Ethinylestradiol differentially interferes with IGF-I in liver and extrahepatic sites during development of male and female bony fish

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Abstract

Growth and sexual development are closely interlinked in fish; however, no reports exist on potential effects of estrogen on the GH/IGF-I-axis in developing fish. We investigate whether estrogen exposure during early development affects growth and the IGF-I system, both at the systemic and tissue level. Tilapia were fed from 10 to 40 days post fertilization (DPF) with 17 α -ethinylestradiol (EE₂). At 50, 75, 90, and 165 DPF, length, weight, sex ratio, serum IGF-I (RIA), pituitary GH mRNA and IGF-I, and estrogen receptor α (ER α) mRNA in liver, gonads, brain, and gills (real-time PCR) were determined and the results correlated to those of *in situ* hybridization for IGF-I. Developmental exposure to EE₂ had persistent effects on sex ratio and growth. Serum IGF-I, hepatic IGF-I mRNA, and the number of IGF-I mRNA-containing hepatocytes were significantly decreased

at 75 DPF, while liver ER α mRNA was significantly induced. At 75 DPF, a transient decline of IGF-I mRNA and a largely reduced number of IGF-I mRNA-containing neurons were observed in the female brain. In both sexes, pituitary GH mRNA was significantly suppressed. A transient down-regulation of IGF-I mRNA occurred in ovaries (75 DPF) and testes (90 DPF). In agreement, *in situ* hybridization revealed less IGF-I mRNA signals in granulosa and germ cells. Our results show for the first time that developmental estrogen treatment impairs GH/IGF-I expression in fish, and that the effects persist. These long-lasting effects both seem to be exerted indirectly via inhibition of pituitary GH and directly by suppression of local IGF-I in organ-specific cells.

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Introduction

Insulin-like growth factor I (IGF-I) plays a central role in the complex system that regulates growth, differentiation, and reproduction. It selectively promotes mitogenesis and differentiation and inhibits apoptosis (Jones & Clemmons 1995, Reinecke & Collet 1998). IGF-I is mainly produced in liver – the principal source of circulating (endocrine) IGF-I – under the influence of growth hormone (GH). IGF-I released from the liver into the circulation acts on a variety of target cells. In addition, IGF-I is also expressed in extrahepatic sites and most likely stimulates organ-specific functions by paracrine/autocrine mechanisms. There is increasing evidence that GH stimulates the expression of IGF-I also in extrahepatic sites (Vong *et al.* 2003, Biga *et al.* 2004).

Among the non-mammalian vertebrate classes, bony fish are the most studied with respect to IGF-I (Duan 1998, Plisetkaya 1998, Wood *et al.* 2005, Reinecke 2006) mainly due to their unique development from larval to adult life and to their high importance in aquaculture. Thus, there is a rising interest in the significance of IGF-I in fish development,

growth, and reproduction. In this respect, some evidence has been presented (Riley *et al.* 2004, Carnevali *et al.* 2005, McCormick *et al.* 2005) that estrogens may acutely influence synthesis and/or release of IGF-I from adult fish liver. Very recently, the influence of exposure to estrogen (E₂) on several genes, including IGF-I, has been investigated in adults of the fathead minnow (Filby *et al.* 2006). In contrast, possible effects of estrogens on the IGF-I system in developing fish have not been studied to date, although it is well documented that prolonged exposure of developing fish to exogenous estrogens is associated with impaired growth (Jobling *et al.* 2002, Rasmussen *et al.* 2002, Fenske *et al.* 2005). This suggests an influence of estrogen on the IGF-I system during development. In contrast to the activational effects of estrogens on the IGF system as reported for adult fish, developmental estrogen exposure may induce organizational alterations of the IGF-I system what could lead to persistent changes in growth and reproduction.

The present study aims to investigate the potential influence of 17 α -ethinylestradiol (EE₂) on IGF-I when applied to developing fish. A population of tilapia

(*Oreochromis niloticus*) was fed with EE₂ during the period of gonad sexual differentiation (10–40 days post fertilization, DPF) at the optimal dosage to induce functional feminization. We assessed whether EE₂ treatment during this development stage exerts lasting effects on gonad differentiation, growth, and crucial parameters of the GH/IGF-I system. In addition, in order to assess potential lasting effects of developmental EE₂ treatment on the estrogen system and the possible association with changes in the GH/IGF-I system, we measured estrogen receptor α (ER α) expression. The following parameters were determined after termination of estrogen treatment at 10, 35, 50, and 125 days posttreatment (50, 75, 90, and 165 DPF): sex ratio, body length (BL) and body weight (BW), serum IGF-I levels, and mRNA expression levels of IGF-I, GH, and ER α . Furthermore, we studied EE₂-related alterations in IGF-I mRNA cellular localization in the potential target organs by *in situ* hybridization. The organs studied included those of the central GH/IGF-I axis, i.e., pituitary and liver. Although fish liver is the major source of circulating IGF-I, IGF-I also occurs in extrahepatic sites (Reinecke *et al.* 1997) where it is particularly expressed during development (Duguay *et al.* 1996, Perrot *et al.* 1999, Radaelli *et al.* 2003, Berishvili *et al.* 2006a). Thus, the alterations in IGF-I mRNA expression were also determined in organs showing high IGF-I expression during ontogeny, i.e., gills and brain. We also examined the influence of EE₂ exposure on IGF-I expression in the gonads. Gonads of developing fish express IGF-I mRNA (Berishvili *et al.* 2006b), although the functional role of IGF-I expression in the gonad, and its response to estrogen exposure are unknown yet. The examination of the IGF-I response to EE₂ treatment in the various target organs and cells of male and female fish was accompanied by measuring expression of ER α mRNA in liver, pituitary, brain, gill filaments, and gonads, in order to evaluate whether the potential changes of the GH/IGF-I system are associated with an activation of the ER-signaling pathway.

Materials and Methods

Fish culture and hormone treatment

Balanced populations of tilapia (*O. niloticus*) were fed in September during a period, i.e., 10–40 DPF, covering the sensitive period with EE₂ at the optimal dosage (125 μ g EE₂/g food) to induce functional feminization in most individuals. Principles of animal care and specific national laws were followed. Hormonally treated salmonid food was prepared by the ethanol evaporation method (0.6 l of 95% ethanol/kg food). EE₂ (Sigma) was dissolved in 95% ethanol and the solution was sprayed over the food. Control food was prepared in the same way without EE₂. Three batches of 500 fry each were used for the treatment.

