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 (E_2) *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 E_2 *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.

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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 (*Oncorhynchus 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 (Miura *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

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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 spermatozoa 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 (E_2) *in vivo*, and in zebrafish testicular tissue exposed to E_2 *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 E_2*

Male zebrafish were exposed to either 0·3 nM E_2 (Sigma-Aldrich), 10 nM E_2, 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 K_d-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 E_2 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 E_2 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 Supplemental 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 E_2*

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*. 2009*a*). 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 E_2 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*. 2009*a*). The E_2 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-Labortechik, 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 E_2 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*. 2009*a*). The 6 and 12 days incubation periods were selected in order to evaluate possible medium- to long-term effects of E_2 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 E_2 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*. 2009*a*), which allowed examination of possible, direct effects of E_2 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 E_2-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 E_2, 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* E_2 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,

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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 E_2 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 $6{\cdot}21 \pm 0{\cdot}54$ to $4{\cdot}68 \pm 0{\cdot}32$ 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 E_2 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 $\sim$19% of control levels in the case of spermatids), whereas the mass of type A spermatogonia increased to $\sim$220% of control levels (Fig. 1E). Expression levels of synaptonemal complex protein 3 like (*sycp3l*) 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* E_2 exposure (Fig. 1F). No differences were observed in the mass of spermatozoa or other cell types between treatment 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 E_2-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 G_2-phase cell cycle marker phosphorylated histone H3 (PH3) was used to assess proliferative activity after 6 days of E_2 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 E_2-treated testes. Quantification of this observation showed that treatment with E_2 reduced the number of proliferating single germ cells as well as cysts of spermatogonia/spermatocytes significantly (to $\sim$53 and $\sim$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 E_2 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 $\sim$1%), *fshr* (to $\sim$10%) and *star* (to $\sim$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 E_2, acute *ex vivo* androgen release was significantly down-regulated between $\sim$2·6 and $\sim$9·9 fold in all the conditions assayed (i.e. basal, forskolin- and Lh-stimulated); oestrogen-exposed testis explants maintained a limited steroidogenic response to forskolin ($P < 0{\cdot}05$), but not to recombinant zebrafish Lh (Fig. 2D).

Effects of ex vivo *exposure to E_2 on zebrafish testicular physiology*

Morphological evaluation of testicular tissue explants exposed to E_2 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* E_2-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 E_2, 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*. 2009*a*). Hence, it seems more appropriate to state that E_2 further enhanced the spontaneous downregulation of *star* and *cyp17a1* mRNA levels observed *ex vivo* (Fig. 3A,

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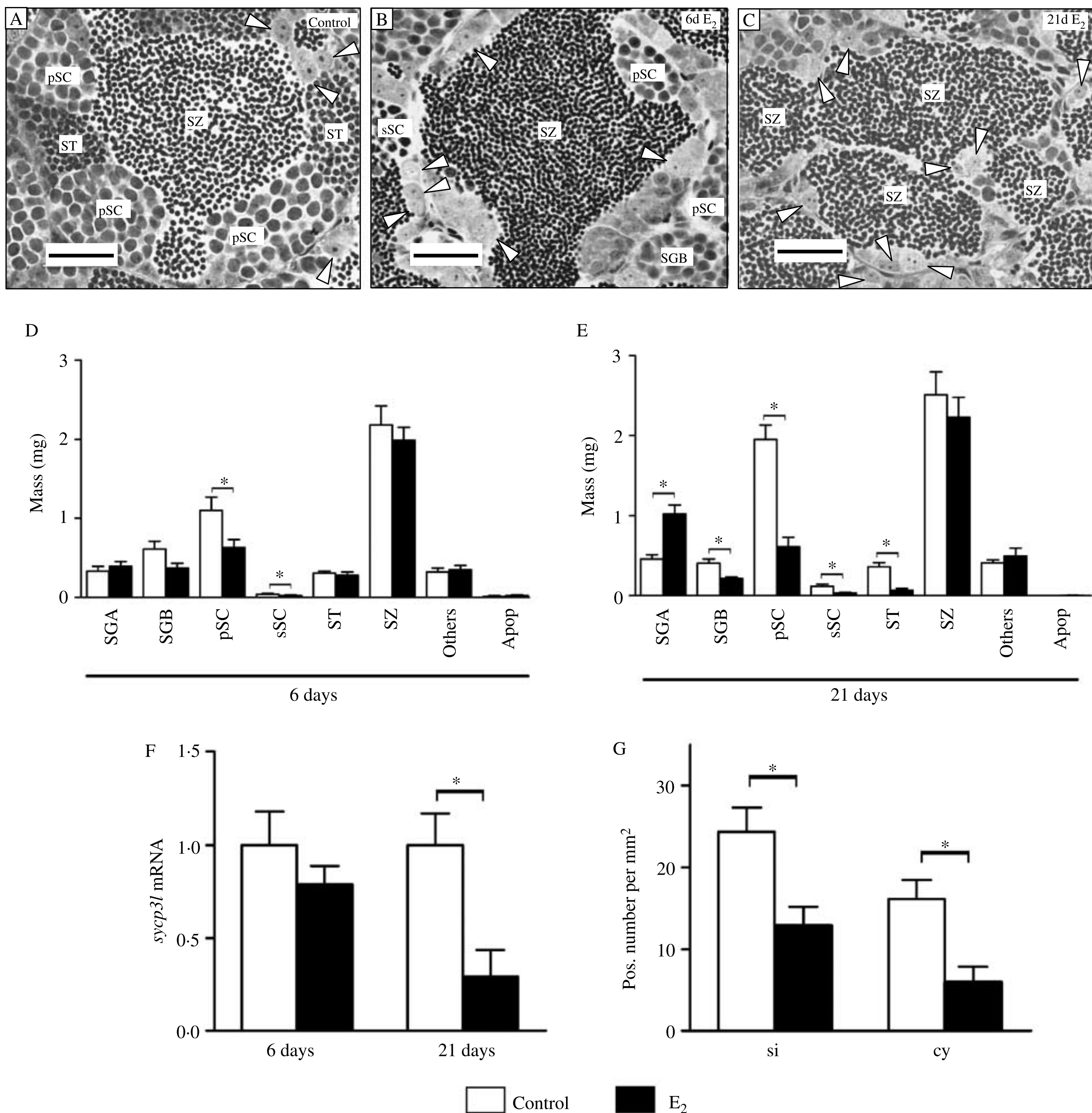

