Oestrogen-induced androgen insufficiency results in a reduction of proliferation and differentiation of spermatogonia in the zebrafish testis

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Abstract

Androgens can induce complete spermatogenesis in immature or prepubertal teleost fish. However, many aspects of the role of androgens in adult teleost spermatogenesis have remained elusive. Since oestrogens inhibit androgen synthesis, we used an oestrogen-induced androgen depletion model to identify androgen-dependent stages during adult zebrafish spermatogenesis. Exposure to 10 nM 17β-oestradiol (E2) in vivo at least halved the mass of differentiating germ cells (from type B spermatogonia to spermatids), while type A spermatogonia accumulated. Studies on the cellular dynamics revealed that a reduction of spermatogonial proliferation together with an inhibition of their differentiation to type B spermatogonia were the basis for the oestrogen-mediated disturbance of spermatogenesis. The capacity of the zebrafish testis to produce 11-ketotestosterone as well as the expression of steroidogenesis-related genes was markedly decreased after in vivo oestrogen exposure. Moreover, the androgen-release response to recombinant zebrafish Lh was lost after oestrogen exposure. We conclude that oestrogen exposure caused a state of androgen insufficiency in adult male zebrafish. Since the downregulation of the steroidogenic system as well as the disturbance of spermatogenesis in testicular explants exposed to E2 ex vivo was much less severe than after in vivo exposure, the main inhibitory effect appears to be exerted via feedback inhibition of gonadotropin release. This experimental set-up helped to identify spermatogonial proliferation and their differentiation as androgen targets in adult zebrafish spermatogenesis. *Journal of Endocrinology* (2009) 202, 287–297

Introduction

Oestrogen signalling is involved in many processes in male reproduction, and is essential to achieve normal fertility, as indicated in mammals in studies conducted with oestrogen receptor (ER) knockout mice (Eddy et al. 1996, Hess et al. 2000, Akingbemi et al. 2003, Gould et al. 2007). Furthermore, oestrogenic feedback on the hypothalamus and pituitary controls the synthesis and release of gonadotropins in mammals, and hence the testicular synthesis of androgens (Jong et al. 1975, Lindzey et al. 1998, Turner et al. 2000). Leydig cells express ER (Zhou et al. 2002), and direct oestrogenic inhibition of steroidogenesis has been reported as well (Bartke et al. 1977).

Three types of ER exist in fish, namely ERα, ERβ1 and ERβ2 (Hawkins et al. 2000, Menuet et al. 2002, Choi & Habibi 2003, Halm et al. 2004, Filby & Tyler 2005); the types are designated as, (gene/protein) esr1/Esr1, esr2b/Esr2b and esr2a/Esr2a respectively, following the Official Zebrafish Nomenclature Guidelines (http://zfin.org). All types of ER are expressed in testis (Hawkins et al. 2000, Menuet et al. 2002, Choi & Habibi 2003, Halm et al. 2004, Filby & Tyler 2005). Studies describing their cellular localization in testis indicate a heterogeneous pattern of mRNA and protein expression, for example, Esr1 protein has been found in the interstitial tissue of rainbow trout (*Omorhynchus mykiss*) testis (Bouma & Nagler 2001), esr1 and esr2 mRNA expressions were found in Japanese common goby (*Acanthogobius flavimanus*) and Japanese eel (*Anguilla japonica*) Sertoli cells (Miuara et al. 1999, Ito et al. 2007), while Esr1 and Esr2 proteins were produced by meiotic and post-meiotic germ cells in channel catfish (*Ictalurus punctatus*) testis (Wu et al. 2001). Functionally, oestrogens stimulate spermatogonial stem cell renewal in immature Japanese eel
testis probably via Sertoli cells (Miura et al. 1999, 2003). In amphibians, which have a cystic organization of spermatogenesis similar to fish, oestrogens promote proliferation of spermatogonia (Minucci et al. 1997, Cobellis et al. 1999, Chieffi et al. 2000). Previous studies have reported detrimental effects of oestrogenic compounds on fertility at all levels of the brain–pituitary–gonad axis in teleost fish (Tsai et al. 2005, Filby et al. 2006, Zhang et al. 2008), including impairment of spermatogenesis (Van der Ven et al. 2003, 2007, Van den Belt et al. 2004, Pawlowski et al. 2004), and inhibition of androgen synthesis, either directly on Leydig cells (Loomis & Thomas 2000, Govoroun et al. 2001, Baron et al. 2005) or via feedback on hypothalamus and pituitary to control synthesis and release of gonadotropins (Dickey & Swanson 1998, Kobayashi et al. 2001, Huggard-Nelson et al. 2002, Banerjee & Khan 2008). However, it is not known exactly which step(s) in the developmental sequence constituting spermatogenesis is/are affected by oestrogens in fish. This may be partially related to the fact that quantitative morphometry has not yet been applied to the evaluation of oestrogenic treatment effects on fish testis.