Fish sampling and tissue preparation

All experiments were performed three times. At the age of 50, 75, 90, and 165 DPF, fish were anesthetized with 2-phenoxy ethanol (Sigma) added to water and measured in weight and length. Blood samples were obtained from control (male: 75 DPF $n=7$, 90 DPF $n=6$, 165 DPF $n=9$; female: 75 DPF $n=7$, 90 DPF $n=7$, 165 DPF $n=12$) and EE₂-treated (male: 75 DPF $n=7$, 90 DPF $n=6$, 165 DPF $n=7$; female: 75 DPF $n=8$, 90 DPF $n=8$, 165 DPF $n=9$) tilapia. For real-time PCR, control (male: 50 DPF $n=12$, 75 DPF $n=9$, 90 DPF $n=9$, 165 DPF $n=9$; female: 50 DPF $n=15$, 75 DPF $n=12$, 90 DPF $n=14$, 165 DPF $n=12$) and EE₂-treated (male: 50 DPF $n=12$, 75 DPF $n=10$, 90 DPF $n=12$, 165 DPF $n=11$; female: 50 DPF $n=12$, 75 DPF $n=12$, 90 DPF $n=14$, 165 DPF $n=11$) tilapia were used. For *in situ* hybridization, tissue specimens of liver, brain, pituitary, gonads, and gill filaments sampled from three control and EE₂-treated fish per sex and point of time were used.

RIA for IGF-I

Blood was collected from the caudal vein using a heparinized 1 ml syringe, centrifuged for 15 min at 4 °C at 10 000 *g*. Serum was removed and stored at –20 °C. Serum IGF-I levels were determined in undiluted samples by RIA after SepPak C18 chromatography (Waters Corp., Milford, MA, USA), as described earlier (Zapf *et al.* 2002). In brief, 0.15 ml PBS containing 0.2% human serum albumin (HSA), pH 7.4, were added to 0.1 ml serum. All samples were acid treated and run over Sep-Pak C18 cartridges (Immunonuclear, Stillwater, MN, USA). After reconstitution with 1 ml PBS/0.2% HSA tilapia, serum samples were assayed as already described (Eppler *et al.* 2007): three different dilutions (1:5, 1:10, and 1:20) in 0.2 ml samples or standards (fish IGF-I from GroPep, Adelaide, Australia) and 0.1 ml IGF-I antiserum (final dilution 1:20 000, GroPep) were preincubated for 24 h at 4 °C. To the final incubation volume (0.4 ml), 25 000–35 000 c.p.m. ¹²⁵I-IGF-I (Anawa, Wangen, Switzerland, specific activity 300–400 μ Ci/ μ l) were added. The reaction mixture was incubated for another 24 h followed by precipitation with goat anti-rabbit γ -globulin antiserum. After centrifugation, the pellet was counted in a γ -counter.

Design of primers and probes for real-time PCR

Based on the mRNA sequences of *O. mossambicus* IGF-I (Reinecke *et al.* 1997), *O. niloticus* GH (Ber & Daniel 1992), and β -actin (Hwang *et al.* 2003), specific primers and probes for real-time PCR were created as described (Caelters *et al.* 2004, 2005) for IGF-I (sense TCTGTGGAGAGCGAGGC-TTT, antisense CACGTGACCGCCTTGCA, probe ATTT-CAATAAACCAACAGGCTATGGCCCCA), GH (sense TCGACAAACACGAGACGCA, antisense CCCAGGACT-CAACCAGTCCA, probe CGCAGCTCGGTCCTGAAGC-TG), and β -actin as a house-keeping gene (sense GC

CCCACCTGAGCGTAAATA, antisense AAAGGTGGA-CAGGAGGCCA, probe TCCGTCTGGATCGGAGGCTT-CATC). Using this method, new primers and probe (sense CAAGTGGTGGAGGAGGAAGATC, antisense CTCAG-CACCTTGGAGCAG, probe CTGATCAGGTGCTCCTC) were designed for ER α based on the *O. niloticus* ER α sequence (Chang *et al.* 1999) with Primer Express software version 1.5 (PE Biosystems, Foster City, CA, USA).

Real-time PCR quantitation of IGF-I, ER- α , and GH expression

Total RNA was extracted from specimens stored in 1.5 ml RNeasy (Ambion, Austin, TX, USA) using TRIzol reagent (Invitrogen) and treated with 1 U RQ1 RNase-free DNase (Catalys AG, Wallisellen, Switzerland). cDNA was synthesized from 800 ng total RNA using 1 $\times$ TaqMan RT Buffer, MgCl₂ (5.5 mM), 1.25 U/ μ l murine leukemia virus (MuLV) reverse transcriptase, 2.5 μ M random hexamers primers, 0.4 U/ μ l ribonuclease inhibitor, and 500 μ M each dNTP (Applied Biosystems, Rotkreuz, Switzerland) for 10 min at 25 °C, 30 min at 48 °C, and 5 min at 95 °C. From 10 ng/ μ l total RNA, 2 μ l cDNA were obtained and were subjected, in triplicates, to real-time PCR using Absolute QPCR low ROX Mix (ABgene, Hamburg, Germany) including Thermo-Start DNA Polymerase, 300 nM of each primer, 150 nM of the fluorogenic probe. Amplification was performed with 10 μ l in a MicroAmp Fast Optical 96-well reaction plate using the ABI 7500 Fast Real-Time PCR System (Applied Biosystems) with the conditions: 15 min at 95 °C, followed by 40 cycles for 15 s at 95 °C and for 1 min at 60 °C.

Relative quantification of treatment effects using the $\Delta\Delta C_T$ method

The comparative threshold cycle ($\Delta\Delta C_T$) method (Livak & Schmittgen 2001) was used to calculate relative gene expression ratios between EE₂-treated and control groups. Data were normalized to β -actin as the reference gene. Efficiency tests for β -actin and IGF-I assays (Caelers *et al.* 2004) and ER α assay (data not shown) permitted the accurate use of the $\Delta\Delta C_T$ method. Relative changes induced by EE₂ feeding were calculated by the formula $2^{-\Delta\Delta C_T}$, with $\Delta\Delta C_T = \Delta C_T$ (treated group) – ΔC_T (untreated control), and $\Delta C_T = C_T$ (target gene) – C_T (reference gene). All data are expressed as *n*-fold changes of gene expression in the experimental group relative to the control group, displayed in the graphs as 2 log scale, a common way of presenting qPCR data (e.g., Dzidic *et al.* 2006). Statistical significance was calculated using Mann–Whitney rank sum test, with an exact *P* value. Statistical analyses were performed with GraphPad Prism 4 (GraphPad, San Diego, CA, USA).

