Multiple molecular effect pathways of an environmental oestrogen in fish

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Abstract

Complex interrelationships in the signalling of oestrogenic effects mean that environmental oestrogens present in the aquatic environment have the potential to disrupt physiological function in fish in a more complex manner than portrayed in the present literature. Taking a broader approach to investigate the possible effect pathways and the likely consequences of environmental oestrogen exposure in fish, the effects of 17 β -oestradiol (E₂) were studied on the expression of a suite of genes which interact to mediate growth, development and thyroid and interrenal function (growth hormone GH (*gh*), GH receptor (*ghr*), insulin-like growth factor (IGF-I) (*igf1*), IGF-I receptor (*igf1r*), thyroid hormone receptors- α (*thra*) and - β (*thrb*) and glucocorticoid receptor (*gr*)) together with the expression analyses of sex-steroid receptors and ten other genes centrally involved in sexual development and reproduction in fathead minnow (fhm; *Pimephales promelas*). Exposure of adult fhm to 35 ng E₂/l for 14 days induced classic oestrogen biomarker responses (hepatic *oestrogen receptor 1* and plasma vitellogenin), and impacted on the reproductive axis, feminising 'male' steroidogenic enzyme expression profiles and suppressing genes involved in testis differentiation. However, E₂ also triggered a cascade of responses for *gh*, *ghr*, *igf1*, *igf1r*, *thra*, *thrb* and *gr* in the pituitary, brain, liver, gonad and gill, with potential consequences for the functioning of many physiological processes, not just reproduction. Molecular responses to E₂ were complex, with most genes showing differential responses between tissues and sexes. For example, *igf1* expression increased in brain but decreased in gill on exposure to E₂, and responded in an opposite way in males compared with females in liver, gonad and pituitary. These findings demonstrate the importance of developing a deeper understanding of the endocrine interactions for unravelling the mechanisms of environmental oestrogen action and predicting the likely health consequences.

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Introduction

It is now firmly established that a wide range of natural and anthropogenic chemicals present in the aquatic environment have the capacity to disrupt the endocrine system and, in turn, alter physiological function (Tyler *et al.* 1998). Many of these effects are as a consequence of exposure to chemicals with oestrogenic activity, so-called environmental oestrogens (for a review, see Sonnenschein & Soto 1998). To date, studies on endocrine disruption have focused heavily on the ability of environmental oestrogens, acting via the oestrogen receptor, to alter reproductive function. This research focus has been driven by the finding that wild fish populations in UK rivers exposed to environmental oestrogens have altered sexual development (Jobling *et al.* 1998) and reduced fertility (Jobling *et al.* 2002a,b).

There are, however, highly complex interrelationships, both within and between body tissues, mediating the endocrine control of reproduction, growth, development and other physiological processes in fish. Therefore, environmental oestrogens, and other

endocrine-disrupting chemicals, have the potential to disrupt physiological function far more widely than directly through reproductive pathways in the gonad alone. Indeed, laboratory-based studies have shown that some environmental oestrogens not only affect reproduction of fish but can also impact on other endocrine-mediated processes, including somatic growth (e.g. 17 α -ethinyloestradiol (EE₂; Versonnen & Janssen 2004, Van den Belt *et al.* 2003), 4-*tert*-nonylphenol (NP; Dreze *et al.* 2000, Magliulo *et al.* 2002), methoxychlor (Magliulo *et al.* 2002)), osmoregulation (e.g. 17 β -oestradiol (E₂) and NP (Madsen *et al.* 1997, Vijayan *et al.* 2001, Arsenaault *et al.* 2004, Madsen *et al.* 2004, McCormick *et al.* 2005)), immune function (e.g. E₂ (Wang & Belosevic 1994, Hou *et al.* 1999, Law *et al.* 2001), EE₂ (Law *et al.* 2001)), the stress response (e.g. E₂ (Pottinger *et al.* 1996), NP (Magliulo *et al.* 2002), methoxychlor (Magliulo *et al.* 2002)) and embryonic development (e.g. E₂ (Rasmussen *et al.* 2002), EE₂ (Van den Belt *et al.* 2003)). The mechanisms by which these effects occur, however, are not fully known.

A mechanistic understanding of the interplay between components of the endocrine system together

with an appreciation of the wider effects of environmental oestrogens in the body are fundamental to better assess the potential health implications of exposure to environmental oestrogens. Molecular approaches (reviewed in Rotchell & Ostrander 2003) provide tools for investigating the multiple pathways of chemical effect in the body and, potentially, for assessing responses to toxicants across entire biochemical pathways. Furthermore, changes in the expression of genes that play fundamental roles in development can signal for subsequent (and often latent) tissue- and organism-level effects. Thus, molecular approaches potentially provide rapid and sensitive diagnostic tools for subsequent physiological impacts.

In this work, we utilised a molecular approach to study the effects of an environmental oestrogen, E₂, on the physiology of fish in a broader context than via reproductive pathways in the gonad alone. E₂ was chosen for this work as it is used widely as a 'model' oestrogen to study the mechanisms of environmental oestrogen action and is one of the principal components responsible for oestrogenic activity in sewage treatment works effluents (Desbrow *et al.* 1998) and, therefore, has environmental relevance. The effects of exposure to E₂ were determined on the expression of a suite of cDNAs for key hormones and receptors which interact to mediate somatic growth, development and thyroid and interrenal function (processes which involve endocrine axes that are known to interact with endogenous sex-steroid hormones, including oestrogens) together with analyses of the expression of sex-steroid receptors and genes that play central roles in reproduction. The study species used for this work was fathead minnow (fhm; *Pimephales promelas*), a model species for endocrine disruption research (reviewed in Ankley & Villeneuve 2006).

Genes chosen for this study and that mediate somatic growth, development and thyroid and interrenal function were growth hormone (*gh*), growth hormone receptor (*ghr*), insulin-like growth factor-I (*igfI*), insulin-like growth factor-I receptor (*igfIr*), thyroid hormone receptor- α (*thra*), thyroid hormone receptor- β (*thrb*) and glucocorticoid receptor (*gr*). Growth hormone (GH), synthesised predominantly by the somatotrophs of the anterior pituitary, is best known for its growth-promoting capabilities (Cavari *et al.* 1993), which are believed to be initiated principally through an intimate association with hepatic insulin-like growth factor-I (IGF-I), following GH-binding to membrane-bound GH receptors (GHRs) (reviewed in Kopchick & Andry 2000). However, GH participates in almost all major physiological processes in fish including osmo- and iono-regulation (Sakamoto & Hirano 1993), immune function (Perez-Sanchez 2000), reproduction (LeGac *et al.* 1993) and behaviour (Bjornsson 1997). Furthermore, both GH and IGF-I additionally

function in an autocrine/paracrine manner and independently of one another in some target tissues (Jones & Clemmons 1995, Harvey *et al.* 1998, Butler & Le Roith 2001). Thyroid hormone receptors (THRs) mediate the effects of the thyroid hormones, thyroxine (T₄) and 3,3',5-triiodo-L-thyronine (T₃) in fish (Lazar 2003) and, as for GH, have the ability to regulate a wide range of cellular functions, including growth, development, differentiation, metabolism and maintenance of homeostasis, in virtually every tissue (Brent 1996). In fish, especially crucial roles of THRs have been recognised in early development and metamorphosis (Power *et al.* 2001). Glucocorticoid receptors (GRs) are the principal receptors mediating the effects of glucocorticoids, the principle one of which in fish is cortisol (F). In fish, the well-established roles of F are in metabolism (many of which characterise the stress response; Wendelaar Bonga 1997), but it also has roles in growth, reproduction, larval development, cognition and immune function, and it mediates some of the processes traditionally thought to depend on mineralocorticoids, such as salt balance (reviewed in Mommsen *et al.* 1999).