Our present knowledge on the role of androgens in fish spermatogenesis is mainly based on studies conducted in prepubertal individuals. In immature Japanese eel testis containing type A and a few type B spermatogonia only, androgens can induce rapid proliferation of spermatogonia and their terminal differentiation into spermatooza under tissue culture conditions (Miura et al. 1991). Androgen treatment of juvenile male African catfish (Clarias gariepinus) induced precocious testis growth, spermatogonial proliferation and entry into meiosis (Cavaco et al. 1998), and the rise of androgen plasma levels in pubertal Chinook salmon (Oncorhynchus tshawytscha) coincided with the start of rapid spermatogonial proliferation (Campbell et al. 2003). However, scarce information is available on the role of androgens in adult spermatogenesis in fish. In mammals, it is well known that the first (pubertal) wave of spermatogenesis differs from adult spermatogenesis in both regulation and timing; for example, androgen requirements differ between pubertal start and adult maintenance of spermatogenesis (Handelsman et al. 1999).

An oestrogen-induced decrease of testosterone levels has given valuable information on androgen-dependent stages in rodent spermatogenesis, revealing a slower conversion of round to elongated spermatids (O’Donnell et al. 1994). A similar approach was used in the current report to investigate the role of androgens in adult fish spermatogenesis. We studied androgen release and expression of steroidogenesis-related genes in the testis of adult zebrafish (Danio rerio) after exposure to 17β-oestradiol (E2) in vivo, and in zebrafish testicular tissue exposed to E2 ex vivo. Moreover, we determined the changes in absolute weight of the different germ cell stages and we quantified germ cell proliferation and apoptosis.

Material and Methods

Fish stocks

Unless otherwise stated, adult (>90 dpf) male Tübingen AB strain zebrafish were used for experimental purposes in the current study. Animal culture, performed using standard conditions for this species (Westerfield 2000), handling and experimentation were consistent with the Dutch national regulations, and were approved by the Life Science Faculties Committee for Animal Care and Use in Utrecht (The Netherlands).

In vivo exposure to E2

Male zebrafish were exposed to either 0·3 nM E2 (Sigma–Aldrich), 10 nM E2, or control conditions (same volume of deionized water only; see below) for either 6 or 21 days during in vivo exposure experiments. The dose of 0·3 nM was chosen since it is close to the Kd-values of the zebrafish Esr proteins (Menuet et al. 2002), while the dose of 10 nM was selected based on its reported capacity to disturb adult zebrafish spermatogenesis (Van der Ven et al. 2003). Since 0·3 nM E2 did not elicit any deleterious effect on spermatogenesis after 21 days of exposure (see Suppl. Figure S1, see Supplementary data in the online version of the Journal of Endocrinology at http://joe.endocrinology-journals.org/content/vol202/issue2/), we decided to continue our studies with the 10 nM dose only. Sample sizes for the different experiments or types of analyses varied from 4 to 13 fish per treatment (see respective figure legends). A 10 μM E2 stock solution was prepared in deionized water by extensive stirring at 40°C, which was then further diluted to 10 nM in aquarium water. Exposure was performed in glass tanks containing 18 liters water maintained at 27.1 ± 0.2°C under constant aeration. Fish were transferred to the experimental tanks 24 h before initiating each exposure period. The exposure environment was refreshed everyday by moving the fish to a second set of identically prepared tanks. After exposure, fish were euthanized in ice water and total body weight was measured. Both testes of each animal were excised, weighed and the gonadosomatic index (GSI; i.e. the ratio between testis weight and body weight) was calculated. Testis samples were used for acute ex vivo steroid release bioassays or processed for histological evaluation, morphometrical quantification, immunohistochemistry, Western blot or gene expression analysis (for detailed experimental procedures, see Supplementary Information, see Supplementary data in the online version of the Journal of Endocrinology at http://joe.endocrinology-journals.org/content/vol202/issue2/).

