These cells were traced to determine the migration route of the PGCs. Unlike zebrafish and medaka, there were two areas where PGCs appeared. One area (area 1) was at the dorsal anterior region along the embryonic body and the other (area 2) at the dorsal part of the blastoderm margin. The PGCs in area 1 made a cluster and came closer to the anterior part of the trunk region and migrated ventrally toward the central line of the embryonic body at 16 hpf, then this cluster moved to the posterior along the embryonic body. The PGCs in area 2 aligned along the embryonic body around 16- to 20-somite at 19 hpf, then migrated ventrally towards the central line of the embryonic body at 23 hpf just before hatching. After hatching, these PGCs migrated axially towards the gonadal region and became a single cluster on Day 2.

Conclusion

In the present study, we demonstrated that anchovy PGCs could be visualized by microinjection of *gfp-nos*3 3'UTR mRNA. GFP-labeled anchovy PGCs displayed a unique pattern of migration during embryonic development. This anchovy model is of value in the developmental study of marine fish PGCs.

GONADAL SEX DIFFERENTIATION OF THE ENDANGERED CYPRINID FISH, LAKE MINNOW *EUPALLASELLA PERCNURUS* (PALLAS, 1814)

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Introduction

The lake minnow *Eupallasella percnurus* is a tiny multi-spawning cyprinid fish. It is not threatened globally, but in Poland it is considered as endangered with extinction so strictly protected by law and included in the Polish Red Data Book of Animals. It is also a priority species in the European Ecological N2000 Network, which requires designation of special areas of conservation and active protection plans including production of its stocking material to initiate new populations. These actions involve extensive studies of lake minnow reproduction biology including gonadal sex differentiation. The aim of the present study was to determine the course of the anatomical differentiation of ovaries and testes, as well as cytological specialization of germ cells present in both types of gonads.

Methods

Lake minnow larvae, offspring of wild individuals caught in May 2012 in a water body in Zielonka near Warsaw, Poland, were reared at 20°C in a recirculation system. Live *Artemia* nauplii were fed to larvae. For histological examination, every 10 DPH 15 fish were sacrified and fixed in Bouin's solution, subsequently dehydrated in ethyl alcohol (75-96% concentration), fixed in xylene and embedded in paraffin. The sections were cut using microtome RM 2155 (LEICA Microsystems, Germany) into pieces 4-5 μm thick and stained with haematoxyline and eosin. Histological cross-sections were analyzed for the shape, size of gonads and the type of germ cells present in gonads under a light microscope LEICA DM 3000 using micro image computer analysis software LEICA QWin Pro (LEICA Microsystems AG, Switzerland).

Results and Discussion

The first symptoms of gonadal sex differentiation of lake minnow appeared on 30th DPH when fish reached the mean body weight of 47.5 ± 6.0 mg. Spindle-shaped gonads attached to the peritoneum on both sides (future ovaries) and pear-shaped gonads attached with a single mesentery string (future testes) were observed. The cytological differentiation of lake minnow took place earlier in females than in males. On 50th DPH the first female germ cells – oogonia with the diameter range 13.9-19.1 μm appeared in ovaries. Spermatogonia located in seminiferous tubules were found in testes on 70th DPH.

Conclusion

Histological studies revealed that lake minnow is characterized by diversified gonochorism. Microscopic changes observed during gonadal sex differentiation were ongoing faster than in other native cyprinid species. These results can be useful in the production of lake minnow stocking material to be used in active protection of this species.

SEX DIFFERENTIATION IN THE ENDANGERED NEOTROPICAL SPECIES *STEINDACHNERIDION PARAHYBAE* (SILURIFORMES: PIMELODIDAE): A CYTOGENETIC AND MORPHOLOGICAL STUDY

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Introduction:

Fish are extremely versatile and with various patterns of sex determination/differentiation, including gonocorists and hermaphrodite animals. Several fish species with temperature sex determination (TSD) have been proposed, and may be regarded as biomarkers of biological impacts on global warming, since changes in temperature induced sex ratio shifts, which are a direct response to the environmental thermal fluctuations. *Steindachneridion parahybae* is a freshwater catfish endemic to the Paraíba do Sul River Basin, Brazil, classified as an endangered Neotropical species. We investigated the sex differentiation in *S. parahybae* during the first hundred days after hatching (DAH), reared in different temperatures (19º, 24º and 29ºC).

Methods:

Artificial breeding of *S. parahybae* broodstock was performed at the CESP fish farm and the fertilized eggs transferred to an aquarium with water recirculation in the IB-USP biotherium, with controlled temperature (19º, 24º and 29ºC) and photoperiod (14:10, L:D). During the first ten DAH, five animals were sampled; thereafter, five animals were sampled every three DAH, until the one hundreth DAH. The animals sampled were fixed in Bouin solution; serial sections (8µm) were obtained and then stained with haematoxylin/eosin, periodic-acid-Schiff (PAS)/haematoxylin/metanil yellow. Additionally, karyotypic data were obtained from 2 females and 2 males of *S. parahybae*.

Results and Discussion:

This species showed a conserved diploid number of 2n = 56 chromosomes, with no morphological evidence of differentiated sex chromosomes or supernumerary chromosomes. The best temperatures for the development of this catfish were 24º and 29ºC and the lower temperature (19ºC) was limited by stage of the development of this species, since abnormal development observed. Canibalism rates increased at higher temperatures and decreased at lower temperatures. Undifferentiated gonads were observed until the sixtieth to the sixty fifth DAH (29ºC); until the seventieth to the eightieth DAH (24ºC); and until the sixty seventh DAH (19ºC). The beginning of the ovarian differentiation was identified from the seventieth to the ninetieth DAH (29º) and from the seventieth to the eightieth (24ºC), with initial formation of the ovarian cavity (extention of ovary wall). Furthermore, no extention of gonad wall was observed in testes differentiation (eightieth to ninetieth DAH (29ºC); and seventieth to seventy eighth DAH (24ºC)). Additionally, when rearing in a different temperature, *S. parahybae* showed a different sex ratio. On the other hand, probably no "thermo sensitive period" was observed.

Conclusion:

It can be considered that this catfish evolved genotypic sex determination but with influence of temperature in this process. Furthermore, this data provide important tools for further investigations of reproduction of this threatened catfish.

LOSS OF FOLLICLE-STIMULATING HORMONE RECEPTOR FUNCTION CAUSES MASCULINIZATION AND SUPPRESSION OF OVARIAN DEVELOPMENT IN GENETICALLY FEMALE MEDAKA

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Introduction

Follicle-stimulating hormone (FSH), a glycoprotein hormone, is circulated from the pituitary and functions by binding to a specific FSH receptor (FSHR). FSHR is a G-protein-coupled, seven-transmembrane receptor linked to the adenylyl cyclase or other pathways and is expressed in gonadal somatic cells. In some non-mammalian species, *fshr* expression is much higher in the ovary than in the testis during gonadal sex differentiation, suggesting that FSHR is involved in ovarian differentiation in non-mammalian vertebrates. However, little is known of FSHR knockout phenotypes in these species. In this study, we established a *fshr* mutant medaka (*Oryzias latipes*) line and analyzed the phenotypes of the fishes.

Methods

We screened for *fshr* mutations from a medaka target-induced local lesion in the genomes (TILLING) library and analyzed the phenotypes of *fshr* mutant medaka using quantitative real-time PCR and enzyme immunoassay. In addition, we rescued FSHR function in the mutant by transgenic over-expression of *fshr*.

Results and Discussion

We screened for *fshr* mutations from a medaka TILLING library and identified one nonsense mutation located in the BXXBB motif, which is involved in G protein activation. Next, we analyzed the phenotypes of *fshr* mutant medaka. The *fshr* mutant male medaka displayed normal testes and were fertile, while the mutant female fish displayed small ovaries and were infertile because vitellogenesis was inhibited. The mutant females also have suppressed expression of *ovary-type aromatase* (*cyp19a1*), a steroidogenic enzyme responsible for the conversion of androgens to estrogens, resulting in decreased 17β-estradiol levels. Moreover, loss of FSHR function caused female-to-male sex reversal in some cases. In addition, transgenic over-expression of *fshr* in *fshr* mutants rescued FSHR function. These findings strongly suggest that in medaka, FSH regulates ovarian differentiation and development mainly by elevation of estrogen levels.

Conclusion

Loss of follicle-stimulating hormone receptor function caused masculinization and suppression of ovarian development in genetically female medaka. This is the first presentation of the knockout phenotype of FSHR in a non-mammalian vertebrate.

CHARACTERIZATION OF MOLECULAR SEX DIFFERENTIATION AND THE SEXUALLY LABILE PERIOD OF GONADAL DEVELOPMENT IN SABLEFISH

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Introduction

Sablefish (*Anoplopoma fimbria*) is an emerging marine aquaculture species native to the North Pacific. Female sablefish grow faster and to a larger size than males and thus there is interest in producing all-female and/or sterile populations for aquaculture. Our recent work has characterized the timing and stages of histological differentiation of sablefish gonads and established several molecular markers of ovarian and testis development. The goal of this study was to characterize early molecular events of sex differentiation to better understand the timing of this process and ultimately identify genes that can be manipulated for sex control.

Methods

Next-generation sequencing (NGS, 454 pyrosequencing) was conducted on mRNAs isolated from histologically differentiated ovaries and testes of juvenile sablefish. Bioinformatics and RNA-seq were performed to reveal genes of interest and putative differentially expressed genes. Selected genes, including some transcription factors, steroidogenic enzymes and growth factors were further analyzed during ontogenetic development of the gonads in genetic females and males by quantitative PCR. Gonadal histology and gene expression profiles of juveniles treated with dietary 17α-methyltestosterone (MT) or 17β-estradiol (E2) for 2 or 4 months were assessed to determine the sexually labile period.

Results and Discussion

NGS and downstream analyses revealed numerous differentially expressed genes between developing ovaries and testes of sablefish. Ontogenetic analyses of selected genes during gonadal differentiation and gametogenesis demonstrated dramatically elevated *foxl2* and *cyp19a1a* mRNA levels in differentiating ovaries relative to testes and elevated *fshr*, *dmrt1*, *amh* and *cyp11b* mRNA levels in differentiating testes relative to ovaries. Increases in mRNA levels of early molecular markers of sex differentiation, such as *dmrt1*, began in fish 40-50 mm in length. This preceded histological differentiation of the gonads, which was first apparent in females ~75 mm long. Unlike the above genes that appeared to coincide with the period of sex differentiation, other genes like *cldn29* were markedly delayed in their onset of expression in ovaries and coincided with the appearance of perinucleolar oocytes. This suggested potential involvement in oogenesis or formation of the ovarian follicle. E2 treatment spanning the period of molecular and early histological differentiation effectively feminized genetic male sablefish, which was also reflected by an inversion of the gonadal gene expression profile to that of ovaries. However, complete masculinization via MT required a longer duration of treatment.

Conclusion

This study established that events related to molecular sex differentiation are initiated in juvenile sablefish 40-50 mm in length. Gonads of juveniles treated with MT or E2 exhibited gene expression profiles and morphology that reflected either partial or complete sex inversion depending on the steroid and duration of treatment. We are now interested in tying events of sex differentiation to sex determination, which appears to be under strict genetic control in this species.

STEROID LEVELS AND GONADOTROPIN GENE EXPRESSION DURING LARVAL DEVELOPMENT AND GONAD DIFFERENTIATION IN SENEGALESE SOLE (*SOLEA SENEGALENSIS*), REARED UNDER INTENSIVE AND MESOCOSM CONDITIONS

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Introduction

The Senegalese sole is a priority species for aquaculture diversification, but it shows critical reproductive problems. Cultured, but not wild-caught, breeders are unable to produce fertilized spawning. We have hypothesized that this could be caused by a deficient early establishment of the reproductive brain-pituitary-gonad axis, associated to "non-optimal" larval culture conditions. This study aimed to investigate the ontogeny of the endocrine system (steroid levels and gonadotropin gene expression) in sole larva and the influence of different culture conditions.

Methods

Intensive culture was done on 400 L tanks, at a density of 40 larva/l and fed with rotifers and artemia nauplii. Mesocosm was done on 3000 L tanks, at a density of 3 larva/L, on "green water" (mixed phytoplankton and zooplankton species) and artemia. Both systems were fed on dry food at weaning (80 days post-hatching (dph) onwards). Fish were sampled from 0 to 147 dph, which included the following periods: pre-metamorphosis (0-8 dph), metamorphosis (8-22 dph), post-metamorphosis (22-98 dph) and gonad differentiation (98-147 dph). Weight, size and survival rate were determined. Sex steroids (testosterone (T), estradiol (E2), 11-ketotestosterone (11kt) and 17α,20β-dihydroxy-4-pregnen-3-one (17,20β-P)) were analyzed in whole body homogenates by ELISA. Gene expression of gonadotropin subunits (α, *fshβ* and *lhβ*) were analyzed by qPCR.

Results and Discussion

Weight and size of larva were higher in mesocosm than intensive groups until weaning, but were similar from 98 dph onwards. T and E2 levels were first detectable at 8 and 18 dph, respectively. Levels remained constant until a sharp rise on 98 dph (T=1.5 pg/mg tissue, E2=1 pg/mg), coinciding with the initiation of the gonad differentiation period and then decreased, but remained high (0.3-0.6 pg/mg), till the end of the differentiation period (147 dph). 11kt was undetectable through larval and post-larval development (0-84 dph), it sharply appeared on 98 dph (around 0.2 pg/mg) and remained constant till 147 dph. Levels of 17,20β-P were first detectable at 8 dph, they increased constantly to peak levels at post-metamorphosis (around 9 pg/mg) and, in contrast to estrogens and androgens, remained constant till 105 dph, decreasing thereafter. Gene expression of all three gonadotropin subunits was detectable in all sampling points and followed similar profiles. Levels were low and constant from 0 to 56 dph (slight increase at metamorphosis) and highly increased (20-, 40- and 25-fold for α, *fshβ* and *lhβ*, respectively) at gonad differentiation (98-147 dph).

Conclusion

Different levels and secretion profiles between the studied steroids suggest significant by different biological functions during larval development. The gonad differentiation period is characterized by high increases in steroid and gonadotropin levels. In general, there were no differences in all these parameters in larva grown under intensive or mesocosm conditions.

BOTH AROMATASE ISOFORMS ARE EXPRESSED IN ALL GONADS DURING SEXUAL DIFFERENTIATION IN ZEBRAFISH

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Introduction

In several gonochoristic fish species, aromatase, the enzyme responsible for the synthesis of estrogens, has been shown to play a critical role in the process of sexual differentiation. In zebrafish, that undergoes a juvenile hermaphrodite stage, there is still a lack of knowledge regarding the precise localization and expression of the two isoforms of aromatase, Cyp19a1a and Cyp19a1b, within the gonads impeding the clear determination of the role of cyp19a1 genes in this process. To fulfill these gaps, we investigated the spatio-temporal expression of both cyp19a1 isoforms either in gonads of wild-type or in gonads of novel *cyp19a1a*-GFP and *cyp19a1b*-GFP transgenic zebrafish strains.

Methods

The localization of expression of gonadal Cyp19a1 proteins was determined at 15, 20, 30, 40 and 60 days post fertilization (dpf) either by means of immunohistochemistry in AB strain using specific zebrafish Cyp19a1a and Cyp19a1b antibodies or by measuring GFP expression driven by the zebrafish *cyp19a1a* or *cyp19a1b* promoters, *in vivo* on whole animals or on frozen sections of gonads of transgenic fish.

Results and Discussion

Cyp19a1 immuno-reactive cells were found in gonads of zebrafish as early as 15 dpf. In undifferentiated gonads (15-20 dpf), dense punctiform fluorescence labelings was observed for Cyp19a1b within the cytoplasm of cells resembling to gonocytes while for Cyp19a1a fluorescence was observed in the entire cytoplasm. At 30 dpf, immune-reactive cells were observed in presumptive testes and in ovary to testis transforming gonads. In these latter individuals, Cyp19a1 expression was localized in gonocytes and in primary oocytes. At 40 and 60 dpf, Cyp19a1a and Cyp19a1b expressing cells in ovary corresponded to peri-oocytes cells and cytoplasm of oocytes at various stages of development. In testis, expression of Cyp19a1 proteins was localized in germ cells and for Cyp19a1a expression was also found in Leydig cells.

To gain further information related to the quantitative expression of *cyp19a1* genes, novel *cyp19a1a*-GFP and *cyp19a1b*-GFP transgenic zebrafish lines were used. In these models GFP mimics the expression of the endogenous aromatase proteins. Preliminary results indicated that *cyp19a1a* is mostly expressed in ovaries than in testis while for *cyp19a1b* no obvious difference was noticed.

Conclusion

Altogether, this preliminary study provides new and relevant data on the localization and expression patterns of aromatases in zebrafish gonads during development. Moreover, *cyp19a1a*-GFP and *cyp19a1b*-GFP zebrafish lines have been characterized and will be useful tools to further study the role of Cyp19a1 in sexual differentiation.

AROMATASE INHIBITOR INDUCED SEX CHANGE IN THE TEMPERATE WRASSE *NOTOLABRUS CELIDOTUS*

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Introduction

The temperate reef fish *Notolabrus celidotus* is a small protogynous wrasse found in abundance around shallow coastal shores in New Zealand. Since these fish also adapt well to captivity, they provide a convenient model species in which to study the mechanisms of sex change across the brain pituitary gonad (BPG) axis. In this study, we set out to characterize the physiological events occurring at the lower end of the BPG axis. We chemically induced sex change in adult female *N. celidotus* in order to examine the changes of germ cell structure and their arrangement within the gonad.

Methods

Sixty female and six male adult *N. celidotus* were collected from the Tauranga Harbour in the Bay of Plenty, New Zealand and housed in 500L recirculating sea water tanks. Each tank contained 1 male and 10 female fish. The latter were implanted with either a sham or a slow release pellet containing 150 µg of the aromatase inhibitor (AI), Fadrozole. Fish were held at 16° C and sampled at 10 – 20 day intervals over a period of 90 days. Gonad tissue was fixed for standard histological processing and then analysed using a light microscope.

Results and Discussion

Evidence of oocyte atresia was clear in AI treated fish within 20 days. This was associated with an increase in spermatogonial germ cells and somatic tissue within the gonad. Approximately 20 days later few oocytes remained in the gonads of fish undergoing sex change. These transitional gonads were characterized by numerous cysts of spermatogenic germ cells located throughout the remnant ovarian lamellae. Fish with fully spermatogenic testes were evident at 90 days. The testes of these fish contained all stages of germ cell development from spermatogonia to spermatozoa, arranged in a clear lobular structure. These terminal stage gonads contained a remnant central lumen and had developed sperm collecting ducts around their periphery. In the course of this trial transitional gonads were only found in the AI treated fish. This indicates that interruption of estrogen production through inhibition of the aromatase enzyme is sufficient to initiate sex reversal in *N. celidotus*. Furthermore, once initiated, this process appears to continue through to complete gonadal restructure to produce a functional testis.

Conclusion

Gonadal restructure of *N. celidotus* follows a similar pattern to that of other protogynous wrasse. This starts with the degeneration of ovarian germ cells followed by male germ cell proliferation, and resulting in a testis with an organized cystic, lobular structure. This process can be functionally completed within 90 days.

ESTROGEN PRODUCED BY THE OVARY STIMULATES THE MENINGES TO EXPRESS A NOVEL HEME-BINDING PROTEIN GENE, *hebp3*, IN A FEMALE-BIASED MANNER IN MEDAKA

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Introduction

Phenotypic sex of teleosts can be manipulated by treatment with exogenous steroid hormones even after sexual maturation. Furthermore, a large number of teleost species spontaneously undergo phenotypic sex reversal in response to social and physiological events. These phenomena indicate that teleosts have a unique mechanism of brain sexual differentiation, which enable them to maintain marked sexual plasticity throughout life. However, very little is known about sexual differentiation of the teleost brain. We have been using medaka as a model species to understand the mode of sexual differentiation of the teleost brain. In this study, we screened for genes exhibiting sexually differentiated patterns of expression in the medaka brain. One of the genes identified in our screen as being preferentially expressed in female was a novel heme-binding protein 3 (*hebp3*).

Methods

We performed a subtractive hybridization between the male and female brain and identified *hebp3* as a female-biased gene. The spatial expression of *hebp3* in the brain was examined using *in situ* hybridization. To clarify the effects of genetic and hormonal factors on *hebp3* expression, real-time PCR was performed using the brain of sex-reversed fish (XX males and XY females) and gonadectomized fish with or without hormone replacement. Luciferase assay was also conducted to evaluate the ability of estrogen to directly stimulate the *hebp3* transcription and determine the cis-element mediating this process.

Results and Discussion

hebp3 expression was exclusively detected in the meninges, which is the membranes enveloping the brain, with higher levels in females; no expression was detected anywhere in the brain parenchyma. The levels of *hebp3* expression coincided with phenotypic sex, but not genetic sex, and estrogen produced by the ovary had a stimulatory effect on *hebp3* expression in a reversible manner. We also confirmed the expression of estrogen receptors in meninges and provided evidence that estrogen directly stimulates *hebp3* transcriptional activity through an estrogen-responsive element on the proximal upstream region of *hebp3*.

Conclusion

Female-predominant expression of *hebp3* in the meninges results from a direct stimulating action of ovarian estrogen. Meninges have traditionally been viewed as specialized membranes that protect the brain parenchyma and reabsorb cerebrospinal fluid. However, there is accumulating evidence that the meninges play critical roles in brain development by releasing several soluble factors. Because *hebp3* encodes a soluble form of the heme-binding protein and exhibits sexually dimorphic expression under the control of estrogen, it may be involved in sexual differentiation of the brain.

THE ESSENTIAL ROLE OF FIGLA IN THE OVARIAN DIFFERENTIATION IN TILAPIA WAS DEMONSTRATED BY TALENS

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Introduction

In mammals, FIGLA (*f*actor *i*n the *g*erm *l*ine, *a*lpha), encoding a germ cell-specific transcription factor, was a central regulator of oocyte-specific genes and played an important roles in folliculogenesis, fertilization, and early development. Mutation of *figla* in mice resulted in failure of primordial follicles formation, massive depletion of oocytes and female sterility. In black porgy (*Acanthopagrus schlegeli* Bleeker), *figla* might play a critical role in the sex-reserval from male to female by controling the transformation from Sertoli cell into follicle-like cells. However, no direct evidence was available of the function of *figla* in fish ovarian differentiation and maintainance. Therefore, we cloned *figla* cDNA from the ovary and designed efficient TALENs for *figla* gene targeting in Nile tilapia (*Oreochromis niloticus*) with the aim to figure out its true functions in fish.

Methods

Tialpias were kept in recirculating freshwater tanks at 26°C. Tissue distribution analysis by real-time PCR was carried out to check the expression of *figla* gene in various tissues of adult fish. Then, we selected and constructed specific TALEN targeting sites for *figla* gene. Finally, mRNAs of TALENs were synthesized by *in vitro* transcription and used for microinjection into mono-sex fertilized eggs. Positive targeting individual was screened by genomic PCR, endogenous restriction and subsequent sequencing. Finally, the morphological and molecular changes were investigated by H.E. staining, immunocytochemistry (IHC) and real-time PCR analysis.

Results and Discussion

In this study, the open reading frame of tilapia *figla* encoding 198 potential amino acids with typical basic helix-loop-helix structure in DNA binding domain was obtained from the ovary. Tissue distribution analysis revealed that tilapia *figla* was exclusively expressed in ovary, and it was barely detected in other tissues. Intriguingly, TALENs successfully targeted and disrupted the tilapia *figla* gene confirming by genomic PCR and sequencing. The induced indel mutations with maximum efficiencies up to 66% at the targeted loci were detected. Furthermore, deficiency of *figla* gene in XX fish led to partial sex reversal revealing by severe degeneration or complete loss of oocytes and ectopic proliferations of somatic cells in the ovary. Amazingly, it was shown by IHC that male specific marker genes of Sertoli cells (Dmrt1) and Leydig cells (Cyp11b2) in the testis were detected around the remanent oocytes in *figla* mutated XX gonads. However, very few Cyp19a1a-positive follicular cells were detected at the same areas. Real-time PCR analysis demonstrated consistent results with IHC.

Conclusion

In summary, our data indicated that mutation of *figla* led to the degeneration of oocytes and differentiation of prefollicle cells into steroli and leydig cells in XX fish. Therefore, we speculated that *figla* might play an essential role in the ovarian differentiation and maintenance in fish.

SEX DIFFERENTIATION AND HERMAPHRODITISM IN SHARP-SNOUT SEA BREAM, *DIPLODUS PUNTAZZO* IN CAPTIVITY

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Introduction

Sex differentiation in fishes is a labile process, depending on genetic and environmental factors, and hermaphroditism is very common. The aim of the present study was to acquire information on sex differentiation and sex strategy of sharpsnout seabream *Diplodus puntazzo* in captivity during the first three years of life. Such information is necessary for managing captive broodstocks in commercial aquaculture facilities, and ensuring optimal egg production.

Methods

A population of fish was studied for 2 years in order to describe sex differentiation and puberty. Growth and gonadal differentiation and development were examined every 50 days during the two years. Another population was studied for 3 years in order to monitor the sex strategy and hermaphroditism during consecutive reproductive seasons. Growth and gonadal development were examined in this population during the reproductive seasons at ages 1+, 2+ and 3+. The reproductive status of the gonads was examined through histological processing.

Results and Discussion

Gonadal differentiation in sharpsnout seabream commenced at 152 dph with the formation of the ovarian cavity. Male differentiation commenced later around 300 dph, when the first bisexual gonads were observed. Until the second year, fish were identified as either females (F) or bisexual. Bisexual fish were further classified as “mf” with gonads consisting of equal amounts of testicular and ovarian tissue, “Mf” with predominantly testicular or “mF” with predominantly ovarian tissue. Males (M) were observed for the first time after 2 years. Bisexual individuals were observed throughout the study, but in 3-year-old fish they were always of the Mf classification. The overall sex ratio of functional males (M and Mf classes): functional females (mF and F classes) were 1.1, 2.0 and 1.7 for 1+, 2+, and 3+ year class fish, respectively.

Conclusion

The data support the existence of rudimentary hermaphroditism in sharpsnout seabream. Bisexual Mf individuals develop into males, mF into females and mf are possibly the ones changing sex by the degeneration of the testicular and the development of the ovarian tissue. The presence of individuals exhibiting bisexual gonads with regressed testicular tissue indicates the occurrence of partial protandry. The study will continue until fish are 5+ year old, but so far it seems that the sex ratio of cultured sharpsnout seabream broodstock may stabilize after their third year of life.

APOPTOSIS AND ANTI-MÜLLERIAN HORMONE GENE EXPRESSION DURING GONADAL SEX DIFFERENTIATION IN PEJERREY *ODONTESTHES BONARIENSIS*

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Introduction

The pejerrey is a gonochorist teleost with strong temperature-dependent sex determination (TSD) and an excellent model to study the interactions of environment and genome during gonadal sex differentiation. Histological sex differentiation in pejerrey follows a cephalocaudal, left-to-right gradient and intense apoptosis in the anterior region of the right gonad is associated with testis differentiation. The sex-related genes *amha* and *amhy* are thought to play important roles in pejerrey sex differentiation but their expression profiles and relation to gonadal apoptosis are still unclear. The purpose of this study was to clarify the relations between gonadal apoptosis, expression of *amh* genes, and water temperature during gonadal sex differentiation in pejerrey.

Methods

A progeny from the cross between an $amhy^{-/-}$ female and an $amhy^{+/-}$ male was reared for 14 weeks at mixed-sex producing (25°C) and masculinizing (29°C) temperatures. Sampling was performed biweekly between 2 and 10 weeks after hatching (wah) for the analyses of apoptosis by TUNEL assay, *amh* mRNA expression by *in situ* hybridization (ISH) with a probe that recognizes both *amha* and *amhy*, and the onset of histological gonadal differentiation and sex ratios by light microscopic histology. The presence/absence of *amhy* gene was taken as an indication of genotypic sex.