Genes chosen for study that mediate sexual function included sex-steroid receptors, steroidogenic enzymes and other genes known to play roles in sexual differentiation and sexual development. The sex-steroid receptor genes were the three oestrogen receptor subtypes: oestrogen receptor 1 (*esr1*; formerly *oestrogen receptor* α), oestrogen receptor 2a (*esr2a*; formerly *oestrogen receptor* β 2 or γ) and oestrogen receptor 2b (*esr2b*; formerly *oestrogen receptor* β) and the androgen receptor (*ar*), which function as ligand-dependent transcription factors to regulate the expression of oestrogen and androgen target genes (reviewed in Tsai & O'Malley 1994). The genes that play roles in sex-steroid synthesis were cytochrome P450 17 (*cyp17*), cytochrome P450 19a (*cyp19a*) and cytochrome P450 19b (*cyp19b*), steroidogenic acute regulatory protein (*star*), hydroxysteroid 11- β -dehydrogenase 2 (*hsd11b2*) and hydroxysteroid 17- β -dehydrogenase (*hsd17b*) and the others with established roles in sex differentiation and reproduction were anti-Müllerian hormone (*amh*), vasa homologue (*vasa*), doublesex and mab-3 related transcription factor 1 (*dmrt1*) and nuclear receptor subfamily-5 group A member 2 (*nr5a2*; formerly fushi tarazu factor 1 (*ftzfl*) or steroidogenic factor 1 (*sf1*)). There is no official gene nomenclature system for the fhm so, in this paper, the official gene and protein designations for the zebrafish (*Danio rerio*; <http://zfin.org>) have been adopted. In parallel with the effects of E₂ on expression of the gene targets studied, induction of plasma vitellogenin (Vg) and somatic weight and gonad growth were quantified as phenotypic effect measures of the oestrogen treatment.

Table 1 Nucleotide sequences of real-time PCR primers, NCBI GenBank accession numbers, real-time PCR product sizes, annealing temperatures, and efficiencies (*E*) for target genes

	GenBank	Sense primer (5'–3')	Antisense primer (5'–3')	Product size (bp)	Annealing temperature (°C)	<i>E</i>
Target mRNA						
<i>esr1</i>	AY775183	CACCCACCAGCCCTCAG	CACCTCACACAGACCAACAC	155	60.5	2.09
<i>esr2a</i>	AY929613	GTGACGGATGCTTTGGTATG	GGTGCCTTATGTGGGAGAG	130	61.8	2.14
<i>esr2b</i>	AY566178	GCCACCTCCAGATTACG	CACGACTCTCCACACCTTCAG	106	62.5	1.99
<i>ar</i>	AY727529	ATGGGAGTGATGGTCTTTG	AGGTCTGGAGCGAAATAC	80	56.0	2.10
<i>gh</i>	AY643399	GGGATGTTTGGATGGTCAAC	GCTCTCTCTGAGGCTGTTT	103	60.5	1.99
<i>ghr</i>	DQ007448	CGGCTCTGATACACAACAC	GAATCGTCGTCGCTTTTG	81	57.0	2.14
<i>igf1</i>	AY533140	CAACGGCACACGGACATC	CCTCGGCTTGAGTTCTTCTG	98	60.0	2.08
<i>igf1r</i>	AY566179	TAGCCGAGCAGCCTTACC	CCAGCACATCCGCATCAG	125	60.0	1.99
<i>thra</i>	DQ074645	ATGACCCAGAGAGCGAGAC	CATCAGACACCACTCCTAACC	93	59.0	1.87
<i>thrb</i>	AY533142	GCCAACCAGTCAGGTCAAG	AGGAACAGAAATGAGGAACAGAG	93	60.0	1.97
<i>gr</i>	AY533141	GAAAGTCCTTCTGCTCCTGAG	AGTTCTCCTCTCTTTCACAATG	125	60.5	2.11
<i>cyp17</i>	AJ277867	AAAGGAAGATGAATGGATTTG	GCAGTTGTTGAAAGAAGATG	126	59.0	1.87
<i>cyp19a</i>	AF288755	GCGGCTCCAGATACTC	ACTCTCCAGAATGTTTAACC	159	56.5	2.29
<i>cyp19b</i>	AJ277866	CCTGTAGATGACTTGATGATG	CTGGGACTGCTGATTGG	160	60.0	1.91
<i>hsd11b2</i>	DT297975	AGACATCACCCAGCCTCAG	ACACACAATCCAGCGTTATTTAC	105	61.0	1.92
<i>hsd17b</i>	DT161033	ACAGCCAGCCGTAGAC	TCCTAAAGCCAGTGATGAC	140	56.5	1.90
<i>nr5a2</i>	DT285288	GGACGGTTCAGAATAACAAGAG	CGACGCTCAGGCATTC	114	61.0	1.86
<i>star</i>	DQ360497	TGTCCGCTGTGCCAAAC	GGCTTTACAAATCCTTTCTCTC	96	58.0	1.95
<i>vasa</i>	DT292437	GGCAGCAAGCAAGTTCAG	GCACACAGGTCCCAAATG	119	59.0	1.94
<i>amh</i>	DT344811	TCGGTTCTGCTGCTGAAG	ACTGACTGGGCGTTGATG	109	59.0	1.97
<i>dmrt1</i>	DT306411	ACTACCAGCAATACCAGATG	GCAGCCGTGTCAATCC	121	59.5	2.01
<i>rpl8</i>	AY919670	CTCCGCTCTTCAAAGCCCATGT	TCCTTCACGATCCCCTTGATG	162	60.0	2.14

Materials and methods

Test species

The fhm used in this study were bred at the Brixham Environmental Laboratory, Brixham, Devon, UK. Fish were maintained under flow-through conditions in dechlorinated water at 25.0 ± 1 °C with a 16 h light:8 h darkness photoperiod. Fish were fed adult *Artemia* sp. twice daily and Ecostart 17 1.0 mm fish food pellets (Biomar Ltd., Brande, Denmark) once daily. All animal-use protocols were carried out ethically in accordance with UK Home Office guidelines.

E₂ exposure

Duplicate tanks of adult (>150 days post hatch) male and female fhm (eight males and eight females per tank) were exposed to 35 ng E₂/l (98% purity; lot 70K1206; Sigma) under flow-through conditions for a period of 14 days. The test concentration adopted in this work was within the concentration range found in European effluents (Desbrow *et al.* 1998, Ternes *et al.* 1999, Baronti *et al.* 2000, Rodgers-Gray *et al.* 2001). Duplicate tanks of the same numbers of fish were maintained in dilution water as controls.

To confirm the oestrogenic activity (E₂ equivalent) in the tanks, water samples were collected from the centre of

each tank (350 ml/tank) on a daily basis and the samples from the duplicate tanks were pooled to provide a composite sample (700 ml/treatment). The sample was spiked with 0.5% methanol and extracted via peristalsis (5 ml/min) onto preconditioned solid-phase extraction columns. E₂ was eluted from the columns using 5 ml methanol and stored at –20 °C for subsequent analysis. At the time of analysis, the methanol was removed under a stream of nitrogen and the extracts were resuspended in 5 ml ethanol. The concentrated extracts were analysed using the recombinant yeast oestrogen screen (as described in Routledge & Sumpter 1996). The E₂ equivalents in the extracts were derived by comparison to a reference E₂ standard curve. The limit of detection for the reference E₂ standard curve was 10 ng/l E₂.

Fish were sacrificed by a lethal overdose of anaesthesia (500 mg/l MS-222 (3-aminobenzoic acid ethyl ester) buffered to pH 7.4; Sigma) and a blood sample was collected from the heart of each fish into chilled heparinised syringes. The blood samples were centrifuged at 28 000 *g* for 5 min and the plasma removed and stored at –80 °C for subsequent measurement of Vg, a biomarker of oestrogen exposure. Vg was measured in the plasma of all fish using a carp Vg ELISA validated for use with fhm (1 ng/ml detection limit; Tyler *et al.* 1999). All fish were measured for total length (mm) and wet weight (mg) and gonads were removed and weighed (to the nearest 0.1 mg) for the

determination of gonadosomatic index (GSI) ($\text{GSI} = (\text{gonad weight/body weight}) \times 100$). Tissue samples (gonad, brain, pituitary (complete on lower skull plate), liver, gill and intestine) were collected from each fish from a single replicate tank, snap-frozen in liquid nitrogen and stored at -80°C until RNA extraction (within 1 month) for subsequent gene expression analyses.