Figure 1 Zebrafish testicular physiology after exposure to 10 nM E_2 *in vivo*. Zebrafish testis sections from control fish (A), or from E_2-treated fish 6 days (B) or 21 days (C) after the start of exposure. Morphometric analysis (n=5–7 fish per group) of zebrafish testis sections, presenting data as mass (mg) of testicular cell types, 6 days (D) or 21 days (E) after the start of exposure to 10 nM E_2. (F) Relative amounts of *sycp3l* mRNA, normalized to 18S rRNA, in control and E_2-exposed males (n=6–13 fish per treatment). (G) Germ cell proliferation after 6 days of E_2 exposure (n=4 fish per treatment). Phosphohistone H3 (PH3)-positive number of single nuclei of type A spermatogonia (si) and cysts (cy) containing ≥4 PH3-positive nuclei of spermatogonia/spermatocytes per mm^2 were counted. Arrowheads and SGA, type A spermatogonia; SGB, type B spermatogonia; pSC, primary spermatocytes; sSC, secondary spermatocytes; ST, spermatids; SZ, spermatozoa; apop, apoptotic cells. Bars marked with * are significantly different from their respective controls ($P<0·05$). Scale bars in (A), (B) and (C) =25 µm.

left panel). However, the continuous presence of 0·5 µM forskolin (Fig. 3A, right panel) reduced the additional down-regulatory effect of E_2 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 E_2 (Fig. 3B,