Results and Discussion

Sex ratios at 25 and 29ºC were 64.4% and 100% male, respectively. Testicular differentiation started at 5 and 7 wah at 29 and 25ºC, respectively, whereas ovarian differentiation at 25ºC was first observed at 4 wah. Apoptosis was detected first in the anterior region and was largelly confined to the right gonads at 25°C; at 29°C both gonads had apoptosis but with less intensity in the left side. Apoptosis ocurred from 4 wah in most $amhy^{+/-}$ and part of the $amhy^{-/-}$ at 25ºC and from 2 wah in all animals at 29°C. *amh* mRNA transcripts were detected between 2 and 10 wah at both temperatures; they were clearly more abundant in the left gonads and generally absent from the anterior region of the right gonads. *amh* transcription was observed in all $amhy^{+/-}$ and 40% of $amhy^{-/-}$ fish at 25ºC and in all fish regardless of genotype at 29°C. Intense apoptosis in the right gonad and *amh* expression in both gonads correlated closely with testicular differentiation.

Conclusion

The results suggest that a process involving *amha* expression and apoptosis leads to testis formation in pejerrey and that either warm temperatures or *amhy* can trigger this process. Further, apoptosis might be implicated in temporarily excluding *amh*-expressing cells from the anterior region of the right gonad in accordance with the gradient of histological sex differentiation.

ROLE OF ALTERNATIVELY SPLICED ISOFORMS OF *IFNγ2* IN GONADAL DEVELOPMENT OF MEDAKA, *ORYZIAS LATIPES*

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Introduction

The reproductive system is unique as its primary role is to assure the continuity of the species, while the immune system provides internal protection and facilitates continued health and survival. Sex steroids are known to regulate the possible crosstalk between reproductive and immune systems. An important mechanism by which estrogen regulates the immune system is by altering the secretion and/or response to cytokines, mainly Interferon gamma (IFNγ). Sex biased *IFNγ* expression has been reported in many instances. Recently, sex specific expression of alternatively spliced isoforms has been reported in medaka. *In silico* genome analysis demonstrated the existence of alternative splicing in medaka *IFNγ2*. The present work was aimed to characterize the significance of the alternatively spliced isoforms of *IFNγ* in the gonadal sex development in medaka.

Methods

The QurtE strain of medaka was used for *IFNγ2* isolation and functional analysis. HEK-293 cells over-expressed with *IFNγ2* mini-genome construct was used to confirm the steroid-dependent induction of alternative splicing. Steroid treatment was carried out to determine the effects on alternative splicing *in vivo*. BRDU treatment was used to study the effects of estradiol-17β (E2) -induced *IFNγ2b* expression on gonadal cell division.

Results and Discussion

The IFNγ2a and 2b were found to be clustered with fish specific IFNγ. We found that *IFNγ2* possesses several potential steroid responsive element sites in the promoter. Moreover, *in vitro* analysis suggests that alternative splicing of *IFNγ2* is dependent on the availability of E2 or testosterone. Tissue distribution analysis depicted a ubiquitous expression of *IFNγ2a*, while *IFNγ2b* expression was gonad specific. Real time PCR also showed female-dominated expression of *IFNγ2b*. *In vivo* testosterone treatment induced *IFNγ2a* expression in females while E2 accelerated the *IFNγ2b* expression in male gonads, further confirming the steroid-dependent splicing. However, *ISH* data suggests that *IFNγ2b* is expressed exclusively in germ cells while *IFNγ2a* is expressed in all types of gonadal cells. We also found that *IFNγ2b* expression was specifically induced in the BRDU-positive cells. This suggests that *IFNγ2b* might be associated with germ cell maintenance which was confirmed by mRNA over-expression of *IFNγ2b* in the medaka embryos.

Conclusion

Although IFNγ is long known as a cytokine related gene, our data suggest that IFNγ also has potential effects on gonadal development, and that the new role of *IFNγ2b* is associated with steroid-dependent induction of alternative splicing in medaka. This study provides new insights into the study of gonadal development in teleosts.

TRANSDIFFERENTIATION OF DIFFERENTIATED OVARY AND GENE EXPRESSION PROFILES IN NILE TILAPIA DURING AROMATASE INHIBITOR INDUCED SEX REVERSAL

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Introduction

The sex of fish is affected by both genetic and environmental factors. For gonochoristic fish, sex reversal of fish with undifferentiated bipotential gonads could be achieved by drug administration before or during the narrow window of sex determination. Once the fish grow beyond the stage, artificially induced sex reversal is difficult or even impossible to accomplish. In the present study, our arm is to achieve the sex reversal of differentiated ovary of Nile tilapia to a functional testis, and to demonstrate the process of transdifferentiation of ovary and the molecular mechanism of sex reversal.

Methods

All-XX fish of 90 dah (days after hatching) were divided into three groups, female control group, treated group and rescued group. All-XY fish of 90 dah were set as male control group. Treated group fish were fed with a diet containing Fadrozole at a concentration of 200μg.g^{-1}diet; while female and male control group fish were fed with a normal diet. Rescued group fish were fed with a diet containing Fadrozole (200μg.g^{-1}diet) and 17β-estradiol (200μg.g^{-1}diet). The drug treatments were lasted for 90 days. Gonadal histology, immunochemistry, transcriptome and serum steroid level were analyzed during sex reversal.

Results and Discussion

The differentiated ovary of female was transdifferenciated into functional testis with fertile sperms, we named it as secondary sex reversal (SSR). The Fadrozole induced sex reversal could successfully rescued by supplement of exogenous 17β-estradiol. The spermatogonia in reversed gonads were transformed from oogonia or germ stem cell like cells distributed in germinal epithelium; while Leydig and Sertoli cells probably came from the interstitial cells and granulosa cells of the ovarian tissue, respectively. The transdifferentiation of somatic cells, as indicated by the appearance of Dmrt1 (pre-Sertoli cells) and Cyp11b2 (pre-Leydig cells) positive cells in the ovary, provided micro-niche for the transdifferentiation of germ cells. Decrease of serum 17β-estradiol was detected earlier than increase of serum 11-ketotestosterone, indicating decrease of estrogen was the cause while increase of androgen was the consequence of SSR. The sex reversed gonad displayed more similarity in morphology and histology with a testis while the global gene expression profiles remained closer to the female control. Detailed analysis indicated that transdifferentiation was driven by suppression of female pathway genes and activation of male pathway genes.

Conclusion

SSR provided a good model for study of sex reversal in teleosts, and for understanding of sex determination and differentiation in non-mammalian vertebrates. Our study suggests that a differentiated ovary can be transdifferentiated into a functional testis. Down-regulation of endogenous estrogen, which resulted in suppression of female pathway genes and activation of male pathway genes, is the prerequisite for the ovarian transdifferentiation. Up-regulation of endogenous androgen is the consequence of SSR in tilapia.

amh EXPRESSION DURING SEX DIFFERENTIATION OF THE SIBERIAN STURGEON, *ACIPENSER BAERII*

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Introduction

The anti-Müllerian hormone (Amh/amh) is produced by embryonic Sertoli cells from the onset of mammalian male sex differentiation and causes the regression of the Mullerian ducts. In fish, *amh* is expressed in somatic cells from the onset of sex differentiation but its role during male sex differentiation is unclear. Sturgeons are basal species in which sex differentiation is poorly studied at the molecular level. In order to determine if *amh* can be involved in gonad sex differentiation we amplified and characterized it in *A. baerii* and studied its expression before and after the sex differentiation period.

Methods

amh was amplified from immature testes (only gonias) of *A. baerii*, using primers designed to target conserved regions in fish. The nucleotide sequence was searched by blastX using the protein DB at NCBI in the Chordata phylum but not the Tetrapoda superclass, then in the Tetrapoda superclass only. Data was retrieved from Ensembl amh families (ENSFM00250000019125 and ENSFM00600000921621) in fish and tetrapods. Sequences were aligned using Muscle alignment software. Redundancies were removed, as well as sequences not aligned with the sturgeon sequence. ML tree was calculated with PhyML. The tissue expression of *amh* was studied in gills, kidney, liver, muscle, brain and gonads. The histological analysis and *amh* expression was studied in gonads sampled at 3, 4, 8, 9, 10, 11 and 14 months old.

Results and Discussion

The phylogenetic analysis suggests that there is no duplication of this gene, except the *amh-Y*, a paralogous gene found specifically in *O. hatcheri*. Overall, the phylogeny of this gene matches that of the species, the mammalian lineage being distinct from the other Sarcopterygii. The tissue expression showed that *amh* is well expressed in brain, gonads and kidney. During gonadal development, the *amh* showed a bi-modal expression. A very high expression level has been found in gonads of fish at undifferentiated stage (3 and 4 months old). Afterwards, when the gonad differentiation starts (8 and 9 months old), a huge decrease was observed (6000 fold). A new increase has been detected at 10 months old. During the differentiated period (>8 months old) the gonads showed a significant ($p<0.05$) difference in *amh* expression between male and female. The expression increased 3,6 to 20 fold in males 9 and 10 months old when compared to females of the same ages. This sex dimorphic expression is lost in fish at 11 and 14 month old.

Conclusion

In summary, we amplified and characterized the *amh* for the first time in any sturgeon, and the results suggest that the male pathway is preceded by an increase in *amh* as it was described in teleost fish, with a possible implication in the regulation of post-differentiated gonads.

COMPARATIVE TRANSCRIPTOMIC ANALYSES OF GENE EXPRESSION PROFILE IN SOUTHERN CATFISH (*SILURUS MERIDIONALIS*) GONAD

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Introduction

Southern catfish (*Silurus meridionalis*) is an important commercial fish endemic to China. The sex ratio of the feral catfish was 1:1, while the fry obtained by artificial propagation were all female. In our previous study, we demonstrated that estrogens paly decisive role in the ovarian differentiation of the Southern catfish though the mechanism remained elusive. In order to elucidate this phenomenon and the roles of sex steroids in southern catfish sex differentiation and maintenance, transcriptome analyses of XX and XY juvenile gonads (1 year) were performed using Illumina Hiseq^{TM} technology.

Methods

Sequence reads pools were generated on the Illumina HiSeq^{TM} 2000 instrument of Beijing Genomics Institute at Shenzhen (BGI Shenzhen, China) from 8 cDNA libraries, respectively. After filtering dirty raw reads, short reads assembling programs–SOAPdenovo and Trinity were used for assembly of the clean reads. BLASTX searches of the assembled unigenes were performed against NCBI non-redundant (NR), Swiss-prot and KEGG protein databases with a cut off E-value of 1e-5. "FDR≤10-3 and |log2(XX_RPKM/XY_RPKM)|≥1" was used to identify sex-biased unigenes.

Results and Discussion

Totally, 77846558 and 77030102 clean reads were obtained from XX, XY gonad, respectively. The reads assembled into 91778 unigenes with an average length of 689 bp, of which, 64744 in XX, 88101 in XY gonad. There are 49086 (54.3%) unigenes having matches to known protein sequences in NR database with E-values 1e-5. More unigenes were found to be expressed in XY gonad compared with XX gonad. Similarly, 49551 and 7953 unigenes were found to be XY- and XX-gonad dominant, respectively, while 62957 unigenes co-expressed in both XX and XY gonads. Gene ontology and annotation analysis suggested that many of these sex-biased unigenes were involved in steroid synthesis, reproductive process, vitellogenesis/spermatogenesis, and possibly sex determination and maintenance.

Conclusion

The present study will allow better understanding on the steroidogenic pathway and regulation mechanism, provide basic data for sex determination and differentiation in catfish and help to elucidate the all female phenomenon by artificial propagation.

RSPO1/β-CATENIN SIGNALING PATHWAY INVOLVING IN THE OVIARIAN DIFFERENTIATION IN A TELEOST, TILAPIA (*OREOCHROMIS NILOTICUS*)

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Introduction

In mammalian species, R-spondin1 (Rspo1) can activate canonical Wnt signaling and β-catenin-dependent gene expression, which plays a key role in the female sex determination/differentiation. However, the role of Rspo1/Wnt/ β-catenin signaling pathway in the fish gonads is largely unknown.

Methods

The tilapias for this study were reared in large tanks with a re-circulating aerated freshwater system (26 ºC). To elucidate the molecular mechanism of female sex determination/differentiation, we carried out the molecular cloning and expression analysis of *Rspo1* and *β-catenin* of Nile tilapia (*Oreochromis niloticus*). Finally, the TALENs targeting *Rspo1* and *β-catenin* were designed to investigate the potential roles of Rspo1/Wnt/β-catenin pathway in ovarian differentiation.

Results and Discussion

In our present study, *Rspo1* and two *β-catenin* (*β-cat-1, -2*) were cloned from the gonad of tilapia (*Oreochromis niloticus*). Phylogenetic analysis showed that tilapia β-cat-1 and -2 were specifically clustered into distinct clads. Tissue distribution analysis revealed that tilapia *Rspo1* was ubiquitously expressed in all examined tissues, while *β-cat-1* and *-2* were expressed in brain, pituitary, gill, heart, kidney, intestine and gonads. In the gonads, the expression levels of *Rspo1* and *β-cat-1* display sexual dimorphism with higher expression level in the ovary. *In situ* hybridization demonstrated that *Rspo1* was expressed in the oogonia and early vitellogenic oocytes in the ovary, spermatogonia and spermatocytes in the testis. Immunohistochemistry revealed that β-cat-1 and -2 was expressed in the germ cell and somatic cells at 10 dah, and it mainly was found in the nuclear of oocytes and follicular cells at 70, 90, 120 dah ovary. Ontogenetic analysis revealed that both *β-cat-1* and *-2* were significantly up-regulated at 10dah, peaked at 30dah in female gonad. However, both were barely detectable in male gonads. Gene knockout by TALEN showed the deficiency of *Rspo1*, *β-cat-1*, *β-cat-2* caused partial sex reversal revealing by severe degeneration of oocytes and ectopic proliferations of somatic cells in the ovary.

Conclusion

Our present data indicated that fish Rspo1 signaling pathway might play key role the ovarian differentiation and maintenance.

EXPRESSION PROFILE OF *amhy* and *amha* GENES AT HIGH AND LOW TEMPERATURES DURING EARLY LARVAL DEVELOPMENT IN PEJERREY *ODONTESTHES BONARIENSIS*

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Introduction

Sex determination in pejerrey *Odontesthes bonariensis* is characterized by a very strong temperature dependence (TSD). However, a homologue of the *O. hatcheri* Y-linked anti-Müllerian hormone gene *amhy*, a testis determinant in that species, has been found also in pejerrey and shows a correlation with maleness at intermediate temperatures. In this study, we investigated the effects of temperature on the expression of *amhy* and the autosomal *amh* (*amha*) during the sex determination/differentiation period to evaluate their relationship with TSD and testicular formation in this species.

Methods

The expression of *amhy*/*amha* was analyzed in the progeny from an $amhy^{+/-}$ male and $amhy^{-/-}$ female that were reared at 17, 25, or 29ºC (female-, mixed-sex-, and male-promoting temperatures, respectively) until 10 weeks after hatching (wah). The sampling covered the estimated period of thermolabile sex determination in pejerrey. Larvae were sampled weekly and fins and trunks were used for *amhy*-based genotyping and real-time PCR expression analyses of *amhy*/*amha*, respectively. The remaining larvae from each temperature were sampled at 14 wah for the determination of sex ratios by light microscopy.

Results and Discussion

Analysis of the phenotypic sex and genotype at 14 wah showed that all $amhy^{-/-}$ fish (n=38) at 17ºC were females whereas the $amhy^{+/-}$ were either males or females (n=16 and 11, respectively). At 25ºC, all $amhy^{+/-}$ fish developed as males (n=33) while the $amhy^{-/-}$ were either females or males (n=21 and 13, respectively). At 29ºC all larvae developed as males regardless of *amhy* genotype. *amhy* expression showed a transient peak at 2 wah at 17ºC and at 1 wah at both 25 and 29ºC. *amha* expression in $amhy^{+/-}$ fish was low from 1 wah to 10 wah at 17ºC whereas at 25 and 29ºC it was up-regulated between 3 and 8 wah. All $amhy^{-/-}$ individuals at 17ºC had low *amha* expression throughout the experiment whereas at 29ºC a notable increase was observed between 3 and 9 wah. Fish at 25ºC showed low *amha* expression until 3 wah and thereafter an approximately bimodal distribution of values resembling either those at 29ºC or 17ºC. Overall, *amhy* expression levels seemed to be temperature-independent whereas those of *amha* clearly increase with increasing temperature. These results suggest that *amhy* is a genetic testis determinant in pejerrey, in spite of its sexual thermolability, and that *amha* is more directly involved in testicular differentiation.

Conclusion

This study demonstrated that both *amh* genes might be involved in the sex determination process of pejerrey but likely with different roles. Thus, *amhy* probably acts as a trigger, although more or less subservient to water temperature, whereas the temperature-modulated *amha* likely directs histological differentiation of the testis.

GERM CELL TRANSPLANTATION FROM THE JUNDIÁ CATFISH (*RHAMDIA QUELEN*) INTO THE NILE-TILAPIA (*OREOCHROMIS NILOTICUS*)

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Introduction

Spermatogonial stem cell (SSCs) transplantation has proved to be the most fascinating technique for investigating spermatogenesis and stem cell biology. Besides that, this technique presents several important potential applications such as for instance the production of transgenic animals, assisted reproductive technologies in aquaculture, and the preservation of endangered species. Because all the necessary approaches important for syngeneic SSCs transplantation in adult tilapia were successfully developed in our laboratory, resulting in donor spermatogenesis and fertile sperm, the establishment of xenogeneic germ cell transplantation using spermatogonia from commercially valuable fish would be of great interest. In the present study we investigated the viability of adult Nile tilapia as a recipient for xenogeneic spermatogonial transplantation, using donor cells from Jundiá (*Rhamdia quelen*) that belongs to a different taxonomic order.

Methods

Ten young sexually mature male tilapia (*Oreochromis niloticus*) had their endogenous spermatogenesis depleted with the chemotherapeutic drug busulfan (Sigma, MO, USA) (18 and 15mg/kg/BW) associated with the temperature of 35°C. These fish, received through the common spermatic duct the donor catfish germ cells labeled with PKH26. The presence and development of catfish germ cells were investigated in the Nile tilapia seminiferous tubules at several different time periods (weeks) post-transplantation.

Results and Discussion

Couple weeks post-transplantation, the fluorescence microscopy analyses showed the presence of several PKH26 labeled spermatogenic cysts in different phases of development in the tilapia seminiferous epithelium. These important preliminary results suggest that tilapia Sertoli cells were able to functionally interact with the catfish *Rhamdia quelen* germ cell during the first steps of spermatogenesis. We are currently evaluating additional samples in order to verify if functional sperm from *Rhamdia quelen* will eventually be formed the Nile tilapia testes.

Conclusion

Different from mammals and corroborating other results found in our laboratory, these preliminary results confirm that the Nile tilapia testis microenvironment is very plastic. These findings provide a new scenario for fish bioengineering and for the preservation of germplasm from endangered species or those carrying commercially valuable traits.

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METHODICAL STANDARDIZATION OF MOTILITY ASSESSMENT AND SHORT-TERM STORAGE IN EURASIAN PERCH (*PERCA FLUVIATILIS*)

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Introduction

Eurasian perch (*Perca fluviatilis*) is a freshwater species with increasing interest in European aquaculture. There is a limited knowledge available about the quality assesments and short-term storage of perch sperm. Development of these techniques could support and improve production of perch in aquaculture.

Methods

Perch males were injected with 500 IU kg^{-1} of hCG. Sperm was collected from 10 anesthetized males using catheter. In Experiment 1, sperm prediluted in 3 different extenders [„Grayling extender" (GE), modified Tanaka extender (TE) and modified Lahnsteiner's immobilizing solution (LI)] was stored for up to 6 hours at 4 ºC and motility was measured with CASA following activation with 50 mmol/l NaCl (common perch activating solution, CA) every 2 hours. In Experiment 2.1, motility of sperm diluted in LI was measured following activation with 4 different solutions [CA, modified Lahnsteiner's activating solution (LA), activating medium for cyprinids (AS) and Woynarovich-solution (WS)] at 10, 30, 60, 90, and 120 seconds after activation. In Experiment 2.2, motility of prediluted sperm was measured 10 and 20 seconds after activation. In Experiment 3, undiluted sperm was stored for up to 6 hours at 4 ºC and motility was measured every hour.

Results and Discussion

In Experiment 1 sperm diluted in LI showed higher motility at 0, 2, 4 and 6 h of storage than sperm diluted in TE and GE (Fig. 1). Motility showed lower decrease in case of LI during 6 hours storage than in TE or GE. In Experiment 2.1 sperm activated with AS (78±11%), LA (75±16%) and WS (76±13%) showed higher motility at 10 seconds after activation than with CA (68±16%). At 30 seconds after activation motility decreased with every activating solutions below 5%. In Experiment 2.2 highest motility was measured at 20 seconds with LA (74±24%). Motility showed lower decrease in case of at 20 seconds after activation than in the case of AS, CA and WS. In Experiment 3 motility significantly decreased after 2 hours of storage. From 2 to 6 hours the reduction of motility was less apparent.

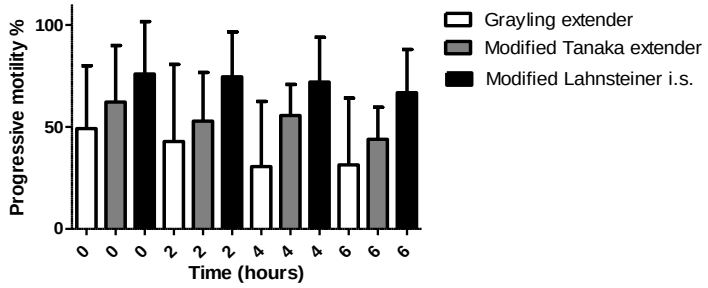

Fig. 1 Motility of Eurasian perch sperm activated following 2, 4 or 6 hours of storage in 3 different extenders.

Conclusion

LI succesfully prevented sperm from activation for up to 6 hours. LA is recommended to be used for motility assesment compared to other frequently used activating solutions. These results correspond with earlier findings that increase of NaCl concentration in the activating solution prolongs spermatozoa movement. Perch sperm is able to move without significant reduction for 20 seconds post activation. Undiluted sperm of perch is sensitive to short-term storage. Perch sperm is recommended to be prediluted with immobilizing medium during short-term storage.

MOTILITY PARAMETERS OF CRYOPRESERVED SPERM FROM SEX-REVERSED DUSKY GROUPER *EPINEPHELUS MARGINATUS* EVALUATED BY COMPUTER-ASSISTED SPERM ANALYSIS (CASA)

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Introduction

The dusky grouper, *Epinephelus marginatus***,** is a vulnerable species of high economic value. *E. marginatus* is a protogynous hermaphrodite teleost characteristic of rocky bottoms. In many tropical and temperate regions, overfishing and environmental degradation have been depleting wild dusky grouper populations and this species has been included in the Red List of Threatened Species of the International Union for the Conservation of Nature (IUCN) since 1996. Our aim was to evaluate the post-thaw quality of cryopreserved sperm of *E. marginatus* using the Computer-Assisted Sperm Analysis (CASA).

Methods

Five fish received an aromatase inhibitor (AI; letrozole) dose of 100 mg/kg intraperitoneally to promote sexual change. Spermiation was induced administrating a single dose (200UI/kg) of human chorionic gonadotropin (hCG, Ovidrel, Serono) and after 24 hours, the sperm volume (µL) from each male was measured using micropipette. Sperm were cryopreserved in 0.5ml straws in a 1:4 dilution (sperm: extender with 10% dimethylsulfoxide (DMSO). The open source ImageJ software and the plugin CASA (Computer-Assisted Sperm Analysis) was used to determine the sperm parameters in thawed samples. In the fresh sperm, the motility rate was evaluated by an analytical manual method. Fresh sperm analysis was performed activating 1 µl of sperm in the Neubauer chamber (cell mL^{-1}) with 30 µl of seawater; the post-thaw analysis was performed activating 10 µl of sperm + cryoprotectant, also in the Neubauer chamber (cell mL^{-1}) with 300 µl of seawater. For each sperm sample analyzed, the following CASA parameters were considered: Motility rate (MOT), curvilinear velocity (VCL), average path velocity (VAP), straight line velocity (VSL), Straightness (STR) = (VSL/VCL x 100%), and number of spermatozoa analyzed (NSPZ).

Results and Discussion

In our preliminary results of the sperm volume collected ranged from 20 to 500 µl. In the fresh sperm, MOT was 85-90%, while in the post-thawing, sperm MOT was 40-50%, both evaluated by the analytical manual methods. The post-thaw sperm parameters (mean ± standard deviation), evaluated by CASA were: MOT= 25.30 ± 5.09%, VAP= 39.90 ± 9.24 µm/s, VCL = 78.69±7.96 µm/s, VSL = 37.05 ± 8.82 µm/s, STR= 92.69 ± 0.96 %, NSPZ= 153± 60.

Conclusion

Our preliminary results show that CASA reduces inter-observer variability and improves accuracy compared to the analytical manual methods. The development of methods that improve the characterization of sperm quality without inter-observer variability is required and would contribute to dusky grouper reproduction in captivity.

GENETIC VARIANTS IN THE *aqp1ab* GENE ARE ASSOCIATED WITH EGG QUALITY IN THE GILTHEAD SEABREAM (*SPARUS AURATA*)

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Introduction

In the aquaculture of marine pelagic teleosts, egg buoyancy is the most widely used phenotypic marker of egg quality. The buoyancy of pelagic eggs and early embryos is acquired by the accumulation of lipids and water, the latter resulting from a massive hydration of the oocyte occurring during meiotic maturation. This process is mediated by the aquaporin-1ab (*aqp1ab*) gene which is tightly regulated both at the transcriptional and post-translational level during oogenesis. Given the essential requirement for the timely expression, trafficking and function of the Aqp1ab water channel during oocyte hydration, we hypothesized that dysfunctions in the Aqp1ab pathway might underlie poor egg quality in cultured marine teleosts. To test this hypothesis, in the present study we investigated whether allelic variants of *aqp1ab* may be associated with egg buoyancy in gilthead seabream (*Sparus aurata*) reared in captivity.

Methods

During the natural spawning season, eggs were collected during two consecutive days from one tank containing 8 functional (spermiating) males and 8 females raised in captivity. Buoyant and sinking eggs were recovered separately, and samples from each group containing 90-95% embryos at the 4-18-cell stage and 5-10% embryos at later stages (blastula, gastrula and neurula) were used for mRNA and protein extraction. For each sample, the full-length Aqp1ab cDNA was amplified by PCR and sequenced, and Western blot was carried out using a seabream Aqp1ab specific antibody. Wild-type *aqp1ab* and allelic variants identified in buoyant and sinking eggs were functionally characterized by ectopic expression in *Xenopus laevis* oocytes.

Results and Discussion

The *aqp1ab* mRNA levels in buoyant and sinking eggs were similar, but non-phosphorylated and phosphorylated Aqp1ab polypeptides appeared to be reduced in sinking eggs. Sequence analysis of 115 different clones indicated the expression of 14% and 43% of *aqp1ab* allelic variants, showing one or two non-synonymous single-nucleotide polymorphisms (SNPs), in buoyant and sinking eggs, respectively. The mutations were more abundant in the transmembrane helices and connecting loops of the encoded protein, whereas a few mutations were detected in the C terminus. Expression of the 28 different alleles identified in *X. laevis* oocytes showed that 43% and 100% of the variants from buoyant and sinking eggs, respectively, encoded channels with lower permeability than the wild-type due to partial or complete retention and/or degradation of the channel in the oocyte cytoplasm.

Conclusion

These data demonstrate that broodstock seabream raised in captivity can carry aberrant alleles in the *aqp1ab* gene that can modify channel trafficking. Our results suggest that *aqp1ab*-based polymorphisms may be employed as novel molecular markers of egg quality in marine teleosts.

