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 17β-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. 17α-ethinylestradiol (EE2; Personen & 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. 17β-oestradiol (E2) and NP (Madsen et al. 1997, Vijayan et al. 2001, Arsenault 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 (igf1), insulin-like growth factor-I receptor (igf1r), thyroid hormone receptor-\(\alpha\) (thra), thyroid hormone receptor-\(\beta\) (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 ionic-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 (THR)s mediate the effects of the thyroid hormones, thyroxine (T\(_4\)) and 3,3',5-triiodo-L-thyronine (T\(_3\)) 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 THR 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 \(\alpha\)), oestrogen receptor 2a (esr2a; formerly oestrogen receptor \(\beta\) 2 or \(\gamma\)) and oestrogen receptor 2b (esr2b; formerly oestrogen receptor \(\beta\)) 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-\(\beta\)-dehydrogenase 2 (hsd11b2) and hydroxysteroid 17-\(\beta\)-dehydrogenase (hsd17b) and the others with established roles in sex differentiation and reproduction were anti-Mullerian hormone (amh), vasa homologue (vasa), doublesex- and mab-3 related transcription factor 1 (dmt1l) and nuclear receptor subfamily-5 group A member 2 (nr5a2; formerly fushi tarazu factor 1 (ftz/f1) 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

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<th>Target mRNA</th>
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<th>Sense primer (5′–3′)</th>
<th>Antisense primer (5′–3′)</th>
<th>Product size (bp)</th>
<th>Annealing temperature (°C)</th>
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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 ± 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 number of fish were maintained in dilution water as controls.

To confirm the oestrogenic activity (E2 equivalent) in 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) \((GSI = \text{gonad weight/body weight}) \times 100\). 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 \(-80^\circ\text{C}\) 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 \((A_{260}\text{ nm}; \text{GeneQuant; Amersham})\) 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 esr1 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 \((E; E = 10^{(-1/slope)}; Rasmussen 2001)\) 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 primer. 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 \(\mu\)g RQ1 DNase-treated (Promega) total RNA using random hexamers (MWG-Biotech) and Moloney-murine leukemia 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 \(35.2 \pm 14.5\) ng/1 \(E_2\) equivalent, compared with \(4.9 \pm 0.94\) ng/1 \(E_2\) 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 \(2.10 \pm 0.11\) g (males) and \(1.00 \pm 0.03\) g (females), which was not significantly different from the weight of control fish (males, \(2.09 \pm 0.12\) g; females, \(1.07 \pm 0.05\) g). On day 14, the total length of \(E_2\)-treated fish was \(58 \pm 1\) mm (males) and \(46 \pm 1\) mm (females), which was not significantly different from the...
total length of control fish (males, 58±1 mm; females, 45±1 mm). GSI was unaffected by the short-term E2 treatment in males (1·2±0·12 in control males; 1·20±0·12 in E2-treated males). However, ovary growth was reduced in E2-treated females (15·60±1·04 in control females; 9·35±0·88 in E2-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 E2 (from 27·16±7·54 ng/ml in control males to 55·30±3·84 µg/ml in E2-treated males; from 408·75±26·92 µg/ml in control females to 617·21±66·66 µg/ml in E2-treated females).

Expression of target genes following exposure to E2

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

Expression to E2 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 E2 (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 E2-exposed fish. Expression of ar was down-regulated in E2-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 E2. Gonadal ar was down-regulated by E2, 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 E2-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 E2 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

Expression to E2 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 E2-exposed males (to 16% of the control level; P=0·020), but highly (fivefold) up-regulated in E2-exposed females (P=0·049). In contrast, cyp19a was between two- and threefold up-regulated in both E2-exposed males (P=0·025) and females (P=0·004), but E2 had no effect on cyp19a. E2 exposure resulted in a decrease in the expression of hsd11b2 (P=0·023) in males but had no effect in females. E2 also decreased hsd17b (P=0·001) expression in males, but while hsd17 was 3·5-fold higher in E2-exposed females, a high degree of variation between individuals meant that this was not statistically significant. Expression of star in E2-exposed males was 30% of that in controls (P=0·046), amh in both E2-exposed males (P=0·038) and females (P=0·033) was only 50% of that in controls, and nr5a2 in E2-exposed females was only 10% of that in control females (P=0·005). There were no statistically significant effects of E2 on expression of vasa or dmrt1 in either males or females.

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

Expression to E2 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 gh, thrb and gr remained unchanged following 14 days exposure to E2. Hepatic expression of igfl appeared to decrease (males, to 40% of the control level; females, to 80% of the control level) following exposure to E2, 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 igfl following exposure to E2, but this was only significant in females (P<0·001). Hepatic thrb expression was induced (4·3-fold, P=0·012) by exposure to E2 in
exposed to E2. In contrast, gonadal results are represented as means (females (control and treated fish for each sex for each tissue type are denoted by an asterisk (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 asterix (*).

Expression was determined as the ratio of target gene mRNA/0 level, P

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 gh, igf1r, thrb and gr, but the effects of E2 on these genes were sex specific. In males, there was suppressed pituitary expression of gr (to 47% of the control level, P=0·024), igf1r (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 gr (4-fold, P=0·045), igf1r (3-fold, P=0·017), thrb (3·6-fold, P=0·03) and gr (2·4-fold, P=0·041).

In the gill of E2-exposed fish (Fig. 3F), igf1r expression was undetectable in 25% of fish (igf1r was detectable in all of the control fish) and, in the fish in which igf1r 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 thrb (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. Goweroun et al. 2001, Halm et al. 2002, Baron et al. 2005).
For example, E2-treated males had increased expression of cyp17, normally higher in female fhm (Filby & Tyler 2005), decreased expression of cyp17 and hsd11b2, key enzymes in the production of androgens in males (Aräi & 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 (Gvorou 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 nrs2a (in females). Nrs2a 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 fhm, 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 fhm; 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 GHR, 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 fhm 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 fhm gh, ghr, igf1, igfr1, 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) fhm following waterborne exposure to 35 ng 17β-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 presented as means ± 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 asterix (*P < 0.05, Student’s t-test).
the active thyroid hormone (e.g. Cyr et al. 1988, Merculze 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 bioactive 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 (Schmutzer 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 expressions were highly sex- and tissue-specific and, for ths, different for each thr subtype. Measurements of plasma T3 and F levels in fhm 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 fhm.

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 esr, 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 igflr. 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 igflr 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 igfl1, 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 fish 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
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target genes was affected by E₂, but there was a clear down-regulation in expression of intestinal esr2a (formerly known as oestrogen receptor β2/γ). 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 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 thr is 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 (Yadetie & 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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