RNA extraction

Total RNA was extracted from each tissue sample using Tri Reagent (Sigma) following the manufacturer's instructions. Total RNA concentration was estimated from absorbance at 260 nm ($A_{260\text{ nm}}$; GeneQuant; Amersham) and RNA quality was verified by electrophoresis on ethidium bromide-stained 1.5% agarose gels and by $A_{260\text{ nm}}/A_{280\text{ nm}}$ ratios > 1.8 .

Real-time PCR

Development of real-time PCR assays for target genes

The assays for the fhm *esrs* were performed as previously described (Filby & Tyler 2005). Primers specific for the other target cDNAs were designed with Beacon Designer 3.0 Software (Premier Biosoft International, Palo Alto, CA, USA) according to the manufacturer's guidelines and purchased from MWG-Biotech (Ebersburg, Germany). Assays were optimised and validated for real-time quantitative PCR using SYBR Green chemistry as described previously (Filby & Tyler 2005). Assays had detection ranges of at least five orders of magnitude. Specificity of primer sets throughout this range of detection was confirmed by the observation of single amplification products of the expected size and melting temperature (T_m) and sequence. All assays were quantitative with standard curve (mean threshold cycle (C_t) vs log cDNA dilution) slopes of between -2.783 and -3.722 , translating to high efficiencies (E ; $E = 10^{(-1/\text{slope})}$; Rasmussen 2001) of 1.86 – 2.29 . Over the detection range, the linear correlation (R^2) between the mean C_t and the logarithm of the cDNA dilution was > 0.99 in each case. Primer sequences, NCBI GenBank accession numbers, PCR product sizes, PCR efficiencies and annealing temperatures are shown in Table 1.

Real-time PCR analyses of the expression of target genes

To analyse the expression of the target genes following exposure to E_2 , cDNA was synthesised from $1\text{ }\mu\text{g}$ RQ1 DNase-treated (Promega) total RNA using random hexamers (MWG-Biotech) and Moloney-murine leukaemia virus reverse transcriptase (Promega), following the manufacturer's instructions. Real-time PCR using SYBR Green chemistry was performed for target genes with the iCycler iQ Real-time Detection System (Bio-Rad) as described previously (Filby & Tyler 2005), using the appropriate annealing temperatures (Table 1). Relative quantitation via normalisation to a 'housekeeping' gene, which was measured in each sample, was performed as described previously (Filby & Tyler 2005). We have previously validated the use of fhm *ribosomal protein 18* (*rpl8*) for normalisation between control and E_2 -treated fhm (AL Filby & CR Tyler, unpublished observations) and applied this system here. Assays had a high level of precision and reproducibility with intraassay coefficient of variation (CV) of 2.42% ($n = 96$). Interassay CV values were not measured because all of the samples for each tissue type for each exposure were run on the same plate.

Data analysis

Statistical differences in relative mRNA expression between experimental groups were assessed by Student's *t*-test. Non-normally distributed data were log-transformed prior to statistical analysis. All statistical analyses were performed using SigmaStat 2.03 Software (Jandel Scientific Software Chicago, IL, USA). All experimental data are shown as the mean $\pm$ S.E.M. Differences were considered statistically significant at $P < 0.05$.

Results

The mean oestrogenic activity in the E_2 -treated tanks over the 14-day exposure period was $35.2 \pm 14.5\text{ ng/l}$ E_2 equivalent, compared with $4.9 \pm 0.94\text{ ng/l}$ E_2 equivalent in the control tanks. There were no effects of E_2 on fish growth. On day 14, wet weight of E_2 -treated fish was $2.10 \pm 0.11\text{ g}$ (males) and $1.00 \pm 0.03\text{ g}$ (females), which was not significantly different from the wet weight of control fish (males, $2.09 \pm 0.12\text{ g}$; females, $1.07 \pm 0.05\text{ g}$). On day 14, the total length of E_2 -treated fish was $58 \pm 1\text{ mm}$ (males) and $46 \pm 1\text{ mm}$ (females), which was not significantly different from the

Figure 1 Expression of key sex steroid receptors (fathead minnow (fhm) *esr1*, *esr2b*, *esr2a*, *ar*) in (A) liver, (B) gonad, (C) brain, (D) pituitary, (E) intestine and (F) gill of adult male (m) and female (f) fhm following waterborne exposure to 35 ng 17β -oestradiol (E_2)/l. The results are represented as means $\pm$ S.E.M. and expressed as fold-increase in relative mRNA expression from the control. Relative mRNA expression was determined as the ratio of target gene mRNA/*rpl8* mRNA. Each treatment group consisted of eight male and eight female fish and each fish was analysed in duplicate. Statistically significant differences in fold-changes in relative gene expression levels between control and treated fish for each sex for each tissue type are denoted by an asterisk ($P < 0.05$, Student's *t*-test).

total length of control fish (males, 58 ± 1 mm; females, 45 ± 1 mm). GSI was unaffected by the short-term E_2 treatment in males (1.2 ± 0.12 in control males; 1.20 ± 0.12 in E_2 -treated males). However, ovary growth was reduced in E_2 -treated females (15.60 ± 1.04 in control females; 9.35 ± 0.88 in E_2 -treated females; $P < 0.001$). There was a significant induction of plasma Vg in both male ($P < 0.001$) and female ($P = 0.031$) fish exposed to E_2 (from 27.16 ± 7.54 ng/ml in control males to 55.30 ± 3.84 µg/ml in E_2 -treated males; from 408.75 ± 26.92 µg/ml in control females to 617.21 ± 66.66 µg/ml in E_2 -treated females).

Expression of target genes following exposure to E_2

Expression of target genes involved in reproduction, growth, development and thyroid and interrenal function, was determined by real-time PCR and compared with expression in untreated fish. Expression of target genes were measured in six tissues with the exception of *gh* expression that was only analysed in pituitary and gonad, because it was undetectable in the other tissue types studied, and *cyp17*, *cyp19a*, *cyp19b*, *hsd11b2*, *hsd17b*, *star*, *amh*, *vasa*, *dmrt1* and *nr5a2* that were measured only in gonad.

Expression of sex-steroid receptors

Exposure to E_2 was associated with changes in expression of fhm sex-steroid receptors in all tissues examined, except brain (Fig. 1). In liver (Fig. 1A), there was a significant induction (approximately fivefold) of *esr1* in male fish exposed to E_2 ($P = 0.041$). There appeared to be a similar induction of hepatic *esr1* in female fish, but this difference was not statistically significant. Hepatic expression levels of *esr2a* and *esr2b* remained unchanged in E_2 -exposed fish. Expression of *ar* was down-regulated in E_2 -exposed fish, but this was only significant in males (50% down-regulation, $P = 0.003$).

As in the liver, in the testis (Fig. 1B), there was a significant induction (2.8-fold, $P = 0.04$) of *esr1*. In contrast, in ovary, *esr1* was down-regulated ($P = 0.002$) to 30% of the level observed in control fish. *esr2b* was also down-regulated (60%, $P = 0.03$) in the gonads of both male and female fish exposed to E_2 . Gonadal *ar* was down-regulated by E_2 , but this was only significant in female fish, where *ar* expression was 40% of that in control females ($P < 0.001$).

In the pituitary (Fig. 1D), there was a large (sevenfold) up-regulation ($P = 0.001$) in *esr1* expression in female fish but not in males. There was also an up-regulation in expression of both pituitary *esr2a* (twofold, $P = 0.04$) and *esr2b* (3.5-fold, $P = 0.017$) in E_2 -exposed females. This contrasts with that which occurred in males where there was a 60% down-regulation ($P = 0.012$) of

pituitary *esr2a* and no significant change in expression of pituitary *esr2b*.

In the intestine (Fig. 1E), the only statistically significant changes in expression of steroid hormone receptors following exposure to E_2 were for *esr2a* which was down-regulated in both males ($P = 0.017$) and females ($P = 0.025$), to 23 and 27% of their control levels respectively. In the gill (Fig. 1F), there was a down-regulation of *esr1*, but this was only significant in male fish (to 40% of its level in control fish, $P = 0.03$). Expression levels of all other branchial steroid hormone receptors remained unchanged.