Ex vivo exposure to E2

To determine whether oestrogens are able to exert direct effects on zebrafish spermatogenesis and testicular androgen production, an ex vivo organ culture system for zebrafish


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testis was used (Leal et al. 2009a). In this system, the influence of interindividual variation is reduced by incubating the two testes of each fish in parallel, such that one testis served as control for the contra-lateral one. Basal culture medium was supplemented with 50 ng/ml recombinant gilthead seabream (Sparus aurata) insulin-like growth factor 1 (Igf1; Prospec-Tany Technogene, Rehovot, Israel), which was added to support spermatogenesis in an androgen-independent manner (Leal et al. 2006). This setting was chosen to study the direct effects of E2 on the testicular steroidogenic system while avoiding dramatic changes in the cellular composition of the testis explant in the absence of compounds supporting spermatogenesis (Leal et al. 2009a). The E2 stock solution used in these experiments was prepared as described above. Incubation took place in a humidified air atmosphere at 25°C, and medium was refreshed every 3–4 days. When androgen release by testicular tissue was to be measured, the culture environment was refreshed with the same frequency by transferring the nitrocellulose membranes supporting the tissue explants to fresh culture wells (and both medium and agarose blocks were stored together at −25°C). After incubation (exposure periods are specified for each experiment performed; see below), testis tissue explants were collected, weighed and processed for morphological evaluation or gene expression analysis (for detailed experimental procedures, see Supplemental Information), while both medium and agarose blocks were stored together at −25°C for quantification of 11-ketotestosterone (11-KT) levels by RIA (Schulz et al. 1994). For that purpose, incubation media and agarose cylinders were transferred to a glass tube for homogenization (Ultra-turrax T25; Janke & Kunkel Ika-Labortechnik, Staufen, Germany) and steroid extraction with diethyl ether (four times with 5 ml solvent each). Recovery studies using tritiated androgens showed that only the steroids in the incubation medium, but not those associated with the agar cylinder, were effectively extracted, resulting in a relatively low recovery of 47 ± 1% (n=8). The results were corrected accordingly, and are expressed as pg 11-KT released per mg of testis tissue incubated.

In a first series of experiments, testicular tissue explants were exposed to either 10 nM E2 or control conditions (basal medium only) for either 30 h (studies on steroid release only; n=9 explants per treatment), 6 days (studies on steroid release, morphology and gene expression; n=6–9 explants per treatment) or 12 days (studies on morphology only; n=6–9 explants per treatment). The 30 h incubation period was selected based on our recent studies showing that after 48 h under basal conditions, the activity of the steroidogenic system had decreased to <10% of the starting level while at 30 h the responsiveness to an acute stimulation was intact (Leal et al. 2009a). The 6 and 12 days incubation periods were selected in order to evaluate possible medium- to long-term effects of E2 on zebrafish spermatogenesis.

In a second series of experiments, testicular tissue explants were exposed to 0.5 μM of the adenylate cyclase activator forskolin (prepared in DMSO; Sigma–Aldrich) alone or in the presence of 10 nM E2 for 6 days (studies on steroid release and gene expression; n=8 explants per treatment). This experimental setting was chosen according to recent studies demonstrating the capacity of forskolin to partially prevent down-regulation of the zebrafish testicular steroidogenic system under culture conditions (Leal et al. 2009a), which allowed examination of possible, direct effects of E2 on the activity of the steroidogenic system in a medium-term time frame.