SEX DISCRIMINATION IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) USING GENETIC MARKERS

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Introduction

Identification of phenotypic sex in rainbow trout is only possible in sexually mature fish during the spawning season. For producing slaughter stock, late maturing females are preferred because the earlier maturing males tend to turn restless, stop feeding and undergo deterioration of meat quality once they have reached puberty. There is thus a demand for a practicable means of identifying genetic sex in rainbow trout, preferably at an early stage of development. There are few markers reported to be associated with genetic sex in rainbow trout. The present study addresses the suitability of two genetic markers (*OmyP9 Rsa*I and *sdY*) for identifying sex in various genetically divergent rainbow trout strains.

Methods

Genomic DNA was extracted from blood samples or fin clips collected from 25 sexually mature phenotypic males and females from each of seven genetically distinct rainbow trout strains originating from various parts of Europe that are being maintained at the Rainbow Trout Breeding Unit of Goettingen University. Polymerase chain reaction was applied to study *sdY* and *OmyP9* markers. With the aid of *Rsa*I restriction analysis of *OmyP9* sequence five different variants (AA, BB, AB, AC and BC) were identified.

Results and Discussion

In 4 of 7 strains studied, fish possessing the restriction variant A of the *OmyP9* sequence were almost exclusively (96-100%) of male sex. In 4 out of 7 strains, 84 to 100% of the fish lacking variant A were females. In the remaining strains this was true for 44 to 76% of the fish. Using the *sdY* marker, in 3 strains 100 % males and females were correctly identified, whereas in the other 4 strains, it was correct in 96% of fish. The differences among various strains reflect the genetic divergence encountered in cultivated rainbow trout. In many situations geographical isolation, restricted exchange of brood stock and artificial reproduction has led to a multitude of isolated strains. It is known that the lack of heteromorphic sex chromosomes in some strains of rainbow trout is not unusual.

Conclusion

It may be concluded that, although both markers investigated are associated with sex of rainbow trout, the *sdY* marker was found to be more reliable. This marker may serve as a valuable asset for identifying genetic sex without having to wait for the fish to reach sexual maturity. It may also be useful to clarify situations where phenotypic and genetic sex does not necessarily agree which occurs naturally or might have been brought about by biotechnological intervention.

CHILLED STORAGE OF EYED RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) EGGS IN SEALED POLYETHYLENE (PE) BAGS

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Introduction

Fertilized rainbow trout eggs are commonly shipped in the eyed stage. Moulded polyurethane foam trays are filled with eyed eggs wrapped in wet cheesecloth and kept cool by packaging between trays of ice to keep the eggs cool and moist. They are transported in shipping cases with perforations at the top for air supply. Duration of shipment should not exceed 48 h. Recently it has been shown that unfertilized rainbow trout eggs sealed in PE bags with no air space or oxygen supply may be stored in a chilled state for extensive periods of time. The present investigation was an attempt to apply this method to eyed eggs.

Methods

Fertilized rainbow trout eggs pooled from several ripe females were incubated at 10±1°C to the eyed stage. Eyed eggs were filled into PE bags, 7×3 cm or 7×8 cm in size. The smaller bags contained 50 eggs and 30 mL water; larger size bags contained 100 eggs and 60 mL water. Bags were sealed and stored at 2±1°C for 1, 2, 4 or 6 days. Thereafter, eggs were returned to the incubator where they remained until hatching. Control eggs underwent the same handling but were returned to the incubator without a storage period. The experiment was conducted with 5 replications. Differences in the proportion of eggs hatching were assessed using one-way ANOVA followed by Scheffé-test ($P<0.05$).

Results and Discussion

Relative to the controls (90±0.8%), hatching rates after storage in smaller sized PE bags for 1, 2 and 4 days were 100±0.1%, 98±1.6% and 94±2.3%, respectively; in the larger bags the respective values were 98±0.4%, 96±0.7% and 95±2.3%. After 6 days of storage a moderate but significant decline to 77±9.1% in small bags and 82+3.1% in large bags was recorded. From preliminary experiments it is known that survival of eyed eggs sealed in 7×3 cm bags for 10 or 20 days was very low. Bag size and, correspondently, difference in egg number, had no effect on hatching rate ($P>0.05$). This makes sense since, when filled with eggs, different sized bags were equally thick (1.5 cm), which, according to earlier findings, is relevant for CO_2 disposal and, thus, stability of pH.

Conclusion

Chilled storage of eyed rainbow trout eggs sealed in PE bags for 4 days, with slight reservation even 6 days, is a viable alternative to conventional means. The method is convenient and space-saving without the necessity for the provision of an air space or oxygen supply and no risk of spillage or dehydration.

GERM CELL-SPECIFIC EXCISION OF THE *LOXP*-FLANKED TRANSGENE IN RAINBOW TROUT: TOWARD THE ESTABLISHMENT OF CELL ABLATION METHOD IN FARMED FISH

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Introduction

The escape of highly domesticated cage-reared salmon into the natural environment could potentially result in a decrease in wild salmon populations through ecological competition. In addition, introgressive hybridization between these farmed salmon and their wild counterparts would also cause genetic pollution. Since farming with infertile salmon could resolve these problems, we are planning to ablate germ cells by using a toxin gene. Although toxin genes driven by germ cell specific-promoters can be microinjected into one-cell-stage embryos to render the developing fish infertile, mass production of such embryos is not be possible as infertile fish cannot produce offspring. Therefore, we focused on conditional ablation method of germ cells using Cre/*loxP*. However, there have been no reports regarding Cre/*loxP* used in aquaculture species. In this study, we aim to examine the feasibility of Cre/*loxP* in germ cells of rainbow trout.

Methods

We established a stable transgenic fish line Tg (*vasa-cre*) carrying the *cre* gene driven by the *vasa* promoter. We also established a stable reporter transgenic line, Tg (*heat shock cognate71:loxP-DsRed-loxP-EGFP* [*hsc:LRLG*]), which carried the transgene consist of a ubiquitous promoter, the *DsRed* gene flanked by two *loxP* sites, and the *EGFP* gene. The double transgenic fish (wTg) produced by crossing the male Tg (*vasa-cre*) with the female Tg (*hsc:LRLG*) are expected to induce excision of the *DsRed* gene and expression of the *EGFP* gene specifically in the germ cells. We then performed PCR, fluorescence, and immunohistochemistry assay to clarify the effect of excision of the germ-cell-specific *DsRed* gene in wTg. To analyze the frequency of the germ cells lacking the *DsRed* gene, we crossed a wTg male with a wild type female and examined the fluorescence phenotypes of the F1 progeny.

Results and Discussion

The results of PCR analysis indicated that the *loxP*-flanked gene was only excised in the gonads of wTg fish. Although no GFP-positive gonadal somatic cells were observed, some GFP-positive cells were observed in the germ cells of wTg gonads, indicating that activity of the Cre/*loxP* system was specific to germ cells. To overcome the low excision efficiency of the *DsRed* gene in wTg germ cells (≤3.97%), we established another wTg line that we derived from the new Tg (*vasa-cre*) line. Although an extremely low level of *DsRed* gene excision was observed in some somatic tissues of wTg, excision was primarily restricted to germ cells with high efficiency (≤89.25%).

Conclusion

We demonstrated *in vivo* excision of the *loxP*-flanked sequence specifically in germ cells of rainbow trout. In order to advance infertile rearing technology, we are attempting to establish a new *cre* line with improved excision efficiency and specificity.

SEMINAL PLASMA AFFECTS POST-THAW SPERM MOTILITY AND CURVILINEAR VELOCITY BUT NOT MEMBRANE INTEGRITY OF *PROCHILODUS LINEATUS* (PISCES, CHARACIFORMES)

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Introduction

Prochilodus lineatus is a native fish species to South America and has been used as a model species for research in a number of studies addressing genetic diversity, health, nutrition and reproduction. Cryopreservation of fish sperm is an important tool to optimize reproduction. However, this process reduces post-thaw sperm quality, as cells are exposed to toxic substances, oxidative stress, osmotic shock and intracellular freezing. Seminal plasma possesses proteins and antioxidants that protect cells from cryoinjuries. The aim of this study was to evaluate the protective effects of seminal plasma by comparing post-thaw quality between semen and spermatozoa (after centrifugation) of *P. lineatus*.

Methods

Semen from *P. lineatus* (n=9) was collected after hormone injection. One aliquot of each sample was diluted in 327 mOsm/Kg glucose and methylglycol, loaded into duplicate 0.25 mL straws and frozen in a nitrogen vapor vessel. The other aliquot was centrifuged and seminal plasma was removed. Sperm pellets were diluted in the same freezing medium and frozen. Straws were thawed in a water bath at 60°C for 3 s and sperm motility and curvilinear velocity (VCL) were determined using a Computer-Assisted Sperm Analyzer (SCA™, Microptics). The percentage of spermatozoa with intact membrane following eosin-nigrosin staining and sperm concentration inside each straw were also calculated.

Results and Discussion

The concentration inside the straws was 2.8 x 10^9 spermatozoa/ml for frozen semen samples and 0.9 x 10^9 spermatozoa/ml for frozen spermatozoa samples. No difference on membrane integrity was observed between semen (89% intact) and spermatozoa (81% intact). Motility and VCL were always higher in semen samples (61% motility and 184 µm/s of VCL) compared to spermatozoa (37% motility and 135 µm/s of VCL). Seminal plasma plays an important role on the sperm protection against cryoinjuries. Although lower motility and VCL were observed on spermatozoa frozen without seminal plasma, the percentage of sperm with intact membrane was not affected. These findings suggest that the absence of seminal plasma affects mainly the spermatozoa structures related to motility.

Conclusion

Seminal plasma protects *P. lineatus* sperm during cryopreservation. Further studies to better understand the seminal plasma cryoprotective effects on P. *lineatus* sperm are necessary.

PARENTAGE ASSIGNMENT AND RELATIVE FEMALE/MALE PARTICIPATION IN THE SPAWNING PERIOD AND DIFFERENTIAL MORTALITY DURING REARING IN RED PORGY, *PAGRUS PAGRUS* AND COMMON PANDORA, *PAGELLUS ERYTHRINUS*).

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Introduction

The pedigree information is an important knowledge for the aquaculture industry and modern selective breeding programmes. In the current study, the objective was to use genetic markers to determine the relative female-male breeders' participation to daily spawning, as well as to investigate whether there is a temporal differential mortality in the offspring, in two sparid species, the red porgy *Pagrus pagrus* and the common Pandora *Pagellus erythrinus*. These fishes have a high commercial value and have been considered as candidates for aquaculture diversification in the Mediterranean area.

Methods

Broodstocks of red porgy and common Pandora were established with 12 females and 5 males, and 4 females and 3 males, respectively. Eggs from daily spawning were collected and evaluated for fertilization success, and were stored in EtOH for later DNA extraction. Spawning kinetics were assessed based on polymorphic microsatellite markers previously developed in other sparid species (multiplexes of 11 loci in red porgy and 8 loci in common Pandora). For red porgy, 438 eggs coming from 9 spawning events equally covering the end of the spawning season were genotyped; for the grow-out period, 273 larvae (at the flexion stage) from 3 mesocosms and 189 fish (at ~3gr) were genotyped. For common Pandora, 463 eggs were genotyped so far from 5 spawning events in the first month, since fish have just recently ceased spawning in IMBBC's installations. The two microsatellite multiplexes proved to be functional and efficient for parentage assignment and allowed to unambiguously allocate more than 90% of the progeny to a single parental pair. Genotyping data were analyzed and interpreted using FAP, exclusion-based software developed by J. Taggart (2007).

Results and Discussion

Egg fecundity and fertilization success was variable during the reproductive season for both red porgy and common Pandora. In red porgy, only one (M03) monopolized spawning. Similarly, in common Pandora only one male (M06) is the male-parent in over 98% of the offspring. The results were also indicating that for both species not all females contributed to the next generation. In red porgy, 10 out of 12 females seemed to spawn and two of them were by far the most successful (F12 and F15 with 23 and 50%, respectively). In common Pandora, all females participated with two of them contributing marginally (<1%) and the other two having the biggest contribution. For the red porgy grow-out, there was no evidence of differential mortality through time at the tail flexion stage or the 3-gr fish, but instead genotyping uncovers contributing fish not seen at the egg sampling.

Conclusion

Due to obvious monopolization of only one male and the unequal participation of females in both species in the breeding season studied, we concluded that for future breeding programmes in the species, in which a great number of families is needed, methods controlling and achieving equal contribution, such as the use of exogenous reproductive hormones or "manual striping", have to be applied.

Acknowledgements
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APPLICATION OF RECOMBINANT DNA AND ORAL DELIVERY TECHNOLOGIES TO MANIPULATE FISH REPRODUCTION IN AQUACULTURE

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Introduction

Current techniques in manipulating reproduction of captive fish usually involve handling that can cause undue stress. Our on-going project aims to develop non-invasive methods for manipulating the reproductive process by oral delivery of key hormones and peptides either as an expression construct or in synthetic form. The specific developmental stages that we are targeting are onset of puberty, sex inversion, final oocyte maturation and spawning. We intend to apply the technology in two species that are late maturing and considerably huge in size at maturity: (1) giant grouper (*Epinephelus lanceolatus*), a protogynous hermaphrodite; and (2) Southern Bluefin Tuna (*Thunnus maccoyii*). We will also utilise the technique to advance the onset of puberty in fish that have been selected for certain traits, such as growth in Yellowtail Kingfish (*Seriola lalandi*).

Methods

We are generating recombinant hormones (tuna GnRH and LH; kingfish FSH) using the *Pichia pastoris* expression system. We are testing liposome and gelatin-based diet as oral delivery systems of recombinant hormones as well as synthetic peptides. Since we are targeting female fish, vitellogenin and oestradiol immunoassays are the main assessment methods.

Results and Discussion

We have generated a construct of the tuna GnRH in pPIC3.5 vector, which allows intracellular expression in *P. pastoris* with the intention of using the yeast itself as part of the diet. We have also generated two single chain tuna LH expression constructs (linked either by carboxy terminal peptide or glycine). We have produced a single chain recombinant kingfish FSH. Western blot analysis of the deglycosylated glycoprotein showed the 23 kDa band, which is the expected size of our recombinant kingfish FSH. We have tested the efficiency of incorporating a GnRHa analog in liposome and observed minimal loss of the peptide. *In vivo* trials will determine the optimal method/s of orally delivering recombinant hormones or synthetic peptides.

Conclusion

Our work in progress addresses the challenge of preventing handling stress when administering hormones or peptides for reproductive manipulation of captive broodstock, particularly those that are late maturing and considerable in size at maturity.

SPERM QUALITY AFTER DILUTION AND FREEZING PROCESSES IN *PROCHILODUS LINEATUS* AND *BRYCON ORBIGNYANUS* (CHARACIFORMES)

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Introduction

The cryopreservation of fish sperm provides a tool by which reproduction is optimized and larval production is increased, thereby improving breeding and fish conservation programs. However, the cryopreservation process reduces post-thaw sperm quality, as cells are exposed to toxic substances (cryoprotectants), osmotic shock and intracellular freezing. The aim of this study was to evaluate the loss of sperm quality during handling and freezing processes by comparing the quality among fresh, pre-freezing and post-thaw sperm of *Prochilodus lineatus* and *Brycon orbignyanus*.

Methods

Sperm from *P. lineatus* (n=21) and *B. orbignyanus* (n=18) was collected after hormone injection, diluted in a freezing medium (5.2% glucose and methylglycol for *P. lineatus*, and 5% BTS™ and methylglycol for *B. orbignyanus*), loaded into straws and frozen in nitrogen vapour vessel (dry-shipper). Fresh and sperm diluted in the freezing medium (pre-freezing sperm) were evaluated for subjective motility rate and motility quality score ranging from 0 (no movement) to 5 (rapidly swimming sperm) from 0 to 60 sec post-activation. Post-thaw sperm was evaluated for motility rate and curvilinear velocity (VCL) using a Computer-Assisted Sperm Analyzer (SCA™, Microptics) from 10 to 70 sec post-activation.

Results and Discussion

Motility rate of *P. lineatus* sperm was similar between fresh and pre-freezing sperm, and decreased little after thawing. Motility significantly decreased after 60 sec of activation in both fresh (from 99 to 84%; from score 4.9 to 3.6) and pre-freezing sperm (from 92 to 73%; from score 4.3 to 2.9), and after only 20 sec in frozen samples (from 73 to 54%; VCL from 277 to 126 $\mu m.s^{-1}$). In our laboratory, high post-thaw sperm quality is defined by motility rate above 60% and VCL above 140 $\mu m.s^{-1}$. Thus the methodology described above is suitable for the cryopreservation of *P. lineatus* sperm. Motility rate of *B. orbignyanus* sperm significantly decreased from fresh to pre-freezing sperm, and then dropped after thawing. Motility significantly decreased after 60 sec of activation in both fresh (from 99 to 88%; from score 4.9 to 3.7) and pre-freezing sperm (from 81 to 66%; from score 3.9 to 2.6), and after only 20 sec in frozen samples (from 42 to 25%; VCL from 160 to 101 $\mu m.s^{-1}$). Although this freezing medium has been tested with better results compared to other media, it seems inadequate for *B. orbignyanus* as a decrease on sperm quality was observed after dilution, even before freezing.

Conclusion

The methodology described above is efficient and maintain good post-thaw sperm quality in *P. lineatus*; however, other freezing media should be tested in *B. orbignyanus* sperm as motility decreased after dilution, even before freezing. Fertilization of oocytes using frozen sperm should be carried out with greater attention as sperm quality reduces significantly fast after thawing.

CAN CORTICAL REACTION BE A COMMERCIALLY APPLICABLE EGG QUALITY INDICATOR IN FRESHWATER CYPRINIDS?

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Introduction

Egg quality determination is one of the main challenges in aquaculture. However, there are still scarce information about the methods allowing objective evaluation of egg quality prior to fertilization, although it could significantly facilitate commercial production and scientific research, especially, where selective breeding, genome manipulation or fertilization of eggs with cryopreserved sperm is planned. Up to date, there is no data related to the estimation of egg quality in cyprinids.

It was recently suggested that the intensity of cortical reaction was an useful method for estimation of egg quality. Therefore, an effort was made in this study on the investigation of this phenomenon, as a potential egg quality indicator, in a model cyprinid species.

Methods

Eggs obtained from 15 females of goldfish (*Carassius auratus*) were used in this study. Each egg sample (three samples from each female) was exposed for 7.5 min to a distilled water and dried immediately afterwards with compressed-air. Next, egg samples were placed in test tubes, tightly closed and stored at 4°C until further analysis. This allowed the exposure of eggs to water only for 7.5 min (the time chosen according to the set of initial experiments). The samples were then homogenized and centrifuged (2500 g for 10 min). Next, supernatant was collected with a pipette, placed in eppendorf tubes and stored at -80°C prior to further analysis. Osmolality (Vapro 5600, Wescor), pH and calcium (Catalyst Dx™) content were analysed in each sample. Additionally, just after stripping, egg samples (n=3 from each female) were *in vitro* fertilized in Petri dishes. Embryonic survival was determined at eyed stage. The correlation of the embryonic survival with the osmolality, pH and calcium was performed.

Results and Discussion

Recorded osmolality, pH and calcium values ranged between 119-49 mOsm kg^{-1}, 6.29-6.48 and 0-0.54 mmol L^{-1}, respectively. Results have shown a strong negative correlation ($r < -0.7$) between the embryonic survival rate and osmolality as well as calcium content. Whenever the osmolality was below 100 mOsm kg^{-1} and calcium below 0.3 the survival rate of embryos was above 80%. This suggest that the cortical reaction (after the contact of eggs with water) has proceeded more intensively in the higher quality eggs. This resulted in higher water influx into previtelline space during the initial phase of swelling process. A similar phenomenon was reported previously for pikeperch, however the observations were based on morphological features.

Conclusion

The obtained results indicates that the measurement of the cortical reaction intensity, as described in this study, may be the first objective indicator of the egg quality in cyprinid species.

IN VITRO EFFECTS OF RECOMBINANT GONADOTROPINS ON GONAD MATURATION IN PRE-PUBERTAL ATLANTIC BLUEFIN TUNA THUNNUS THYNNUS (L.)

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Introduction

In some farmed fish species the attainment of puberty requires several years with remarkable costs and risks since potential broodstock has to be maintained for prolonged time until maturation. Thus, the development of species-specific techniques to advance/induce puberty control in farmed fishes are often necessary. Atlantic Bluefin tuna *Thunnus thynnus* (BFT) is a high commercial value pelagic fish candidate to aquaculture, whose age at first maturity in the wild is around 4-5 years. The objective of the present study was to produce BFT recombinant FSH and LH (*rbft*-FSH and *rbft*-LH) and evaluate their effect on the *in vitro* developmental potential of gonad samples derived from pre-pubertal individuals reared in captivity.

Methods

Atlantic bluefin tuna FSHβ and LHβ were sequenced and *rbft*-FSH and *rbft*-LH were produced in the methylotrophic yeast, *Pichia pastoris*. Juveniles BFT were caught in the Mediterranean Sea, reared for one year in the north Adriatic Sea and sampled in July 2009 and June 2010, when they were 3 and 4 years old. After fish death, testis and ovary samples were rapidly removed and small pieces (average of 100±5 mg/piece) were preincubated using culture plate containing 3 ml of ice-cold incubation medium. In order to eliminate endogenous steroids, three consecutive washes were performed and then the gonadal fragments were challenged (16h; 18°C) with fresh ice-cold medium containing *rbft*-FSH (50 and 500 ng ml^{-1}) or *rbft*-LH (25 and 250 ng ml^{-1}). The samples were then fixed in 10% buffered formalin and embedded in paraffin, sectioned and stained with haematoxylin-eosin. Proliferating male germ cells were immunolocalized with monoclonal anti PCNA (proliferating cell nuclear antigen) antibodies.

Results and Discussion

In testis samples, the presence of all the spermatogenetic stages as well as of a limited amount of luminal spermatozoa was observed. Testis samples treated with *rbft*-FSH and *rbft*-LH at all doses showed seminiferous lobules significantly larger than controls. A significant increase of testis surface occupied by proliferating germ cells was found in samples treated with the highest *rbft*-LH concentration. In ovary samples, only oocytes at perinucleolar stage were observed. Treatment with both *rbft*-FSH doses resulted in a significant increase of oocyte diameter.

Conclusion

The present study showed positive effects of *rbft*-FSH and *rbft*-LH on testicular and ovarian development in pre-pubertal BFT, thus laying the bases for a hormone-based therapy to advance puberty in captive-reared juveniles.

HYPOTHALAMIC AND PITUITARY FACTORS CONTROLLING REPRODUCTION IN ATLANTIC SALMON

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Introduction

In mammals, the KissS/Gpr54 system is pivotal for integrating internal (nutrition/growth and gonadal status) and external input (photoperiod cues, behavior) relevant for the initiation of puberty: Kiss/Gpr54 activates the hypothalamic GnRH system to stimulate pituitary gonadotropin release. The Kiss/Gpr54 system had not been characterized in Atlantic salmon, and the pituitary relevant Gnrh receptor (Gnrhr) form still has to be found. This study (part of the European Union-funded project LIFECYCLE, no. FP7-222719) aimed to characterize these factors in Atlantic salmon in the context of different feeding and photoperiod treatments that modulate puberty.

Methods

Atlantic salmon brain and pituitary RNA was extracted and converted into cDNA, and 3'- or 5'-RACE ready cDNA, and full-length versions of the cDNA sequences for the various isoforms of Kiss, Gpr54 and Gnrhr were obtained. To pharmacologically characterize these Gpr54 and Gnrhr proteins, the relevant constructs were co-transfected into HEK293T cells with a luciferase-reporter gene able to detect increases in intracellular IP_3 levels. Transfected cells were stimulated with increasing concentrations of various molecular forms of Kiss and Gnrh and luciferase activity was measured.

Results/Discussion

Two different cDNAs (*kiss2a* and *kiss2b*) were cloned, while no expressed *kiss1* homologs were identified, consistent with findings in rainbow trout. Similarly, two distinct cDNAs both coding for type 2 Gpr54 proteins were cloned and pharmacologically characterized: both responded well to Kiss2 peptides ($8.1<pEC_{50}<9.0$), but less well to the Kiss1 peptides, human Kiss and zebrafish Kiss1 ($6.8<pEC_{50}<7.8$). Altogether our observations suggest that only the Kiss2 receptor pathway may be functional in salmon. Finally, four *gnrhr* cDNAs (*gnrhr1a*, *gnrhr1b*, *gnrhr2* and *gnrhr4*) have been identified, of which *gnrhr1a* and *gnrhr1b* represent duplicated isoforms. Pharmacologically, all four receptors responded similarly to Gnrh2 ($7.9<pEC_{50}<8.9$). However, Gnrhr4 responded ~100 fold stronger ($7.6<pEC_{50}<9.5$) to various Gnrh's (excluding Gnrh2) than Gnrhr1a ($5.5<pEC_{50}<6.2$), Gnrhr1b ($5.1<pEC_{50}<7.0$) and Gnrhr2 ($6.7<pEC_{50}<7.1$). Moreover, Gnrhr4 showed highest homology to, and the molecular motifs specific of, the pituitary-relevant form, as identified in coho salmon and rainbow trout. Only *gnrhr4* gene expression was significantly up-regulated in pituitary during pubertal development.

Conclusion. The key players controlling reproduction in Atlantic salmon have been cloned and their functional relevance was identified based on their pharmacology, *in situ* hybridization and gene expression profile.

LOOKING CLOSER TO THE ORGANIZATION OF AROMATASE-POSITIVE RADIAL GLIAL CELLS IN THE BRAIN OF ZEBRAFISH

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Introduction

An astonishing feature of the brain of fish is the massive expression of the cyp19a1b gene, encoding aromatase B, in radial glial cells (RGC), a unique cell type of the astroglial lineage. Such cells are actually progenitors capable of generating new neurons in both developing and adult brain. Most of the information on radial glia comes from studies in mammals in which the majority of RGC disappear at birth to become star shaped-astrocytes. Astrocytes in mammals carry major functions and establish special relationships between them, with synapses, brain blood vessels and the meninx. In this study we aimed at providing more information on the morphology of RGC and their relationships with other brain elements using immunohistochemistry and confocal microscopy.

Methods

Both wildtype and tg(*cyp19a1b*-GFP) adult zebrafish were used in this study. Brains were fixed and cut using a vibratome (100 microns) or a cryostat (14 microns). SYTOX Orange or DAPI were used for nuclei labeling and rabbit anti-beta-catenin for immunohistochemical staining of adherens junctions. Confocal Olympus BX61WI and Zeiss AxioImager Z1 were used for high-resolution image acquisition. Pictures were treated using the image processing software ImageJ.

Results and Discussion

As shown before, *cyp19a1b*-GFP is exclusively localized in RGCs of the whole zebrafish brain, but expression was stronger and most abundant in the telencephalon, preoptic and mediobasal hypothalamic areas. GFP-expressing RGC send a proximal process towards the ventricle and these processes form a continuous barrier along the ventricles. Moreover, we find that beta-catenin is expressed in cells lining the ventricles suggesting that adherens junctions connect RGCs. The long distal processes of RGC project to the meninx and also appear to form a continuous barrier around the brain periphery. However, according to our 3D analyses *cyp19a1b*-GFP positive RGC processes also surround a large majority of blood vessels in the brain parenchyma. Large blood vessels and small capillaries are also consistently surrounded by beta-cathenin positive cells, suggesting that both *cyp19a1b*-RGC and endothelial cells participate in the establishment of the blood-brain-barrier (BBB) in the brain of teleosts.

Conclusions

The present results show that the forebrain parenchyma is virtually entirely surrounded by *cyp19a1b*-GFP positive RGC end-feet lining the brain ventricles, the blood-vessels and the meninx. Furthermore, adherens junctions are observed along all brain ventricles and most blood vessels with the possible exception of the ventral diencephalon. This suggests that aromatase-positive RGC in fish play a role equivalent to astrocytes in other vertebrates in providing biochemical support to endothelial cells and participating to the establishment of the BBB. The role of estrogens in these mechanisms remains open to speculation

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ISOLATION OF THE α- AND ß-SUBUNIT GENES OF FOLLICLE-STIMULATING HORMONE (FSH) OF THE GIANT AMAZONIAN FISH PIRARUCU (*ARAPAIMA GIGAS*)

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Introduction

Arapaima gigas (pirarucu) is one of the largest freshwater fish of the world reaching 2m of length and weighing up to 250kg. It lives mainly in the Amazon basin, is very important for the alimentation and commercial breeding of the region, being considered an overexploited and endangered species. For better studying its reproductive physiology and improve its breeding we cloned for the first time the α- and ß-subunit of *Arapaima gigas* follicle-stimulating hormone (ag-FSH), for subsequent synthesis, purification and characterization.