Expression of genes involved in reproduction

Exposure to E_2 was associated with changes in the gonadal expression of seven of the ten genes involved in reproduction (Fig. 2). For those genes involved in gonadal steroidogenesis, *cyp17* was down-regulated in E_2 -exposed males (to 16% of the control level; $P = 0.020$), but highly (fivefold) up-regulated in E_2 -exposed females ($P = 0.049$). In contrast, *cyp19b* was between two- and threefold up-regulated in both E_2 -exposed males ($P = 0.025$) and females ($P = 0.004$), but E_2 had no effect on *cyp19a*. E_2 exposure resulted in a decrease in the expression of *hsd11b2* ($P = 0.023$) in males but had no effect in females. E_2 also decreased *hsd17b* ($P = 0.001$) expression in males, but while *hsd17* was 3.5-fold higher in E_2 -exposed females, a high degree of variation between individuals meant that this was not statistically significant. Expression of *star* in E_2 -exposed males was 30% of that in controls ($P = 0.046$), *amh* in both E_2 -exposed males ($P = 0.038$) and females ($P = 0.033$) was only 50% of that in controls, and *nr5a2* in E_2 -exposed females was only 10% of that in control females ($P = 0.005$). There were no statistically significant effects of E_2 on expression of *vasa* or *dmrt1* in either males or females.

Expression of target genes involved in growth, development and thyroid and interrenal function

Exposure to E_2 was associated with changes in expression of genes involved in growth, development and thyroid and interrenal function in all tissues examined except intestine (Fig. 3). In liver (Fig. 3A), the expression of *ghr*, *thra* and *gr* remained unchanged following 14 days exposure to E_2 . Hepatic expression of *igf1* appeared to decrease (males, to 40% of the control level; females, to 80% of the control level) following exposure to E_2 , but this decrease was only significant in males ($P < 0.05$). There appeared to be an induction (males, 1.2-fold; females, 3.2-fold) of hepatic *igf1r* following exposure to E_2 , but this was only significant in females ($P < 0.001$). Hepatic *thrb* expression was induced (4.3-fold, $P = 0.012$) by exposure to E_2 in

Figure 2 Expression of genes involved in sexual development and reproduction (fhm *cyp17*, *cyp19a*, *cyp19b*, *hsd11b*, *hsd17b*, *star*, *amh*, *vasa*, *dmrt1*, *nr5a2*) in gonad of adult male (m) and female (f) fhm following waterborne exposure to 35 ng 17 β -oestradiol (E₂)/l. The results are represented as means $\pm$ S.E.M. and expressed as fold-increase in relative mRNA expression from the control. Relative mRNA expression was determined as the ratio of target gene mRNA/*rpl8* mRNA. Each treatment group consisted of eight male and eight female fish and each fish was analysed in duplicate. Statistically significant differences in fold-changes in relative gene expression levels between control and treated fish for each sex for each tissue type are denoted by an asterisk ($P < 0.05$, Student's *t*-test).

male fish, but decreased (to 37% of the control level, $P = 0.003$) in exposed females.

In gonad (Fig. 3B), the expression of all target genes with the exception of *igf1r* was altered by exposure to E₂ in at least one sex of fish. There were up-regulations in the expression of gonadal *gh* and *thrb* in both males (*gh* 3.5-fold, $P = 0.005$; *thrb* 2.8-fold, $P = 0.009$) and females (*gh* 2.7-fold, $P = 0.003$; *thrb* 2.1-fold, $P = 0.016$) exposed to E₂. In contrast, gonadal *ghr* (to 31% of the control level, $P = 0.029$), *igf1* (to 14% of the control level, $P = 0.009$), *thra* (to 27% of the control level, $P < 0.001$) and *gr* (to 28% of the control level, $P = 0.003$) were down-regulated by E₂, but in females only.

In brain (Fig. 3C), the expression of all genes remained unaltered following exposure to E₂ for 14 days, with the exception of *igf1* which was significantly induced in both males (1.8-fold, $P = 0.011$) and females (fivefold, $P = 0.042$). In pituitary (Fig. 3D), the expression of *gh*, *igf1r* and *thrb* remained unchanged following exposure to E₂. There were, however, changes in the expression of *ghr*, *igf1*, *thra* and *gr*, but the effects of E₂ on these genes were sex specific. In males, there was suppressed pituitary expression of *ghr* (to 47% of the control level, $P = 0.024$), *igf1* (to 25% of the control level, $P = 0.015$) and *gr* (to 51% of the control level, $P = 0.016$). In females, in contrast, there was increased pituitary expression of *ghr* (4-fold, $P = 0.045$), *igf1* (3-fold, $P = 0.017$), *thra* (3.6-fold, $P = 0.03$) and *gr* (2.4-fold, $P = 0.041$).

In the gill of E₂-exposed fish (Fig. 3F), *igf1* expression was undetectable in 25% of fish (*igf1* was detectable in all of the control fish) and, in the fish in which *igf1* expression was detectable, there was a down-regulation in expression in males (to 21% of the control level, $P = 0.006$) and females (to 26% of the control level, $P = 0.029$). There were also down-regulations in the expression of *igf1r* (to 18% of the control level, $P < 0.001$) and *thra* (to 43% of the control level, $P = 0.039$) in gills of male fish exposed to E₂.

Discussion

As expected, the exposure of fhm to the environmental oestrogen E₂ caused the induction of classic biomarkers of oestrogen exposure, most notably the hepatic *esr1* gene (in males) and the female yolk protein precursor Vg (in both sexes) (Sumpter & Jobling 1995, MacKay *et al.* 1996). Moreover, in the gonad, E₂ treatment was associated with alterations in the expression of sex-steroid receptors and genes centrally involved in reproductive function in a manner consistent with that expected for exposure to an oestrogen. In male fhm, the exposure to E₂ was associated with feminisation of the expression profiles for sex-steroid receptors and steroidogenic enzymes, consistent with published data on fhm and other teleost species (e.g. Gavoroun *et al.* 2001, Halm *et al.* 2002, Baron *et al.* 2005).

For example, E₂-treated males had increased expression of *esr1*, normally higher in female fhms (Filby & Tyler 2005), decreased expression of *cyp17* and *hsd11b2*, key enzymes in the production of androgens in males (Arai & Tamaoki 1967, Conley & Bird 1997) and increased expression of *cyp19b*, the enzyme responsible for the conversion (aromatisation) of androgens to oestrogens (reviewed in Simpson *et al.* 1994). Suppression of 'male' steroidogenic enzymes by oestrogens may be due to a negative feedback of E₂ on follicle-stimulating hormone (Fsh), but data from studies on trout instead supports a direct effect of E₂ on the testis (Govoroun *et al.* 2001, Baron *et al.* 2005). In female fish, a stimulatory effect of E₂ on gonadal E₂ synthesis is implied by the increased expression of *cyp17* (which, through its role in androgen production, is also indispensable for the production of oestrogens by *cyp19*) and increased expression of *cyp19b*. This is consistent with increased gonadal E₂ production and plasma E₂ levels in female mummichog exposed to environmentally relevant levels of oestrogen (MacLatchy *et al.* 2003).

Further, reproductive effect pathways of E₂ are highlighted by down-regulation of *amh* (in males and females) and *nr5a2* (in females). Nr5a2 acts as a transcription factor to regulate many enzymes involved in steroid production (including *cyp19*), controls pituitary expression of the *fsh* gene, and is a likely regulator of *amh* (reviewed in Liu *et al.* 1997), so its modulation by E₂, therefore, has implications for both steroidogenesis and sex differentiation. Amh is best known for its role in males during early life in inhibiting the development of female primordial internal genitalia and diverting the steroidogenic pathway from oestrogens to androgens through inhibition of Fsh-stimulated *cyp19a* expression (reviewed in Josso *et al.* 1998). However, it also has roles in later life in folliculogenesis in females (Durlinger *et al.* 2002) and in negatively influencing development of the adult testis in males (Josso *et al.* 1998). As for *amh*, another testis differentiation gene, *dmrt1*, was also apparently decreased by E₂ in this study, consistent with current data showing *dmrt1* down-regulation by feminising agents (Marchand *et al.* 2000), but due to high variability the difference was not statistically significant in our study.