Acute ex vivo steroid release bioassays

The steroidogenic capacity of testicular tissue after 6 days of in vivo oestrogen exposure was evaluated using an acute ex vivo steroid release bioassay previously described for African catfish (Schulz et al. 1994) and adapted for zebrafish testis explants. For this experiment, adult outbred zebrafish were used. The testes of either 10 nM E2-treated or control fish (n=10–12 fish per group) were collected after a 6 day in vivo oestrogen exposure period and the two testes were immersed in D-PBS+. One testis was incubated in basal medium (the same as used for ex vivo exposure to E2, but excluding retinoic acid and Igf1), serving as control for the contra-lateral testis, which was challenged with either 0.5 μM forskolin or a 1/10 dilution of single-chain, recombinant zebrafish Lh stock solution (preparation of recombinant zebrafish Lh is described in Supplemental Information). Testis tissue was immersed in 200 μl medium in 96-well flat-bottom plates (Corning Inc., New York, NY, USA) in a humidified air atmosphere at 25°C for 18 h. After incubation, testis explants were weighed and discarded, and the incubation medium was heated at 80°C for 1 h, centrifuged for 30 min (16 000 g; 4°C) and the supernatant was stored at −25°C until direct quantification (i.e. without extraction) of 11-KT levels by RIA as reported (Schulz et al. 1994). The results were expressed as pg 11-KT released per mg of testis tissue incubated.

Statistical analysis

For the in vivo experiments, differences between treatments were analysed by the Student t-test with two-tailed P value (in some cases, data were log transformed to achieve an equal variance), except for the gene expression analysis after 21 days in vivo E2 exposure in which, due to differences in the number of individuals between groups, the Mann–Whitney non-parametric test was used. Comparison of groups in the acute 11-KT release assay for both in vivo and ex vivo experiments was done with one-way ANOVA followed by the Student–Newman–Keuls test. For the ex vivo gene expression experiments, differences between treatments were tested for statistical significance using the paired t-test (when necessary,
data were log transformed to achieve an equal variance). A significance level (P) of 0.05 was applied in all the statistical analyses, which were performed using the Prism4 software package (GraphPad software, San Diego, CA, USA).

Results

Effects of in vivo exposure to E2 on zebrafish testicular physiology

In vivo exposure to oestrogen, either for 6 or 21 days, did not induce significant changes in body weight or GSI (data not shown), while the decrease in total testis weight (from 6.21 ± 0.54 to 4.68 ± 0.32 mg) observed after 21 days reached statistical significance.

Qualitative morphological (Fig. 1A–C) and quantitative morphometric analysis (Fig. 1D and E) of testis tissue samples collected after both 6 and 21 days in vivo exposure to E2 revealed a significant inhibitory effect on zebrafish spermatogenesis. On day 6 the main changes in the morphometric analysis were recorded among type B spermatogonia, primary spermatocytes, and secondary spermatocytes, which decreased to 54–60% of control levels (Fig. 1D). After 21 days of oestrogen exposure, the mass of type B spermatogonia, primary and secondary spermatocytes and spermatids decreased further and significantly (e.g. down to ~19% of control levels in the case of spermatids), whereas the mass of type A spermatogonia increased to ~220% of control levels (Fig. 1E). Expression levels of synaptosomal complex protein 3 like (syn3p3) mRNA, a zebrafish homologue to a marker for primary spermatocytes (De la Fuente et al. 2007), correlated well with the reported decrease in the presence of primary spermatocytes in zebrafish testis after in vivo E2 exposure (Fig. 1F). No differences were observed in the mass of spermatooza or other cell types between control groups at both sampling times (Fig. 1D and E). A very low incidence of apoptotic germ cells (strongly condensed, darkly stained nuclei in toluidine-blue-stained sections), and no difference between control and E2-treated groups at both sampling times were observed by morphometrical analysis (Fig. 1D and E).