Tissue preparation of paraffin sections and in situ hybridization

Samples were fixed by immersion in Bouin's solution for 4 h at room temperature, dehydrated in ascending series of

ethanol and routinely embedded in paraplast (58 °C). Sections were cut at 4 μ m and processed for *in situ* hybridization using the sense (5'-GTCTGTGGAGAGCGAGGCTTT-3') and antisense (5'-AACCTTGGGTGCTCTTGGCATG-3') probes corresponding to the tilapia IGF-I B and E domains, as described (Schmid *et al.* 1999, Berishvili *et al.* 2006a,b). After dewaxing and rehydration, the sections were postfixed with 4% paraformaldehyde and 0.1% glutaraldehyde in PBS. The following steps were carried out with diethylpyrocarbonate (DEPC)-treated solutions in a humidified chamber. The sections were digested with 0.02% proteinase K in 20 mM Tris–HCl (pH 7.4), 2 mM CaCl₂ for 10 min at 37 °C and treated with 1.5% triethanolamine and 0.25% acetic anhydride for 10 min at room temperature. Slides were incubated with 100 μ l prehybridization solution per section for 3 h at 54 °C and hybridized overnight at 54 °C with 50 μ l hybridization buffer containing 200 ng sense (negative control) or antisense probes previously denatured for 5 min at 85 °C. Slides were washed for 15 min at room temperature in 2 $\times$ SSC and for 30 min at the specific hybridization temperature at descending concentrations of SSC (2 $\times$, 1 $\times$, 0.5 $\times$, and 0.2 $\times$). The sections were incubated with alkaline phosphatase-coupled anti-digoxigenin antibody diluted (1: 4000) in 1% blocking reagent (Roche Diagnostics) in buffer P1 for 1 h at room temperature in the darkness. After washing in buffer P1, sections were treated with buffer P3, 5 mM levamisole, and Nitroblue tetrazolium chloride (NBT)/5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt (BCIP) stock solution (Roche Diagnostics). Color development was carried out overnight at room temperature and stopped by rinse of the slides in tap water for 15 min.

Results

Specificity of in situ hybridization

Specificity of the probes as previously demonstrated for adult tilapia male and female gonads (Schmid *et al.* 1999, Berishvili *et al.* 2006b) and liver (Schmid *et al.* 1999) was reassured on adjacent sections of 50 DPF male brain with IGF-I antisense (Fig. 1A) and sense (Fig. 1B) probes and gills. *In situ* hybridization revealed positive signals with the antisense probe whereas no signals were present in the negative controls.

Sex ratio

After EE₂ treatment, the sex ratio had shifted from 47.2 $\pm$ 8.5% females in control to 86.5 $\pm$ 14.1% females at 165 DPF.

Body length and weight

EE₂ treatment caused a progressive decrease in BL and BW in both sex (Fig. 2). Both parameters were significantly lowered from 90 DPF onwards (90 DPF – BL: –16.9% in males, –13.4% in females; BW: –47.4% in males, –32.3% in females; 165 DPF – BL: –19.5% in males, –15.2% in females; BW: –46.2% in males, –40.1% in females).

Figure 1 Specificity of the *in situ* hybridization technique shown on two adjacent sections of 50 DPF male tilapia brain hybridized with (A) an IGF-I antisense probe and (B) an IGF-I sense probe.

Serum IGF-I level

While at 50 DPF the fish were too small to allow blood drawing, blood could be taken at 75, 90, and 165 DPF. At 75 DPF, in males (Fig. 3G), the IGF-I serum level was

significantly ($P=0.002$) decreased in the EE₂-treated group (5.65 ± 1.18 ng/ml) when compared with the controls (9.70 ± 2.06 ng/ml), while in females (Fig. 3H) there was only a trend to reduce IGF-I serum level (control 11.20 ± 2.18 versus EE₂-treated 8.54 ± 2.26 , $P=0.08$). At the later stages, there was no significant difference between the treated group and the controls (Fig. 3G and H).

IGF-I and ER α mRNA levels in liver

Hepatic IGF-I mRNA was significantly reduced by EE₂ feeding with the effect becoming evident in females (Fig. 3C) later than in males (Fig. 3A). At 50 DPF, IGF-I mRNA in liver was lowered in males by 7.1-fold ($P=0.05$), at 75 DPF in males by 3-fold ($P=0.01$) and in females by 2.7-fold ($P=0.002$), at 90 DPF in males by 1.7-fold ($P=0.02$) and in females by 5-fold ($P=0.0003$) and almost recovered in both sex at 165 DPF (Fig. 3A and C). *In situ* hybridization revealed a markedly reduced number of hepatocytes containing IGF-I mRNA after EE₂ treatment at 50 (Fig. 3E and F) and 75 DPF in male liver and at 75 and 90 DPF in female liver. Hepatic ER α mRNA was significantly increased to 6.1-fold in males after EE₂ feeding ($P=0.01$) and 2.6-fold ($P=0.004$) in females at 50 DPF (Fig. 3B and D). At 75 DPF, ER α mRNA was raised to 2.2-fold in males ($P=0.02$) and 37-fold in females ($P=0.002$). In male liver, ER α was back at the

Figure 2 Influence of EE₂ exposure on fish growth throughout the experimental period. Body length (in cm) and weight (in g) in (A and C) male and (B and D) female control (white columns) and EE₂-treated (black columns) tilapia. X-axis is labeled as DPF. Columns denote mean values and bars denote s.d. Significance levels: * $P<0.01$, ** $P<0.005$, *** $P<0.0005$.

Figure 3 Influence of EE₂ exposure on IGF-I and ERα gene expression in liver and on IGF-I peptide levels in serum. Relative changes (log 2) of (A and C) IGF-I and (B and D) ERα mRNA expression in EE₂-treated tilapia when compared with age-matched control tilapia. Control (male: 50 DPF *n*=12, 75 DPF *n*=9, 90 DPF *n*=9, 165 DPF *n*=9; female: 50 DPF *n*=15, 75 DPF *n*=12, 90 DPF *n*=14, 165 DPF *n*=12) and EE₂-treated (male: 50 DPF *n*=12, 75 DPF *n*=10, 90 DPF *n*=12, 165 DPF *n*=11, female: 50 DPF *n*=12, 75 DPF *n*=12, 90 DPF *n*=14, 165 DPF *n*=11) tilapia were used. Normalization was performed with β-actin as housekeeping gene. *In situ* hybridization with IGF-I antisense probe of tilapia liver specimens in 50 DPF old (E) control and (F) EE₂-treated male tilapia. White arrows point to IGF-I mRNA-expressing hepatocytes. IGF-I peptide concentrations in serum determined by a fish-specific RIA in control (white columns) and EE₂-treated (black columns) (G) male and (H) female fish. Blood samples were obtained from control (male: 75 DPF *n*=7, 90 DPF *n*=6, 165 DPF *n*=9; female: 75 DPF *n*=7, 90 DPF *n*=7, 165 DPF *n*=12) and EE₂-treated (male: 75 DPF *n*=7, 90 DPF *n*=6, 165 DPF *n*=7; female: 75 DPF *n*=8, 90 DPF *n*=8, 165 DPF *n*=9) tilapia. X-axis is labeled as DPF. Columns denote mean values and bars denote s.d. Significance level: **P*=0.05, *P*=0.02, *P*=0.01; ***P*=0.002, *P*=0.004; ****P*=0.0003.

normal level at 90 DPF. In female liver, ERα at 90 DPF was still raised to 2.5-fold (*P*=0.05) of the control mRNA and at about the normal level at 165 DPF.