Methods

Pituitary glands were removed immediately following decapitation of sexually mature *A.gigas* and frozen in liquid nitrogen or stored in RNALater^{R}. Total RNA was extracted using a specific purification kit, primers were designed first with basis on conserved regions of several FSH fish sequences. Once obtained by RT-PCR a partial sequence of the gene, with basis on specific sequences of *A.gigas,* the complete cDNA sequence was obtained by rapid amplification of cDNA ends (RACE). The complete amino acid sequence of the hormone subunits was determined and the alignment of the mature peptide sequence with those of known species of fish, carried out by using appropriate softwares. The phylogenetic tree and related analyses were also conducted by using appropriate computer programs based on the Neighbor-Joining method and rooted with 3 *Acipenseriformes* species as the outgroup.

Results and Discussion

Considering that the α-subunit gene sequence, common also to luteinizing (LH) and thyroid-stimulation (TSH) hormones, had already been obtained in previous work, this project was dedicated to the isolation of the ß-subunit cDNA of ag-FSH. After having confirmed the same sequence in at least three different hypophysis, we concluded that ag-FSH ß-subunit cDNA sequence is 917 bp in total length and has an open reading frame of 381 bp beginning with the first ATG codon at position 79 bp, the stop codon (TAA) at position 437 and a 450 bp 3'UTR. The coding region translates into a peptide of 126 amino acids, while the cleavage site for the putative signal peptide was found between amino acid 18 and 19. The phylogenetic tree, carried out by using n=76 FSH ß-subunit peptide sequences, placed A.gigas as the most basal species of all analyzed teleosts.

Conclusion

The ag-FSH ß-subunit gene was repeatedly sequenced and confirmed. Together with the α-subunit gene, already defined in previous work, we can now construct expression vectors and proceed to hormone biosynthesis. A phylogenetic study was also carried out.

CLONING AND CHARACTERIZATION OF BRAIN AND OVARIAN CYTOCHROME P450 AROMATASE GENES IN THE CATFISH *HETEROPNEUSTES FOSSILIS*: SEX, TISSUE AND TEMPORAL VARIATIONS IN GENE EXPRESSION

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Introduction

Estrogens are female sex hormones involved in sexual differentiation, ovarian growth and development, and reproductive behaviour. They are synthesized from androgens through aromatization catalyzed by an aromatase enzyme complex comprising of a cytochrome P450 reductase and a monooxygenase (cytochrome P450 aromatase, P450arom). P450 aromatase gene has been cloned in vertebrates from fish to mammals. A single gene has been isolated in amphibians, reptiles, birds, humans, mammals, rats and mice. However, two or more genes have been cloned in pigs and peccaries. Among fishes, while a single gene has been characterized in cartilaginous fishes, and Japanese and European eels, two forms have been distinguished in many species including catfishes. These are designated as the ovarian (*cyp19a*) and brain (*cyp19b*) types. *Heteropneustes fossilis* (Order: Cypriniformes; Family Heteropneustidae or Saccobranchidae), the lone member of the family is an economically important edible catfish and its flesh is rich in protein and poor in fat content and esteemed for its invigorating qualities. In the present study, *cyp19a* and *cyp19b* were isolated and sex, tissue and temporal expression patterns of these genes were evaluated.

Methods

For cloning, adult female *H. fossilis* (40–50 g) were collected and acclimatized. Brain and ovary were used for total RNA extraction, cDNA synthesis through degenerate PCR and RACE using standard protocols. The full length sequences were analyzed *in silico* for determining homology and phylogenetic relationships. For gene expression studies, tissue samples from both male and female fish in different reproductive phases (resting, preparatory, prespawning and spawning) were used for semi-quantitative PCR using gene-specific primers. The PCR products were separated on 2% agarose gel stained with ethidium bromide. Images of the gel were scanned with a Typhoon FLA 7000 and the signal intensity was quantified with Image Quant TL Plus. Catfish β-actin gene was used as the control.

Results and Discussion

Full length cDNAs 2,006 bp and 1,913 bp were isolated, respectively, from the brain and ovary (GenBank No: JN399998 and KF164286). The ovarian type (*hfcyp19a)* has an ORF of 1,575 bp and the brain type (*hfcyp19b*) has an ORF of 1,488 bp. The ORFs encoded a protein of 496 (brain) and 525 (ovary) amino acid residues. Both genes showed differential expressions in tissues with reference to sex and reproductive stage, and brain regional differences.

Conclusion

In the catfish two distinct types aromatase genes were identified, implying its inclusion in the teleost taxon with two forms. The differential expressions of the genes suggest different functional roles.

DEVELOPMENT OF A HOMOLOGOUS ENZYME-LINKED IMMUNOSORBENT ASSAY FOR SENEGALESE SOLE FSH USING A RECOMBINANT CHIMERIC GONADOTROPIN

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Introduction

The Senegalese sole (*Solea senegalensis*) is a highly valued flatfish in Southern European aquaculture, but the industrial production of this species is still impaired due to poor knowledge of its reproductive endocrinology. The main endocrine regulators of fish gametogenesis are the pituitary gonadotropins (GTHs), follicle-stimulating hormone (Fsh) and luteinizing hormone (Lh), which are composed by a specific beta chain (Fshb, Lhb) and a common alpha chain (Cga). Although cDNAs encoding both GTHs have been isolated in some flatfishes, no specific assay has been developed in these species to measure their physiological levels. Since immunoassays for GTH dimer proteins may be more sensitive than those using isolated subunits, in this study we developed a specific enzyme-linked immunosorbent assay (ELISA) for Senegalese sole Fsh using a chimeric protein containing homologous Fshb coupled with chicken (*Gallus gallus*) Cga.

Methods

The methylotrophic yeast *Pichia pastoris* was used to produce a chimeric recombinant single-chain Fsh containing the coding sequence of the sole mature Fshb (SsFshb), followed by six His residues, the carboxyl-terminal peptide sequence of human chorionic gonadotropin b subunit as a linker, and the mature sequence of the chicken Cga (GgCga). Homologous recombinant single-chains of Fsh (SsFsh) and Lh (SsLh) were produced in CHO cells. Polyclonal antibodies against the chimeric construct were generated in rabbits, and specific SsFshb antibodies were further purified by affinity chromatography on GgCga-bound columns. Preadsorbed and GgCga adsorbed antibodies were tested in ELISA using SsFshb-GgCga for coating and standard curve generation.

Results and Discussion

ELISA using the preadsorbed antiserum showed a crossreactivity with GgCGa and SsLh of 46.0% and 10.1%, respectively, which was respectively reduced to 0.5% and 0.05% after purification. Using the purified antiserum, the ELISA standard curves for SsFshb-GgCga paralleled those of serially diluted plasma and pituitary extracts, as well as that of SsFsh. The sensitivity of the assay for Fsh was <10 pg/ml, and the intra- and inter-assay coefficients of variation were respectively 4.2% and 3.9 %, respectively. The use of this ELISA assay for monitoring the plasma levels of Fsh during the reproductive cycle in Senegalese sole and in other flatfish species is being assessed.

Conclusion

The development of an ELISA using a chimeric recombinant hormone proved to be highly sensitive and allowed specific and accurate measurements of Senegalese sole Fsh plasma levels. This approach may be useful to develop Fsh immunoassays for other teleosts.

THE DEVELOPMENT OF THE KISSPEPTIN SYSTEM IN ZEBRAFISH BRAIN

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Introduction

The important role of kisspeptin in the regulation of the hypothalamic-pituitary-gonadal (HPG) axis has been extensively investigated since its discovery in 2003. How kisspeptin regulates sexual maturation and fertility is of great interest and yet to be fully revealed, particularly in fish that possess two forms of kisspeptin. Our research focuses on the development of the kisspeptin system and the dynamics of interaction between the kisspeptin and the GnRH systems in the context of reproduction.

Methods

Our animal model is the zebrafish, which is amenable to genetic manipulation such as transgenesis, genes knockout and ablation of specific neurons. Transgenic fish lines expressing fluorescent protein in kisspeptin neurons are currently being generated from recombinant bacterial artificial chromosomes (BAC) kisspeptin clones. These lines will provide accurate spatio-temporal organization of the kisspeptin systems. Kisspeptin knockout is carried out by utilizing two recent technologies, CRISPR and TALEN, which were found to be efficient in inducing targeted genetic modifications in zebrafish. Conditional ablation of kisspeptin neuronal system is conducted using transgenic fish co-expressing the suicidal enzyme nitroreductase, which converts the pro-drug into a cytotoxin, specifically in kisspeptin neurons.

Results and Discussion

(1) Transgenic lines: The DNA sequence encoding green fluorescent protein (GFP) and enzyme nitroreductase were inserted into the BAC plasmid of kiss1 and kiss2 in order to facilitate co-expression under the control of the kiss promoters. The two constructs were microinjected into one-cell stage zebrafish embryos and the progeny of founder (F0) fish are being screened for GFP-positive embryos. Whole mount in situ hybridization analysis and GFP expression examination in injected embryos show a bilateral expression of kiss1 in the olfactory region and kiss2 in the hypothalamus during the first 7 days of development. These lines will be crossed with our existing GnRH2 and GnRH3 transgenic lines to obtain double transgenic lines. This will enable the identification and visualization of the kisspeptin expressing neurons in the brain and characterization of the dynamic interactions between the kisspeptin and GnRH neuronal systems in correlation with specific reproductive stages. (2) Gene knockout: The kiss2 TALEN constructs were microinjected into embryos and sequencing analysis on F1 confirmed mutations in the kiss2 coding region. F2 homozygous line is currently being generated.

Conclusion

At early stage of development, kiss1 is expressed in the olfactory region, unlike what is known in the adult fish, where it is observed exclusively in the habenula. This initial expression pattern may indicate a dynamic kiss1 system, like GnRH3. More information about the expression patterns of both kisspeptin forms and their roles is expected soon.

Gpha, Fshb AND Lhb GONADOTROPIN SUBUNITS IN THE SOUTH AMERICAN FISH *ASTYANAX ALTIPARANAE* (TELEOSTEI, CHARACIFORMES): MOLECULAR CHARACTERIZATION AND THEIR PHYOLOGENETIC RELATIONSHIPS

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Introduction

Fish gonadotropins (FSH and LH) play a crucial role on the regulation of the HPG axis. They are heterodimers formed by two distinct subunits. Glycoprotein a-subunit (Gpha) has identical amino acid (aa) sequences for both hormones, while the b-subunit (Fshb or LHb) is structurally different and responsible for specific biological functions. This is the first study to isolate, clone and characterize the full- cDNA for *agph*, *fshb* and *lhb* subunits in a Characiform species, *Astyanax altiparanae*, a South American freshwater teleost that presents ecological and economic importance for Brazilian Aquaculture.

Methods

Pituitaries from adult females of *A. altiparanae* were collected and destined to total RNA extraction, followed by cDNA synthesis and amplification of a partial cDNA for *gpha, fshb* and *lhb* subunits using degenerated primers. Specific primers were designed and used for RACE PCR. The obtained sequences were cloned and sequenced, and its homology was confirmed by alignment with other sequences in GenBank. Several *in silico* analysis were developed. Multiple alignment and phylogenetic analysis were performed with the aa sequences from several species available on GenBank.

Results and Discussion

The full-cDNA enconding *fshb*, *lhb* and *gpha* of *A. altiparanae* consists of 609, 638 and 744 nucleotides respectively. The deduced sequence for Gpha results in a mature protein with 95 aa that has highly conserved 10 cysteine (cys) residues and two potencial N-glycosylation sites. The Fshb mature sequence has 116 aa and 13 cys as other Ostariophysi species, but only one putative N-glycosylation site in the N-terminal region. Lhb mature sequence is composed by 117 aa with 10 cys and a single N-glycosylation fully conserved in teleost and mammals. *In silico* analysis revealed that all subunits display several putative O- glycosylation sites, especially in Fshb sequence. All gonadotropin subunits in *A. altiparanae* share the highest degree of aa identity with Siluriformes, followed by Cypriniformes and Anguiliformes species. FSHb shares the lowest aa identity as compared to other subunits, corroborating the evidences that FSHb subunit has the fastest diversification during teleost evolution. Phylogenetic trees for Agph, Fshb and LHb displayed a similar topology, corroborating the results of aa identity and the current phylogenetic hypotheses for Ostariophysi.

Conclusion

The full-cDNA sequences of *gpha, fshb* and *lhb* in *A. altiparanae* were cloned and their deduced aa sequences were analyzed. These sequences are now available for further studies related to endocrine control of reproduction in this species and for biotechnological approaches

KISSPEPTINS IN THE BRAIN OF THE EUROPEAN SEA BASS

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Introduction

Kisspeptins are considered the key players in the neuroendocrine control of puberty and reproduction in mammals. Kiss genes, as well as kiss receptor genes, are present in most classes of vertebrates and derived from an ancestral gene that underwent gene duplication followed by gene losses. In most fish species two kiss genes (kiss1 and kiss2) and two kiss receptor genes (kiss1r and kiss2r) are identified but the organization and the functions of these duplicated systems are still poorly understood.

Whit Withthe aim to investigate whether both kiss genes are involved in the neuroendocrine control of reproduction in fish we have characterized the neuroanatomical organization of both systems in zebrafish (*Danio rerio*, Cyprinodontiform), and the European sea bass (*Dicentrarchus labrax*), a highly evolved Perciform fish intensively farmed in Southern Europe. We also have investigated whether Kiss neurons have any special relationships with the GnRH neurons and express estrogen receptors. Finally, the nature of the very abundant cells expressing kiss2r was studied using combination of in situ hybridization and immunohistochemistry.

The main kiss1 expression site is the habenula, whereas kiss2 mRNA is mostly observed at the lateral recess in both species. No evident sexual dimorphism was observed but an additional kiss1 expressing cells population was reported into the mediobasal hypothalamus in sea bass during the spawning period. In both teleosts, few kiss2 cells of the preoptic region could make synaptic contact with the hypophysiotrophic GnRH neurons. In both species, the kiss population sensible to estrogens is the one placed into the ventral hypothalamus corresponding to a kiss1 cell population in sea bass and to kiss2 expression site in zebrafish.

Altogether our findings suggest that *kiss2* in zebrafish and *kiss1* gene in sea bass would be more likely to participate in the regulation of reproduction through the mediobasal hypothalamus kiss population sensitive to estrogens.

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INHIBITORY EFFECT OF CADMIUM ON ESTROGEN SIGNALING IN ZEBRAFISH BRAIN AND PROTECTION BY OLIGOELEMENTS (ZINC)

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Introduction

The toxicity of the heavy metal cadmium (Cd) is well established. We have previously shown that Cd acts as an endocrine disrupting chemical targeting estrogen receptor (ER) by inhibiting ER DNA-binding activity. This study investigates the effects of Cd on the estrogen signaling in the zebrafish brain and to examine whether oligoelements such as zinc (Zn) could protect the inhibitory effect of Cd. To achieve this, the effects of estradiol (E2), Cd and Zn alone or in combination were analysed using i) wild and transgenic zebrafish embryos and ii) an ER-negative glial cell line expressing different zebrafish ER subtypes (esr1, esr2a, esr2b).

Methods

Wild and transgenic embryos tg(*cyp19a1b*-GFP) were maintained at 28°C and treated for 72hpf with E2, Cd and/or Zn. Immunohistochemistry for aromatase B and quantitative real-time PCR, were performed to study the expression of aromatase B gene (*cyp19a1b*) in wild zebrafish using the elongation factor (EF1) as a reference gene.

The glial cells, U251-MG, were maintained at 37°C in 5% CO_2 atmosphere in phenol red-free Dulbecco's Modified Eagle's Medium supplemented with 10% fetal calf serum and transfected with the three ER and the reporter gene (ERE-tk-luc). The luciferase activity was assayed 48 hours later using β-galactosidase activity to normalize transfection efficiency in all the experiments.

Results and discussion

Both assays, the immunohistochemistry and GFP expression in transgenic larvae, demonstrated that the brain aromatase B is well expressed in radial glial cells and upregulated by E2 in the treated animals. Cd treatment (E2+Cd) clearly inhibited this stimulatory effect of E2. Additionally, the presence of Zn (E2+Cd+Zn) seems to restore the normal expression observed in the E2 treated group.

We confirmed these results by quantitative RT-PCR which showed that the expression of cyp19a1b was upregulated by E2 and strongly inhibited by Cd compared to E2 treated group. However, we did not observe the protection effect of Zn supplementary against the toxic effect of Cd.

Using transfection assays in U251-MG cells, we showed that the luciferase activity of the reporter gene was induced with E2 treatment for the three ER subtypes. After Cd exposure, our results showed an inhibition of the luciferase activity about 50% which was abolished by Zn supplement.

Conclusion

Our results showed that Cd act as a potent anti-estrogen *in vivo* and *in vitro*. Importantly, we provide evidences that Cd inhibits the estrogen action in the brain as demonstrated by inhibition of E2-induction of aromatase B. While, the precise mechanisms underlying the effects of Cd as an endocrine disrupting chemical remain to be elucidated, our data suggest that effects of Cd on estrogen signaling in the brain are, in part, due to interference with classical nuclear ER subtypes.

POSSIBLE INVOLVEMENT OF MALE-SPECIFIC, ANDROGEN-INDUCED HYPOTHALAMIC VASOTOCIN EXPRESSION IN AGGRESSIVE BEHAVIOR OF MEDAKA

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Introduction

Vertebrates, including teleost fish, exhibit sex differences in a variety of behavioral and physiological traits, including reproductive behavior, aggression, and endocrine secretion patterns. For example, males generally show more aggression than females. We recently found that vasotocin gene (*vt*), which has been implicated in aggression, is male-specifically expressed in the hypothalamus of medaka. Given that aggression is facilitated by androgens and that their nuclear receptors (androgen receptors; ARs) act as ligand-gated transcription factors, we hypothesized that the male-specific *vt* expression in the hypothalamus is under the control of androgens and mediates their stimulatory effects on aggression. Based on this hypothesis, in this study, we investigated the mechanisms underlying sex differences in the hypothalamic *vt* expression and its possible involvement in aggressive behavior in medaka.

Methods

The effects of gonadectomy and subsequent sex steroid treatments on the male-specific *vt* expression in the hypothalamus was evaluated by quantitative *in situ* hybridization. Expression of ARs (ARα and ARβ) in the male-specific hypothalamic *vt* neurons was examined by double *in situ* hybridization. Reporter assay was performed to assess the androgen responsiveness of the *vt* promoter region. Behavioral analyses were also carried out to evaluate the effects of androgen and vasotocin on each measure of aggressive behavior.

Results and Discussion

The male-specific *vt* expression in the hypothalamus was diminished by removal of the testis and recovered by subsequent treatment with androgen. Meanwhile, this expression could be induced even in females with intact ovaries, when they were treated with androgen. Evidence was also obtained that the male-specific hypothalamic *vt* neurons express ARβ and that androgen, upon binding to ARs, directly activate the transcription of *vt* via androgen-responsive elements in its promoter region. Behavioral analysis revealed that females, when treated with androgen, exhibited not only male-like *vt* expression in the hypothalamus but also male-like aggressive behavior. Females treated with vasotocin also showed male-like aggressive behavior.

Conclusion

Here we show that the male-specific *vt* expression in the hypothalamus arises from the direct transcriptional activation of *vt* by testicular androgens via ARβ and that administration with androgen and vasotocin induces aggressive behavior even in females. It thus seems likely that *vt* expressed male-specifically in the hypothalamus functions to promote aggressive behavior in males under the control of testicular androgens. Moreover, our data provide evidence that the hypothalamic expression of *vt* and aggressive behavior are both sexually plastic.

MOLECULAR IDENTIFICATION AND FUNCTIONAL CHARACTERIZATION OF THE GNIH/GNIHR SIGNALLING IN PROTOGYNOUS ORANGE-SPOTTED GROUPER, *EPINEPHELUS COIOIDES*

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Introduction

Evidences have shown that gonadotropin inhibitory hormone (GnIH) plays a negative role on the reproductive axis by inhibiting luteinizing hormone (LH) synthesis and release via binding to its receptor (GnIHR) in avian, mammalian, as well as several teleost species, however, controversy still exists. The biological significance of the GnIH/GnIHR signalling in hermaphroditic fish is totally unknown. To address this issue, gGnIH and gGnIHR genes have been cloned and functionally characterized in protogynous orange-spotted grouper, *Epinephelus coioides*.

Methods

Full-length cDNA sequences encoding gGnIH and gGnIHR were obtained by the 5'- and 3'-rapid amplification of cDNA ends (RACE) kit (Invitrogen). Peptides corresponding to gGnIH-I, II and III were synthesized by GL Biochem, Shanghai, China. Real-time PCR was performed on a Roche Light Cycler 480 real-time PCR System using the SYBR Green I Kit (Toyobo).

Results and Discussion

The cDNA encoding grouper GnIH (gGnIH) was 882 bp with an open reading frame (ORF) of 597 bp. The gGnIH precursor protein had 199 amino acids (aa) with an N-terminal putative signal peptide sequence of 21aa, and contained three putative LPXRFamide peptides (gGnIH-I, II, III). Phylogenetic analysis showed that gGnIH shared the highest similarity with medaka GnIH. The full-length of grouper GnIHR (gGnIHR) cDNA was 2016 bp with a 1449 bp ORF, encoding a protein of 483aa with an extracellular N-terminus, a seven transmembrane domain and a cytoplasmic C-terminus. Phylogenetic analysis indicated that gGnIHR was clustered into GPR147 clade with most closely related to those of medaka and Nile tilapia. The ligand-receptor interactions assay showed that both gGnIH-I and gGnIH-II could significantly decrease CRE promoter activity, while only gGnIH-I could significantly decrease SRE promoter activity in a dose-dependent manner in COS-7 cells transfected with gGnIHR. gGnIH was highly expressed in hypothalamus, while gGnIHR was widely distributed in all the tissues examined with relative high expression in testis, telencephalon and hypothalamus. Localization of gGnIH mRNA in the brain with *in situ* hybridization showed an intense expression only in the nucleus posterioris periventricularis (NPPv). Synthesized gGnIH peptide (100 nM) had no effects on the expression of both *LHb* and *FSHb* mRNA, but could significantly inhibit synthesis of both GnRH1 and LHRH-A-stimulated (100nM) *LHb in vitro*.

Conclusion

GnIH/GnIHR signalling was identified in a protogynous species, *Epinephelus coioides*, which might be involved in the regulation of reproduction through inhibiting GnRH-stimulated gonadotropin synthesis.

BRAIN IN THE ENDANGERED NEOTROPICAL CATFISH SPECIES – *STEINDACHNERIDION PARAHYBAE* (SILURIFORMES: PIMELODIDAE): MAIN NUCLEUS RELATED TO REPRODUCTION DURING ONTOGENY

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Introduction

The studies on reproductive endocrinology in fish have contributed to understanding the main factors that can influence the entire reproductive process either in both, captive and/or in wild environments. However, baseline studies enabling the basic morphological knowledge of the species are required for further investigations. In this sense, the present work aims to present a cytoarchitectonic study in the brain of the endangered Neotropical catfish species, *Steindachneridion parahybae*, describing the main nuclei related to the reproductive process.

Methods

Fertilized eggs of *S. parahybae* were obtained from artificially induced spawning at CESP (Companhia Energética do Estado de São Paulo) fish farm, and transferred to aquaria in a biotherium at USP (Universidade de São Paulo). During the first ten days after hatching (DAH), five animals were sampled; thereafter, five animals were sampled every three DAH, until the one hundreth DAH. The animals were anaesthetized with benzocaine and then fixed in Bouin's solution for 20 hours. Samples were dehydrated and embedded in Paraplast®. For the analyses of the main encephalic nucleus, serial transverse sections (10μm) were stained with cresyl-violet. Different cell groups were identified, following some criteria: density of cell bodies, staining intensity and pattern of distribution of cell bodies. For the analyses of the slices and documentation of the images, a computer system was used. The identification of the main nuclei was made based on studies related to the identification and localization of the GnRH (gonadotropin-releasing hormone) neurons in catfish.

Results and Discussion

The main encephalic nuclei in *S. parahybae,* which are directly related to reproduction, can be easily distinguished on the 40th DAH. These nuclei were predominant in: forebrain (telencephalon, olfactory bulbs, and diencephalon) and mesencephalon (midbrain tegument). The telencephalic nuclei were divided into dorsal and ventral, which may be still subdivided in: dorsal, ventral, lateral and central part of the dorsal and ventral telencephalon. The diencephalic nucleus was mainly divided into: gigantocellular part of the magnocellular preoptic nucleus (PMgc), anterior periventricular nucleus (NAPv), suprachiasmatic nucleus (NSC) and ventromedial thalamic nucleus (VM) (which are located in the periventricular region); and the inferior lobe of the hypothalamus (IL). Previous studies in *S. parahybae*, as well as in *Clarias gariepinus,* showed the presence of GnRH neurons in this region, which were identified in the midbrain tegument, preoptic area and in areas of the hypothalamus and inferior lobe of the hypothalamus. These last two regions are reported as the main regions related to reproduction in teleosts. The development of the pituitary occurred on the 40th DAH. Additionally, *S. parahybae* showed a well developed cerebellum compared to other teleosts, and a long medium olfactory tract.

Conclusion

The cytoarchitectonic pattern in *S. parahybae* is similar to that observed in other teleosts, except for a larger cerebellum and a longer medium olfactory tract, compared to some teleosts. Thus, this study is most important to understand the basic physiology of this endangered species.

EXPRESSION OF *KISS2* AND *KISS1R* GENES IN SEABREAM *(SPARUS AURATA)* BROODSTOCK AND ONTOGENY

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Introduction

Since its discovery in fish the kisspeptin system has been an area of great interest, potentially linking environmental cues to regulation of reproductive physiology as kiss genes are known to act on GnRH neurons and initiate reproduction. In fish two *Kiss* genes have been identified and are activated through G protein coupled receptor (*Kiss1r*). Oliveira et al previously demonstrated the initiation of the kiss system during the early ontogeny of seabream larvae. Expression of *Kiss2* and *Kiss1r* was significantly elevated from 5 days post hatch (DPH) and decreased after 20 and 30 DPH respectively coinciding with early ontogenetic events. This study expands upon these results. Firstly the ontogeny study investigates the expression of *Kiss2* and *Kiss1r* from post hatch (PH) to 140DPH whilst a second experiment determines expression patterns in seabream broodstock throughout the sex reversal process.

Methods

All fish were reared under standard aquaculture conditions. For the ontogeny study seabream were sampled from 0 to 140 DPH (0,15,30,45,60,75,90,105,120 and 140DPH). For the broodstock experiment brain and gonad tissue samples were collected from fish undergoing the sex reversal process at two sampling points in (October 2012 and in January 2013). Total RNA was extracted from all samples and qPCR was carried out to determine mRNA expression of *Kiss2* and *Kiss1r*.

Results and Discussion

Results for the ontogeny study demonstrate, for the first time, the continuation of kisspeptin expression beyond early larval ontogeny to 140 DPH. *Kiss2* and *Kiss 1r* mRNA was shown to be elevated at 60 and 90 DPH respectively. Moreover the broodstock experiment is the first attempt to characterise kisspeptin gene expression during the different stages of the sex reversal in process in seabream, a fundamental aspect of their production.

Conclusions

In order to gain a better understanding of reproductive physiology in the gilthead seabream determining how the kisspeptin system interacts with external environment and reproductive axis is essential. This study provides an extensive description of *Kiss2* and *Kiss1r* expression during seabream ontogenesis and during the sex reversal process, the first step a better understanding of the kisspetin system in this economically important species.