Searching for a mechanistic basis for the reduced mass of type B spermatogonia and spermatocytes, immunocytochemical detection of the G2-phase cell cycle marker phosphorylated histone H3 (PH3) was used to assess proliferative activity after 6 days of E2 exposure. As shown in Suppl. Figure S2 (see Supplementary data in the online version of the Journal of Endocrinology at http://joe.endocrinology-journals.org/content/vol202/issue2/), proliferating (i.e. PH3-positive) single spermatogonia, as well as cysts containing four or more proliferating germ cells (spermatogonia or spermatocytes), were found in greater numbers on sections from control than from E2-treated testes. Quantification of this observation showed that treatment with E2 reduced the number of proliferating single germ cells as well as cysts of spermatogonia/spermatocytes significantly (to ~53 and ~37% of control levels respectively; Fig. 1G).

We then examined the assumption that in vivo oestrogen exposure inhibited the testicular steroidogenic system. We quantified mRNA and protein levels of cytochrome P450 17α-hydroxylase/17-20-lyase (cyp17a1/Cyp17a1), a steroidogenic enzyme required for androgen production, the mRNA amounts of steroidogenic acute regulatory protein (star), the protein controlling the rate-limiting step in steroidogenesis (i.e. the transfer of cholesterol from the outer to the inner mitochondrial membrane), as well as the mRNA amounts of both gonadotropin receptors (Lh receptor, lhr; and Fsh receptor, fshr). Moreover, we challenged testis explants from animals exposed to oestrogen for 6 days in vivo with stimulators of steroid release. After 6 days of E2 exposure, lhr mRNA levels increased significantly (to 155% of control levels), while no significant changes were observed in fshr mRNA levels. The levels of both star and cyp17a1 mRNA diminished to 50 and 14% of control levels respectively (Fig. 2A). Both western blotting of testis tissue homogenates (inset Fig. 2B and C) and immunohistochemical detection in tissue sections (Fig. 2B and C) demonstrated a clear down-regulation of the levels of Cyp17a1 protein, which is exclusively expressed in Leydig cells. Twenty-one days of oestrogen exposure resulted in strong decreases in the mRNA levels of lhr (to ~1%), fshr (to ~10%) and star (to ~6%) (Fig. 2A). Levels of cyp17a1 mRNA could not be measured at 21 days due to technical problems, although a similarly strong downregulation should be expected. After 6 days in vivo exposure to E2, acute ex vivo androgen release was significantly downregulated between ~2.6 and ~9.9 fold in all the conditions assayed (i.e. basal, forskolin- and Lh-stimulated); oestrogen-exposed testis explants maintained a limited steroidogenic response to forskolin (P<0.05), but not to recombinant zebrafish Lh (Fig. 2D).

Effects of ex vivo exposure to E2 on zebrafish testicular physiology

Morphological evaluation of testicular tissue explants exposed to E2 for 6 and 12 days ex vivo revealed no clear disruptive effect on spermatogenesis (data not shown), in contrast to the results obtained in the testes of in vivo E2-exposed fish. With regards to gene expression, however, lhr mRNA levels showed an increase (to 184% of control levels; Fig. 3A) after 6 days of ex vivo exposure to E2, as observed after 6 days of in vivo exposure, while mRNA amounts of fshr decreased (to 65% of control levels; Fig. 3A). Also the mRNA levels of star and cyp17a1 were decreased to almost half of control levels (Fig. 3A) and responded similarly as in the in vivo experiments, though not as prominently for cyp17a1. However, it should be noted that the steroidogenic system undergoes a spontaneous downregulation under ex vivo culture conditions, reflected by a decrease in cyp17a1 and star mRNA levels (Leal et al. 2009a). Hence, it seems more appropriate to state that E2 further enhanced the spontaneous downregulation of star and cyp17a1 mRNA levels observed ex vivo (Fig. 3A, 202, 287–297).
However, the continuous presence of 0.5 μM forskolin (Fig. 3A, right panel) reduced the additional down-regulatory effect of E2 on star mRNA levels, while it abolished such an effect on cyp17a1 mRNA levels.