IGF-I and ERα mRNA levels in brain

In the male brain, no significant change in the expression of IGF-I mRNA was detected throughout the experimental period (Fig. 4A). In the female brain, however, at 75 DPF IGF-I mRNA was significantly (*P*=0.001) reduced (3.45-fold). At 90 and 165 DPF, IGF-I mRNA was about the normal level (Fig. 4C). At 75 DPF, in all regions of the female brain (Fig. 4F), the number of neurons showing IGF-I mRNA was largely reduced when compared with control (Fig. 4E). Brain ERα mRNA exhibited no significant changes at any experimental stage (Fig. 4B and D).

IGF-I and ERα mRNA levels in male and female gonads

No alteration in the expression of IGF-I was detected at 50 DPF (Fig. 5A and C). At 75 DPF, there was a significant (*P*=0.015) decrease (−2.2-fold) in the IGF-I mRNA level in the female gonad and at 90 DPF, there was a significant (*P*=0.0013) reduction (−2.5-fold) of IGF-I mRNA in the male gonad. At the later stages, IGF-I mRNA reached the normal level. *In situ* hybridization of male gonad at 90 DPF revealed that the number of IGF-I mRNA containing spermatogonia was markedly lower in EE₂-treated (Fig. 5F) fish than in control (Fig. 5E). Less IGF-I mRNA signals were observed in granulosa cells of the ovaries in EE₂-treated fish at 75 DPF (Fig. 5H). Furthermore, IGF-I mRNA in small oocytes as present in controls (Fig. 5G) was largely reduced. In the male gonads, a significant increase in ERα mRNA was found at 50 DPF

Figure 4 Influence of EE₂ exposure on IGF-I and ERα gene expression in brain. Relative changes (log₂) of (A and C) IGF-I and (B and D) ERα mRNA expression in EE₂-treated when compared with age-matched control tilapia. Control (male: 50 DPF *n*=12, 75 DPF *n*=9, 90 DPF *n*=9, 165 DPF *n*=9; female: 50 DPF *n*=15, 75 DPF *n*=12, 90 DPF *n*=14, 165 DPF *n*=12) and EE₂-treated (male: 50 DPF *n*=12, 75 DPF *n*=10, 90 DPF *n*=12, 165 DPF *n*=11; female: 50 DPF *n*=12, 75 DPF *n*=12, 90 DPF *n*=14, 165 DPF *n*=11) tilapia were used. Normalization was performed with β-actin as house-keeping gene. *In situ* hybridization with IGF-I antisense probe of tilapia brain specimens in 75 DPF old (E) control and (F) EE₂-treated female tilapia. In the cell body layer of the periventricular zone (cPZ) and the tectum opticum (TeO), the number of IGF-I mRNA expressing neurons (black arrows) is largely reduced in the EE₂-treated brain. X-axis is labeled as DPF. Columns denote mean values and bars denote s.d. Significance level: ***P*=0.001.

(4.4-fold, *P*=0.015), 75 DPF (4.2-fold, *P*=0.028), and 90 DPF (2.6-fold, *P*=0.009; Fig. 5B). In contrast, in the female gonad, a significant (*P*=0.015) increase in ERα mRNA was obtained only at 50 DPF by 1.78-fold followed by a significant decrease (−1.79-fold, *P*=0.006) at 75 DPF. At 90 DPF, there was only a tendency (*P*=0.11) to decrease ERα mRNA. At 165 DPF, ERα mRNA was at the normal level.

IGF-I and ERα mRNA levels in gill filaments

Significant changes in IGF-I mRNA expression were obtained only at 50 DPF in males where it was decreased by 5.9-fold (*P*=0.02). No significant changes in IGF-I mRNA were detected in males later on or in females at any experimental stage (Fig. 6A and C). Using *in situ* hybridization, the number of IGF-I mRNA containing chloride cells in the gill filament

epithelium was found to be reduced in EE₂-treated males at 50 DPF (Fig. 6E and F). At 50 DPF, branchial ERα mRNA was significantly decreased in both sex, in males by 27-fold (*P*=0.03) and in females by 3.2-fold (*P*=0.03). At the later stages, no significant changes were obtained (Fig. 6B and D).

Pituitary GH and ERα mRNA levels

Pituitaries could be dissected only at 75, 90, and 165 DPF. GH mRNA was significantly decreased after EE₂ treatment in male pituitary at 165 DPF (*P*=0.0571) by 2.33-fold (Fig. 7A) and in female pituitary at 75 DPF by 2.27-fold (*P*=0.0571) and at 90 DPF to 3-fold (*P*=0.0571; Fig. 7C). ERα mRNA was significantly (*P*=0.0061) raised to 3.38-fold at 165 DPF in the male pituitary (Fig. 7B) and in the female pituitary to 2.55-fold at 75 DPF (*P*=0.0159) and at 90 DPF (*P*=0.0012) to 2.7-fold (Fig. 7D).

Figure 5 Influence of EE₂ exposure on IGF-I gene expression in male and female gonad. Relative changes (log 2) of (A and C) IGF-I and (B and D) ERα mRNA expression in EE₂-treated tilapia when compared with age-matched control tilapia. Control (male: 50 DPF $n=12$, 75 DPF $n=9$, 90 DPF $n=9$, 165 DPF $n=9$; female: 50 DPF $n=15$, 75 DPF $n=12$, 90 DPF $n=14$, 165 DPF $n=12$) and EE₂-treated (male: 50 DPF $n=12$, 75 DPF $n=10$, 90 DPF $n=12$, 165 DPF $n=11$; female: 50 DPF $n=12$, 75 DPF $n=12$, 90 DPF $n=14$, 165 DPF $n=11$) tilapia were used. Normalization was performed with β -actin as house-keeping gene. (E–H) *In situ* hybridization with IGF-I antisense probe. (E and F) Male gonad at 90 DPF. Arrows point to IGF-I mRNA-expressing spermatogonia. (G and H) Female gonad at 75 DPF. Fewer IGF-I mRNA signals are found in granulosa cells (black arrow heads) and in small oocytes (white arrows) of (H) EE₂-treated fish than in (G) controls. X-axis is labeled as DPF. Columns denote mean values and bars denote s.d. Significance level: * $P=0.028$, $P=0.015$, ** $P=0.006$, $P=0.009$, $P=0.0013$.

Discussion

Nothing is known about the potential interference of estrogen(s) and IGF-I during fish development. In our study, EE₂ feeding from 10 to 40 DPF, a period that includes the sensitive period of gonad differentiation, led to a lasting decline in growth that was most pronounced at the end of the experiment (165 DPF), i.e. about 3 months after end of the treatment. Then, BW was reduced when compared with controls in males by about 46% and BL by 19.5%, and in females by about 40 and 15% respectively. Thus, EE₂ feeding for about 1 month during the sensitive phase of sexual development resulted in severe and persistent growth impairment in both sexes.