ISOLATION OF THE α- AND ß-SUBUNIT GENES OF LUTEINIZING HORMONE (LH) OF THE GIANT AMAZONIAN FISH PIRARUCU (*ARAPAIMA GIGAS*)

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Introduction

Arapaima gigas (pirarucu) is one of the largest freshwater fish of the world reaching 2m of length and weighing up to 250kg. It lives mainly in the Amazon basin, is very important for the alimentation and commercial breeding of the region, being considered an overexploited and endangered species. For better studying its reproductive physiology and improve its breeding we cloned for the first time the α- and ß-subunit of *Arapaima gigas* luteinizing hormone (ag-LH), for subsequent synthesis, purification and characterization.

Methods

Pituitary glands were removed immediately following decapitation of sexually mature *A.gigas* and frozen in liquid nitrogen or stored in RNALater^{R}. Total RNA was extracted using a specific purification kit, primers were designed first with basis on conserved regions of several FSH fish sequences. Once obtained by RT-PCR a partial sequence of the gene, with basis on specific sequences of *A.gigas*, the complete cDNA sequence was obtained by rapid amplification of cDNA ends (RACE). The complete amino acid sequence of the hormone subunits was determined and the alignment of the mature peptide sequence with those of known species of fish, carried out by using appropriate softwares. The phylogenetic tree and related analyses were also conducted by using appropriate computer programs based on the Neighbor-Joining method and rooted with 3 *Acipenseriformes* species as the outgroup.

Results and Discussion

Considering that the α-subunit gene sequence, common also to follicle-stimulating (FSH) and thyroid-stimulation (TSH) hormones, had already been obtained in previous work, this project was dedicated to the isolation of the ß-subunit cDNA of ag-LH. Up to now about 60% of the nucleotides and amino acid sequence was repeatedly confirmed and we are carrying out 5'-RACE for determining the remaining part of the ag-LH β-subunit sequence. With basis on the data obtained (n=78 amino acids) we could calculate an identity of 73.1% with anguilla, 55.1% with sturgeon and 50.0% with human ß-subunit LH peptide sequences. The phylogenetic tree, carried out by using >70 FSH ß-subunit peptide sequences of teleosts, is being studied, also in comparison with the α- and the FSH β-subunit, and will be discussed.

Conclusion

Part (~60%) of ag-LH ß-subunit gene was repeatedly sequenced and we are in the process of soon obtaining the whole gene sequence. Together with the α-subunit gene, already defined in previous work, we will be able to construct expression vectors and proceed to hormone biosynthesis. A complete phylogenetic study is also being carried out.

EXPRESSION AND PUTATIVE FUNCTION OF KISSPEPTINS AND THEIR RECEPTORS DURING EARLY DEVELOPMENT IN MEDAKA

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Introduction

Kisspeptins (Kiss1 and Kiss2) and their receptors (putatively Gpr54-1 and Gpr54-2) have emerged as key players in vertebrate reproduction owing to their stimulatory effect on the hypothalamus-pituitary-gonadal axis. Virtually nothing is known, however, about their role during embryogenesis. Using medaka (*Oryzias latipes*) as model system, we report for the first time in vertebrates, an early developmental expression and putative function of kisspeptins.

Methods

Relative quantification of gene expression was performed by qPCR. *six3* and *dlx2* whole mount *in situ* hybridization was performed using DIG-incorporated riboprobes. Gene knockdown experiments were performed by injecting various doses of either morpholino or GripNA in medaka embryos at the one-cell stage. Maternal and zygotic expression was specifically knocked down by targeting either the translation initiation start site or the first exon-intron splice site. Knockdown rescue control experiments were performed by simultaneous injection of RNA of the targeted gene.

Results and Discussion

Both ligands (*kiss1* and *kiss2*) and one receptor (*gpr54-1*) gene were expressed maternally in medaka embryos. Gene expression of *gpr54-2* first appeared after commencement of zygotic expression, around stage 15. Expression analyses and knockdown experiments together suggest that early actions of kisspeptins are probably mediated by binding to maternally supplied Gpr54-1 and late action by both Gpr54-1 and Gpr54-2. Knockdown of maternally provided *kiss1* and *gpr54-1* arrested development during gastrulation, before establishment of any germ layers, while knockdown of zygotically provided *kiss1* and *gpr54-1* disrupted brain development. A similar phenotype was observed for *gpr54-2* knockdown embryos, suggesting a critical role for *kiss1*, *gpr54-1*, and *gpr54-2* in neurulation. No particular phenotype was observed following knockdown of *kiss2*, regardless of developmental stage.

Conclusion

These data demonstrate that kisspeptin signaling is active both maternally and zygotically, and is involved in embryonic survival and (brain) morphogenesis.

PLASTICITY OF LUTEINIZING HORMONE BETA-EXPRESSING GONADOTROPES SHOWS THAT HORMONE PRODUCTION IN THE TELEOST PITUITARY IS UNEXPECTEDLY COMPLEX

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Introduction

Gonadotrope cells of the pituitary gland are responsible for the synthesis and release of the pituitary gonadotropins, follicle-stimulating hormone (Fsh) and luteinizing hormone (Lh). Fsh and Lh are key players in the endocrine control of vertebrate reproduction, and each hormone regulates different aspects of gonad growth and maturation. Although the regulation of the individual hormones have been studies in many species, there is limited knowledge concerning the global changes that take place in the gonadotrope cells in order to regulate the differential synthesis and release of these hormones. Using a transgenic line of medaka (*Oryzias latipes*) where expression of Gfp is under the control of the endogenous *lhb* promoter (*lhb*:Gfp), we have investigated the morphological and transcriptional characteristics of *lhb*-expressing gonadotrope cells, and changes that take place from the juvenile to the adult stage.

Methods

From enzymatically dispersed pituitary cells, fluorescent activated cell sorting (FACS) was used to isolate and morphologically analyze Gfp-positive cells from the *lhb*:Gfp transgenic line of medaka. High throughput cDNA sequencing (RNA-seq) was used to analyze and compare the transcriptome of Gfp-positive cells in juvenile and adult female fish, respectively. Flow cytometry was used to investigate changes in cell number and morphology. Single-cell qPCR was used for verification of RNA-seq results.

Results and Discussion

Flow cytometry experiments revealed dramatic changes in the relative pituitary cell number and morphology during the transition from juvenile to adult fish. Furthermore, the expression of all major pituitary hormone encoding genes in addition to *lhb* was discovered in the Gfp-positive cells. We present a detailed investigation of these expression patterns, with the main focus on follicle-stimulating hormone beta (*fshb*). These results provide strong evidence that the expression of several different hormone encoding genes in the *lhb*-expressing cells represents genuine biological results, and are thus opposing the conservative model of the teleost pituitary, where one pituitary cell type produces and secretes one hormone.

Conclusion

The FACS, RNA-seq, and single-cell qPCR experiments performed in this study support the concept of pituitary plasticity, indicating advanced communication between the different pituitary cells. We suggest that the control of hormone production in the teleost pituitary is more complex than previously recognized.

ELECTROPHYSIOLOGICAL DIFFERENCES BETWEEN *fshb*- AND *lhb*-EXPRESSING GONADOTROPES IN PRIMARY CULTURE

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Introduction

Synthesis and release of Fsh and Lh are differentially regulated by Gnrh, but the mechanisms by which this regulation is achieved are not well understood. Teleost fish are powerful models for studying this differential regulation because they have distinct pituitary cells producing either Fsh or Lh.

Methods

By using primary pituitary cultures from Atlantic cod (*Gadus morhua*), we were able to investigate and compare the electrophysiological properties of *fshb*- and *lhb*-expressing cells, identified by single-cell quantitative PCR after recording. Acute responses to Gnrh were assessed by patch-clamp recordings (membrane potential signals) and fura-2 Ca^{2+} imaging (cytosolic Ca^{2+} signals).

Results and Discussion

Both cell types fired action potentials spontaneously. The relative number of excitable cells was dependent on reproductive season but varied in opposing directions according to season in the two cell types. Excitable and quiescent gonadotropes displayed different ion channel repertoires. The dynamics of outward currents and Gnrh-induced membrane responses differed between *fshb*- and *lhb*-expressing cells, whereas inward currents and Gnrh-induced cytosolic Ca^{2+} responses were similar. The cytosolic Ca^{2+} responses consisted of contributions from both extracellular and intracellular Ca^{2+} sources. Expression of Ca^{2+}-activated K^{+} channels also differed with cell type and showed seasonal variation when measured in whole pituitary. The differential presence of these channels corresponds to the differences observed in membrane response to Gnrh.

Conclusion

These results suggest that differences in ion channel expression levels may be involved in seasonal regulation of hormone secretion as well as the differential response to Gnrh in Lh- and Fsh-producing gonadotropes, through differences in excitability and Ca^{2+} influx.

OOGENSIS CONTOL IN MULTI-SPAWNING BLUE GOURAMI AS A MODEL FOR THE ANABANTIDAE FAMILY

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Introduction

In natural habitats, blue gourami (*Trichogaster trichopterus*) reproduction occurs year-round but more rapidly during the heavy rain season. The key hormones involved in regulating oogenesis in the brain are gonadotropin-releasing hormone (GnRH 1 and 3), pituitary adenylate cyclase-activating polypeptide (PACAP) and its related peptide (PRP), pituitary follicle-stimulating hormone (βFSH), luteinizing hormone (βLH), growth hormone (GH) and prolactin (PRL)-ovary steroids.

Methods

The steroids and glucuronides in plasma, gonads and holding water of blue gourami were identified by gas chromatography-mass spectrometry (GCMS), the radioimmunoassay method (RIA) and thin layer chromatography (TLC). The cDNA sequences of the full-length blue gourami brain GnRH 1,2 and 3, PACAP and PRP and pituitary βFSH, βLH, GH and PRL and mRNA levels were measured by real-time PCR with oocytes in the maturation stage compared to levels in vitellogenic and non-vitellogenic fish. The effect of all these hormones were studied *in vitro* and *in vivo* during oogenesis.

Results and Discussion

In blue gourami, environment factors affect the oogenesis of mature fish, e.g., water temperature and quality affected the transcription of genes encoded to hormones involved in reproduction.Two hypothalamus-pituitary gonad axes control the oogenesis. The first is the gonad axis. From three GnRH systems of which only two peptides exhibited high mRNA levels in the brain of females during oogenesis, both the GnRH1 and GnRH3 were high in vitellogenesis and the final oocyte maturation stage, and they are induced by βFSH, βLH and GH dictated in both gene transcription and level in the plasma. Many steroids were found in the ovary during oogenesis, however, *17β-estradiol* (E_2) and testosterone (T) increase during vitellogenesis at a relatively high level in the plasma, and 17α,20β-dihydroxy-4-pregnen-3-one (17,20P) during maturation and ovulation in blue gourami. The second is the hypothalamus-pituitary gonad axis of GHRH, GH and gonads. Pituitary adenylate cyclase-activating polypeptide (PACAP) is synthesized from a precursor, which includes the PACAP related peptide (PRP; formerly known as GHRH-like peptide). PRP-PACAP mRNA levels were higher in females with oocytes in the final maturation stage than in vitellogenic individuals. Stimulation of pituitary cells with blue gourami PRP (bgPRP) caused an increase in bLH subunit transcription levels only in females, and an increase in GH mRNA levels and PRP receptor in the pituitary cells.

Conclusion

In blue gourami females under non-reproductive conditions, vitellogenesis occurs through the GnRH1→ FSH → E2 and GH → IGF-1 pathways. Under reproductive conditions, FOM is promoted by GnRH3 and PRP → LH → 17,20P and vitellogenesis is promoted by the synergistic effect of PACAP and GnRH → FSH and GH → E2 and IGF-1.

EXPLORING POSSIBLE ROLES OF GONADAL KISSPEPTINS IN THE REPRODUCTIVE DEVELOPMENT OF SABLEFISH *ANOPLOPOMA FIMBRIA*

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Introduction

The kisspeptin system plays a pivotal role in the regulation of vertebrate reproduction. Currently, the best understood function of the teleost kisspeptin system involves its stimulation of GnRH neurons in the brain, thereby regulating many key reproductive events. Although the expression of kisspeptins has also been documented in other reproductive tissues, such as the gonads, their function in these tissues is largely unknown. Thus, we sought to characterize the expression of gonadal kisspeptins and address their possible roles during early sexual development in a teleost species, sablefish (*Anoplopoma fimbria*).

Methods

The specific goals of this study were to confirm the presence of kisspeptin genes in sablefish, sequence the mRNA coding regions, reveal their tissue distribution, and analyze their gonadal expression profiles during development. Coding sequences for the *kiss*-related genes (*kiss1, kiss2* and the receptors *kiss1ra* and *kiss1rb*) were obtained by PCR using degenerate primers, rapid amplification of cDNA ends (RACE) PCR, and cloning. Semi-quantitative PCR assays were used to compare *kiss* and *kiss1r* mRNA levels across sablefish tissues. Using quantitative PCR assays, *kiss* mRNA levels were measured in the gonads of sablefish undergoing sexual differentiation and early gametogenesis. These gene expression data were then analyzed based on histological stage of the gonads. In situ hybridization (ISH) was conducted to localize *kiss* mRNAs in immature ovaries.

Results and Discussion

The four *kiss*-related genes were successfully cloned from sablefish ovary and brain. The sequences exhibited a high degree of homology (84% - 98%) to orthologs in other marine fishes. Gene expression analyses revealed that the *kiss* genes were mainly expressed in the brain, pituitary, and gonads and show sexually dimorphic expression in both juvenile and adult gonads. Transcripts for *kiss2*, the predominately expressed *kiss* gene in fishes, were most abundant in the ovary. Analysis of *kiss2* mRNA levels during ovarian development revealed that it is expressed at low levels during ovarian and testicular differentiation, and then significantly increases as the proportion of oocytes at the perinucleolus stage increase. Results of ISH demonstrated that *kiss2* mRNA is strongly localized in the ooplasm of perinucleolar oocytes of juvenile sablefish.

Conclusion

Our results suggest that gonadal kisspeptins are unlikely to play a role in sexual differentiation, but instead may be involved in oogenesis and/or the formation of the ovarian follicle. Kiss2 synthesized within the oocyte may act in an autocrine or paracrine manner within the oocyte or ovary, or may be secreted to act on other reproductive tissues.

EXPRESSION PROFILES OF FOLLICLE-STIMULATING HORMONE AND LUTEINIZING HORMONE GENE EXPRESSION DURING OOCYTE DEVELOPMENT IN CULTIVATED YELOWTAIL, *SERIOLA QUINQUERADIATA*

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Introduction

Yellowtail is one of the most commercially valuable marine species in Japan. Recently, there is a critical need to develop new technologies in order to increase seed production and the ability to induce spawning to optimize production. However, little is known about the reproductive characteristics of yellowtail in captivity. In the present study, as a first step in investigating gonadotropic regulation in female yellowtail, we examined in detail the expression profiles of gonadotropin (GtH) subunit genes during oocyte development.

Methods

Adult (2-yr-old) yellowtail were held at Goto Station, Seikai National Fisheries Research Institute, under natural conditions of photoperiod and temperature in a floating net cage. Fish were sampled once or twice a month from July 2012 from May 2013. Ovaries were classified by maturational stage according to the relative abundance of the most advanced type of oocytes. The mRNA expression of GtH subunit genes, FSHβ, LHβ and glycoprotein-α (GPα), were measured by quantitative real-time RT-PCR.

Results and Discussion

Histological examination of the gonads revealed that previtellogenic oocytes (yolk vesicle stage) were present at the end of January. Vitellogenic oocytes were first detected at the beginning of March, which was accompanied by a significant increase in gonadosomatic index. During the spawning phase in April, oocytes were observed at various developmental stages from the perinucleolus to the tertiary yolk globule stage. The FSHβ and LHβ transcript levels increased prior to at the onset of oocyte secondary growth, and were high from the early stages of vitellogenesis to the spawning season. Furthermore, the levels of LHβ mRNA were more abundant than those of FSH throughout oocyte development. These results suggest that both FSH and LH may regulate vitellogenesis and oocyte maturation in female yellowtail.

Conclusion

The correlation between FSHβ and LHβ transcript levels and the reproductive phases of the female yellowtail suggests that FSH and LH may play important roles in gonadal growth as well as in final maturation.

TWO TELEOST SPECIFIC USF2 ARE INVOLVED IN *fshr* REGULATION IN EUROPEAN SEA BASS

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Introduction

Upstream Stimulatory Factors (USFs) are Helix-Loop-Helix transcription factors involved in a wide range of biological processes. We recently demonstrated that Human Upstream Stimulatory Factors (USFs) could bind to European sea bass *follicle stimulating hormone receptor* (*fshr*) 5' UTR through an E-box to activate its expression. Next, we cloned for the first time in a teleost species, three partial sequences coding for three distinct *usfs* (namely *usf1*, *usf2a* and *usf2b*) in sea bass (*Dicentrarchus labrax*), and described their ubiquitous expression. However, no information is available about their origin or their function in teleosts.

Methods

We isolated sea bass *usf2a* and *usf2b* full length cDNAs from ovarian follicular cells and introduced them in pcDNA3. CHO cells were transfected with these expression plasmids i) to determine by immunohistochemistry the specificity of a heterologous antibody designed for Human USF2 (IgG Z). ii) to produce nuclear extracts enriched in sea bass Usf2a, Usf2b or Usf2a/Usf2b heterodimers. iii) to determine by luciferase assays their effect on *fshr* promoter. EMSAs were performed with *fshr* promoter probes and ovary or nuclear extracts from Usfs-transfected CHO cells.

Results and Discussion

The isolated full length cDNAs code for two nuclear proteins, Usf2a and Usf2b, that can be recognized by the heterologous antibody IgG Z in sea bass follicular cells. Phylogenetic analysis indicates that these genes originated from the teleost-specific whole genome duplication. These two proteins can physically bind to E-boxes located in the *fshr* 5'UTR as demonstrated by EMSA. However, we could not appreciate the formation of Usf2a/Usf2b heterodimers. Finally, luciferase assays show both Usf2a and Usf2b could activate *fshr* promoter through this E-box.

Conclusion

Usf2a and Usf2b are two teleost-specific proteins present in sea bass follicular cells that can activate the *fshr* promoter by binding through an E-box (CACGTG). Although these interactions have to be confirmed *in vivo* these two transcription factors could play a role in teleost reproduction success.

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BROODSTOCK SPAWNING PERFORMANCE OF BALLAN WRASSE (*LABRUS BERGYLTA*)

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Introduction

Sea lice continue to be one of the largest issues for the salmon farming industry and the use of cleaner fish (e.g. ballan wrasse), as a biological control, is considered one of the more sustainable solutions in development. This is only achievable if the wrasse are farmed rather than wild; however, intensive production is still very much in its infancy with limited knowledge about reproductive physiology, spawning behaviour, larval development and general performance in captivity. The aim was to monitor captive breeding populations throughout a spawning season to look at timing and duration of spawning, to quantify egg production, and to look at seasonal changes in egg quality parameters and develop methodologies for degumming.

Methods

Four breeding populations were set up in four 7 m^3 litre tanks with a 1:10 male: female sex ratio. Carpet mats were provided as spawning substrate. Tanks were checked daily between 0900 and 1000 for evidence of spawning. When spawning occurred, egg numbers were estimated and sampled to look at fertilization rate, egg stage, oocyte diameter, gum layer thickness, and fatty acid (FA) content. Fertilization rate and egg stage were assessed by eye under dissecting microscope. 35 eggs per batch were photographed and measured via ImageJ software. FA composition of eggs collected was determined over the spawning season.

Results and Discussion

A clear spawning rhythm was shown with intervals of 5-14 days between spawning events as expected in a batch spawning species. However, parental contribution was unknown and parentage assignment is on going. Fertilization rate remained consistently high (87.5-100%) over the spawning season and did not differ significantly between tanks. Hatch rate was variable, but appeared to peak half way during the spawning season. All eggs sampled were found to be at 'blastula' stage (between 20-29 hours post fertilization at 12.0 ± 2.36°C). Mean oocyte diameter decreased slightly over the spawning season with significant differences between tanks. Mean gum layer thickness decreased by 30 % over the season with no significant difference between tanks. FA profile of eggs remained relatively constant throughout the season and with the exception of high levels of ARA (3.8 ± 0.5% of total FA), the FA profile was similar to that observed in other marine fish species. In addition, EPA:ARA and DHA:EPA ratios observed were consistent with that observed in wild ballan wrasse eggs.

Conclusion

Ballan wrasse is not only a commercially important species for aquaculture, but a very interesting species with unusual reproductive strategies. Basic knowledge of the reproductive characteristics and egg quality parameters is vital to develop broodstock management protocols for commercial success of ballan wrasse farming. This study serves as a baseline measure of spawning performance of a captive ballan wrasse broodstock population and will point us in the direction for future research.

CHARACTERIZATION OF GENES REGULATED BY FOLLICLE-STIMULATING HORMONE DURING EARLY OOGENESIS IN SALMON

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Introduction

Oogenesis in fishes is primarily regulated by pituitary gonadotropins, follicle-stimulating hormone (Fsh) and luteinizing hormone (Lh). Most studies of the biological actions of fish gonadotropins have focused on Lh regulation of steroidogenesis and final oocyte maturation. In contrast, less is known about previtellogenic ovarian growth in fishes and its regulation by Fsh. The goal of this study was to gain a fundamental understanding of how Fsh acts at the level of the ovary to stimulate early oocyte growth.

Methods

To determine the unique functions of Fsh regulating early stages of oogenesis in coho salmon, we first evaluated the *in vitro* effect of recombinant analogs of salmon Fsh and Lh (sFsha and sLha) on cortical alveolus stage follicles. The effect of sFsha and sLha (comprised of human alpha and salmon beta subunits) on ovarian genes was examined at doses of 0, 5, 50 and 500 ng ml^{-1}. The developmental profiles of genes regulated specifically by sFsha were characterized across stages of oogenesis.

Results and Discussion

Both sFsha and sLha were effective in stimulating 17-beta estradiol production, although they differed in their effect on steroidogenesis-related genes. sFsha increased transcripts for *hsd3b* and *cyp19a1a*, while sLha increased *star* and *cyp17a1*. Furthermore, sFsha, but not sLha specifically regulated the expression of ovarian genes involved in cell communication (*connexin 34.3, cx34.3*), survival (*clusterin 1* and *2*, *clu1* and *2*), and differentiation and growth (*wilms tumor protein 2-like*, *wt2l*, and *alcohol dehydrogenase 8-like*, *adh8l*), growth factor signaling (*bone morphogenetic protein 16*, *bmp16*) and extracellular matrix remodeling (*connective growth factor*, *ctgf*). Overall, the effects of sFsha were consistent with the expression of these genes in ovarian follicles during oocyte growth. Transcripts for *cx34.3*, *clu1* and *2*, and *wt2l* increased during secondary oocyte growth, whereas those for *bmp16* were maintained at low levels and peaked during maturation. Contrarily, transcripts for *ctgf* and *adh8l* were downregulated in the ovary by sFsha *in vitro*, but increased naturally during secondary oocyte growth, suggesting that other factors participate in their regulation during oogenesis.

Conclusion

These data suggest Fsh plays a unique role in the development of the ovarian follicle structure prior to vitellogenesis. Hormone therapies that either mimic Fsh activity or stimulate secretion of endogenous Fsh will be required to induce puberty in females. In this regard, future experiments will analyze ovarian changes in response to sFsha in prepubertal salmon. Funded by National Research Initiative Competitive Grant no. 2007-35203-18088.

CHANGES OF EXPRESSION AND CELLULAR LOCALIZATION OF INSULIN-LIKE GROWTH FACTOR I IN OVARIES DURING OVARIAN DEVELOPMENT OF YELLOWTAIL

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Introduction

Numerous studies have described the involvement of somatic growth-related hormones in ovarian development among mammals and some fishes. Recent studies have suggested that most prominent among these hormones is the insulin like-growth factor I (IGF-I), which is expressed in the ovaries and stimulates oocyte and follicular development. However, less attention has been paid to the role of IGF-I in marine fish reproduction, while marine fish are very important for aquaculture. In this study, in order to elucidate the roles of IGF-I in the ovarian development, we characterized the expression and cellular localization of IGF-I in the ovary during the ovarian development of yellowtail *Seriola quinqueradiata*.

Methods

Two-year-old cultured yellowtails were reared from post-spawning (July) to the next spawning (April) under natural conditions, and sampled every month at the Goto station, Seikai National Research Agency, Fisheries Research Agency, Japan. Females were histologically classified into the perinucleolus stage (Pn), yolk vesicle stage (Yv), primary yolk stage (Py), secondary yolk stage (Sy) and tertiary yolk stage (Ty) according to the most advanced type of oocytes. mRNA was extracted from the ovaries and the expression quantities of IGF-I were measured by real-time PCR system. Also, cellular localizations of IGF-I in the ovaries were studied by immunohistochemistry.

Results and Discussion

Transcripts for IGF-I were detected in the ovaries of all developmental stages and the ovarian IGF-I expression tended to decrease from Pn and Yv to Py, and thereafter increased and reached the maximum level in Ty. In immunohistochemistry, immunoreactive IGF-I was found in the theca cell layer of the oocytes from Yv to Ty, although immunoreactive IGF-I was not present in the follicles at Pn. Also, immunoreactive IGF-I was found in a part of the granulosa cells of only Ty. These results indicated that the follicular IGF-I in the ovaries may be potential autocrine/paracrine regulators of the ovarian development of yellowtail.

Conclusion

The expression of the ovarian IGF-I tended to increase with the ovarian development and IGF-I was produced in the ovarian follicular cell layer. These results indicate that follicular IGF-I may be involved in marine fish reproduction by autocrine/paracrine mechanisms.

5S rRNA AND TFIIIA IN GONADS AND THE MOLECULAR IDENTIFICATION OF SEX AND FEMALE REPRODUCTIVE STAGE IN COMMERCIAL FISH SPECIES

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Introduction

The estimation of maturity stage and sex ratio of fish stocks in European waters is a requirement of the EU Data Collection Framework as part of the policy to improve fisheries management. We have recently found that 5S rRNA could provide an easy, inexpensive and rapid way to identify sex in fish, as it is very highly expressed in ovaries of thicklip grey mullets (*Chelon labrosus*) but not in testes. Similar expression profile is shown in gonads by the transcription factor necessary for polymerase III driven 5S rRNA production; TFIIIA. Our aim hereby was to proof the usefulness of 5S rRNA and TFIIIA in the identification of sex in different economically relevant fish species from the Biscay Bay.

Methods

Gonad samples of commercially relevant fish species were collected from local commercial fishing vessels: Atlantic horse mackerel (*T. trachurus*), European hake (*M. merluccius*), pilchard (*Sardina pilchardus*), chub and Atlantic mackerel (*Scomber japonicas* and *S. scombrus*), anchovy (*Engraulis encrasicolus*), blue whiting (*Micromesistius poutassou*) and megrim (*Lepidorhombus whiffiagonis*). All individuals were histologically sexed and gonad RNA extracted. 5S and 18S rRNA concentrations were quantified by capillary electrophoresis and a 5S/18S rRNA index was calculated. cDNA was produced and TFIIIA fragments (≈500 bp) were cloned and sequenced using degenerate primers. In megrim, 5S and 18S rRNA levels were quantified by qPCR in ovaries in different developmental stages.

Results

5S rRNA expression clearly distinguishes ovarian and testicular RNA in all studied species, 5S/18S rRNA index always showing higher values in females than in males. TFIIIA fragments were cloned for all species showing strong transcription levels in ovary but not in testes. In megrim, where ovaries with previtellogenic, early and late vitellogenic oocytes were available among the sampled organisms, 5S/18S rRNA index (calculated by capillary electrophoresis and by qPCR) was able to quantitatively distinguish different female reproductive stages. This is so because 18S rRNA expression only begins with the onset of vitellogenesis, while 5S rRNA expression is very high from the beginning of oocyte development.