After 30 h of tissue culture ex vivo and during the first three days in the continuous presence of forskolin, there was no change in the cumulative testicular release of 11-KT between groups incubated in the absence or presence of E2 (Fig. 3B, left panel).
Despite the protective effect of forskolin on E2-induced down-regulation of star and cyp17a1 mRNA levels, *ex vivo* exposure to E2 for 6 days was reflected in a significantly lower amount of 11-KT released from culture days 3 to 6 (Fig. 3B, right panel).

**Discussion**

Our data demonstrates oestrogen-induced disruption of the spermatogenic process in adult zebrafish. Previous studies reported similar disruptive effects of oestrogens on the testis of sexually mature zebrafish (Van der Ven et al. 2003, 2007, Van den Belt et al. 2004) and other teleost species (e.g. Kinnberg & Toft 2003, Pawlowski et al. 2004, Chaves-Pozo et al. 2007), but the precise stage of inhibition of spermatogenesis was not determined. Therefore, the mechanistic basis of oestrogen-induced disruption of germ cell development in adult male teleost testis has remained unclear.

Different from earlier stereological analyses on testes of E2-exposed zebrafish (Van der Ven et al. 2003, Christianson-Heiska et al. 2004) or guppy (*Poecilia reticulata*; Nielsen & Baatrup 2006), a distinction was made in the spermatogonial compartment between early (type A) and late (type B) spermatogonia in the current study (see Leal et al. 2009b, for a comprehensive description of the spermatogonial generations in zebrafish). Implementation of this distinction, quantification of the absolute masses of the different germ cell types, and analysis of germ cell proliferation and apoptosis enabled us to pinpoint the stages of spermatogenesis affected by the oestrogen treatment. Thus, E2 exposure resulted in two main effects: i) the reduction, but not the abolishment, of proliferation of type A and type B spermatogonia as demonstrated by quantifying PH3-positive germ cells, and ii) the blockade of differentiation of type A into type B spermatogonia, as demonstrated by the accumulation (i.e. increased mass) of type A spermatogonia. Jointly, these effects resulted in a depletion of developmental stages beyond type A spermatogonia, as demonstrated by the significant

Figure 2 Steroidogenic capacity of zebrafish testis after exposure to 10 nM E2 *in vivo*. (A) Relative mRNA levels of fshr, lhr, star and cyp17a1 after either 6 or 21 days of E2 exposure (n=6–13 fish per group). Data are shown as relative values of respective transcript amounts measured in control fish. Cyp17a1 immunodetection on transversal zebrafish testis cryosections obtained from (B) control fish and (C) E2-exposed fish. The inset between B and C shows Cyp17a1 protein amounts detected by Western blot in testicular lysates obtained from both treatment groups. (D) *Ex vivo* acute androgen release in the absence (Ba, basal) or presence of either 0.5 mM forskolin (Fo) or recombinant zebrafish Lh by zebrafish testis tissue obtained from fish exposed to E2 *in vivo* for 6 days (n=5–6 fish per treatment). Androgen release is expressed as amount of 11-KT produced per unit weight of testis tissue incubated. Bars marked with * are significantly different from their respective controls (P<0.05). For each compound in panel (D), different letters denote statistical differences (P<0.05). Scale bars in (B) and (C) = 100 µm.
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Figure 3  Steroidogenic capacity of zebrafish testis after exposure to 10 nM E₂ ex vivo. (A) Relative mRNA levels of fshr, lhr, star and cyp17a1 after 6 days of E₂ ex vivo exposure either in the absence (Ba, basal) or the presence of 0.5 μM forskolin (Fo) (n=8 explants per group). Data are shown as relative values of respective transcript controls, while different letters denote statistical differences in panel bars marked with * are significantly different from their respective treatment. These seemingly contradictory observations, which are reported for the first time in any fish species but also below for a discussion on oestrogen feedback effects on the brain/pituitary level. Moreover, in eel testis ex vivo, the stimulatory effect decreased with increasing (0.01–1 ng/ml) oestrogen doses (Miura et al. 1999), the concentration used in the present study being approximately three times higher than the maximum concentration used for eel testis.