Although growth-reducing effects of continuous estrogen exposure on developing fish have been shown in some studies, less information is available whether estrogen exposure during specific developmental stages leads to altered growth later in life. Treatment of embryonic trout with estrogen-receptor-binding alkylphenols until 21 DPF resulted in a permanently suppressed growth until 400 DPF (Ashfield *et al.* 1998). This finding agrees with our observation that

developmental estrogen exposure evoked a persistent reduction of tilapia growth. The question is if this permanent growth-suppressing effect is caused by an interaction with the GH/IGF-I system, be it directly and/or indirectly. To this end, we analyzed parameters of the GH/IGF-I system, in a series of target and effector organs, and both at the transcriptional and translational level. Early life treatment of tilapia with EE₂ resulted in a significant decrease in circulating serum IGF-I by about 30% at 75 DPF, i.e., about 1 month after end of treatment. The lowered serum IGF-I was paralleled by a high and significant decrease in IGF-I mRNA in liver, the main source of endocrine IGF-I, and a reduced number of IGF-I mRNA expressing hepatocytes as shown by *in situ* hybridization. Further, the decline in hepatic IGF-I synthesis was accompanied by a significant induction of ERα mRNA, which was most pronounced at the time of the strongest decline of hepatic IGF-I expression. ERα mRNA was induced by EE₂ treatment also in other tissues such as pituitary or brain, and this response represents the well-characterized autoregulatory effect of estrogens on their own receptors, as it has been shown for other fish species too (Filby *et al.* 2006). However, it needs to be emphasized that the

Figure 6 Influence of EE₂ exposure on IGF-I and ERα mRNA in gill filaments. Relative changes (log 2) of (A and C) IGF-I and (B and D) ERα mRNA expression in EE₂-treated tilapia when compared with age-matched control tilapia. Control (male: 50 DPF *n*=12, 75 DPF *n*=9, 90 DPF *n*=9, 165 DPF *n*=9; female: 50 DPF *n*=15, 75 DPF *n*=12, 90 DPF *n*=14, 165 DPF *n*=12) and EE₂-treated (male: 50 DPF *n*=12, 75 DPF *n*=10, 90 DPF *n*=12, 165 DPF *n*=11, female: 50 DPF *n*=12, 75 DPF *n*=12, 90 DPF *n*=14, 165 DPF *n*=11) tilapia were used. Normalization was performed with β-actin as house-keeping gene. (E and F) *In situ* hybridization with IGF-I antisense probe in gill specimens of 50 DPF male tilapia. Black arrows point to IGF-I mRNA containing chloride cells in the gill filament epithelium. X-axis is labeled as DPF. Columns denote mean values and bars denote s.d. Significance level: **P*=0.03, *P*=0.02.

association between upregulation of ERα and altered expression of IGF-I, as observed in the present study, does not implicate a mechanistic link between the two observations. However, even without assuming a causative role of the EE₂-induced activation and upregulation of the ER pathway, our results suggest that administration of EE₂ during early development exerts a long-term suppressive effect on hepatic IGF-I expression and synthesis. Previous *in vivo* and *in vitro* studies in adults of different fish species also reported an estrogen-associated decrease in hepatic IGF-I mRNA (Riley *et al.* 2004, Carnevali *et al.* 2005, Filby *et al.* 2006) or in serum IGF-I (Arsenault *et al.* 2004, McCormick *et al.* 2005).

However, those effects occurred during ongoing estrogen treatment while the effects observed in our study represent lasting effects of exposure earlier in life. The EE₂ effect on IGF-I of tilapia was partly gender specific. For instance, the EE₂-induced decrease in liver IGF-I mRNA appeared earlier (50 DPF) in males than in females (75 DPF). Interestingly, this was paralleled by a later increase in ERα mRNA expression in females, suggesting a causative link between these events, although we cannot prove this on the basis of our data. Sex-specific responses of the IGF-I system of fish to estrogens have also been reported by Filby *et al.* (2006), who found that the hepatic expression of IGF-I in adult fathead minnow exhibited a high and significant decrease in males exposed

to E₂ but only an insignificant one in females. Gender differences in the response of IGF-I to estrogens may indicate that the estrogen effects on IGF-I expression in organs such as the liver do not only result from a direct, local crosstalk between the two hormone systems, but also interactions at the hypothalamus–pituitary level and subsequent systemic changes may be involved as well. Interestingly, brain IGF-I mRNA was responsive to developmental EE₂ treatment only in female tilapia. Here the suppression of IGF-I expression obtained by PCR was paralleled by an overall decrease in IGF-I mRNA signals in neurons as found by *in situ* hybridization.

Furthermore, EE₂-treated fish of either sex exhibited a significant decrease in GH mRNA, which was accompanied by a significant upregulation of the ERα. Evidence for effects of estrogens on fish pituitary GH gene is conflicting. While in some studies no effects of E₂ on GH mRNA were revealed (Melamed *et al.* 1998, Filby *et al.* 2006), others report that E₂ stimulated GH synthesis and secretion, but not gene transcription (Holloway & Leatherland 1997, Zou *et al.* 1997). The discrepancy between these studies and the present one may be due to the different estrogens used, to different modes of application or to hormone application at different life stages or physiological states of the fish. The reduction in pituitary GH mRNA as observed here may well be caused by a direct effect of EE₂ on the pituitary GH cells. However, EE₂

Figure 7 Influence of EE₂ exposure on GH and ERα mRNA levels in tilapia pituitary revealed by real-time PCR. Relative changes (log 2) of (A and C) GH and (B and D) ERα mRNA expression in EE₂-treated tilapia when compared with age-matched control tilapia. Control (male: 50 DPF $n=12$, 75 DPF $n=9$, 90 DPF $n=9$, 165 DPF $n=9$; female: 50 DPF $n=15$, 75 DPF $n=12$, 90 DPF $n=14$, 165 DPF $n=12$) and EE₂-treated (male: 50 DPF $n=12$, 75 DPF $n=10$, 90 DPF $n=12$, 165 DPF $n=11$; female: 50 DPF $n=12$, 75 DPF $n=12$, 90 DPF $n=14$, 165 DPF $n=11$) tilapia were used. Normalization was performed with β -actin as house-keeping gene. White columns correspond to controls and black columns to EE₂-treated fish. X-axis is labeled as DPF. Columns denote mean values and bars denote s.d. Significance level: * $P=0.0159$, $P=0.0571$, ** $P=0.0061$, $P=0.0012$.

may also have suppressed GH release at the hypothalamic level because E₂ increased the expression of somatostatin-14 in the goldfish brain (Canosa *et al.* 2002).