While the data on the reproductive axis provide valuable insight into oestrogen effect pathways in the gonad with likely reproductive consequences in the fhms, the effects seen are perhaps not especially

surprising. E₂ treatment, however, was also shown to lead to altered expression of genes involved in growth, development and thyroid and interrenal function throughout the body, indicating wider potential impacts on the physiological function of fish. Moreover, some of the effects seen on gene transcription were highly sex- and tissue-specific.

Although E₂ had no effect on fish growth after 14 days of exposure (consistent with another short-term study on the effects of the E₂-mimic EE₂ on somatic growth in fhms; Panter *et al.* 2002), changes occurred in the expression of key growth-regulating genes in the liver. In particular, hepatic *igf1* expression was down-regulated (in male fish) by E₂, consistent with findings in other teleosts discussed previously. E₂ is, in fact, a powerful regulator of pituitary GH, paradoxically increasing circulating GH whilst inhibiting somatic growth (reviewed in Holloway & Leatherland 1998). In addition, E₂ has direct effects on *igf1* and *insulin-like growth factor-binding protein (igfbp)* expression in hepatocytes (Riley *et al.* 2004). In fhms exposed to E₂, pituitary *gh* expression was unaffected but, in goldfish, E₂ increased pituitary GH levels without any changes in steady-state pituitary *gh* mRNA levels, suggesting that the actions of E₂ on GH are not at the level of transcription (Zou *et al.* 1997). Moreover, the absence of oestrogen-response elements on teleost *gh* genes (Chen *et al.* 1994, Xiong *et al.* 1994) suggests that E₂ does not increase GH through direct genomic effects. Down-regulated hepatic *ghr* expression, rather than plasma GH, has also been attributed to decreased plasma IGF-I by E₂ (McCormick *et al.* 2005). Our observations do not concur with this hypothesis, but the nature of *ghr* translation means a direct correlation between *ghr* mRNA and *ghr* protein cannot be assumed (see Calduch-Giner *et al.* 2003). Since the liver is the main source of circulating IGF-I, decreased hepatic IGF1 synthesis also has the potential for wide-reaching effects in the body via disruption of the actions of plasma IGF-I in other body tissues.

Alterations in the normal functioning of the thyroid hormone and corticosteroid systems may provide an additional pathway by which environmental oestrogens compromise the growth and development of fish. For example, both T₃ (Peng & Peter 1997, Schmid *et al.* 2003) and F (reviewed in Mommsen *et al.* 1999) are regulatory hormones for the GH/IGF system. Suppressive effects of E₂ on thyroidal activity have been shown in fish, most notably through reductions in plasma T₃,

Figure 3 Expression of fhms *gh*, *ghr*, *igf1*, *igf1r*, *thra*, *thrb*, and *gr* in (A) liver, (B) gonad, (C) brain, (D) pituitary, (E) intestine and (F) gill of adult male (m) and female (f) fhms following waterborne exposure to 35 ng 17 β -oestradiol (E₂)/l. Fhm *gh* expression was only analysed in pituitary and gonad because it was undetectable in the other tissues studied. The results are represented as means $\pm$ S.E.M. and expressed as the fold-increase in relative mRNA expression from the control. Relative mRNA expression was determined as the ratio of target gene mRNA/*rpl8* mRNA. Each treatment group consisted of eight male and eight female fish and each fish was analysed in duplicate. Statistically significant differences in fold-changes in relative gene expression between control and treated fish for each sex for each tissue type are denoted by an asterisk ($P < 0.05$, Student's *t*-test).

the active thyroid hormone (e.g. Cyr *et al.* 1988, Mercure *et al.* 2001, Qu *et al.* 2001, McCormick *et al.* 2005), although responses were highly variable between studies. In rat, E₂ also decreased serum T₃ (but not T₄) levels (probably through an inhibitory effect of E₂ on deiodinase conversion of bioinactive T₄ to bioactive T₃), and may influence the hypothalamic/pituitary set-point for the negative feedback effect of thyroid hormone on thyroid stimulating hormone secretion (Schmutzler *et al.* 2004, Seidlova-Wuttke *et al.* 2005). There is also evidence from salmonids that, through contrasting effects at multiple sites on the hypothalamus—pituitary—interrenal axis, E₂ regulates F production from the fish interrenal. *In vitro*, E₂ suppressed the ability of the rainbow trout interrenal to synthesise F (McQuillan *et al.* 2003), but *in vivo* it had a stimulatory effect elevating plasma F through an increase in plasma adrenocorticotrophic hormone (Pottinger *et al.* 1996), the pituitary hormone which is the main secretagogue for F. In this work, E₂ affected *thr* expression in liver, gonad, pituitary and gill (see later), and *gr* expression in gonad and pituitary, the expression of which is auto-regulated by their ligands (e.g. -Tata *et al.* 1993), but these effects were highly sex- and tissue-specific and, for *thrs*, different for each *thr* subtype. Measurements of plasma T₃ and F levels in fhms exposed to E₂, together with a more complete understanding of the differential roles of THR and GR subtypes in fish tissues and their ligand regulation, are therefore required to more fully evaluate the effects of environmental oestrogens on these axes in fhms.

Since reproductive roles for GH (reviewed in LeGac *et al.* 1993), IGFs (Huang *et al.* 1998, Weber & Sullivan 2005), thyroid hormones (Soyano *et al.* 1993, Tambets *et al.* 1997) and F (reviewed in Mommsen *et al.* 1999) have all been demonstrated in fish, gonadal changes in their expression further indicate multiple pathways of oestrogen effect on sexual development. Given the lack of knowledge on the function of locally produced GH and IGF-I, and of the roles of THRs and GRs in the gonad, however, it is difficult to interpret these effects. Gonadal *gh* expression was clearly up-regulated by E₂ in both males and females, which may have been a direct effect of E₂, via changes in the expression of gonadal *ess*, and/or may have been a result of increased plasma GH levels associated with E₂ exposure, since GH treatment increases gonadal *gh* mRNA (Biga *et al.* 2004). Effects of E₂ further downstream in the GH/IGF-I axis were, however, sex specific with inhibition of the IGF system (via decreased gonadal *ghr* and *igf1*) in females, but no further responses in males. In females, decreased gonadal *igf1* expression, combined with decreased hepatic *igf1* expression (and therefore plasma IGF-I levels), may be central to the E₂-inhibited ovarian growth observed as IGF-I drives oocyte maturation (Negatu *et al.* 1998). Treatment of female

seabream with E₂ has been shown to inhibit the IGF system (via a decreased ovarian *igf1* and an increased *igfbp2* expression), but the effects were related to the reproductive phase (Gioacchini *et al.* 2005). While these inhibitory effects on the IGF system were seen in fish exposed during the period prior to sexual maturity, during the reproductive period E₂-induced gonadal *igf1* expression and also expression of gonadal insulin-like growth factor-2 (*igf2*) and *igf1r*. Interestingly, the effects of GH treatment on the ovarian IGF system also varied depending on reproductive status, with GH inducing ovarian *igf1* expression in fish prior to sexual maturity, but inhibiting the ovarian IGF system in reproductively active fish by reducing *igf2* and *igf1r* mRNA and inducing *igfbp2* mRNA (Gioacchini *et al.* 2005). Sex differences in the effects of E₂ on gonadal genes may be due to the differential effects of E₂ on gonadal *esr1* expression in male and female gonad.

For the most part, the neural expression of our target genes (and steroid hormone receptors) was unchanged by exposure to E₂, while, in the pituitary, the target genes were highly responsive to E₂. The exception was for *igf1*, whose expression in the brain was up-regulated by E₂ in both males and females. The widespread localisation of *igf1* expression in the brain in mammals has argued for a general role of brain IGF-I in neuronal proliferation, growth and survival (Bondy & Lee 1993), which is supported by IGF-I and IGF-IR mRNA localisation studies in teleost brain (Perrot *et al.* 1999, Smith *et al.* 2005). E₂ may have a role in regulating these actions. Up-regulation of the *igf1* gene in brain and pituitary may also reflect feedback effects of IGF-I on the hypothalamic pathways which regulate pituitary GH secretion, or directly on GH-producing cells in the pituitary, since IGF-I inhibits pituitary GH transcription and release (Blaise *et al.* 1995, see review in Fruchtman *et al.* 2000). Brain *igf1* up-regulation by E₂ in female fish may additionally be connected with the potentiating effect of IGF-I on pituitary gonadotrophin responses to gonadotrophin-releasing hormone involving paracrine pathways (Weil *et al.* 1999). It is possible that the general lack of effects of E₂ on the brain observed in fhms in this study was due to the fact that we measured whole brain expression of target genes, and this may have masked any effects of E₂ in specific neural regions.

