

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

Paul P de Waal¹, Marcelo C Leal^{1,2}, Ángel García-López¹, Sergio Liarte³, Hugo de Jonge¹, Nathalie Hinfray⁴, François Brion⁴, Rüdiger W Schulz^{1,5} and Jan Bogerd¹

¹Division of Endocrinology and Metabolism, Department of Biology, Faculty of Science, Utrecht University, Hugo R Kruyt Building, Padualaan 8, 3584 CH Utrecht, The Netherlands

²Laboratory of Cellular Biology, Department of Morphology, Institute of Biological Sciences, Federal University of Minas Gerais, Belo Horizonte MG 31270-901, Brazil

³Department of Cell Biology, Faculty of Biology, University of Murcia, 30100 Murcia, Spain

⁴Unité d'évaluation des risques écotoxicologiques, Direction des Risques Chroniques, Institut National de l'Environnement Industriel et des Risques (INERIS), BP 2, F-60550 Verneuil-en-Halatte, France

⁵Research Group Reproduction and Growth, Institute of Marine Research, PO Box 1870, Nordnes, 5817 Bergen, Norway

(Correspondence should be addressed to J Bogerd or R W Schulz at Division of Endocrinology and Metabolism, Department of Biology, Faculty of Science, Utrecht University; J Bogerd; Email: j.bogerd@uu.nl, R W Schulz; Email: r.w.schulz@uu.nl)

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 17 β -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 ER α , ER β 1 and ER β 2 (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

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 17 β -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 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 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 μ M 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 \pm 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

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 synaptonemal complex protein 3 like (*syq3l*) 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 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 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 17 α -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 down-regulated 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,