In the gonads, IGF-I occurs during the juvenile and adult stage in testes in spermatogonia, spermatocytes, Sertoli and Leydig cells (Le Gac *et al.* 1996, Reinecke *et al.* 1997, Berishvili *et al.* 2006b) and in the ovary in small and previtellogenic oocytes and in follicular granulosa and theca cells (Schmid *et al.* 1999, Perrot *et al.* 2000, Berishvili *et al.* 2006b). In Japanese eel cultured testes, IGF-I stimulated spermatogenesis induced by 11-ketotestosterone (Nader *et al.* 1999). In rainbow trout, testicular IGF-I increased after GH treatment (Le Gac *et al.* 1996, Biga *et al.* 2004) and both GH and IGF-I stimulated the incorporation of thymidine into spermatogonia and primary spermatocytes (Loir 1999). In the ovary of different fish species, IGF-I stimulated thymidine incorporation in vitellogenic follicles (Srivastava & Van der Kraak 1994), promoted oocyte maturation (Kagawa *et al.* 1994, Negatu

et al. 1998) and selectively influenced the production of sex steroids in theca and granulosa cells (Maestro *et al.* 1997). Hence, IGF-I most likely acts as a paracrine/autocrine regulator of fish spermatogenesis and oocyte proliferation and maturation in interaction with steroid hormones.

EE₂ treatment caused a significant reduction in IGF-I mRNA in both ovaries and testes. The downregulation occurred earlier in female than in male gonad. In agreement with the PCR results, *in situ* hybridization in testes showed a decrease in the number of IGF-I mRNA containing spermatogonia, and in ovary a reduction of IGF-I expression in small oocytes and granulosa cells after EE₂ treatment. Remarkably, the ERα mRNA showed a long-term induction by EE₂ exposure only in the testes, while in the female gonad a significant downregulation of the ERα mRNA was obtained at 75 DPF after an initial upregulation at 50 DPF. Similarly, in adult fathead minnow exposed to E₂ for 14 days, ERα mRNA was also increased in male and decreased in female gonad (Filby *et al.* 2006). Thus, ER-signaling pathway and IGF-I expression of male and female gonads show differential responses to EE₂ exposure what might indicate that in this case the EE₂ effects on IGF-I are not mediated through the ER pathway.

Overall, from the present results, two options for explaining the impairing effects of estrogens on growth, differentiation, and function of fish gonads (Jobling *et al.* 2002, Rasmussen *et al.* 2002, Fenske *et al.* 2005) are likely: they may be exerted via suppression of IGF-I production in liver resulting in a lowered level of circulating (endocrine) IGF-I and/or by the reduction of autocrine/paracrine IGF-I expression within the gonads.

In gill filaments, only males exhibited a significant decrease in IGF-I mRNA at 50 DPF that is reflected at the cellular level by a reduction of the number of IGF-I mRNA containing chloride cells, while no significant changes were present throughout the experimental period in females. The results at 50 DPF, i.e., 10 days after end of the treatment, agree in part with those obtained in the adult cyprinid fathead minnow exposed to E₂ for 14 days (Filby *et al.* 2006): IGF-I mRNA was amplified only in the gills of some fish and here a down-regulation was found in both sexes. IGF-I seems to have a physiological impact on smoltification. The chloride cells of the filament epithelium not only express Na⁺, K⁺-ATPase (McCormick 1996) but also in developing and adult fish IGF-I mRNA (Reinecke *et al.* 1997, Radaelli *et al.* 2003, Berishvili *et al.* 2006a). In tilapia, the importance of local IGF-I expression is stressed by its very early appearance in chloride cells around 6–7 DPF (Berishvili *et al.* 2006a). In several fish species, evidence has been presented that both circulating (liver-derived) IGF-I (Madsen & Bern 1993, Shepherd *et al.* 1997, Inoue *et al.* 2003) and autocrine/paracrine IGF-I from the chloride cells (Sakamoto & Hirano 1993, Biga *et al.* 2004) mediate the osmoregulatory actions of GH. Previous studies have reported that E₂ impairs osmoregulation in salmonid (Arsenault *et al.* 2004, Madsen *et al.* 2004, McCormick *et al.* 2005) and non-salmonid (Vijayan *et al.* 2001) fish, and suppressed plasma levels of

IGF-I were thought to be the underlying mechanism (McCormick *et al.* 2005). The present results not only support this idea but also suggest a direct effect of estrogens on IGF-I production in gill filaments in addition to the endocrine route. This is not only indicated by the present PCR results but also by the observed decrease of IGF-I mRNA in chloride cells as revealed by *in situ* hybridization.

In summary, the findings from the present study on developing fish are in line with some earlier findings on adult fish that estrogen(s) are able to modulate IGF-I transcription and translation in liver. In addition, the study provides evidence that estrogens applied during early development: a) change the IGF-I system in liver and, concomitantly, circulating IGF-I and, thus, influence the endocrine route of IGF-I action, b) impair local IGF-I in other organs by changing IGF-I expression within the organ-specific cells as shown by *in situ* hybridization, and c) that the IGF-I response is associated with a change of pituitary GH expression. Thus, the estrogen effect on IGF-I seems to involve both direct (autocrine/paracrine) interactions in peripheral organs as well as indirect (endocrine) effects via modulation of the brain-pituitary GH system.

Finally, our results for the first time provide evidence that developmental estrogen exposure can have long-lasting effects on the GH/IGF-I system. As individual growth has consequences for demographic parameters such as age-specific survival, time to maturation or fecundity, our findings point to a potentially important mechanism through which environmental estrogens, in addition to their direct effect on fish reproduction, could alter population growth of fish species.

The present study aimed to reveal whether interactions between the estrogen and the GH/IGF-I system in principal can take place; to this end, we used a dietary EE₂ concentration in the range used for intended feminization of tilapia in aquaculture (for literature see Piferrer 2001). Future research would now have to examine the estrogen-IGF-I interaction at environmentally relevant exposure concentrations. Importantly, (xeno)estrogens are not bioaccumulative like many of the classical environmental toxicants, such as polychlorinated biphenyls (PCBs). Accordingly, food chain transfer of estrogens has not been demonstrated to date and the dietary exposure route appears to play no significant role. Dietary application of estrogens, similar to injection of estrogens that has also often been described, therefore represents an artificial exposure situation, which is useful to provide principal information on mechanisms and targets but not to assess the environmental hazard of estrogens. Future research has to address the environmental relevance of the effects observed in the present study as well as the mechanism(s) through which estrogen(s) act to suppress pituitary GH and local IGF-I expression in liver and extrahepatic sites. In particular, given our findings on the correlation between ER and IGF-I changes, it would be important to examine whether the EE₂ effects on the GH/IGF-I system are mediated through the ER pathway and/or through other mechanisms.