In addition to assessing the effects of E₂ on reproductive tissues (liver, gonad, brain and pituitary), we also looked for possible wider effects of E₂ on other body tissues (gill and intestine) which have received very little, if any, attention in environmental oestrogen research. The fact that exposure to E₂ also affects the expression of key endocrine genes in these tissues supports our proposal that a far broader approach to understanding the effects of environmental oestrogens in the body is required. In the intestine, none of our

target genes was affected by E₂, but there was a clear down-regulation in expression of intestinal *esr2a* (formerly known as oestrogen receptor $\beta 2/\gamma$). We have previously shown that, in fhm, *esr2a* is most highly expressed in intestine (Filby & Tyler 2005), implying important roles in activating E₂-target genes in this tissue. Further work on other oestrogen-regulated genes is required to determine the implications of E₂ exposure on intestinal function.

Suppression of gill *igf1* expression (and potentially plasma IGF-I) due to E₂ treatment highlights the potential for a reduced osmoregulatory ability in fhm exposed to environmental oestrogens. Although the osmoregulatory physiology of fhm is unknown, in other teleost species *igf1* mRNA has been identified in osmoregulatory organs (gill and kidney), and osmoregulatory challenges increase its expression (Sakamoto & Hirano 1993). Furthermore, there is evidence that IGF-I (both plasma and local) is a hormonal mediator of the hypo-osmoregulatory actions of GH (McCormick *et al.* 1991, Madsen & Bern 1993, Sakamoto & Hirano 1993). Previous studies have reported that E₂, and other environmental oestrogens, impact on osmoregulation both in salmonid (Madsen *et al.* 1997, 2004, Stoffel *et al.* 2000, Arsenault *et al.* 2004, McCormick *et al.* 2005) and non-salmonid (Vijayan *et al.* 2001) species, and suppressed plasma levels of IGF-I were identified as the likely mechanism (McCormick *et al.* 2005). Our work supports this theory and provides new evidence for a direct effect of E₂ on local IGF-I production in gill and this is likely to be an additional mechanism of E₂-related osmoregulatory effects. A possible pathway for this may be via the down-regulation of *esr1* observed in the gill. In many teleost species, thyroid hormones also support the hypo-osmoregulatory actions of GH (McCormick 2001), and the down-regulation observed in gill *thra* of fhm exposed to E₂ may also be a pathway for E₂-related osmoregulatory effects, especially since negative effects of E₂ on the thyroid axis have already been implicated in perturbations in osmoregulation (McCormick *et al.* 2005).

To conclude, our data show that exposure to E₂ has multiple and wide-ranging effects on the expression of genes involved in the regulation of a broad range of physiological functions, and not just those central to reproduction. These findings are consistent with the evidence for a high degree of interplay between signalling pathways involved in the control of growth, development, thyroid and interrenal function, and reproduction. The gene responses to E₂ were highly complex (frequently both sex- and tissue-specific), highlighting the importance of a more complete understanding of the roles, and modes of action, of these proteins in each tissue if we are to fully appreciate the health implications of environmental oestrogen exposure for fish. Our data, together with recent

reports in which two other important environmental oestrogens, NP and bisphenol A, have been shown to affect the GH-IGF and/or thyroid hormone systems in fish (Yadetic & Male 2002, Suzuki & Hattori 2003, Arsenault *et al.* 2004), raises further concerns about the presence and potential for detrimental health effects of sex steroids and their mimics/antagonists in aquatic environment.

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References

- Ankley GT & Villeneuve DL 2006 The fathead minnow in aquatic toxicology: past, present and future in press. *Aquatic Toxicology* **78** 91–102.
- Arai R & Tamaoki B 1967 Steroid biosynthesis of 11 β -hydroxytestosterone and 11-ketotestosterone in the testis of rainbow trout, *Salmo gairdneri*. *Canadian Journal of Biochemistry* **45** 1191–1195.
- 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.
- Baron D, Fostier A, Breton B & Guiguen Y 2005 Androgen and estrogen treatments alter steady state messenger RNA (mRNA) levels of testicular steroidogenic enzymes in the rainbow trout. *Molecular Reproduction and Development* **71** 471–479.
- Baronti C, Curini C, D'Ascenzo G, Di Corcia A, Gentili A & Samperi R 2000 Monitoring natural and synthetic estrogens at activated sludge sewage treatment plants and in receiving river water. *Environmental Science and Technology* **24** 5059–5066.
- 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.
- Bjornsson BT 1997 The biology of salmon growth hormone: from daylight to dominance. *Fish Physiology and Biochemistry* **17** 9–24.
- Blaise O, Weil C & LeBail PY 1995 Role of IGF-I in the control of GH secretion in rainbow trout (*Oncorhynchus mykiss*). *Growth Regulation* **5** 142–150.
- Bondy CA & Lee WH 1993 Patterns of insulin-like growth factor and IGF receptor gene expression in the brain. *Annals of New York Academy of Science* **692** 33–43.
- Brent GA 1996 Thyroid hormones (T₄, T₃). In *Endocrinology: Basic and Clinical Principles*, pp 32–67. Ed S Melmed. Totowa: Humana Press.