Conclusion

5S rRNA and TFIIIA allow molecular discrimination of males and females in any fish species. Additionally, the dynamics of rRNA production during oocyte development and growth allow identification of the developmental stage of the ovary through a simple quantification of a 5S/18S rRNA index. This technology could have multiple applications in the study of fish stock dynamics, reproduction and reproductive endocrine disruption.

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LIGAND BINDING PROPERTIES OF OVARIAN LIPOPROTEIN RECEPTORS IN THE CUTTHROAT TROUT

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Introduction

Lipoprotein yolk precursors called vitellogenins (Vtgs) are produced by the liver and deposited in growing oocytes by endocytosis. Recent investigations revealed that salmonids may produce two distinct types of Vtg, VtgAs and VtgC, and that there are multiple ovarian membrane proteins that specifically bind Vtg(s). Although these membrane proteins are expected to be involved in endocytosis of Vtgs, their various identities and ligand-binding properties remained to be discovered. The goal of this study was to develop a better understanding of salmonid yolk formation with respect to the identities and functional characteristics of their multiple Vtg-binding proteins.

Methods

Specific polyclonal antisera were raised in rabbits against two discrete ovary lipoprotein receptors that we have cloned and characterized in cutthroat trout (*Oncorhynchus clarki*). One of these is the Vtg receptor and has eight ligand binding repeats (Lr8: termed Vtgr here) and the other is a novel receptor with 13+1 ligand binding repeats (Lr13+1). These antisera (a-Vtgr and a-Lr13+1) were utilized in Western blotting of ovarian membrane extracts to identify the respective Vtg-binding proteins. Ligand blotting was then performed to test receptor specificity for plasma lipoproteins, including very-low-density lipoprotein (VLDL), low-density lipoprotein (LDL), and high-density lipoprotein (HDL), in addition to VtgAs and VtgC.

Results and Discussion

Western blotting confirmed that Lr13+1 and Vtgr correspond to a 210 kDa protein and three proteins within a 95-110 kDa size range in the ovary membrane, respectively. When digoxygenin (DIG)-labeled VtgAs was used in ligand blotting, at least five bands including the three Vtgrs, the Lr13+1, and an unidentified receptor were visualized. The Vtgrs were found to be the major VtgAs-binding sites. In contrast, DIG-VtgC did not bind these Vtgrs but dominantly bound to the unidentified receptor. The DIG-labeled VLDL, LDL, and HDL did not bind to the Vtgrs.

Conclusion

We confirmed that multiple ovarian receptors have disparate specificities for different types of Vtg in cutthroat trout, although some receptors bound both VtgAs and VtgC. These findings suggest that the ratio of deposited yolk proteins derived from different types of Vtg may be regulated, in part, by their alternative uptake *via* two or more distinct receptors.

CHARACTERIZATION AND GENE EXPRESSION ANALYSES OF GONADOTROPIN RECEPTORS IN GREATER AMBERJACK (*SERIOLA DUMERILI*) OVARIES

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Introduction

The greater amberjack (*Seriola dumerili*) is a multiple-spawning marine fish and has been targeted for aquaculture, mainly in the Mediterranean region and Japan. Understanding the endocrine regulation of reproduction is important for aquaculture; however, the available information on its reproductive physiology is not sufficient. In this study, we molecularly characterized greater amberjack gonadotropin (GtH) receptors (GtHRs; follicle-stimulating hormone receptor [FSHR] and luteinizing hormone receptor [LHR]) and quantitated their mRNA expression levels in the ovaries throughout the seasonal reproductive cycle.

Methods

The full-length cDNA sequences of greater amberjack FSHR and LHR were determined by molecular cloning. We developed methods for quantifying their gene expressions in the gonad and estimated their changes during ovarian development. Adult greater amberjack were reared in sea pens and each 5–7 female fish was sampled regularly between September 2011 and August 2012. Total RNA was isolated from each ovary and cDNA was reverse-transcribed. Real-time PCR was performed using probes and primers designed for greater amberjack *fshr* and *lhr*, respectively. Results were normalized to mRNA levels of elongation factor 1 alpha.

Results and Discussion

In ovaries, *fshr* expression gradually increased between March and April during the early phase of ovarian development, dramatically increased in July during the spawning season and then decreased in August during the post-spawning. On the hand, the higher expression of *lhr* was only observed in the spawning season (June). These results suggest that in the greater amberjack ovary, FSHR is involved in the ovarian growth, while LHR is critical for the oocyte maturation. Further studies on the expression and function of FSHR and LHR will be needed to understand how GtH/GtHR systems regulate oocyte development in greater amberjack.

Conclusion

This study provides the first data on the distinct expression of FSHR and LHR in a *Seriola* species; the increase of *fshr* expression was accompanied by the ovarian growth, while *lhr* expression was high during the spawning season.

ANALYSIS OF HORMONE PROFILE IN *ASTYANAX BIMACULATUS* FEMALES WITH POSITIVE RESPONSE TO REPRODUCTIVE STRESS PROTOCOL IN FISH FARM

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Introduction

Stress can be defined as a condition in which the dynamic equilibrium of the body is disturbed. In this field, several studies have demonstrated the action of some corticosteroid hormones modulate stress situations, such as reproduction. However, the role of corticosteroids in fish reproduction can be either positive or deleterious, depending on many variables. Interestingly, in the *Astyanax* genus, the same stress stimulus can successfully induce the reproduction in *A. bimaculatus*, but not in other species of the genus. Therefore, the aim of this study was to analyze the cortisol and progestogens levels of *Astyanax bimaculatus*, a species that successfully reproduce using stress as a stimulus.

Methods

For this study, a reproductive protocol using stress conditions was used to stimulate spawn. The stress protocol is used regularly and successfully at CESP (Companhia Energética do Estado de São Paulo) for restocking program purposes. This protocol consists of keeping *A. bimaculatus* males and females together in a tank in a very high density (1.5 fish/m^3) and an intense water flow (10L.m^{-1}). We chose 3 experimental groups for testing: Stress (just described); human chorionic gonadotropin (hCG; 5UI.g^{-1}); and Serum (animals injected with NaCl 0.37% in the same hCG volume). To assess the hormonal status of the animals, 5 individuals were collected at 0, 8, 12 and 24 hours after the beginning of the treatment. Plasma samples were collected from the caudal vein to measure 17α-hydroxiprogesterone (17α-OHP) and cortisol (CORT) by ELISA immunoassay; and ovaries were fixed in Bouin solution to analyze the maturation stage of the oocytes, by histological analysis.

Results and Discussion

The experiment lasted 24 hours when females from the hCG and Stress groups successfully spawned. At 12 hours after the beginning of the experiment, there was a sudden increase in plasma levels of CORT in females from the Stress group, but not in the hCG and Serum groups. 17α-OHP did not change during the experiment and also did not differ among groups. Considering that 17α-OHP is a precursor of the maturation-induced steroid (MIS, 17α-20β-dihydroxiprogesterone), we expected to observe a peak of this progestogen closer to the time of ovulation. In the ovaries, the presence of mature oocytes was observed in the first half of the experiment in all groups, and, at 24 hours, a great amount of postovulatory follicles, but there were still cortical alveoli oocytes in the ovaries of females in hCG and Stress groups.

Conclusion

We suggest a possible role for CORT in stimulating the reproductive activity, with a direct influence on ovulation and/or spawning of *A. bimaculatus*, and with a positive action as an inducing agent in the spawning of this species.

INFLUENCE OF ACIDIC pH, ALUMINUM AND MANGANESE ON THE ACTIVITY OF HEPATIC ANTIOXIDANT ENZYMES OF *ASTYANAX BIMACULATUS* (CHARACIFORMES: CHARACIDAE) MALES

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Introduction

Reactive oxygen species (ROS) are molecules normally produced in the body and assist in the elimination of pathogens. The balance between the production and elimination of ROS is mediated by the antioxidant enzymes that neutralize these molecules. This balance can be altered in the presence of metals in the body, leading to oxidative stress and changing the metabolism of the animal. Thus, the aim of this study was to investigate the influence of the aluminum (Al) and manganese (Mn) metals, either isolated or combined, on acidic pH and on the activity of some oxidative stress enzymes in the liver of mature males of the characid *Astyanax bimaculatus*, during an acute exposure of 96 hours followed by an additional period of 96 hours in clean water, to check the possible effects on the recovery of the metals and/or acidic pH effects.

Methods

Mature males of *A. bimaculatus* were submitted to an acute period of exposure in experimental groups: control (pH 7.5), acidic pH (5.5), Al, Mn and Al + Mn combined, with the metal groups kept at pH 5.5. After this period, they were transferred to clean water to check the possible effects on the recovery of the metals and/or acidic pH effects. The enzymatic activities of catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPX) were analyzed, in addition to lipid peroxidation.

Results and Discussion

The exposure of metals to acidic pH was able to cause oxidative stress. The activity of SOD was unchanged throughout the experimental period, suggesting that the production of free radical did not increase superoxide radical production. The activity of CAT was altered, increasing during the acute period in the males in all experimental groups, and only the animals exposed to acidic pH and Al recovered from this effect. GPX increased its activity in acidic pH, Al and Mn in the recovery period, and returned to the initial concentrations at the end of the period. Both enzymes (CAT and GPX) are responsible for neutralizing hydrogen peroxide, the free radical precursor of molecules that cause lipid peroxidation in cell membranes. However, the increased activity of these enzymes was not enough to neutralize these compounds, and resulted in lipid peroxidation in males exposed to acidic pH.

Conclusion

Al and Mn were able to stimulate the production of free radicals in *A. bimaculatus* in the mature period of the reproductive cycle, changing the balance between production and elimination of ROS. Thus, they may act directly on fish metabolism and probably increase the permeability of cell membranes, suggesting an alteration in the concentrations of proteins and lipids, and consequently, the generation of deleterious effects on reproduction.

REPRODUCTION AND SPAWNING OF THE ENDANGERED CHARACID *BRYCON ORTHOTAENIA* DOWNSTREAM A HYDROELECTRIC DAM, SÃO FRANCISCO RIVER, BRAZIL

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Introduction

Species of the *Brycon* genus are widely distributed throughout South and Central America. *Brycon orthotaenia* Günther, 1864, is an omnivorous species endemic to the São Francisco River basin, which migrates upstream during its reproductive period. Its body weight may reach more than 7 kg, and it is an important fish in commercial and sport fishing. This species is endangered in some regions of the São Francisco River basin mainly due to high environmental degradation. Whereas the strategies of life story of *Brycon* still remain poorly understood, the goal of this study was to analyse reproductive parameters of *B. orthotaenia* in two sections of the São Francisco River downstream from the Três Marias dam, southeastern Brazil.

Methods

A total of 312 adult specimens (123 females and 189 males) were captured bimonthly in 2011 and 2012. The fish were sampled in two river sections: immediately downstream from the Três Marias dam (section 1: 18º11'S,45º14'W) and after the confluence with the Abaeté River (section 2: 18º02'S,45º11'W). Biometric data were obtained of each specimen and gonadal samples fixed in Bouin fluid were processed for histological analyses. The physical-chemical parameters of the water were recorded during sampling on both São Francisco River sections. All data were submitted to statistical analyses.

Results and Discussion

Specimens of *B. orthot*aenia from section 2 presented higher values of total length (females = F: 30.7 cm, males = M: 26.4 cm) and body weight (F: 468.8g, M: 256.4g), when compared to section 1 (F: 17.8 cm and 325.4g, M: 23.8 cm and 188.8g). The reproductive period occurred from October to February, with gonad-somatic index (GSI) peaks occurring in November/December. In section 2, the highest GSI means (F: 9.97, M:0.93) were registered, compared with section 1 means (F: 6.15, M: 0.91). Unlike section 2, no spawned females or spent males were captured in section 1. Water temperature, dissolved oxygen and turbidity presented higher values in section 2. Similar results were also observed downstream from other hydroelectric power plants.

Conclusion

These results show that *B. orthotaenia* found favourable conditions for reproduction just in section 2 of the São Francisco River, probably due the suitable water parameters from the tributary Abaeté River, which flows 34 km downstream from the Três Marias dam.

REPRODUCTIVE PHYSIOLOGY OF *ASTYANAX FASCIATUS* (TELEOSTEI: CHARACIFORMES) IN CAPTIVITY

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Introduction

Anthropic actions, such as the construction of reservoirs, alter fish migration affecting the normal function of the brain-pituitary-gonads axis. To mitigate the environmental impact on the biota, fish restocking programs aim to reproduce fish species affected by dams. In the Ponte Nova Fish Farm, in the Upper Tietê River Basin, the migratory fish *Astyanax fasciatus* is routinely produced for restocking with a conventional artificial breeding protocol , but its success varies among the females. A cytogenetic study detected that the number of chromosomes was different among the broodstocks, a common feature in *A. fasciatus*. The purpose of this study is to analyze the reproductive physiology of *A. fasciatus*, hypothesizing that endocrine changes may explain the distinct responses to breeding protocols in females with different chromosome numbers.

Methods

We defined the following experimental groups: G1-46 chromosomes and low success in induced breeding; and G2 - 48 chromosomes and high success in induced breeding. Both groups, composed of wild animals, were sampled in captivity throughout the year. In each season, 6 females from each environment were sampled. The ovaries were excised for calculation of the fecundity and gonadosomatic index and plasma collected to measure estradiol (E_2) levels. The Two-way ANOVA statistical test was accomplished to determine the environmental and seasonal effects on the reproductive activity.

Results and Discussion

Females of G1, in both environments, began the vitellogenic phase of the reproductive cycle in the winter, with an increase in E_2. In the wild animals, the highest percentage of vitellogenic oocytes was maintained in the ovaries from spring to summer, a period in which E_2 levels remained high. On the other hand, in captivity, a higher level of E_2 was observed only in winter. Even so, the females held in captivity maintained the fecundity significantly higher when compared with the wild ones, and also a higher percentage of vitellogenic oocytes, demonstrating the absence of spawning and a delay in the yolk absorption. In group G2, the vitellogenic stage of oocytes in wild females started in the fall, i.e., before the period observed in G1, with a progressive increase in E_2 levels, reaching its peak in spring, and spawning from summer to fall. Contrastingly, in the G2 females in captivity, E_2 levels and fertility rate remained high throughout the year, even higher than in wild females, almost every year.

Conclusion

G1 females maintained a kind of seasonality in captivity, but with a pattern that differs from that originally presented in the wild animals. Thus, the practice of induced breeding can only be successful if animals are handled soon after the beginning of the vitellogenic period, i.e., in the springtime. On the other hand, in G2, when in captivity, females have greater plasticity and do not perceive the environmental tips, losing the seasonal synchrony, but adopting reproductive tactics that keep physiological conditions that enable success in induced spawning almost all year. Molecular systematic studies have been investigating the possibility that these animals belong to different species.

SEXUAL DIFFERENTIATION IN FERTILIZED EGGS OF MEDAKA FOLLOWING EXPOSURE TO ENDOCRINE DISRUPTING HORMONES

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Introduction

It has been recently shown that genotyping sex can be easily changed by a single and brief immersion of the fertilized eggs, in a saline containing heterogeneous sex steroids. However, it is still an open question how exogenously administrated sex steroids can alter the pathway of sex differentiation to either ovary or testis during embryonic development. In this study, potential enhancement of sex reversal and transcript alternations of genes involved in sex steroidogenesis or differentiation were investigated following exposure of fertilized eggs of medaka (*Oryzias latipes*) to androgenic and estrogenic hormones. The key aim was to develop molecular marker(s) determining sex differentiation in the early life stages of medaka.

Methods

Medaka strain of SK-mc2 was used because of the potential to separate male and female embryos on the basis of leucophores at 4 days post fertilization (dpf). The fertilized eggs were exposed to 10 ng.mL^{-1} of Ethinyl estradiol (EE_2, a potent synthetic estrogen) and to 2.5 or 10 ng.mL^{-1} of 17α-methyl testosterone (MT, a potent synthetic androgen) for 24 h and let the eggs grow in tap water until 45 dpf. Samples were taken for transcriptomic and histological analysis.

Results and Discussion

Transcripts of *cyp19b* (enzyme that catalyzes androgens into estrogens) and *vtg* mRNA were increased following 5 and 10 dpf in the EE_2 exposed male embryos, respectively. But no changes were observed in the sex steroid receptor mRNAs. Among genes involved in sex differentiation such as *dmy*, *foxl2* (a marker of ovarian differentiation), and *sox9b* (a marker of testicular development), only *dmy* was changed in male embryos exposed to 2.5 ng/ml MT at 10 dpf. In females, *sox9b* showed slight increase following exposure to 2.5 ng/mL MT. Gonadal histology analyses showed development of spermatogonia in the ovary and appearance of oocyte in testis of female and male embryos.

Conclusion

Our research confirmed that a single 24 h exposure of fertilized eggs to sex steroids can alter the sex determining pathways in medaka following fertilization.

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INFLUENCE OF ACIDIC pH, ALUMINUM AND MANGANESE IN TESTICULAR STEROIDOGENESIS OF *ASTYANAX BIMACULATUS* (CHARACIFORMES: CHARACIDAE)

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Introduction

Metals can cause a wide range of adverse effects on the reproductive functions of animals, mostly on aquatic organisms. They can alter the endocrine system, acting on gonadal steroids, which can affect the reproductive process of fish. We aimed to investigate the effects of the metals aluminum (Al) and manganese (Mn) in acidic pH on the steroidogenesis of sexually mature male individuals of *Astyanax bimaculatus*, after an acute exposure of 96 hours, and evaluate if the animals were able to recover from the possible effects of these metals in clean water.

Methods

Sexually mature *Astyanax bimaculatus* males were exposed to a concentration of 0.5 $mg.l^{-1}$ of Al and Mn, and the experimental groups were maintained at acidic pH (5.5) to keep the metals in the dissolved state. We performed an experiment keeping the samples under an acute exposure for 96 hours, with samplings taken at 24h and 96h, and thereafter, a period of 96h in clean water, with samplings taken at 120h and 192h from the beginning of the experiment. We measured the plasma levels of testosterone (T), 11-ketotestosterone (11KT), 17β-estradiol (E_2) and cortisol (C) using ELISA immunoassay.

Results and Discussion

The metals studied, either alone or combined, in addition to acidic pH, were able to alter plasma concentrations of the androgens T and 11KT. A transient increase (24h) in E_2 levels was also observed, but only in animals exposed to Mn. Moreover, the interaction of Al and Mn had an antagonistic effect on E_2 levels, reducing plasma levels. Exposure to acidic pH and metals, alone or combined, did not trigger changes in C levels. The period of 96 hours in clean water was not enough to detect any form of recovery of the animals, except for 11KT levels, which returned to the baseline values after combined exposure to both metals. On the other hand, the exposure to the acidic pH only provided a recovery of androgen levels in 120 hours in clean water. Generally, Al and Mn, alone or combined, as well as the acidity of water, can act as endocrine disruptors, mainly by stimulating the androgen synthesis, causing changes in the physiological system. However, 24 hours in clean water was sufficient for the animals to recover from the effects of acidity in water. However, even 96 hours were not enough for them to recover from the effects of the metals.

Conclusion

The metals studied, as well as the acidity of water, may be considered endocrine disrupters in males of *A. bimaculatus*. Reproductive tactics were used by the species to trigger changes in testicular steroidogenesis, mainly accelerating the process of spermatogenesis and spermiogenesis, which may interfere with the reproductive dynamics.

STEROID EXPOSURE INFLUENCES SEASONAL VARIATION IN REPRODUCTIVE GENE EXPRESSION IN PITUITARY PRIMARY CULTURE FROM ATLANTIC COD (*GADUS MORHUA*)

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Introduction

Gonadal steroids assert feedback on all levels of the brain-pituitary-gonadal axis, and are involved in the regulation of the production of gonadotropins. In the present study, primary pituitary cultures from Atlantic cod were prepared at different times of the year, representing the different reproductive stages in cod: resting, maturing, mature and spent. The cultures were exposed to different concentrations of either testosterone (T), estradiol (E2) or dihydrotestosterone (DHT) to investigate the effect on follicle-stimulating hormone (*fshb*), luteinizing hormone (*lhb*) and gonadotropin releasing hormone receptor (*gnrhr1b* and *gnrhr2a*) gene expression. In addition, possible effects on cell viability were also investigated.

Methods

Primary pituitary cultures were prepared from both male and female Atlantic cod at different times of the year. Optimized culture conditions regarding pCO_2, osmolality, pH and temperature were applied. Cells were seeded in multi-well plates at a density of $1.5 \times 10^5/cm^2$ and exposed to steroids at day 4. Steroids were dissolved in ethanol and diluted in L-15 medium prior to exposure. An equivalent concentration of ethanol was added to control cell media. At day 7, the cells were harvested and used either for cell viability testing with a combination of two fluorometric assays (Alamar Blue and 5-carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM), detecting metabolic activity and membrane integrity, respectively) or qPCR gene expression analysis.

Results and Discussion

No negative effects on either metabolic activity or membrane integrity were detected after a three day exposure to T, E2 or DHT. All measured responses were either identical or positive compared to control, regardless of reproductive stage of the fish used for culture and of steroid used.

Gene expression of all genes investigated; *lhb*, *fshb*, *gnrh1b* and *gnrh2a*, showed an expected seasonal variation in both control and exposed cells, most notably with high levels of *lhb* expression in the mature stage and high levels of *fshb* in the maturing stage and very low lovels in the spent stage. In general, steroid exposure led to no or positive effects on gene expression compared to control, but with considerable differences between the steroids. Exposure to either E2 or DHT generally showed a more potent stimulatory effect than did T.

Conclusion

Individual steroids had differential effects on the measured mRNA at different reproductive stages. However, all steroids showed similar stimulatory effect on cell viability in all cultures.

TERATOGENIC EFFECTS OF ORGANOPHOSPHOROUS PESTICIDE, CHLORPYRIFOS, ON FRESHWATER CATFISH, *HETEROPNEUSTES FOSSILIS* (BLOCH)

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Introduction

The use of chlorpyrifos {O,O–diethyl–O (3, 5, 6-trichlor-2-pyridyl) phosphorothioate; CPF} organophosphorous pesticide has been suggested for the eradication of number of pests, to increase the crop production. It reaches the aquatic environment mainly by runoff or drainage from treated agricultural lands, inadvertently exposing the non-target organisms especially the fish. Exposure to CPF can affect both the fertility and reproductive outcomes because of its ability to interact with steroid hormones receptors. Our aim was to investigate the extant of teratogenic potential caused by Hilban$^{(R)}$ (20% CPF) on hatchlings of catfish (*Heteropneustes foosilis*).

Methods

The broodstock of *H. fossilis* were collected from commercial fisherman located in Lucknow, U.P., India and acclimatized in the laboratory in glass aquaria. Matured fish were administered with HCG hormone (6.95 IU/g dose of body mass) intramuscularly. After the stripping, the fertilized eggs were transfered in different dilution of Hilban$^{(R)}$ (1/20th, 1/60th, 1/500th, 1/4000th, 1/7000th of 96 h LC_{50}; 0.24, 0.082, 0.009, 0.0012, 0.0007 µl/l) along with control set of fertilized eggs. The teratogenic changes were recorded after 24 hr of hatching.

Results and Discussion

The result showed that chlorpyrifos produced a concentration dependent inhibitory response in normal development of the hatchlings. Among teratogenic changes, larvae of exposed groups were mainly affected by ventral and/or lateral tail flexure coupled with abnormal spinal bending and odema. The major categories of gross morphological deformities were observed viz., irregular head shape and size, loss of eye, reduced barbel, pericardial oedema, yolk sac oedema, notochordal defect, finfold defect, reduction of brain development and reduction of pigmentation. In higher concentrations of sublethal dose hatchlings were recorded with combinations of these abnormalities. These muscular and morphological damages may be related to inhibition of acetylcholinesterase activity.

Conclusion

We speculate that these teratogenic changes showed a clear concentration dependent response of CPF on exposed hatchlings. Results suggest a possible role played by OPs on induction of muscular dystrophy and morphological deformities during development.

TROPHIC TRANSFERENCE OF FATTY ACIDS IN FEMALES OF *ASTYANAX FASCIATUS* (TELEOSTEI: CHARACIDAE), INHABITING A FRESHWATER SYSTEM WITH ANTHROPOGENIC IMPACT

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Introduction

The increasing anthropic actions and continuous population growth in watershed areas have boosted the pollution levels in aquatic ecosystems, mainly due to the increased availability of nutrients. This nutrient load that is discharged daily into the rivers, mainly by sewage, is changing the community of water bodies, due to the excessive production of plankton biomass. Changes in physico-chemical characteristics of water or others modifications of habitats result in alterations of food resource for fish, changing the composition of fatty acids (FA), along the food web. FA are considered important trophic biomarkers, in addition to playing a crucial role in the reproduction of fish. Therefore, this study aims to understand how the trophic transfer of FA occurs in *Astyanax fasciatus* females at the mature gonadal stage, in an impounded environment.

Methods

Adult females were sampled in two reservoirs in the São Paulo State (Brazil) with different Trophic State Indices (TSI): Ponte Nova (PN, a mesotrophic reservoir) and Billings (BIL, an hypereutrophic reservoir) at the mature gonadal stage profile of plankton, stomach content, adipose tissue and ovarian triglycerides (TG), and phospholipids (PL) were analyzed by gas chromatography.

Results and Discussion

Physico-chemical parameters of water were analyzed, showing higher levels of xenobiotics, such as metals in BIL than in PN reservoir. The levels of FA in plankton showed a predominance of C16 PUFA (polyunsaturated FA) (diatoms biomarkers) in the PN reservoir, while C20:5n3 (eicosapentaenoic acid, EPA) and C22:6n3 (docosahexaenoic acid, DHA) (diatoms and dinoflagelatte biomarkers), were higher in BIL. In the stomach content, C18:2n6 (linoleic acid, LA) -a terrestrial plant biomarker-, and C18:1n9 (oleic acid, OA) -a zooplankton biomarker-, were predominant in the PN females, while C18:3n3 (linolenic acid, LNA) -biomarkers of insect larvae-, and EPA were predominant in the stomach content of animals from BIL. Odd FA and C18:1n7, FA from bacterial, were higher in BIL than in PN. The difference of FA levels in plankton, was reflected not only in the adipose tissue but also in mature ovaries, with a predominance of n6 PUFA (LA in TG and PL; C20:4n6 in PL) in the ovaries of females sampled in PN, while a higher percentage of n3 PUFA (EPA and DHA) was observed in females collected in BIL.

Conclusion

The observed differences are the result of the FA levels in the stomach content, besides the incorporation of other autochthonous components such as insects, and allochthonous material such as terrestrial plants. Thus, alterations in n3/n6 in the animal tissues, could alter both, the immune system and the reproductive processes, which depends on the balance between n3 and n6 PUFA.

WIDESPREAD OCCURRENCE OF THE INTERSEX CONDITION AND ASSOCIATED MOLECULAR RESPONSES IN THICKLIP GREY MULLET (*CHELON LABROSUS*) FROM THE BASQUE COAST (SOUTH EAST BAY OF BISCAY)

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Introduction

Thicklip grey mullet (*Chelon labrosus*) belongs to the worldwide distributed gonochoristic family of Mugilidae. Individuals of this family inhabit marine, estuarine and freshwater ecosystems and can endure highly polluted environments such as effluent discharge sites, port areas and highly urbanized sites. Thus, mugilids are used as sentinels of environmental pollution and biomarkers of exposure and stress have been assessed in these organisms. Effects of endocrine disruption have been described in mullet populations, including occurrence of intersex gonads. However, molecular mechanisms underlying the intersex condition in mullets are not well understood. In the present work we aim to present data on intersex condition in mullets from the Basque coast and on the potential association of intersex to molecular endocrine disruption responses such as changes in cyp19 aromatase transcription levels and other estrogen dependent genes.

Methods

Mullets collected in seven sites (Santurtzi, Plentzia, Arriluze, Gernika, Ondarroa, Deba and Pasaia) from of the Basque coast between April 2007 and June 2013 were studied for the presence of intersex gonads by histological assessment. Transcription levels of cyp19a1 and cyp19a2 aromatases, together with other endocrine disruption related genes such as vitellogenin, estrogen receptor and retinoid-x-receptor were quantified by qPCR. Additionally, steroid plasma levels, gonad aromatase activity and transcription levels of genes associated to steroid hormone regulation (star, cyp11b, ugt and sult) were determined.