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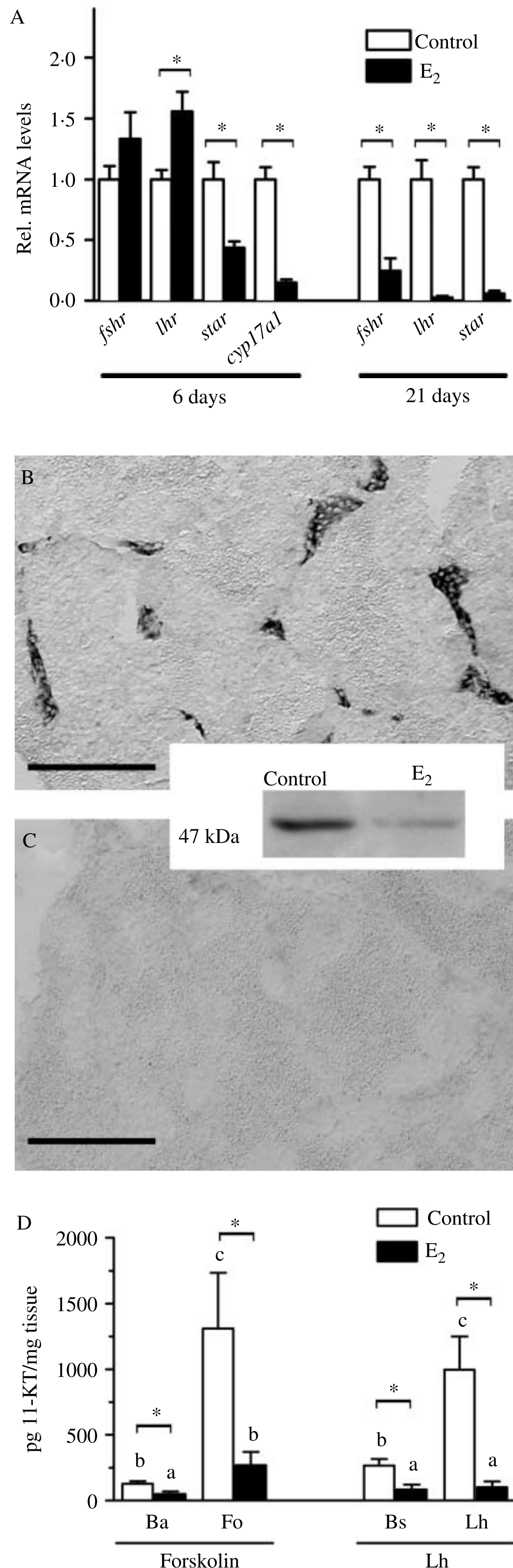

left and middle panel). Despite the protective effect of forskolin on E_2-induced down-regulation of *star* and *cyp17a1* mRNA levels, *ex vivo* exposure to E_2 for 6 days was reflected in a significantly lower amount of 11-KT released from culture days 3 to 6 (Fig. 3B, right panel).

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 E_2-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*. 2009*b*, 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, E_2 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 E_2 *in vivo*. (A) Relative mRNA levels of *fshr, lhr, star* and *cyp17a1* after either 6 or 21 days of E_2 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) E_2-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 μM forskolin (Fo) or recombinant zebrafish Lh by zebrafish testis tissue obtained from fish exposed to E_2 *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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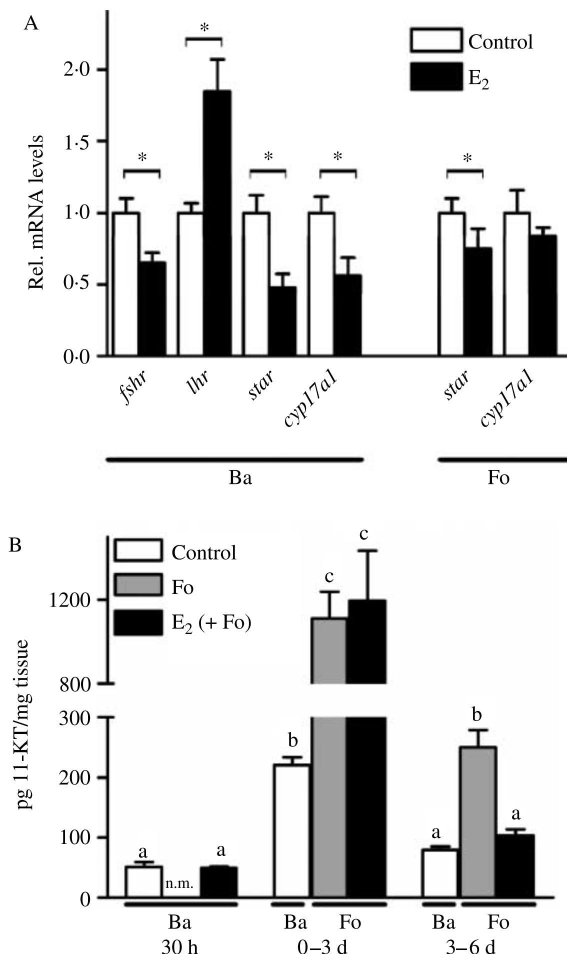

Figure 3 Steroidogenic capacity of zebrafish testis after exposure to 10 nM E_2 *ex vivo*. (A) Relative mRNA levels of *fshr, lhr, star* and *cyp17a1* after 6 days of E_2 *ex vivo* exposure either in the absence (Ba, basal) or the presence of 0·5 μM forskolin (Fo) ($n=8$ explants per group). Data are shown as relative values of respective transcript amounts measured in control fish. (B) Cumulative androgen release after *ex vivo* E_2 exposure for 30 h under basal conditions and for 3 or 6 days (release was measured from 0 to 3 and from 3 to 6 days) in the absence or presence of 0·5 μM forskolin ($n=8$ explants per group). Androgen release is expressed as amount of 11-KT produced per unit weight of testis tissue incubated. In panel (A), bars marked with * are significantly different from their respective controls, while different letters denote statistical differences in panel (B) ($P<0·05$). n.m., not measured.