- Butler AA & Le Roith D 2001 Control of growth by the somatotrophic axis: growth hormone and the insulin-like growth factors have related and independent roles. *Annual Reviews in Physiology* **63** 141–164.
- Calduch-Giner JA, Mingarro M, Vega-Rubin de Celis S, Boujard D & Perez-Sanchez J 2003 Molecular cloning and characterization of gilthead sea bream (*Sparus aurata*) growth hormone receptor (GHR). Assessment of alternative splicing. *Comparative Biochemistry and Physiology Part B* **136** 1–13.
- Cavari B, Funkenstein B, Chen TT, Gonzalez-Villasenor LI & Scharlt M 1993 Effect of growth hormone on the growth rate of gilthead seabream (*Sparus aurata*), and use of different constructs for the production of transgenic fish. *Aquaculture* **111** 189–197.
- Chen TT, Marsh A, Shambloft M, Chan KM, Tang YL, Cheng CM & Yang BY 1994 Structure and evolution of fish growth hormone and insulin-like growth factor genes. In *Fish Physiology XIII – Volume XIII – Bioenergetics and Growth*, pp 179–209. Eds WS Hoar, DJ Randall & JR Brett. San Diego/New York: Academic Press.
- Conley AJ & Bird LM 1997 The role of cytochrome P450 17 α -hydroxylase and 3 β -hydroxysteroid dehydrogenase in the integration of gonadal and adrenal steroidogenesis via the $\Delta 5$ and $\Delta 4$ pathways of steroidogenesis in mammals. *Biology of Reproduction* **56** 789–799.
- Cyr DG, MacLachy DL & Eales JG 1988 The influence of short-term 17 beta-estradiol treatment on plasma T3 levels and in vitro hepatic T4 5'-monooxidase activity in immature rainbow trout, *Salmo gairdneri*. *General and Comparative Endocrinology* **69** 431–438.
- Desbrow C, Routledge EJ, Brightly GC, Sumpter JP & Waldock M 1998 Identification of estrogenic chemicals in STW effluent, 1. Chemical fractionation and in vitro biological screening. *Environmental Science and Technology* **32** 1549–1558.
- Dreze V, Monod G, Cravedi JP, Biagianti-Risbourg S & Le Gac F 2000 Effects of 4-nonylphenol on sex differentiation and puberty in mosquito fish (*Gambusia holbrooki*). *Ecotoxicology* **9** 93–103.
- Durlinger AL, Visser JA & Themmen AP 2002 Regulation of ovarian function: the role of anti-Mullerian hormone. *Reproduction* **124** 601–609.
- Filby AL & Tyler CR 2005 Molecular characterization of estrogen receptors 1, 2a and 2b and their tissue and ontogenic expression profiles in fathead minnow (*Pimephales promelas*). *Biology of Reproduction* **73** 648–662.
- Fruchtman S, Jackson L & Borski R 2000 Insulin-like growth factor I disparately regulates prolactin and growth hormone synthesis and secretion: studies using the teleost pituitary model. *Endocrinology* **141** 2886–2894.
- Gioacchini G, Cardinali M, Maradonna F, Funkenstein B, Mosconi G & Carnevali O 2005 Hormonal control of the IGF system in the sea bream ovary. *Annals of the New York Academy of Science* **1040** 320–322.
- Govoroun M, McMeel OM, Mecherouki H, Smith TJ & Guiguen Y 2001 17Beta-estradiol decreases steroidogenic enzyme messenger ribonucleic acid levels in the rainbow trout testis. *Endocrinology* **142** 1841–1848.
- Halm S, Pounds N, Maddix S, Rand-Weaver M, Sumpter JP, Hutchinson TH & Tyler CR 2002 Exposure to exogenous 17beta-estradiol disrupts p450aromB mRNA expression in the brain and gonad of adult fathead minnows (*Pimephales promelas*). *Aquatic Toxicology* **60** 285–299.
- Harvey S, Johnson CDM, Sharma P, Sanders EJ & Hull KL 1998 Growth hormone: a paracrine growth factor in embryonic development. *Comparative Biochemistry and Physiology Part C* **119** 305–315.
- Holloway AC & Leatherland JF 1998 Neuroendocrine regulation of growth hormone secretion in teleost fishes with emphasis on the involvement of gonadal steroids. *Reviews in Fish Biology and Fisheries* **8** 409–429.
- Hou YY, Suzuki Y & Aida K 1999 Effects of steroid hormones on immunoglobulin M (IgM) in rainbow trout, *Oncorhynchus mykiss*. *Fish Physiology and Biochemistry* **20** 155–162.
- Huang YS, Rousseau K, Le Belle N, Vidal B, Burzawa-Gerard E, Marchelidon J & Dufor S 1998 Insulin-like growth factor I stimulates gonadotropin production from eel pituitary cells: a possible metabolic signal for induction of puberty. *Journal of Endocrinology* **159** 43–52.
- Jobling S, Nolan M, Tyler CR, Brightly G & Sumpter JP 1998 Widespread sexual disruption in wild fish. *Environmental Science and Technology* **32** 2498–2506.
- Jobling S, Coey S, Whitmore JG, Kime DE, Van Look KJ, McAllister BG, Beresford N, Henshaw AC, Brightly G, Tyler CR & Sumpter JP 2002a Wild intersex roach (*Rutilus rutilus*) have reduced fertility. *Biology of Reproduction* **67** 515–524.
- Jobling S, Beresford N, Nolan M, Rodgers-Gray T, Brightly GC, Sumpter JP & Tyler CR 2002b 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** 3–29.
- Josso N, Racine C, di Clemente N, Rey R & Xavier F 1998 The role of anti Mullerian hormone in gonadal development. *Molecular and Cellular Endocrinology* **145** 3–7.
- Kopchick JJ & Andry JM 2000 Growth hormone (GH), GH receptor, and signal transduction. *Molecular Genetics and Metabolism* **71** 293–314.
- Law WY, Chen WH, Song YL, Dufour S & Chang CF 2001 Differential in vitro suppressive effects of steroids on leukocyte phagocytosis in two teleosts, tilapia and common carp. *General and Comparative Endocrinology* **121** 163–172.
- Lazar MA 2003 Thyroid hormone action: a binding contract. *Journal of Clinical Investigation* **112** 497–499.
- LeGac F, Blaise O, Fostier A, Leblail PY, Loir M, Mourot B & Weil C 1993 Growth hormone (GH) and reproduction – a review. *Fish Physiology and Biochemistry* **11** 219–232.
- Liu D, LeDrean Y, Ekker M, Xiong F & Hew CL 1997 Teleost FTZ-F1 homolog and its splicing variant determine the expression of the salmon gonadotropin II beta subunit gene. *Molecular Endocrinology* **11** 877–890.
- MacKay ME, Raelson J & Lazier CB 1996 Up-regulation of estrogen receptor mRNA and estrogen receptor activity by estradiol in liver of rainbow trout and other teleostean fish. *Comparative Biochemistry and Physiology Part C* **115** 201–209.
- MacLachy DL, Courtenay SC, Rice CD & Van der Kraak GJ 2003 Development of a short-term reproductive endocrine bioassay using steroid hormone and vitellogenin end points in the estuarine mummichog (*Fundulus heteroclitus*). *Environmental Toxicology and Chemistry* **22** 996–1008.
- 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, Mathiesen AB & Korsgaard B 1997 Effects of 17 β -oestradiol and 4-nonylphenol on smoltification and vitellogenesis in Atlantic salmon (*Salmo salar*). *Fish Physiology and Biochemistry* **17** 303–312.
- Madsen SS, Skoybolling S, Nielsen C & Korsgaard B 2004 17-Beta estradiol and 4-nonylphenol delay smolt development and downstream migration in Atlantic salmon. *Salmo salar*. *Aquatic Toxicology* **68** 109–120.
- Magliulo L, Schreiberman MP, Cepriano J & Ling J 2002 Endocrine disruption caused by two common pollutants at 'acceptable' concentrations. *Neurotoxicology and Teratology* **24** 71–79.
- Marchand O, Govoroun M, D'Cotta H, McMeel O, Lareyre JJ, Bernot A, Laudet V & Guiguen Y 2000 DMRT1 expression during gonadal differentiation and spermatogenesis in the rainbow trout, *Oncorhynchus mykiss*. *Biochimica et Biophysica Acta* **1493** 180–187.
- McCormick SD 2001 Endocrine control of osmoregulation in teleost fish. *American Zoologist* **41** 781–794.
- McCormick SD, Sakamoto T, Hasegawa S & Hirnao T 1991 Osmoregulatory actions of insulin-like growth factor I in rainbow trout (*Oncorhynchus mykiss*). *Journal of Endocrinology* **130** 87–92.