Results and Discussion

Intersex gonads were detected all along the study and in four out of the seven populations studied. Histological characterization of the intersex condition revealed that in most cases previtellogenic oocytes were scattered in the testicular tissue. The severity of the intersex condition in mullets from the Basque coast could be considered low to moderate, based on the testicular surface occupied by oocytes. Intersex mullets showed up-regulated levels of cyp19a2 in the brain, but no significant differences in ovarian cyp19a1 and aromatase activity were detected, probably due to the low number of oocytes per testicular area. Vitellogenin transcripition levels did not follow a clear pattern in intersex mullets, suggesting that other compounds different from xenoestrogenic pollutants could contribute to the intersex development in mullets.

Conclusion

Intersex condition is widespread in mullets inhabiting the Basque coast, demonstrating exposure to endocrine disrupting compounds. The molecular mechanisms leading to the development of intersex in mullets are not clear, but a potential role of cyp19a2 could be hypothesized. Further works are required in order to elucidate biological mechanisms regulating the development of the intersex condition in this sentinel species.

INFLUENCE OF TIDAL CYCLES ON THE CONTROL OF REPRODUCTIVE ACTIVITY IN COMMON SNOOK (*CENTROPOMUS UNDECIMALIS*)

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Introduction

Research has shown that in temperate fish species, the brain pituitary gonad (BPG) axis is entrained by external cues such as seasonal variation in environmental conditions including photoperiod and temperature. However, other environmental signals (lunar and tidal cycles) appear to be involved in tropical fish species. In the present study, we developed and validated real-time quantitative RT-PCR assays for gonadotropin genes (*fshβ* and *lhβ*) from the pituitaries of sexually mature male and female common snook *Centropomus undecimalis*. Temporal expression patterns of *fshβ* and *lhβ* during the reproductive cycle were then evaluated in relation to sex steroid production, LH blood plasma levels, gonadal development and tidal cycle.

Methods

A total of 38 male and 50 female adult common snook were collected from three locations in Manatee County (Florida, USA). Blood samples were taken for steroid and gonadotropin analyses. Fish were then sacrificed: the pituitary was collected for gene expression profiling of *fshβ* and *lhβ*; gonads were fixed for histological examination. To account for daily or seasonal variation in tidal cycles, data were transformed so sample collection times could be normalized and expressed irrespective of tidal length.

Results and Discussion

The phylogenetic analysis of the deduced amino acid sequence of snook for *fshβ* and *lhβ* revealed strong identity with other teleosts (75-90%). Histological observations of gonads showed a diurnal rhythm of follicular development strongly associated with the tidal cycle. This confirms the key role played by tide on the control of snook gonadal development and spawning. Female snook sampled on the rising tide, were all found to have oocytes that were in the secondary growth stage; whereas those females which had oocytes in the later stages of maturation and ovulation were only observed at high tide or on the falling tide. Additionally, the expression of *fshβ* and *lhβ* increased during vitellogenesis as for LH plasma levels and reached peak expression levels at ovulation in females. Plasma estradiol and testosterone significantly increased in late vitellogenesis and final oocyte maturation respectively. In males, *lhβ* mRNA expression peaked at the mid germinal epithelium (GE) stage as for testosterone and 11-KT in the blood while *fshβ* expression and plasma LH levels peaked at late GE stage.

Conclusion

This study confirms the role played by tidal cycle on the entrainment of gametogenesis of common snook. A better understanding of the environmental control of reproduction in the species will help broodstock management in captivity which remains problematic.

REPRODUCTIVE BIOMARKERS RESPONSES INDUCED BY XENOESTROGENS IN THE CHARACID FISH *ASTYANAX FASCIATUS* INHABITING A SOUTH AMERICAN RESERVOIR: AN INTEGRATED FIELD AND LABORATORY APPROACH

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Introduction

Field studies evaluating the effects of endocrine disruption chemicals (EDCs) on the fish reproduction are scarce worldwide. The goal of this study was to assess hepatic levels of vitellogenin (Vtg), *zona radiata* proteins (*Zr*p) and insulin-like growth factors (IGF-I and IGF-II), and relating them to reproductive endpoints in a wild fish population habiting a reservoir that receive domestic sewage, agricultural and industrial residues.

Methods

Adult fish *Astyanax fasciatus* were sampled during the reproductive season in five sites from the Furnas Reservoir, Grande River, Paraguay-Paraná basin. As a control to field data, fish were experimentally exposed via dietary intake, to oestradiol benzoate (OB) for 7 days.

Results and Discussion

Fish from site with little anthropogenic interference showed hepatic levels of Vtg, *Zr*p and IGF-I and IGF-II similar to those from the non-treated experimental group. In sites located immediately downstream from the municipal wastewater discharges, the water total oestrogen was > 120 ng/l, and male fish displayed increased Vtg and *Zr*p and decreased IGF-I levels similar to OB treated fish. In females, higher levels of Vtg, *Zr*p and low IGF-I suggest an impairment of final oocyte maturation and spawning, as also detected by high frequency of over-ripening, increased follicular atresia and reduced fecundity. At the sites that receive agricultural and industrial residues, the water total oestrogen was < 50 ng/l and females showed decreased *Zr*p and increased IGF-II levels associated to reduced diameter of vitellogenic follicles, indicating an inhibition of ovarian development.

Conclusion

Overall, the current study reports oestrogenic contamination impairing the reproduction of a wild fish from a hydropower reservoir and, the data contribute to improving the current knowledge on relationship between hepatic Vtg, *Zr*p and IGF-I and IGF-II, and reproductive endpoints in a teleost fish.

IMMUNOASSAYS ARE NOT IMMUNE TO ERRORS – EXAMPLES FROM TWO STUDIES MEASURING SEX STEROIDS IN RIVER WATER

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Introduction

In 2007, a scientific publication reported very high concentrations (means of 4 to 79 ng L^{-1}) of three sex steroids (11-ketotestosterone (11-KT), testosterone (T) and oestradiol (E2)) in river water immediately downstream of rainbow and brown trout farms in England. These concentrations are higher than in the output from many sewage treatment works and thus potentially a major source of endocrine disruption. We set out to investigate this finding – our initial hypothesis was that the reported concentrations were falsely high due to matrix effects disturbing the EIAs (=ELISAs) used in the 2007 study.

Methods

Water samples were collected from the same sites in 2010 and extracted in the same way (C18 and aminopropyl solid phase cartridges). The extracts (representing a 1000-fold concentration from the original water sample) were split between four laboratories to be assayed blind for E2, 11-KT and T by both RIA and EIA (to test our hypothesis).

Results and Discussion

Several mistakes occurred in the 2010 study, at three of the four laboratories. Once mistakes had been identified (by comparison between laboratories) and corrected, the differences in concentrations between RIA and EIA (and between laboratories) were negligible, disproving the initial hypothesis. However, the concentrations of steroids in the river water in the 2010 study were far lower than reported in the 2007 study 100-fold for E2 and T, and 1000-fold for 11-KT. Possible massive changes in conditions (e.g. water flow, farmed trout diets) between the two studies were investigated but dismissed. A low extraction efficiency in the 2010 study was also ruled out from spiked samples. The only remaining explanation was that there had been a major calculation error in the 2007 study. Circumstantial evidence suggested that the concentrations reported in the 2007 study were not ng L^{-1} of the original water sample, but ng L^{-1} of assayed extract (i.e. no corrections had been made for any extraction concentration or assay dilution steps). If the 2007 data were recalculated on the basis of this hypothesis, then average steroid concentrations in both studies were similar (all < 1 ng L^{-1} and of no consequence). Although this hypothesis should have been easy to prove, all raw data (lab book information, assay results and calculations) from the 2007 study had been discarded in the intervening period!

Conclusion

Assessment of steroid concentrations in the environment involves many steps (e.g. sample site selection, sample collection, sample storage, extraction, assay, calculation and final interpretation) which provide numerous opportunities for mistakes. To err is human. It is essential that:

- spreadsheets and calculations are independently checked before results are released into the public domain;
- raw data are never discarded before peer reviewers and independent experts have had an opportunity to raise questions about the results.

PLASTICITY OF THE GnRH3 SYSTEM IN DEVELOPING ZEBRAFISH

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Introduction

GnRH3 is the major hormonal system responsible for sexual maturation in zebrafish. It stimulates the synthesis and release of gonadotropins from the pituitary gland, which, in turn, drive gonadal development and maturation. During early development, GnRH3 neurons undergo a long-distance tangential migration, starting from the olfactory region and reaching the hypothalamic preoptic area. Our laboratory previously showed that knocking down GnRH3 and other specific neurofactors, via antisense approaches, completely inhibited the migration of GnRH soma and neurons during early development in zebrafish. Moreover, the early and complete ablation of the GnRH3 soma led to the elimination of the GnRH3 system in adult fish and to full sterility. The aim of our study was to investigate whether a short-term pharmacological approach, using immersion in non-lethal compounds during early stages of development, can irreversibly disrupt the neuromigration of the GnRH precursor cells. It would prevent the establishment of the functional GnRH system and, in turn, the requisite pubertal development of the gonads.

Methods

GnRH3-eGFP transgenic zebrafish were exposed, via immersion, to different compounds beginning immediately before or after fertilization and during the next 5-7 days. The migration of GnRH3 somas/projections was monitored by *in vivo* imaging or immunohistochemistry throughout development. The GnRH3 system pathway and gonad histology were also studied later at sexual maturity.

Results and Discussion

Treatments with compounds like a short GnRH metabolite (GnRH1-5), the gonadotropic inhibiting hormone (GnIH), an IGF signal blocker and CxCR4 antagonists caused similar effects. These chemicals delayed the early migration of GnRH3 neurons for about 10 days, but at the age of 2-3 months the fish GnRH3 system, as well as their gonads, appeared normally developed and no obvious differences from control fish were observed.
Interestingly, when embryos were exposed to estrogenic compounds as well as to cobalt chloride (a chemical HIF-1 inducer), the GnRH3 somas and projections were advanced and increased in number. This effect is evident as soon as at 48 hpf.

Conclusion

Our findings show that an early and short exposure to several compounds is able to disrupt or delay the migration of GnRH3 somas and afferents neurons in developing embryos. However, these effects are soon lost since the normal GnRH3 neuronal pathway is observed in 2-3 month old brains. This is evidence of the great developmental plasticity of the GnRH system in zebrafish, which is able to recover from a severe early disruption. This plasticity assures sexual maturation and a normal reproductive axis even when fish have been exposed to stressful conditions or events during early development.

ASSESSING REPRODUCTIVE AND DEVELOPMENTAL TOXICITY IN ZEBRAFISH AFTER LONG-TERM EXPOSURE TO GALLIC AND PELARGONIC ACIDS

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Introduction

Gallic acid (3,4,5 trihydroxybenzoic acid) and pelargonic acid (nonanoic acid) are naturally found in a variety of plants or common food products. Despite their extensive use in a growing number of man-made applications including industrial, agrochemical, pharmaceutical and health perspectives, little is known regarding their occurrence and fate in natural surface water or artificial ponds. In particular, the associated toxicological risks subsequent to passive exposure of fish in these environments remain to be investigated. The aim of this work was to assess the biological effects of long-term exposure of zebrafish to these two organic acids under flow-through conditions. Because breeding success is of primary importance for fish population sustainability, special focus was made on reproductive parameters and embryo-larvae development.

Methods

Adult specimens of *Danio rerio* (TU strain) were acclimatized in the laboratory for two weeks in dechlorinated tap water. During this period, fish reproduction and development were regularly checked. Then, groups of randomly selected 5 males and 5 females were placed into 10 L (1 fish.L^{-1}) glass tanks and exposed to gallic or pelargonic acid at different concentrations (0; 0.05; 0.5; 5 mg.L^{-1}). 70% daily water exchange was made using peristaltic pumps to maintain a steady dose of pollutant. After one month of exposure, fish were sacrificed, liver and blood were sampled for assessment of usual biomarkers (biotransformation and oxidative stress related enzymes), vitellogenin and sexual-hormone levels. Gonads were fixed in Bouin's solution for histopathological examination. Reproductive performance, embryo-larvae development and larval resistance to starvation were evaluated following regular spawning trials.

Results and Discussion

During all the exposure period, no mortality neither abnormal behavior could be reported. The exposure of fish to both type of organic acids affected to various extent their reproductive performance. Moreover, delay in median hatching time associated with developmental abnormalities could be observed, particularly from middle to high level of doses depending on the tested compound. Measurement of fish physiological parameters with histological examination allowed a better understanding of the potentially involved toxicity mechanisms. Finally, there was also clear assumption that direct egg exposure to contaminated water could impair further embryo development.

Conclusion

Chronic exposure of fish to low doses of naturally occurring and commonly used gallic and pelargonic acids may induce adverse biological effects depending on the dose. Additional investigations are needed to better assess the ecotoxicological risks that may arise in the context of exposure to multiple environmental contaminant stressors.

EFFECT OF THERMAL REGIME ON VITELLOGENESIS, OVULATION AND LARVAL DEVELOPMENT OF EUROPEAN EEL

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Introduction

European eel perform a long reproductive migration at high depth and low temperatures while water temperature in the spawning grounds in Sargasso sea is warm (18-20 ºC). Also, it is known that eels perform daily vertical migrations, and then experience daily thermal changes during their migration (Aaerestrup *et al*., 2009). Our objective has been to test if thermal regimes more similar to the natural ones could improve vitellogenesis and ovulation in this species.

Methods

Three experiments were performed to study the effect of temperature on sex development of female European eels. All female eels were hormonally matured with weekly injections of carp pituitary extract (CPE, 20 mg*kg^{-1}). In the first experiment, eels were kept in a variable thermal regime (from 10 to 17 ºC) and in a constant regime (20 ºC). In the second experiment, eels were kept under 3 thermal regimes, 2 variable (T10: 10 ºC 6 weeks +15 ºC 8 weeks; T15: 15 °C 10 weeks + 20 °C 4 weeks), and one constant (T18) at 18 ºC. In the third experiment, eels were maintained first 12 weeks under 2 regimes: VAR (weekly changes 10-15 ºC) and CTE (15 ºC), and then temperature was elevated to 18 ºC. Steroid levels were measured by ELISA (T, 11-KT) or RIA (E2). Specific qPCR analyses for selected genes were performed.

Results and Discussion

In the first experiment the low variable thermal regime promoted the early vitellogenesis stages through increased E2 levels, but high constant temperatures caused acceleration in the last vitellogenic stages. That was confirmed in the second experiment, in which the low E2 levels at high temperatures (T18) were related with low *cyp191a* expression. Low 11-KT levels, previously related to oocyte previtellogenic growth, were also observed in group T18. This group also showed lower pituitary expression of *lhβ* and *gnrhr2*. Those results suggest that early ovarian development can be stimulated at low temperatures of 10-15 ºC. However, late vitellogenesis was only observed in eels maintained at 18 ºC (4 weeks exp. 3, 12 weeks exp. 2). In the third experiment, both regimes originated high quality eggs, (72-75 % fertilization rate), but VAR group originated higher egg buoyancy and embryo development.

Conclusion

Our results indicates that before hormonal treatment, low temperatures promote early vitellogenesis in eel through changes in steroidogenesis and the expression of some key genes in brain and pituitary. Thus, by combining hormonal treatment with thermal regimes starting at low temperature and increasing it later, egg quality can be improved, as indicated by our last results.

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THE PITUIRARY ADENYLATE CYCLASE-ACTIVATING PEPTIDE (PACAP) IN THE GONADS OF *CHIROSTOMA HUMBOLDTIANUM*

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Introduction

Chirostoma humboldtianum is an endemic fish from central highland and western of Mexico. Traditionally, *Chirostoma* genus has been used as food since prehispanic times. This genus includes the silverside fish.

Pituirary adenylate cyclase-activating peptide (PACAP) is well known for its stimulating role in GH release from pituitary. Less known are our knowledge about the presence of PACAP in the gonads of teleost.

The aim of this work was determinate the PACAP peptide in the testis and ovary from *Chirostoma humboldtianum.*

Methods

Fish from Zacapu lagoon, Mexico, were collected during recrudescent and mature phase of sexual cycle. Fish were anesthetized, dissected and the gonads were removed. One of them was frozen in dry ice until their arrival to laboratory. Total RNAs were extracted throughout Trizol reagent. The integrity of RNAs was corroborated by Agarose gels. cDNAs were made through out RT-PCR using First Strand kit. For PCR, the primers used were two set originally described to search catfish. The annealing at 54°C. The products were purified, and sequenced in ABY system 3600 sequencer. Another gonad was fixed in Bouin, dehydrated and included in paraffin. The gonads were sectioned at 5 μm. The immunostain was made with commercial antiPACAP27 antibodies in dilution 1: 300.

Results and Discussion

Our results one product of 432 bp (G.B. JN157843.1) previously reported for this specie is expressed in both gonads in both phases of the sexual cycle, recrudescent and mature. Also, in the testis the spermatogia and primary spermatocytes were immunopositive to PACAP. On the ovary, granulosa and theca cells were reactive to PACAP antibody during exogenous vitellogenesis.

Conclusion

PACAP seems participate in the early gametogenesis and steroidogenesis in *C. humboldtianum*.

RELATIONSHIP BETWEEN SEXUAL PLASTICITY AND BODY GROWTH IN THE OVARY OF KOI CARP (*CYPRINUS CARPIO*)

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Introduction

Recently, it had been shown that inhibition of estrogen biosynthesis induces complete masculinization in adult female of some gonochoristic teleosts, indicating that sexual bipotentiality is maintained far beyond the embryonic labile period. Workable methods for sex control is desired in aquaculture of koi carp (coloured carp), a major ornamental fish, because females have higher commodity value. However, growth rate of carp is highly variable depending on population density and feeding rate, and has been inferred to influence on gonadal sex differentiation. In this study, we investigated the relationship between sexual plasticity and body growth.

Methods

Genetically controlled female (all-XX) populations of koi carp with different growth rate were fed a diet containing exemestane (1mg/g diet), an aromatase inhibitor (AI), from 2, 3, 4 and 6 months after hatching (MAH). A part of fish were sacrificed at 4 months and subjected to histological analysis.

Results and Discussion

In the higher growth rate (low density with normal feeding) groups, administration of AI from 2, 3 and 4 MAH induced well developed testes by 4 months, whereas ovarian tissues coexisted with testicular tissues by administration from 6 MAH. In the lower growth rate (high density with restricted feeding) groups, complete masculinization was observed by administration from 2, 3 and 4 MAH, while testicular tissues did not well developed suggesting the pubertal suppression under restricted growth. The lower growth rate group for administration from 6 MAH was lost accidentally. These findings suggest that sexual bipotentiality in female carp gonad is maintained after ovarian differentiation. This investigation is ongoing to elucidate the alteration of plasticity with maturation.

Conclusion

Sexual plasticity in carp ovary is maintained after ovarian differentiation, while gradually attenuated in the process of puberty. Further investigations will be required to fully clarify the mechanism underlying sexual plasticity.

CANDIDATE GENES FOR STERILITY IN ATLANTIC SALMON (*SALMO SALAR*)

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Introduction

Early sexual maturation and genetic interference with wild stocks are major challenges in salmon aquaculture, and production of sterile individuals would overcome these issues. Sterile zebrafish has previously been produced by ablation of primordial germ cells (PGCs), which may be a promising method to induce sterility in other commercially important species. However, limited information exists on which factors that are essential for PGC migration and survival within the developing gonads of salmon, and if knock-out of genes encoding them will interfere with other processes than gonad development. Our aim was to study spatial and tissue specific gene expression of known "sterility genes" previously identified in model species like zebrafish and mouse, with the main aim to identify salmon sterility candidates expressed exclusively in gonads and in PGCs in embryos.

Methods

Based on homology with other species, a number of candidate genes (candidates 1-9) were predicted in the most recent salmon genome assembly (Acc. No. AGKD00000000.1). These genes were measured by PCR in the following salmon tissues: testis (T), ovary (O), brain (B), pituitary (Pit), skin (S), muscle (M), liver (L), vertebrae (V), gut (G), eye (E), heart (H), 30 and 700 day degrees (d°) embryo. *In situ* hybridisation of candidate genes was performed on sections of testis and ovary from immature Atlantic salmon.

Results and Discussion

Due to the partial tetraploidy of Atlantic salmon several of the selected genes, candidates 1, 3, 5 and 6, displayed paralogous genes within the genome. The following genes were expressed exclusively in gonads and early embryos: candidates 2, 3 (variant 2), 6 (variant 2), and 7. All of these were expressed in both T and O. In addition, candidates 3 (variant 2) and 7 were expressed in 30 d° embryo, and candidate 6 (variant 2) was expressed in both 30 d° and 700 d° embryo. These genes are therefore good candidates for gene knock-out experiments aiming at disrupting gonad development. Ongoing *in situ* analyses have so far confirmed that candidate 3 (variant 2) and 7 are expressed in immature testis and ovary. Candidate genes from this study have been included in gene knock-out experiments. If this proves successful, resulting in sterile salmon, it may have large implications in salmon aquaculture. Vaccine trials will then be initiated, with the goal to eliminate the problems of early sexual maturation and genetic interference with wild salmon.

Conclusion

We have identified four candidate genes for sterility in salmon. Ongoing functional studies will reveal if we can successfully produce sterile salmon.

ALTERNATIVE REPRODUCTIVE TACTCIS IN SNAIL-BROODING CICHLIDS DIVERGE IN ENERGY RESERVE MANAGEMENT

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Introduction

When individuals pursue alternative reproductive tactics (ARTs), their reproductive investment patterns may diverge substantially as bourgeois and parasitic males are likely exposed to different limitations and trade-offs. The Lake Tanganyika cichlid fish *Lamprologus callipterus* shows three ARTs involving an extreme size dimorphism between different male types that is determined by a Mendelian genetic polymorphism. Large males construct nests of empty snail shells in which females breed, and invest heavily in courtship and nest defence. In contrast, dwarf males do not invest in courtship and brood care, while attempting to parasitize the reproductive effort of nest males. A third, conditional and opportunistic sneaking tactic is performed by males of all sizes. Only few studies have focused on variation in intraspecific energy allocation patterns, although they are of particular interest when individuals pursue ARTs. Our aim was to study whether male energy reserve management diverges in dependence of the pursued tactic and the associated limitations and trade-offs.

Methods

After observation of the behaviour of nest males in the field over an experimentally chosen nest holding period ranging from 1 to 32 days, evisceral and visceral energy stores of focal nest males and of randomly chosen sneaker, dwarf and immature males were obtained using Soxhlett extractions. The two different fat fractions were used to compare long and short-term energy stores of different male types.

Results and Discussion

The energy reserve management of different male types differs as predicted. Sneaker males show the highest amount of accumulated long- and short-term energy stores. Individuals close to nest male status typically accumulate high amounts of fat stores, apparently in preparation of future reproduction as a nest owner. The energy-demanding nest holding period of bourgeois nest males resulted in a steady depletion of previously accumulated energy reserves, conforming to a capital breeding strategy. In dwarf males, the absence of costly courtship and brood care allows them to reproduce without reserve accumulation, as they can feed while searching for opportunities to reproduce. Hence, dwarf males pursue an income breeding strategy.

Conclusion

Intraspecific energy allocation patterns are important correlates of life history variation and represent crucial functional components of ARTs. Our data demonstrate that males pursuing ARTs may diverge substantially in the energy accumulation and management of energy stores.

Controlled feeding rescues the growth reduction associated with spawning in farmed yellowtail (*SERIOLA QUINQUERADIATA*)

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Introduction

Growth initiates sexual maturation in fish, but the onset of maturity also leads to product the risks associated with spawning, such as a marked loss in body weight. To reduce the production risks, fish farmers of yellowtail have in the past attempted to restrict the diets of their farmed fish. In our present study, we show through endocrinological and physiological analysis of harm-cultured yellowtails that the growth of these fish can be regulated through dietary control.

Methods

The fish of the control group (6673 fish in 12 m^3 cage) were fed to satiety. The fish in the experimental group (6379 fish in 12 m^3 cage) received a restricted diet. For calculation of feeding rate, the total number of fish and the feed volumes were counted. During the experimental periods, with one month-intervals, 50 yellowtails from each groups were measured for total fork length and body weight (BW) to analyze the growth differences. These 15 fish were collected bloods, gonads to analyze the gonad somatic index (GSI) and serum steroid hormones. These muscle samples were measured the contents of protein, fat, moisture, ash and lipase activity. Then, all fish were assessed for sexual maturation by histological sections.

Results and Discussion

Our study findings indicate the BW and fork lengths of a diet restricted group gradually increases through the breeding season compared with the controls. Eventually these of the diet restricted group surpassed the control groups. In the spawning season, the diet restricted group had smaller gonads than the controls in both sexes. However, the GSI rapidly increased in the control group compared with the restricted group and histological observations of the controls further indicated that the testes contained sperm, and that the female gonads contained numerous oocytes that had accumulated yolk. These findings confirm that a restriction of the diet suppresses gonad development during the yellowtail breeding season. The sex steroid hormone, 17α, 20β-diOHprog, was found to be associated with this dietary control of gametogenesis as it showed decreased levels in the restricted groups compared with the controls. To further investigate the functions of 17α, 20β-diOHprog on fish growth, this hormone was administered intraperitoneally to juvenile yellowtails. Implanted fish showed a remarkable increase in BW compared with the control group and the lipase activities of the dorsal and ventral muscles were also higher than those in the control group. These findings suggest that 17α, 20β-diOHprog is a candidate factor related to BW reduction in breeding yellowtail.

Conclusion

We conclude from our data that the growth inhibition and/or BW reduction caused by sexual maturation in farmed fish may be avoided by inhibiting gametogenesis through dietary regulation.

GRAVID-SPOT AS AN EXTERNAL INDICATOR OF EMBRYONIC DEVELOPMENT IN A LIVE BEARING FISH *GAMBUSIA HOLBROOKI*

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Introduction

In live bearing fish the gravid-spot is an excellent marker to identify brooding females, however its utility to predict progress of embryonic development, brood size and timing of parturition remain unexplored. Most livebearers, including *Gambusia holbrooki* exhibit asynchronous embryonic development and parturition with variable clutch size - often limiting an ability to predict reproductive output in wild fish or conduct experiments such as sex reversal in the laboratory. Furthermore, viviparity compels sacrifice of brooding mothers precluding multiple time point observations (i.e. pre and post parturition). To overcome these limitations we investigated the relationship between gravid-spot (size and intensity) and embryonic development, to predict the timing of parturition and clutch size.

Methods

Brooding females (n=100; 20–50 mm SL) were euthanased and images captured along with a colour (B&W) reference card using a mounted DSLR camera. The images were analyzed in ImageJ to quantify the gravid spot intensity and size. The intensity was numerically quantified in 8 bits/pixel format (256 shades of grey; 0 – black, 255 – white). Arbitrarily, the observed intensity values (IV) were categorized into four groups (groups 1-4) of 20 units ranging from 20–100 and the fish assigned to the respective groups. Each fish was dissected and the number of eggs and embryos counted. Staging of embryos was as described previously in *G. affinis*. Statistical analyses were carried out using SPSS. Parturition was observed in a separate group of fish.

Results and Discussion

The observed IVs and size of the gravid spots ranged between 28-92 and 0.002- 0.068 cm^2 respectively. Embryos or fertilized eggs were found in 51 females, falling into six broad categories: stage 2-7 (6-11 days post fertilization); stage 11 (9-14 dpf); stage 13 (9-14 dpf); stage 18 (11-17 dpf); stage 22 (15-18 dpf) and stage 25 (16-19 dpf). There was a significant difference ($P<0.05$) in the number of embryos in each stages between the intensity groups. The number of embryos in late developmental stage (stages 22 and 25; near parturition) was significantly higher in group 1 (IV 20–40; size 0.023-0.053 cm^2) compared to group 2 (IV 40–60; size 0.013-0.043 cm^2) - the former parturating within days and latter taking over a week. Groups 3 (IV 60–80) and 4 (IV 80–100) only possessed embryos at early developmental stages taking between 4-5 weeks to parturate. A combination of intensity and size of gravid spot is a convenient means to predict the status of embryonic development. We routinely use this to predict clutch size and timing of parturition.