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.

Our results on oestrogen-mediated reduction of germ cell proliferation are in accordance with previous observations in fish and amphibian species (Song & Gutzeit 2003, Tsai *et al.* 2005, Chaves-Pozo *et al.* 2007), although these studies did not determine the changes in germ cell masses (i.e. provided data on relative changes in the germ cell compartment), and did not always identify the specific stage of spermatogenesis affected. Interestingly, Miura *et al.* (2003) reported an E_2-triggered molecular mechanism, which involves up-regulation of platelet-derived endothelial cell growth factor, and subsequently stimulates self-renewal divisions of spermatogonial stem cells, the first generation of type A spermatogonia, in immature Japanese eel testis *ex vivo*. Although we found an increased mass of type A spermatogonia, this was associated with a reduced proliferation activity of these cells. In conjunction with no change in the low rate of apoptosis, this can be explained by a block of differentiation, and ensuing accumulation of type A spermatogonia. Therefore, juvenile eels may respond differently to oestrogens than adult zebrafish (see 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.

Different from previous reports, where an increased incidence of germ cell apoptosis was proposed as an important cause for oestrogen-induced impairment of spermatogenesis in other fish species (Weber *et al.* 2004, Chaves-Pozo *et al.* 2007), 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 E_2 *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 E_2 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

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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 E_2-treated fish than from control fish, we speculate that oestrogens may target one or more components of the $G\alpha_S$ – 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 E_2 on testis physiology using a recently developed tissue culture system for zebrafish testis explants (Leal *et al.* 2009*a*). 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 E_2 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.* 2009*a*). Hence we further studied the *ex vivo* effects of E_2 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.* 2009*a*), 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.* 2009*a*). 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 E_2 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 E_2. In Atlantic croaker (*Micropogonias undulatus*), 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 E_2 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 testis were comparatively weak and possibly mediated via a nuclear Er, considering the prolonged time required for them to become evident, while the binding of E_2 to zebrafish Gper, although of high affinity (K_d=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 E_2-exposed zebrafish *in vivo*, morphological and/or morphometrical evaluation of testis tissue exposed to E_2 *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 E_2-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, Huggard-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*; Huggard-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 ovariectomized 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 recrudescent goldfish of mixed sex with E_2 suppressed GnRH-mediated elevation of *fshb* and *lhb* expression both *in vivo* and *in vitro* (Huggard-Nelson *et al.* 2002). The latter has been reported recently in medaka (*Oryzias latipes*; Zhang *et al.* 2008) as well. These data indicate that E_2 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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(Kah *et al.* 1992), or the recently described Kiss1/Gpr54 system (Elizur 2009) may be influenced by estrogens in teleost fish. Therefore, E_2-induced gonadotropin insufficiency seems the main factor contributing to the observed downregulation of androgen production in the zebrafish testis, which in turn results in an interruption of spermatogenesis.

Androgen depletion or blocking of androgen signalling had a marked effect on meiotic and postmeiotic events of rodent spermatogenesis, whereas effects on spermatogonial proliferation were limited (O'Donnell *et al.* 1994, França *et al.* 1998, De Gendt *et al.* 2004). This differs from the situation reported in different fish species. For instance, in juvenile testes containing only spermatogonia, androgen treatment stimulated rapid spermatogonial proliferation both in tissue culture experiments (Miura *et al.* 1991) and *in vivo* (Cavaco *et al.* 1998). However, it was not known if, after the first (pubertal) wave of spermatogenesis, also spermatogonial proliferation during adult spermatogenesis depends on androgens. In mice, for example, more androgen is required for the first than for subsequent waves of spermatogenesis (Handelsman *et al.* 1999). The present study shows that oestrogen-induced androgen insufficiency reduced proliferation of type A spermatogonia as well as their differentiation into type B spermatogonia in adult zebrafish testis, and strongly reduced the number of meiotic and haploid germ cells produced. To our knowledge, this is the first study localizing specific, androgen-dependent germ cell stages during adult spermatogenesis in teleost fish. This observation correlates well with both the high level of *ar* mRNA expression in Sertoli cells contacting type A spermatogonia in zebrafish testis (De Waal *et al.* 2008) and the elevation of circulating androgen levels in Chinook salmon at the time when seasonal spermatogonial proliferation starts (Campbell *et al.* 2003).

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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