our morphometric analysis could not confirm this observation for zebrafish.
Based on the data available from mammalian models (Bartke et al. 1977, Akingbemi et al. 2003, Gould et al. 2007), we hypothesized that oestrogen treatment inhibits androgen synthesis, thereby indirectly affecting spermatogenesis. Indeed, exposure of adult zebrafish to E₂ in vivo suppressed the testicular steroidogenic capacity, as reflected by down-regulation of key steroidogenesis-related genes (cyp17a1 and star) and the lower acute testicular 11-KT release (under basal conditions). Moreover, the E₂ treatment suppressed the androgen release response to an acute gonadotropic stimulation with biologically active recombinant zebrafish Lh even though lhr mRNA levels were elevated and fshr mRNA showed no significant changes after oestrogen treatment. These seemingly contradictory observations, which are reported for the first time in any fish species but have been previously reported in oestrogen-exposed mice (Fukuzawa et al. 2004), can be reconciled assuming that decrease in the masses of type B spermatogonia, spermatocytes and spermatids. Germ cell depletion was increasingly prominent with their progressive differentiation (reductions to 53, 31, 31 and 19% of control values for type B spermatogonia, primary, secondary spermatocytes, and spermatids respectively). This may indicate that oestrogen-mediated inhibition of spermatogenesis is based on (at least) two components, the reduced availability of type B spermatogonia, as demonstrated in the present study, and inhibition of germ cell entry into or progress through meiosis and spermiogenesis. To clarify the second component, however, other experiments will be required. In any case, the absence of a clear inhibitory effect of oestrogen on meiosis/spermiogenesis ex vivo renders it unlikely that oestrogen affects these stages directly, while the presence of meiotic and spermiogenic stages shows that meiosis and spermiogenesis are compatible with elevated oestrogen concentrations in zebrafish.
oestrogen exposure interfered with the signalling pathways activated by Lh, but downstream of its receptor, in steroidogenic cells. Since forskolin, an activator of the adenylate cyclase, also induced a much weaker response in tissue from E2-treated fish than from control fish, we speculate that oestrogens may target one or more components of the GαS – adenylate cyclase – protein kinase A (PKA) pathways. Therefore, we conclude that oestrogenic treatment induced a state of androgen-insufficiency in zebrafish by disturbing the testicular steroidogenic system at different levels, as previously suggested for other teleost fish species (Sohn et al. 1998, Loomis & Thomas 2000, Andersen et al. 2006, Filby et al. 2006, Chaves-Pozo et al. 2007, Meier et al. 2007, Arukwe 2008, Blum et al. 2008, Jukosky et al. 2008, Zhang et al. 2008).