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References

- Arsenault JT, Fairchild WL, MacLatchy DL, Burrige L, Haya K & Brown SB 2004 Effects of water-borne 4-nonylphenol and 17 β -estradiol exposures during parr-smolt transformation on growth and plasma IGF-I of Atlantic salmon (*Salmo salar* L). *Aquatic Toxicology* **66** 255–265.
- Ashfield LA, Pottinger TG & Sumpter JP 1998 Exposure of female juvenile rainbow trout to alkylphenolic compounds results in modification to growth and ovosomatic index. *Environmental Toxicology and Chemistry* **17** 679–686.
- Ber R & Daniel V 1992 Structure and sequence of the growth hormone-encoding gene from *Tilapia nilotica*. *Gene* **113** 245–250.
- Berishvili G, Shved N, Eppler E, Clota F, Baroiller J-F & Reinecke M 2006a Organ-specific expression of IGF-I during early development of bony fish as revealed in the tilapia, *Oreochromis niloticus*, by *in situ* hybridisation and immunohistochemistry: Indication for the particular importance of local IGF-I. *Cell Tissue Research* **325** 287–301.
- Berishvili G, D'Cotta H, Baroiller J-F, Segner H & Reinecke M 2006b Differential expression of IGF-I mRNA and peptide in the male and female gonad during early development of a bony fish, the tilapia *Oreochromis niloticus*. *General and Comparative Endocrinology* **146** 204–210.
- Biga PR, Schelling GT, Hardy RW, Cain KD, Overturf K & Ott TL 2004 The effects of recombinant bovine somatotropin (rbST) on tissue IGF-I, IGF-I receptor, and GH mRNA levels in rainbow trout, *Oncorhynchus mykiss*. *General and Comparative Endocrinology* **135** 324–333.
- Caelers A, Berishvili G, Meli ML, Eppler E & Reinecke M 2004 Establishment of a real-time RT-PCR for the determination of absolute amounts of IGF-I and IGF-II gene expression in liver and extrahepatic sites of the tilapia. *General and Comparative Endocrinology* **137** 196–204.
- Caelers A, Maclean N, Hwang G, Eppler E & Reinecke M 2005 Expression of endogenous and exogenous growth hormone (GH) in a GH-transgenic tilapia (*Oreochromis niloticus*). *Transgenic Research* **14** 95–104.
- Canosa LF, Lin X & Peter RE 2002 Regulation of expression of somatostatin genes by sex steroid hormones in goldfish forebrain. *Neuroendocrinology* **76** 8–17.
- Carnevali O, Cardinali M, Maradonna F, Parisi M, Olivetto I, Polzonetti-Magni AM, Mosconi G & Funkenstein B 2005 Hormonal regulation of hepatic IGF-I and IGF-II gene expression in the marine teleost *Sparus aurata*. *Molecular Reproduction and Development* **71** 12–18.
- Chang XT, Kobayashi T, Todo T, Ikeuchi T, Yoshiura Y, Kajiura-Kobayashi H, Morrey C & Nagahama Y 1999 Molecular cloning of estrogen receptors alpha and beta in the ovary of a teleost fish, the tilapia (*Oreochromis niloticus*). *Zoological Science* **16** 653–658.
- Duan C 1998 Nutritional and developmental regulation of insulin-like growth factors in fish. *Journal of Nutrition* **128** (2 Suppl) 306S–314S.
- Duguay SJ, Lai-Zhang J, Steiner DF, Funkenstein B & Chan SJ 1996 Developmental and tissue-regulated expression of IGF-I and IGF-II mRNAs in *Sparus aurata*. *Journal of Molecular Endocrinology* **16** 123–132.
- Dzidic A, Prgomet C, Mohr A, Meyer K, Bauer J, Meyer HHD & Pfaffl MW 2006 Effects of mycophenolic acid on inosine monophosphate dehydrogenase I and II mRNA expression in white blood cells and various tissues in sheep. *Journal of Veterinary Medicine A* **53** 163–169.
- Eppler E, Caelers A, Shved N, Hwang G, Rahman AM, Maclean N, Zapf J & Reinecke M 2007 Insulin-like growth factor I (IGF-I) in a growth-enhanced transgenic (GH-overexpressing) bony fish, the tilapia (*Oreochromis niloticus*): indication for a higher impact of autocrine/paracrine than of endocrine IGF-I. *Transgenic Research* **16** 479–489.