- McCormick SD, O'Dea MF, Moeckel AM, Lerner DT & Bjornsson 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.
- McQuillan HJ, Lokman PM & Young G 2003 Effects of sex steroids, sex and sexual maturity on cortisol production: an *in vitro* comparison of Chinook salmon and rainbow trout interrenals. *General and Comparative Endocrinology* **133** 154–163.
- Mercure F, Holloway AC, Tocher DR, Sheridan MA, Van der Kraak G & Leatherland JF 2001 Influence of plasma lipid changes in response to 17 β -oestradiol stimulation on plasma growth hormone, somatostatin, and thyroid hormone levels in immature rainbow trout. *Journal of Fish Biology* **59** 605–615.
- Mommsen TP, Vijayan MM & Moon TW 1999 Cortisol in teleosts: dynamics, mechanisms of action, and metabolic regulation. *Reviews in Fish Biology and Fisheries* **9** 211–268.
- 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.
- Panter GH, Hutchinson TH, Lange R, Lye CM, Sumpter JP, Zerulla M & Tyler CR 2002 Utility of a juvenile fathead minnow screening assay for detecting (anti-)estrogenic substances. *Environmental Toxicology and Chemistry* **21** 319–326.
- Peng C & Peter RE 1997 Neuroendocrine regulation of growth hormone secretion and growth in fish. *Zoological Studies* **36** 79–89.
- Perez-Sanchez J 2000 The involvement of growth hormone in growth regulation, energy homeostasis and immune function in the gilthead sea bream (*Sparus aurata*): a short review. *Fish Physiology and Biochemistry* **22** 135–144.
- 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.
- Pottinger TG, Carrick TR, Hughes SE & Balm PHM 1996 Testosterone, 11-ketotestosterone, and estradiol-17 β modify baseline and stress-induced interrenal and corticotropic activity in trout. *General and Comparative Endocrinology* **104** 284–295.
- Power DM, Llewellyn L, Faustino M, Nowell MA, Bjornsson BJ, Einarsdottir IE, Canario AVM & Sweeney GE 2001 Thyroid hormones in growth and development of fish. *Comparative Biochemistry and Physiology Part C* **130** 447–459.
- Qu X, Nagae M, Adachi S & Yamauchi K 2001 Effect of estradiol-17 β on pituitary-thyroidal axis of Japanese eel (*Anguilla japonica*). *Acta Oceanologica Sinica* **20** 585–596.
- Rasmussen R 2001 Quantification on the LightCycler. In *Rapid Cycle Real-Time PCR, Methods and Applications*, pp 21–34. Eds S Meuer, C Wittwer & K Nakagawara. Heidelberg: Springer Press.
- 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.
- 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 Part C* **138** 177–186.
- Rodgers-Gray TP, Jobling S, Kelly C, Morris S, Brighty G, Waldock MJ, Sumpter JP & Tyler CR 2001 Exposure of juvenile roach (*Rutilus rutilus*) to treated sewage effluent induces dose-dependent and persistent disruption in gonadal duct development. *Environmental Science and Technology* **35** 462–470.
- Rotchell JM & Ostrander GK 2003 Molecular markers of endocrine disruption in aquatic organisms. *Journal of Toxicology and Environmental Health Part B* **6** 453–495.
- Routledge EJ & Sumpter JP 1996 Estrogenic activity of surfactants and some of their degradation products assessed using a recombinant yeast screen. *Environmental Toxicology and Chemistry* **15** 241–248.
- 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, Lutz I, Kloas W & Reinecke M 2003 Thyroid hormone stimulates hepatic IGF-I mRNA expression in a bony fish, the tilapia *Oreochromis mossambicus*, *in vitro* and *in vivo*. *General and Comparative Endocrinology* **130** 129–134.
- Schmutzler C, Hamann I, Hofmann PJ, Kovacs G, Stemmler L, Mentrup B, Schomburg L, Ambrügger P, Gruters A, Seidlöva-Wuttke D, Jarry H, Wuttke W & Kohrle J 2004 Endocrine active compounds affect thyrotropin and thyroid hormone levels in serum as well as endpoints of thyroid hormone action in liver, heart and kidney. *Toxicology* **205** 95–102.
- Seidlöva-Wuttke D, Jarry H, Christoffe J, Rimoldi G & Wuttke W 2005 Effects of bisphenol-A (BPA), dibutylphthalate (DBP), benzophenone-2 (BP2), procymidone (Proc), and linurone (Lin) on fat tissue, a variety of hormones and metabolic parameters: A 3 months comparison with effects of estradiol (E2) in ovariectomized (ovx) rats. *Toxicology* **213** 13–24.
- Simpson ER, Mahendroo MS, Means GD, Kilgore MW, Hinshelwood MM, Graham-Lorence S, Amarneh B, Ito Y, Fisher CR & Michael MD 1994 Aromatase cytochrome P450, the enzyme responsible for estrogen biosynthesis. *Endocrine Reviews* **15** 342–355.
- Smith A, Chan SJ & Gutierrez J 2005 Autoradiographic and immunohistochemical localization of insulin-like growth factor-I receptor binding sites in brain of the brown trout, *Salmo trutta*. *General and Comparative Endocrinology* **141** 203–213.
- Sonnenschein C & Soto AM 1998 An updated review of environmental estrogen and androgen mimics and antagonists. *Journal of Steroid Biochemistry and Molecular Biology* **65** 143–150.
- Soyano K, Saito T, Nagae M & Yamauchi K 1993 Effects of thyroid hormone on gonadotropin-induced steroid production in medaka, *Oryzias latipes*, ovarian follicles. *Fish Physiology and Biochemistry* **11** 265–272.
- Stoffel MH, Wahli T, Friess AE & Burkhardt-Holm P 2000 Exposure of rainbow trout (*Oncorhynchus mykiss*) to nonylphenol is associated with an increased chloride cell fractional surface area. *Schweizer Archiv für Tierheilkunde* **142** 263–267.
- Sumpter JP & Jobling S 1995 Vitellogenesis as a biomarker for estrogenic contamination of the aquatic environment. *Environmental Health Perspectives* **103** 173–178.
- Suzuki N & Hattori A 2003 Bisphenol A suppressed osteoclastic and osteoblastic activities in the cultured scales of goldfish. *Life Sciences* **73** 2237–2247.
- Tambets M, Molsa H & Saat T 1997 Effects of triiodothyronine on *in vitro* maturation of vendace (*Coregonus albula*) oocytes under unfavourable influences. *Annales Zoologici Fennici* **34** 213–216.
- Tata JR, Baker BS, Machuca I, Rabelo EM & Yamauchi K 1993 Autoinduction of nuclear receptor genes and its significance. *Journal of Steroid Biochemistry and Molecular Biology* **46** 105–119.
- Ternes TA, Stumpf M, Mueller J, Haberer K, Wilken RD & Servos M 1999 Behaviour and occurrence of estrogens in municipal sewage treatment plants. I. Investigations in Germany, Canada and Brazil. *Science of the Total Environment* **225** 81–90.
- Tsai MJ & O'Malley BW 1994 Molecular mechanisms of action of steroid/thyroid receptor superfamily members. *Annual Reviews in Biochemistry* **63** 451–486.
- Tyler CR, Jobling S & Sumpter JP 1998 Endocrine disruption in wildlife: a critical review of the evidence. *Critical Reviews in Toxicology* **28** 319–361.
- Tyler CR, van Aerle R, Hutchinson TH, Maddix S & Trip H 1999 An *in vivo* testing system for endocrine disruptors in fish early life stages using induction of vitellogenin. *Environmental Toxicology and Chemistry* **18** 337–347.

- Van den Belt K, Verheyen R & Witters H 2003 Effects of 17 α -ethinylestradiol in a partial life-cycle test with zebrafish (*Danio rerio*): effects on growth, gonads and female reproductive success. *The Science of the Total Environment* **309** 127–137.
- Versnoren BJ & Janssen CR 2004 Xenoestrogenic effects of ethinylestradiol in zebrafish (*Danio rerio*). *Environmental Toxicology* **19** 198–206.
- Vijayan MM, Takemura A & Mommsen TP 2001 Estradiol impairs hypoosmoregulatory capacity in the euryhaline tilapia, *Oreochromis mossambicus*. *American Journal of Physiology* **281** R1161–R1168.
- Wang R & Belosevic M 1994 Estradiol increases susceptibility of goldfish to *Trypanosoma danilewskyi*. *Developmental and Comparative Immunology* **18** 377–387.
- Weber GM & Sullivan CV 2005 Insulin-like growth factor-I induces oocyte maturational competence but not meiotic resumption in white bass (*Morone chrysops*) follicles *in vitro*: evidence for rapid evolution of insulin-like growth factor action. *Biology of Reproduction* **72** 1177–1186.
- Weil C, Carre F, Blaise O, Breton B & Le Bail RY 1999 Differential effect of insulin-like growth factor I on *in vitro* gonadotropin (I and II) and growth hormone secretions in rainbow trout (*Oncorhynchus mykiss*) at different stages of the reproductive cycle. *Endocrinology* **140** 2054–2062.
- Wendelaar Bonga SE 1997 The stress response in fish. *Physiological Reviews* **77** 591–625.
- Xiong F, Suzuki K & Hew CL 1994 Control of teleost gonadotropin gene expression. In *Fish Physiology XIII – Volume XIII – Bioenergetics and Growth*, pp 135–158. Eds WS Hoar, DJ Randall & JR Brett. San Diego/New York: Academic Press.
- Yadete F & Male R 2002 Effects of 4-nonylphenol on gene expression of pituitary hormones in juvenile Atlantic salmon (*Salmo salar*). *Aquatic Toxicology* **58** 113–129.
- Zou JJ, Trudeau VL, Cui Z, Brechin J, Mackenzie K, Zhu Z, Houlihan DF & Peter RE 1997 Estradiol stimulates growth hormone production in female goldfish. *General and Comparative Endocrinology* **106** 102–112.

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