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

Natallia Shved, Giorgi Berishvili, Helena D’Cotta1, Jean-François Baroiller1, Helmut Segner2, Elisabeth Eppler and Manfred Reinecke

Division of Neuroendocrinology, Institute of Anatomy, University of Zürich, Winterthurerstr. 190, CH-8057 Zürich, Switzerland
1CIRAD-EMVT UPR20, Campus International de Baillarguet, F-34398 Montpellier, France
2Centre for Fish and Wildlife Health, University of Bern, Länggasse 122, CH-3012 Bern, Switzerland
(Correspondence should be addressed to M Reinecke; Email: reinecke@anatom.uzh.ch)

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 17α-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 (ERα) 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 ERα 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.


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/auto-crine 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, Plisetskaya 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-I system as reported for adult fish, developmental estrogen exposure may induce organizational alterations of the IGF-I system which could lead to persistent changes in growth and reproduction.

The present study aims to investigate the potential influence of 17α-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 gonadal 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 (ERα) 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 ERα. 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 (Duguy 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 ERα 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 μg 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-phenoxo 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, Wängen, Switzerland, specific activity 300–400 μCi/μl) 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 already described (Caelters et al. 2004, 2005) for IGF-I (sense TCTGTGAGAGCGAGGCTTTT, antisense CACGTGACCGCCTTGGCA, probe ATTTCGAATAAACCAACAGGGCTATGGCCCA), GH (sense TGAGACAAACAGAGCGCA, antisense CCCAGGACTCAGCAGCTCCA, probe CGCAGCTGCTCCTGAGGCTG), and β-actin as a house-keeping gene (sense GC
CCCACCTGAGGCTAAA, antisense AAAGGTTGGA-
CAGAGGCC, probe TTCGCTGAGATCGAGGCTT-CATC). Using this method, new primers and probe (sense CAAGTTTTGGAGGAGGATGTC, antisense TCTAG-
CAGCTGATCGAGGAGAGATC, probe CTGATCGAGGCTTCCCT) were designed for ERα based on the O. niloticus ERα 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 RNAlater (Ambion, Austin, TX, USA) using TRIzol reagent (Invitrogen) and treated with 1 U RQ1 RNase-free DNase (Promega, Madison, WI, USA) using TRIzol reagent with an exact significance was calculated using Mann–Whitney rank sum test, way of presenting qPCR data (e.g., Dzidic et al. 2006) and a common 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). Efficiency tests for each gene were performed using PCR primer and probe (sense and antisense) previously denaturated for 5 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 ΔΔC_{\text{T}} method

The comparative threshold cycle (ΔΔC_{\text{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 ERα assay (data not shown) permitted the accurate use of the ΔΔC_{\text{T}} method. Relative changes induced by EE2 feeding were calculated by the formula 2^{\text{-ΔΔC}_{\text{T}}} with ΔΔC_{\text{T}} = C_{\text{T}} (treated group) − C_{\text{T}} (untreated control), and ΔC_{\text{T}} = C_{\text{T}} (target gene) − C_{\text{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 paraplast 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 μm and processed for in situ hybridization using the sense (5’-GCTTGAGGAGGCTTCCCT-3’) and antisense (5’-AACCTGGGTGCTTGGCATG-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 μl prehybridization solution per section for 3 h at 54 °C and hybridized overnight at 54 °C with 50 μl hybridization buffer containing 200 ng sense (negative control) or antisense probes previously denaturated for 5 min at 85 °C. Slides were washed for 15 min at room temperature in 2× SSC and for 30 min at the specific hybridization temperature at descending concentrations of SSC (2×, 1×, 0·5×, and 0·2×). 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 ± 8·5% females in control to 86·5 ± 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).

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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 ($5.65\pm1.18$ ng/ml) when compared with the controls ($9.57\pm2.06$ ng/ml), while in females (Fig. 3H) there was only a trend to reduce IGF-I serum level (control $11.20\pm2.18$ versus EE2-treated $8.54\pm2.26$, $P=0.08$). At the later stages, there was no significant difference between the treated group and the controls (Fig. 3G and H).

**IGF-I and ERα 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 ERα 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, ERα mRNA was raised to 2.2-fold in males ($P=0.02$) and 37-fold in females ($P=0.002$). In male liver, ERα was back at the
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 (2.2-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. 4D), 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. 5G). 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.

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**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 = 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 tilapia. 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 < 0.01, *** P < 0.001.
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 (P=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 sexes, 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<0.006) 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).
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
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 decline in males exposed to EE2 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
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-ketosterone (Nader et al. 1999). In rainbow trout, testicular IGF-I increased spermatogenesis induced by 11-ketosterone after GH treatment (Le Gac et al. 1999). 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

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.e. Significance level: *P = 0.0159, **P = 0.0061, ***P = 0.0012.
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 and in muscle. In addition, the study provides evidence that estrogens applied during early development: a) change the IGF-I system in liver and, concomitantly, circulating IGFI-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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