- Fenske M, Maack G, Schäfers C & Segner H 2005 An environmentally relevant concentration of estrogen induces arrest of male gonad development in zebrafish, *Danio rerio*. *Environmental Toxicology and Chemistry* **24** 1088–1098.
- Filby AL, Thorpe KL & Tyler CR 2006 Multiple molecular effect pathways of an environmental oestrogen in fish. *Journal of Molecular Endocrinology* **37** 121–134.
- Le Gac F, Loir M, Le Bail P-Y & Ollitrault M 1996 Insulin-like growth factor I (IGF-I) mRNA and IGF-I receptor in trout testis and in isolated spermatogenic and Sertoli cells. *Molecular Reproduction and Development* **44** 23–35.
- Holloway AC & Leatherland JF 1997 Effect of gonadal steroid hormones on plasma growth hormone concentrations in sexually immature rainbow trout, *Oncorhynchus mykiss*. *General and Comparative Endocrinology* **105** 246–254.
- Hwang GL, Rahman AM, Razak AS, Sohm F, Farahmand H, Smith A, Brooks C & Maclean N 2003 Isolation and characterisation of tilapia β -actin promoter and comparison of its activity with carp β -actin promoter. *Biochimica et Biophysica Acta* **1625** 11–18.
- Inoue K, Iwatani H & Takei Y 2003 Growth hormone and insulin-like growth factor I of a Euryhaline fish *Cottus kazika*: cDNA cloning and expression after seawater acclimation. *General and Comparative Endocrinology* **131** 77–84.
- Jobling S, Beresford N, Nolan M, Rodgers-Gray T, Brighty GC, Sumpter JP & Tyler CR 2002 Altered sexual maturation and gamete production in wild roach (*Rutilus rutilus*) living in rivers that receive treated sewage effluents. *Biology of Reproduction* **66** 272–281.
- Jones JI & Clemmons DR 1995 Insulin-like growth factors and their binding proteins: biological actions. *Endocrine Reviews* **16** 3–34.
- Kagawa H, Kobayashi M, Hasegawa Y & Aida K 1994 Insulin and insulin-like growth factors I and II induce final maturation of oocytes of red seabream, *Pagrus major*, in vitro. *General and Comparative Endocrinology* **95** 293–300.
- Livak KJ & Schmittgen TD 2001 Analysis of relative gene expression data using real-time quantitative PCR and the $2(-\Delta\Delta C_t)$ method. *Methods* **25** 402–408.
- Loir M 1999 Spermatogonia of rainbow trout: II. in vitro study of the influence of pituitary hormones, growth factors and steroids on mitotic activity. *Molecular Reproduction and Development* **53** 434–442.
- Madsen SS & Bern HA 1993 In vitro effects of insulin-like growth factor I on gill Na^+ , K^+ -ATPase in coho salmon, *Oncorhynchus kisutch*. *Journal of Endocrinology* **138** 23–30.
- Madsen SS, Skovbølling S, Nielsen C & Korsgaard B 2004 17- β estradiol and 4-nonylphenol delay smolt development and downstream migration in Atlantic salmon, *Salmo salar*. *Aquatic Toxicology* **68** 109–120.
- Maestro MA, Planas JV, Moriyama S, Gutierrez J, Planas J & Swanson P 1997 Ovarian receptors for insulin and insulin-like growth factor I (IGF-I) and effects of IGF-I on steroid production by isolated follicular layers of the preovulatory coho salmon ovarian follicle. *General and Comparative Endocrinology* **106** 189–201.
- McCormick SD 1996 Effects of growth hormone and insulin-like growth factor I on salinity tolerance and gill Na^+ , K^+ -ATPase in Atlantic Salmon (*Salmo salar*): interaction with cortisol. *General and Comparative Endocrinology* **101** 3–11.
- McCormick SD, O'Dea ME, Moeckel AM, Lerner DT & Björnsson BT 2005 Endocrine disruption of parr-smolt transformation and seawater tolerance of Atlantic salmon by 4-nonylphenol and 17 β -estradiol. *General and Comparative Endocrinology* **142** 280–288.
- Melamed P, Rosenfeld H, Elizur A & Yaron Z 1998 Endocrine regulation of gonadotropin and growth hormone gene transcription in fish. *Comparative Biochemistry and Physiology. Part C, Pharmacology, Toxicology and Endocrinology* **119** 325–338.
- Nader MR, Miura T, Ando N, Miura C & Yamauchi K 1999 Recombinant human insulin-like growth factor I stimulates all stages of 11-ketotestosterone-induced spermatogenesis in the Japanese eel, *Anguilla japonica*, in vitro. *Biology of Reproduction* **61** 944–947.
- Negatu Z, Hsiao SM & Wallace RA 1998 Effects of insulin-like growth factor-I on final oocyte maturation and steroid production in *Fundulus heteroclitus*. *Fish Physiology and Biochemistry* **19** 13–21.
- Perrot V, Moiseeva EB, Gozes Y, Chan SJ, Ingleton P & Funkenstein B 1999 Ontogeny of the insulin-like growth factor system (IGF-I, IGF-II, and IGF-IR) in gilthead seabream (*Sparus aurata*): expression and cellular localization. *General and Comparative Endocrinology* **116** 445–460.
- Perrot V, Moiseeva EB, Gozes Y, Chan SJ & Funkenstein B 2000 Insulin-like growth factor receptors and their ligands in gonads of a hermaphroditic species, the gilthead seabream (*Sparus aurata*): expression and cellular localization. *Biology of Reproduction* **63** 229–241.
- Piferrer F 2001 Endocrine sex control strategies for the feminisation of fish. *Aquaculture* **197** 229–281.
- Plisetskaya EM 1998 Some of my not so favorite things about insulin and insulin-like growth factors in fish. *Comparative Biochemistry and Physiology. Part B, Biochemistry and Molecular Biology* **121** 3–11.
- Radaelli G, Domeneghini C, Arrighi S, Bosi G, Patruno M & Funkenstein B 2003 Localization of IGF-I, IGF-I receptor, and IGFBP-2 in developing *Umbra cirrosa* (Pisces: Osteichthyes). *General and Comparative Endocrinology* **130** 232–244.
- Rasmussen TH, Andreassen TK, Pedersen SN, Van der Ven LTM, Bjerregaard P & Korsgaard B 2002 Effects of waterborne exposure of octylphenol and estrogen on pregnant viviparous eelpout (*Zoarces viviparus*) and her embryos in ovario. *Journal of Experimental Biology* **205** 3857–3876.
- Reinecke M 2006 Insulin-like growth factor I and II in fish. In *Fish Endocrinology*, vol 1, pp 87–130. Eds M Reinecke, G Zaccone & BG Kapoor. Enfield, Jersey, Plymouth: Science Publishers.
- Reinecke M & Collet C 1998 The phylogeny of the insulin-like growth factors. *International Review of Cytology* **183** 1–94.
- Reinecke M, Schmid A, Ermatinger R & Löffing-Cueni D 1997 Insulin-like growth factor I in the teleost *Oreochromis mossambicus*, the tilapia: gene sequence, tissue expression, and cellular localization. *Endocrinology* **138** 3613–3619.
- Riley LG, Hirano T & Grau EG 2004 Estradiol-17 β and dihydrotestosterone differentially regulate vitellogenin and insulin-like growth factor-I production in primary hepatocytes of the tilapia *Oreochromis mossambicus*. *Comparative Biochemistry and Physiology. Toxicology and Pharmacology* **138** 177–186.
- Sakamoto T & Hirano T 1993 Expression of insulin-like growth factor I gene in osmoregulatory organs during seawater adaptation of the salmonids fish: possible mode of osmoregulatory action of growth hormone. *PNAS* **90** 1912–1916.
- Schmid AC, Naef E, Kloas W & Reinecke M 1999 IGF-I and IGF-II in the ovary of a bony fish *Oreochromis mossambicus*, the tilapia: in situ hybridisation, immunohistochemical localisation, northern blot and cDNA sequences. *Molecular and Cellular Endocrinology* **156** 141–149.
- Shepherd BS, Sakamoto T, Nishioka RS, Richman NH III, Mori I, Madsen SS, Chen TT, Hirano T, Bern HA & Grau EG 1997 Somatotrophic actions of the homologous growth hormone and prolactins in the euryhaline teleost, the tilapia, *Oreochromis mossambicus*. *PNAS* **94** 2068–2072.
- Srivastava RK & Van der Kraak G 1994 Regulation of DNA-synthesis in goldfish ovarian follicles by hormones and growth factors. *Journal of Experimental Zoology* **270** 263–272.
- Vijayan MM, Takemura A & Mommsen TP 2001 Estradiol impairs hypoosmoregulatory capacity in the euryhaline tilapia, *Oreochromis mossambicus*. *American Journal of Physiology* **281** R1161–R1168.
- Vong QP, Chan KM & Cheng CH 2003 Quantification of common carp (*Cyprinus carpio*) IGF-I, IGF-II mRNA by real-time PCR: differential regulation of expression by GH. *Journal of Endocrinology* **178** 513–521.
- Wood AW, Duan C & Bern HA 2005 Insulin-like growth factor signaling in fish. *International Review of Cytology* **243** 215–285.
- Zapf J, Gosteli-Peter M, Weckbecker G, Hunziker EB & Reinecke M 2002 The somatostatin analog octreotide inhibits GH-stimulated, but not IGF-I-stimulated, bone growth in hypophysectomized rats. *Endocrinology* **143** 2944–2952.
- Zou JJ, Trudeau VL, Cui Z, Brechin J, Mackenzie K, Zhu Z, Houlihan DF & Peter R 1997 Estradiol stimulates growth hormone production in female goldfish. *General and Comparative Endocrinology* **106** 102–112.

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