Conclusion

The gravid-spot can be used as an external marker to predict the stages of embryonic development, timing of parturition and size of clutch in *Gambusia holbrooki*. This will be useful in multiple applications such as reproductive studies both in wild and in the laboratory.

L-TRYPTOPHAN SUPPLEMENTED DIET ENHANCES BRAIN SEROTONERGIC ACTIVITY AND REDUCES STRESS THRESHOLD: THE ROLE OF SEROTONIN IN TWO SOUTH AMERICAN CICHLID FISH

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Introduction

Recent studies have been focused on the lessening effect over aggression and stress of an enhanced serotonergic activity, by means of L-tryptophan (Trp) supplemented diets. These factors are detrimental to farming of comestible and ornamental fish, particularly in highly aggressive and densely reared species. *Cichlasoma dimerus (Cd)* and *Australoheros facetus (Af)* are South American cichlids easy to maintain and breed under laboratory conditions, and exhibit salient social hierarchies with agonistic interactions. Thus, they arise as excellent models to evaluate preliminary serotonin (5-HT) effects. Our first objective was to perform a morphological characterization of the brain serotonergic system in *Cd* and *Af*. Secondly, this study aimed to investigate if a Trp enriched diet could affect brain monoamine (5-HT and dopamine) levels, stress and sex hormones and growth in both species.

Methods

The distribution of 5-HT cell bodies and fibers was described by means of an immunofluorescence technique using anti-5-HT antibodies. Experiments were performed with fish held in separated aquaria (21 L) for 35 days. During the first week (acclimation period) animals were fed with a control diet (no added tryptophan -Trp0X). For the next 28 days, half of the individuals continued to be fed with control pellets, whereas the other half was fed with Trp enriched ones (Trp8X). Body weight and length were assessed and blood samples taken at days 7 and 35. The following plasma steroids were examined by ELISA: cortisol, 11-ketotestosterone, testosterone and estradiol. At day 35, brains were dissected, separated in forebrain and hindbrain and homogenised. Brain monoamines, dopamine (DA) and 5-HT and their metabolites, DOPAC and 5-HIAA were measured by HPLC-EC.

Results and Discussion

We found two brain regions with immunoreactive cell bodies: the raphe and the ventral forebrain; in the latter, somata appeared in the NPPv, NRLd, NRLv and NRP. Abundant fibers were present throughout the whole brain, few of them entering the pituitary gland. *Cd* males, but not females, showed a greater forebrain serotonergic activity (5-HIAA/5-HT) (approx. 20%) when fed with Trp8X; although not significant, a tendency was observed towards higher forebrain 5-HIAA/5-HT (15%) in *Af* males fed with Trp8X. Dopaminergic activity (DOPAC/DA) did not vary within the experimental groups. The decrease in cortisol levels was higher in *Cd* males (about 30%), females (20%) and *Af* males (20%) fed with TRP8X. Body weight, length or sex hormones change did not differ between Trp0X vs. Trp8X fed animals.

Conclusion

TRP enriched diets increased brain serotonergic activity and reduced basal cortisol plasma concentrations but had no effect on growth or sexual steroid plasma levels.

FOOD RESTRICTION STIMULATES FINAL MATURATION OF SPERMATOGENESIS AND INDUCES VARIATION IN LEPTIN-A EXPRESSION IN THE CICHLID FISH *CICHLASOMA DIMERUS*

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Introduction

In vertebrates, reproduction is affected by different factors including food availability. Leptin has been proposed in mammals as the link between nutritional status and reproduction, however in teleosts more studies are necessary to elucidate leptin function. There are many reports studying the effects of feeding on the reproductive axis of female fish, but fewer studies have been undertaken in males. In this context, the aim of the present study was to examine the effects of fasting on *leptin-a* expression and spermatogenesis in the South American cichlid fish *Cichlasoma dimerus*.

Methods

Cichlasoma dimerus pairs at the same reproductive stage (two days after spawning) were separately maintained for three weeks in small aquaria. Body weight and total length were recorded at the beginning of this experimental period. The fish were then daily fed with commercial pellets at 1.5% of their body weight or completely starved. At the end of the three weeks period, morphological parameters were recorded and the gonadosomatic and hepatosomatic index (GSI and HSI, respectively) were calculated. Testicular histology was performed and the proportion of the different spermatic cells was calculated along the testis. Gonadotropin releasing hormone I (GnRH I, the hypophysiotropic GnRH variant in this species), luteinizing hormone (LH) and follicle-stimulating hormone (FSH) mRNA levels were evaluated by real time PCR in the brain and pituitary gland respectively. In addition, leptin-a mRNA levels were studied in brain, liver and gonads. A Randomized Blocking Design ANOVA was used to evaluate differences between treatments.

Results and Discussion

The three weeks fasting resulted in a reduction of 6.5 % of the body weight. Fasted fish presented lower HSI with respect to fed ones, but no differences in GSI were observed between both groups. Nevertheless a great variation in the percentage of the different cell types in the testis was obtained. Starved males presented a statically greater proportion of spermatozoa (54.1% *vs.* 23.8%) but a lower proportion of primary spermatocytes (27.2% *vs.* 57%) comparing with fed males. However, no differences were obtained in the other testis cells proportions. Additionally, an increment in LH and FSH mRNA levels was observed in starved males, with no differences in GnRH I expression levels. With respect to *leptin-a* expression, starvation induced an up regulation in the liver, but a down regulation in the gonads and the brain.

Conclusion

Three weeks of starvation highly stimulates final maturation in *C. dimerus* and probably decreases the possibility of a new spermatogenetic cycle. Interestingly, in organs related with feeding and reproduction, *leptin-a* expression responses differentially to fasting protocol.

EVOLUTION OF THE GALINERGIC SYSTEM AND THEIR POSSIBLE ROLE IN SEA BASS REPRODUCTIVE FUNCTION

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Introduction

The activation of the hypothalamus-pituitary-gonadal (HPG) axis at the onset of puberty is a key requirement for enabling the production of sex steroids and gametogenesis to proceed. In vertebrates, the central role of kisspeptins and its receptors as key regulators of the activation of gonadotropin-releasing hormone (GnRH) production and pulsatile release and/or gonadotropin (LH/FSH) release at puberty is well demonstrated. However, the signals that trigger this process are far from being identified. In the European sea bass (*Dicentrarchus labrax*), a species with photolabile pubertal activation, precocious puberty of larger males is thought to be associated to differences in metabolic status signaling to the brain and anticipating the HPG axis activation. One of the candidate neuroendocrine systems that may be involved in translating an inducing signal to the neuroendocrine axis is the galinergic system. We have recently characterized four galanin receptors (GALR1a, GALR1b, GALR2a and GALR2b) in sea bass and highlighted the photolabile regulation of brain GALR1b gene expression and androgen induced gene expression in the testes of pre-pubertal fish. The aim of this study was to isolate the sea bass galanin gene, to identify novel receptors and to characterize their evolution and its possible roles in sea bass reproductive function.

Methods

Vertebrate and invertebrate genome and protein databases were searched using sea bass galanin receptors and the Nile tilapia galanin gene. Multiple sequence alignments and phylogenetic analysis were done to identify and cluster the different receptors. Primers were designed to amplify the sea bass transcripts for a novel galanin receptor and for the galanin peptide. Their expression in different tissues was characterized by quantitative PCR.

Results and Discussion

We have isolated from sea bass brain two transcripts for galanin (a longer form encoding the full-length GAL protein and a shorter splice variant) and one transcript for a novel and uncharacterized galanin receptor (GALR1-like). Vertebrate GALRs phylogenetic analysis highlights the existence of two separate clades (GALR1/GALR-like and GALR2/3) that appear to have arisen from a common ancestral gene and the novel receptor is present from lamprey to higher vertebrates, except in mammals. Expression for the two GAL transcripts and the novel GALR was detected in several tissues of male and female sea bass, with apparent sex dimorphisms.

Conclusion

We have identified and characterized a novel GALR in vertebrates. Sexual dimorphic expression of galanin forms and its receptors suggests a role in the reproductive function of sea bass and possibly in other fish species.

INSULIN-LIKE GROWTH FACTORS IN GONAD MATURATION OF A NEOTROPICAL FISH

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Introduction

In vertebrates, the insulin-like growth factor (IGF) system regulates a variety of cellular processes including growth, proliferation, survival, migration, and differentiation. The expression of these proteins has been demonstrated in gonads of some fish species, but their role in regulating gonadal development is not yet been completely elucidated. In this context, the present study reports, for the first time, a gonad maturity stages-specific assessment of levels and cellular localisation of IGF-I and IGF-II peptides in a Neotropical Characid fish.

Methods

A total of 50 adult specimens (25 females and 25 males) of *A. fasciatus* were caught during the reproductive season peak (January 2011) at Turvo site in the Furnas Reservoir, Grande River, Paraguay-Paraná basin. A transversal section of the middle region of the left gonad was obtained from each fish, then fixed in Bouin's fluid for 8 -12 h and submitted to immunohistochemistry with human polyclonal-rabbit anti-IGF-I and anti-IGF-II primary antibodies (Santa Cruz Biotechnology, Inc. USA) at 1:100 dilution for females and 1:200 for males. Gonad samples from fish (n=5/gonad maturity stage) were frozen in liquid nitrogen and preserved at -80ºC for ELISA assays using anti-IGF-I and anti-IGF-II primary antibodies at 1:1000 dilution. For validation of the ELISA assays, dilution curves of samples homogenates were performed for each protein analysed.

Results and Discussion

The data acquired in this work indicated that the souces of expression of IGF-I and IGF-II in *A. fasciatus* are highly dependent on gonad maturity stages. Ovarian levels of IGF-I were markedly increased in stages rest and totally spent stages occurring, mainly in oogonia, whereas the levels of IGF-II increased significantly according to the gonad maturity stages, reaching the highest levels at partially and totally spent stages. The testicular immunolocalisation for IGF-I was marked in spermatogonia and discrete in spermatocytes, mostly in ripening and ripe fish. IGF-II expression showed two characteristic histological patterns: surrounding spermatids cysts and the entire lumen of the seminiferous tubules in ripening and in spermatogia germ cells in spent fish. The data obtained in this study gave us clues that besides controlling the processes of germline cells differentiation and proliferation, the balance between gonadal levels of IGF-I and IGF-II is extremely important in regulation of gonadal maturation. In rest ovary there is a predominance of IGF-I, with proliferative action, while ripening, ripe and post-spawning stages are marked by the predominance of IGF-II, probably promoting ovarian maturation. Differently, in males there is a predominance of IGF-I at pre-spawning stages, stimulating testicular maturation, whereas in the spent stage there is a prevalence of IGF-II, stimulating spermatogonial self-renewal.

Conclusion

The data obtained in this study contribute to understanding the role of IGF's in fish reproduction, especially, addressing the balance between IGF-I and IGF-II levels and its possible role in the regulation of gonadal maturation in Neotropical fishes.

PROFILE OF SEX STEROIDS DURING SPAWNING INDUCTION IN DUSKY GROUPER *EPINEPHELUS MARGINATUS*

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Introduction

The manipulation of reproduction in captivity is fundamental to increase aquaculture production. Hormonal therapies to induce reproduction in females are focused mainly on the induction of final oocyte maturation and ovulation. The dusky grouper, *Epinephelus marginatus*, is considered a potential candidate for the development of aquaculture. Thus, a thorough understanding of the endocrine control under hormonal therapies is imperative for the proper management of reproduction of this species in captivity. Therefore, the aim of this study was to analyze the profile of sex steroids in females with different oocyte diameters, before, during and after breeding induction with human chorionic gonadotropin (hCG).

Methods

During the breeding season, two experiments were realized. In the first experiment, six females with mean oocyte diameter below 300 µm were divided into two groups: 1) females induced with two doses of 1000 IU hCG/kg at intervals of 24 hours; 2) females administered saline (control). During this experiment, blood samples were collected at zero, 24, 48 and 72 hours after the first induction. The diameter of a subsample of oocytes was measured at the first and last blood sampling. In the second experiment, three females with mean oocyte diameter of 850 µm were induced with three doses of 1000 IU hCG /kg administered at 20h intervals from the first to the second dose; the third dose was performed 34hs after the second dose. Blood samples were collected at the same time as the doses adminstration and just after ovulation. In all experiments, the oocytes were measured at the beginning and end of the blood collection. Plasma levels of steroids estradiol (E2), 17α-hydroxiprogesterone (17α-OHP), testosterone (T) and 11-ketotestosterone (11KT) were determined by elisa imunoassay. Hormone levels of each animal were compared between groups and treatments using Repeated Measures (RM) ANOVA.

Results and Discussion

In the first experiment (below 300µm) only 17α-OHP increased after hCG stimulation; the remaining steroids did not change after hormonal induction. However this increase was not reflected in the diameter of the oocytes and animals did not ovulate. In the second experiment, three females ovulated within 24 to 32 hours after the third hCG doses, but showed two different patterns of spawning: a complete spawning or spawning in multiple small batches. Plasma levels of E2, T and 17α-OHP presented a progressive increase throughout the experiment in all females of the second experiment, while 11KT showed no changes in plasma levels.

Conclusions

The investigations in this preliminary study show that the use of hCG in grouper artificial breeding is effective, however, doses and time of ovulation must be investigated.

EFFECT OF DELAYED SPAWNING ON EGG VIABILITY RATES AND THE OCCURRENCE OF MALFORMATIONS AND PLOIDY ANOMALIES IN PIKE PERCH (*SANDER LUCIOPERCA* L.*)*

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Introduction

Oocyte ageing is one of the most important factors affecting egg quality of several fish species. The time period during which eggs remain viable after ovulation has been reported from a few minutes to a few weeks according to the fish species and the storage temperature. One of the most important problems in the artificial breeding of pikeperch is that the females release the eggs after ovulation and therefore the eggs cannot be easily obtained for stripping and artificial propagation. The present study was performed to identify the effect of post-ovulatory oocyte ageing caused by suturing female genital papilla on egg quality in pikeperch at least during 12 hours after ovulation.

Methods

Pikeperch brood fish were treated with a single muscular injection of Human Chorionic Gonadotrophin HCG (500 IU/kg). Then the genital papilla of females were sutured. Each pair of injected fish was placed in to a separate tank and kept at 15 ± 0.5 °C until spawning. Females were examined for ovulation every 3 hours starting 24 hours after injection. While ovulation occurred, unfertilized eggs were allowed to be retained inside the fish body for 0-3, 3-9, 6-12 and 12-18 Hours Post Ovulation (HPO) by adjusting the time of cutting sutures. Fertilization, hatching and embryo mortality rates as well as the occurrence of the malformations and ploidy anomalies were considered as indexes for egg quality.

Results and Discussion

The fertilization and hatching rates remained almost constant, around 80%, for the eggs retained in the ovarian cavity between 0-18 hours after ovulation. Post ovulatory oocyte ageing did not affect the embryo mortality as well as the occurrence of the malformations and ploidy anomalies at least during the 12-hour experimental period.

Although do not differ significantly ($P > 0.05$), the highest fertilization and hatching rates (89.3 ± 2.7% and 87.7 ± 3.4% respectively) and the lowest embryo mortality (1.6 ± 1%) were observed for the eggs fertilized 3-9 hours after ovulation while no larvae malformation and ploidy anomalies were detected in this group. Such an initial increase in egg quality and viability rates has been also documented in some other species. A slight asynchrony between the processes of meiotic maturation and ovulation has been reported to be the most likely reason for this trend.

Conclusion

Unfertilized eggs of pikeperch can retain their viability up to around 80% during at least 12 hours after ovulation. This appears to be helpful for pikeperch breeding programs providing synchronous artificial egg insemination using a suture on female genital papilla and eventually ease of hatchery management.

Acknowledgements

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OLFACTION, GROWTH AND MATURATION IN FARMED SEA BASS *DICENTRARCHUS LABRAX*

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Introduction:
Chemical communication is a widespread phenomenon in the animal kingdom and, in fish, olfaction constitutes a powerful channel for both social and physical environment cues. In many freshwater fish, chemical cues are one of the most important signals to trigger reproduction. However, in marine teleosts this question remains poorly studied. Here we investigate the role of olfaction on growth and pubertal processes of immature European seabass *Dicentrarchus labrax* in aquaculture conditions, using anosmia in two long-term experiments (3 and 8 months) and two different time windows.

Methods:
Anosmia was achieved by blocking the nostrils with Vinilpolisiloxane through the anterior nostril in pre-pubertal fish. The effectiveness of this method was assessed through olfactory nerve. In the first experiment, anosmia was performed in December (fork length= 15cm, weight= 50g) and the fish were sampled in March (3 months). In the second experiment, anosmia was performed in July (fork length= 12cm, weight= 21g) and the fish were sampled in March (8 months). A feeding test was performed to confirm that the anosmic and control fish were consuming the same amount of food. Fish sex, length, weight, gonad maturation state and gonado-somatic index were compared between groups. The effect of anosmia on the gene expression of KISS1, GnRH1 and gonadotropins (LH and FSH) were analysed in the brain and pituitary by real time qPCR.

Results:
Olfactory nerve recordings showed that the anosmia procedure was effective. There were no differences in food consumption, nor in any of the morphological or histological variables. The frequency of individuals in each gonad maturation state in the end of both experiments did not differ. However, the analysis of the gene expression suggested a link between olfactory sensing and the reproductive endocrine system as anosmic pre-pubertal fish presented significantly higher KISS1 and gonadotropin (LH and FSH) mRNA levels than control fish, but no effect was detected in GnRH1 mRNA levels.

Conclusions:
In European sea bass, olfactory cues do not appear to be important for gonadal development at the time of puberty. However, conspecific odorants may be relevant to modulate gonadotrophin production (LH and FSH), possibly via the kisspeptin system.

Funded by the European Community's Seventh Framework Programme (FP7/ 2007- 2013) under grant agreement n° 222719 – LIFECYCLE.

PRODUCTION, GENE STRUCTURE AND CHARACTERIZATION OF TWO ORTHOLOGS OF LEPTIN AND A LEPTIN RECEPTOR IN TILAPIA

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Introduction

Leptin is known as an important hormone in the regulation of food intake, energy expenditure and reproduction in mammals. In contrast, information regarding non-mammalian and in particular fish leptins is scarce. Due to the large differences at both gene and protein levels between mammalian and teleost leptin, preparation of species-specific leptin is crucial in order to characterize the function of leptin in fish.

Methods

Full-length cDNA encoding for two Leptin (tLepA and tLepB) and one Leptin receptor (tLepR) sequences were identified in tilapia (*Oreochromis niloticus*). The cDNAs of tLepA and tLepB were 486 bp and 459 bp in length, encoding for proteins of 161 aa and 152 aa, respectively. The cDNA for tLepA and tLepB was cloned and synthetic cDNA optimized for expression in *Escherichia coli*. The tLepA and tLepB, expressing plasmids were transformed into *E. coli* and expressed as recombinant protein upon induction with nalidixic acid, which was found almost entirely in the insoluble inclusion bodies (IBs). The proteins were solubilized, refolded and purified to homogeneity by anion-exchange chromatography. The full-length cDNA of tLepR was 3423 bp, encoding a protein of 1140 amino acid (aa) which contained all functionally important domains conserved among vertebrate leptin receptors. Twenty four male tilapia fish (body weight 41.7±1.64g, GSI=0.05±0.015) were divided into two groups, one was fed *ad libitum* and the other was fasted for 26 days. The tLepA, tLepB and tLepR expression in the brain was analysed by real-time PCR.

Results and Discussion

The three-dimensional (3D) structures modeling of tLepA and tLepB predicts strong conservation of tertiary structure with that of human leptin, comprising of four helixes. Moreover, using synteny, the tLep's were found neighboring common genes, such as IMPDH1 and LLRC4. The recombinant tLeps were biologically active in promoting proliferation of BAF/3 cells stably transfected with the long form of human leptin receptor (hLepR), but their activity was four orders of magnitude lower than that of mammalian leptin. Furthermore, the recombinant tLeps were biologically active in promoting STAT-LUC activation in COS7 cells transfected with the identified tLepR but not in the cells transfected with hLepR. tLepA was more active than tLepB. Such low/no activity are likely results from low identity (9-22%) to mammalian leptins. In *ad libitum* versus fasting *in vivo* experiment, there was no significantly difference in the expression of tLepA, tLepB or tLepR in the brain.

Conclusion

In the present study we identified novel sequences of tilapia leptin receptor and two leptins and prepared two leptin like biologically active recombinant proteins.

SMALL RNAS IN IMMATURE AND MATURE TESTIS OF ATLANTIC SALMON (*SALMO SALAR* L.)

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Introduction

Previous studies have revealed a differential expression of small RNA repertoires between immature and mature testis, however no such global small RNA expression studies have been performed in salmonides. Since both precocious maturation and fertility are current problems in salmon aquaculture, increased knowledge about molecular regulation might lead to better protocols which can prevent maturation or induce sterility in aquaqulture of salmon. To study the regulation of micro-RNAs during early maturation in salmonides we used a material which were represented by testes which displayed an (1) immature status, (2) "just starting to mature" stage, characterized by slow stem cell renewal and (3) a more mature phase characterized by single cell proliferation of spermatogonia.

Methods

Small RNA library preparation was performed according to the Truseq small RNA library preparation kit. The small RNA sequencing libraries were then sequenced on an Illumina hiseq instrument to a depth of approximately 20 million reads per library. The Fastq files generated were processed through a customized galaxy work-flow, were any proceeding 5'-end adapter and/or preceding 3'-end adapter were removed using FastqMCF. The small RNAs were mapped with BWA against known Salmon Salar microRNAs and thereafter the sequence abundance in each library was quantified and compared using DeSeq.

Results and Discussion

The resulting small RNA libraries were analyzed by FastQC and displayed a bimodal size distribution after adapter removal. Intriguingly a similar size distribution has been described also for small RNA libraries from testis samples of other species where the relatively small peak centered around 20nt corresponds to microRNAs (miRNAs) and the more prominent peak centered around 26nt represents a large class of piwi interacting RNAs (piRNAs) that have shown to be important in controlling transposable elements in the germline. Differential expression analysis of miRNAs at the above mentioned maturation stages (1-3), show that a small but consistent number of miRNAs are regulated during initial stages of maturation in salmon testis. Considering the important roles of miRNAs as global gene expression regulators, we hypothesize that these differential expressed miRNAs might play a key role in testicular germ cell maturation. We are currently mapping our small RNA libraries against Rfam in order to also identify differentially expressed homologues of other noncoding RNA species.

Conclusion

We do observe a broad peak of small RNAs that based on size and abundance in testicular tissue most probably represents piRNAs which are known to be indispensable for germ cell maturation in other species. Also it was detected differential expression of a few micro-RNA species during early maturational stages in Atlantic salmon testis. Further studies will reveal if small RNAs are indeed involved in salmon maturation.

PUBERTY VARIANCES (MALE x FEMALE) DRIVE HARVEST WEIGHT IN THE AMAZONIAN TAMBAQUI (*COLOSSOMA MACROPOMUM)*

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Introduction

In Brazil, the aquaculture industry is growing continuously as a consequence of the reduction of natural stocks, the constant increase of fish meat demand and the huge potential of the country with its large coast and abundant in land waters. The Amazonian characid tambaqui (*Colossoma macropomum*) is the most commercially important native fish in Brazil, however, its farming is still very traditional and no technologies have been specifically adapted or optimized for the species. For instance, this is the first study aiming at understanding the cellular events that occur during the tambaquis´ first maturation and relating this data with the difference in body weight between males and females at market size.

Methods

200 tambaquis were collected from November 2011 to September 2012 (±12/month) from a commercial farm in Manaus, Amazonas. Fish were from the same batch and were 2 months old at the beginning of the experiment, weighing 280 gr. Prior to sampling, all fish were anesthetized with Benzocaine 10% and biometrical measurements were made individually. Fragments of gonads were fixed in Bouin´s solution and embedded in paraffin. Slides were stained with hematoxylin-eosin for histological analysis. For the last sampling, 40 fish were analyzed for comparison between harvest weights of males and females.

Results and Discussion

All fish were immature until 4 months (mo) old, and gonads were then small and transparent. Spermatogenesis commenced in February (rainy season; fish 5 mo old and 750 gr weigh) and did not take long to be completed, as in less than two months testis full of free spermatozoa in the tubular lumen were common among the males. However, the volume of semen of this pubertal wave was too small (as well as the GSI 0.033 ± 0.02) to be released at abdominal pressure. Three months later males were maturing again, which confirms that in the North of Brazil tambaqui can mature all year long, as opposed to other regions were the breeding season is restricted to the rainy season. Females were immature until the end of the study (3.0kg; harvest weight), and entered meiosis with the formation of diplotene (present still in nests with oogonia) at weight of approximately 1.5 kg (8 mo old). With further folliculogenesis and oocyte primary growth, new oocytes were continuously recruited; however, they did not surpass the perinucleolar phase. At harvest, females were significantly heavier (gutted weight) than males, in the order of 21%.

Conclusion

There is an asynchronic incidence of puberty between males and females of tambaqui reared on a commercial scale. While males commence spermatogenesis earlier and finish it quicker than females, the later do not reach maturation at harvest size (3kg). This divergence seems to be responsible for the difference of body weight between females and males of farmed tambaqui, rendering the former the most profitable gender to be cultivated in terms of aquaculture industry.

ADDENDUM
Poster Session: Brain and Pituitary
Poster P075

MOLECULAR CHARACTERIZATION OF EUROPEAN SEA BASS SIALYLTRANSFERASES

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Introduction

FSH is synthesized and secreted by the pituitary as differently glycosylated forms that differ in their oligosaccharide structure. In mammals the bioactivity of less sialylated FSH is higher than that of more sialylated counterparts and these FSH glycosylation variants have complementary and specific actions on the developing follicles. The endocrine milieu likely regulates the composition of FSH carbohydrate moiety and GnRH and sexual steroids have been proposed as the main regulators of FSH microheterogeneity. Several enzymes are involved in the post-translational FSH glycosylation processing; particularly the sialyltransferases (STs) that transfer sialic acid residues into the FSH carbohydrate chain. Our aim was to clone the ST genes of the European sea bass (*Dicentraruchus labrax*) and to and study their expression patterns in pituitary at different reproductive stages

Methods

The amino acid sequences of the stickleback STs were used as queries in TBLASTN searches against the sea bass genome database. The complete coding sequences of ST3GAL3 (2 variants), ST3GAL4, ST6GAL1 and ST8SIA2 were amplified by PCR from sea bass brain and pituitary cDNA libraries, and then cloned in the pGEM-T Easy vector and sequenced. Primary cell culture of adult sea bass pituitaries was treated with two doses of LHRHa (10 and 100 mM), Estradiol (E2) and Testosterone (T) (50 and 1000 mM). Gene expression of STs was analysed by qPCR in these cultures and in pituitaries of juvenile and adult sea bass.

Results and Discussion

Five sea bass ST cDNAs potentially involved in the addition of sialic acid to Fsh were isolated and cloned. Previously we observed that Fsh bioactivity during male and female early development was higher in females. Assuming that less sialylated Fsh has higher bioactivity, we studied the pituitary expression of the STs in 300 days old juvenile males and females. We observed that, albeit with different intensities, the expression was always lower in females. Results of treated pituitary cells suggest that high doses of LHRHa and E2 stimulate the expression of ST3GAL4 and ST6GAL1 whereas it was inhibited by high doses of T. Pituitary expression analysis of ST3GAL4 and ST6GAL1 along the reproductive cycle showed that ST6GAL1 has maximum expression during vitellogenesis and maturation in females and late recrudescence in males. While, ST3GAL4 had low expression levels during vitellogenesis and maturation in females and high levels at spermation and spermiogenesis in males.

Conclusion

In sea bass, Fsh bioactivity could be dependent on the content of sialic acid, likely regulated by the endocrine milieu, adapting the function of Fsh to the moment needs.

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