In search of a mechanism explaining the oestrogen-induced disruption at the testicular level, we thereafter studied possible direct effects of E2 on testis physiology using a recently developed tissue culture system for zebrafish testis explants (Leal et al. 2009a). After 6 days ex vivo oestrogen treatment, zebrafish testis showed significantly reduced star and cyp17a1 mRNA levels as compared with control levels suggesting a direct inhibition of E2 on testicular steroidogenic system. However, it should be mentioned that the expression of steroidogenic enzymes, and thus the steroidogenic capacity, shows a spontaneous downregulation under prolonged ex vivo culture conditions (Baron et al. 2005, Leal et al. 2009a). Hence we further studied the ex vivo effects of E2 exposure on the steroidogenic potential of zebrafish testis i) within the first 30 h of culture, when the zebrafish testicular steroidogenic system is still responsive to short-term acute stimulations, and thus can be considered relatively intact (Leal et al. 2009a), and ii) during 6 days of culture in the presence of forskolin, which partially prevents the aforementioned downregulation of the system under culture conditions, probably by upregulating the expression of several steroidogenesis-relevant genes through the cAMP/PKA pathway (Schwartz & Roy 2000, Manna et al. 2003, Leal et al. 2009a). Under these culture conditions, oestrogen exposure was only able to decrease androgen release from day 3 to 6 possibly in association with the slight, but significant, downregulation of star mRNA transcription observed at the end of the incubation period. These results suggest that the direct effects exerted by oestrogens in the zebrafish testicular steroidogenic system may be relatively minor under the conditions tested in the current study, since E2 exposure could neither decrease androgen output when the system was still intact at 30 h of culture nor at 3 days of treatment. Previous studies in other fish and amphibian species, however, have reported significant inhibitory effects of oestrogenic compounds on both testicular androgen release and steroidogenic enzymes expression in tissue culture exhibiting, therefore, direct suppressive actions of oestrogens on testicular androgenesis (Pierantoni et al. 1986, Loomis & Thomas 2000, Baron et al. 2005). Notably, such effects were only evident at very high oestrogen concentrations (>367 nM), making these results difficult to interpret in context with physiological oestrogen concentrations or to compare with our observations in the present study using 10 nM E2. In Atlantic croaker (Micropogonias undulates), direct suppression of testicular androgen production by very high oestrogen concentrations (>36.7 μM) was shown to be rapid (within 5 min) and was transduced by a membrane-associated ER (Loomis & Thomas 2000). Recently, a membrane-associated ER, homologous to mammalian GPER, has been cloned from zebrafish, and is expressed in testis (Liu et al. 2009). The E2 concentration used in the current study (10 nM) could have been sufficient to activate the zebrafish Gper. However, the direct effects of oestrogen in the zebrafish testes were comparatively weak and possibly mediated via a nuclear ER, considering the prolonged time required for them to become evident, while the binding of E2 to zebrafish Gper, although of high affinity (Kd=2-8 nM), was characterized by both rapid association and dissociation (Liu et al. 2009).

In contrast to the results obtained in the testes of E2-exposed zebrafish in vivo, morphological and/or morphometrical evaluation of testis tissue exposed to E2 ex vivo revealed no clear disruption of spermatogenesis which, together with the minor direct effect exerted by oestrogen on the steroidogenic system, suggests that the E2-induced inhibitory effects on zebrafish testis functions mainly involve feedback mechanisms on the hypothalamus–pituitary system. Sex steroid, either androgens or oestrogens, feedback on gonadotropin synthesis and release is well established in fish (Dickey & Swanson 1998, Kobayashi et al. 2001, Hug-Nelson et al. 2002, Banerjee & Khan 2008). In zebrafish, information on oestrogen effects on gonadotropin levels (either subunits mRNA expression levels or plasma levels) is scarce. Recently, Lin & Ge (2009) described direct oestrogenic stimulation of fshb and lhb transcript levels in primary zebrafish pituitary cell culture, similar to previous findings in the closely related goldfish (Carassius auratus; Hug-Nelson et al. 2002). While no information is available on in vivo oestrogen feedback effects on gonadotropin subunit mRNA levels in zebrafish, respective data (as well as gonadotropin plasma amounts) are available in goldfish. For this species, oestrogen treatment abolished the upregulation of both pituitary fshb mRNA levels and Lh plasma levels observed in ovarietomized individuals (Kobayashi et al. 2001). Furthermore, treatment of sexually mature goldfish with an ER antagonist increased circulating Lh levels (Billard & Peter 1977). Finally, pre-treatment of late recrudescence goldfish of mixed sex with E2 suppressed GnRH-mediated elevation of fshb and lhb expression both in vivo and in vitro (Hug-Nelson et al. 2002). The latter has been reported recently in medaka (Oryzias latipes; Zhang et al. 2008) as well. These data indicate that E2 inhibited the GnRH/GnRH-receptor signalling system, and that these effects apparently overrule the direct, stimulatory effects of oestrogens on gonadotropin subunit expression. Also signalling pathways towards GnRH neurons, like γ-aminobutyric acid
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Declaration of interest

The authors declare there is no conflict of interest that could have prejudiced the impartiality of the research reported.

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