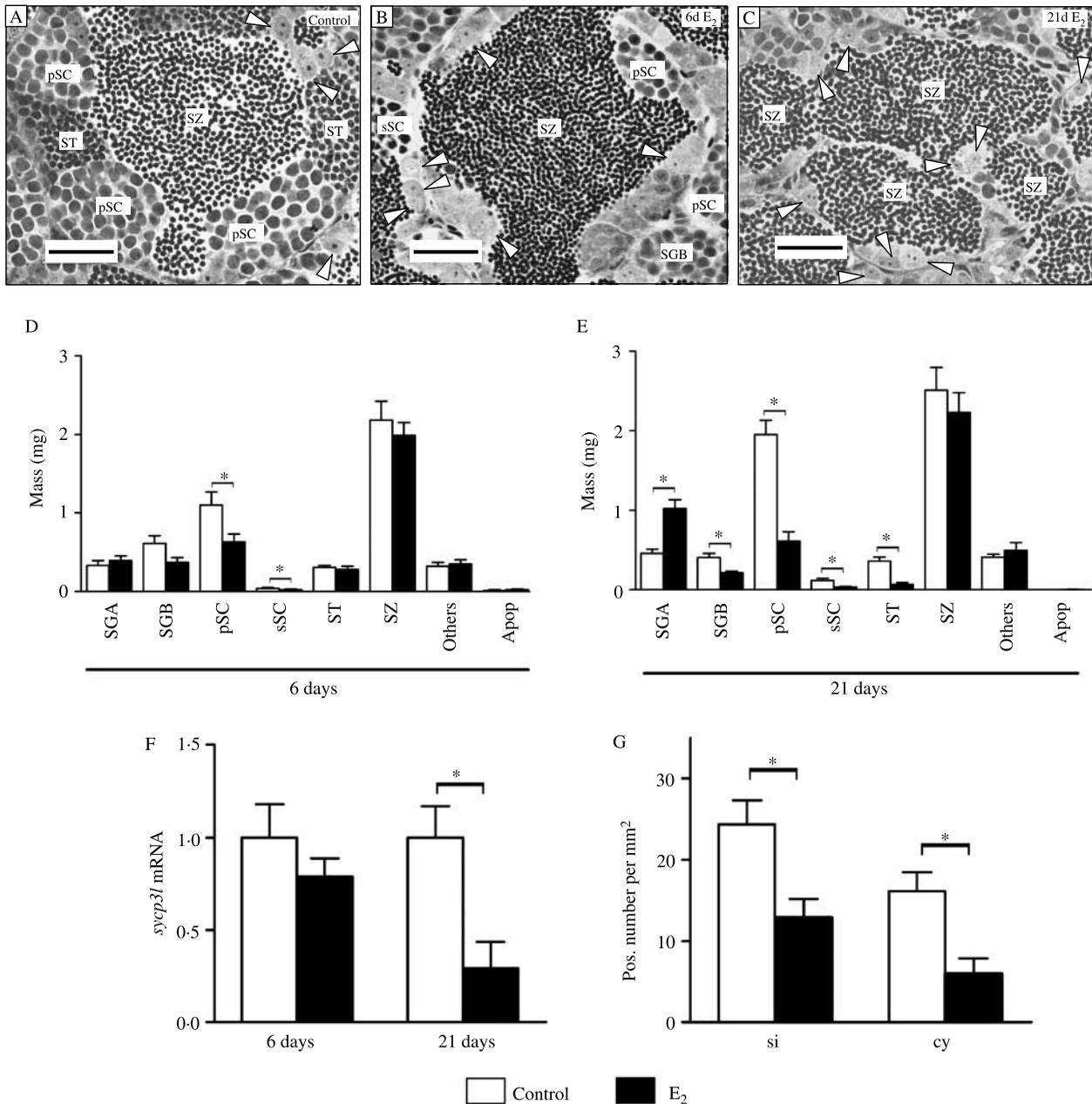
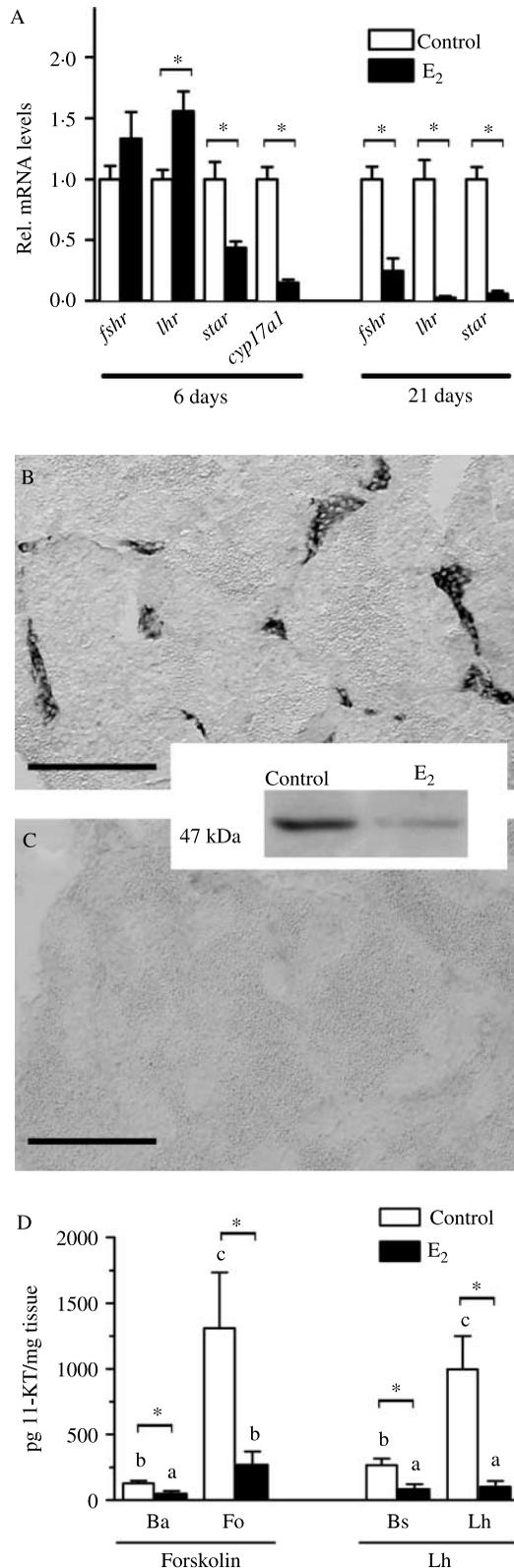


Figure 1 Zebrafish testicular physiology after exposure to 10 nM E₂ *in vivo*. Zebrafish testis sections from control fish (A), or from E₂-treated fish 6 days (B) or 21 days (C) after the start of exposure. Morphometric analysis ($n=5-7$ fish per group) of zebrafish testis sections, presenting data as mass (mg) of testicular cell types, 6 days (D) or 21 days (E) after the start of exposure to 10 nM E₂. (F) Relative amounts of *sycp31* mRNA, normalized to 18S rRNA, in control and E₂-exposed males ($n=6-13$ fish per treatment). (G) Germ cell proliferation after 6 days of E₂ 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 mm² 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 μ m.

left panel). However, the continuous presence of 0.5 μ M 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 and middle 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 μ M 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 μ m.

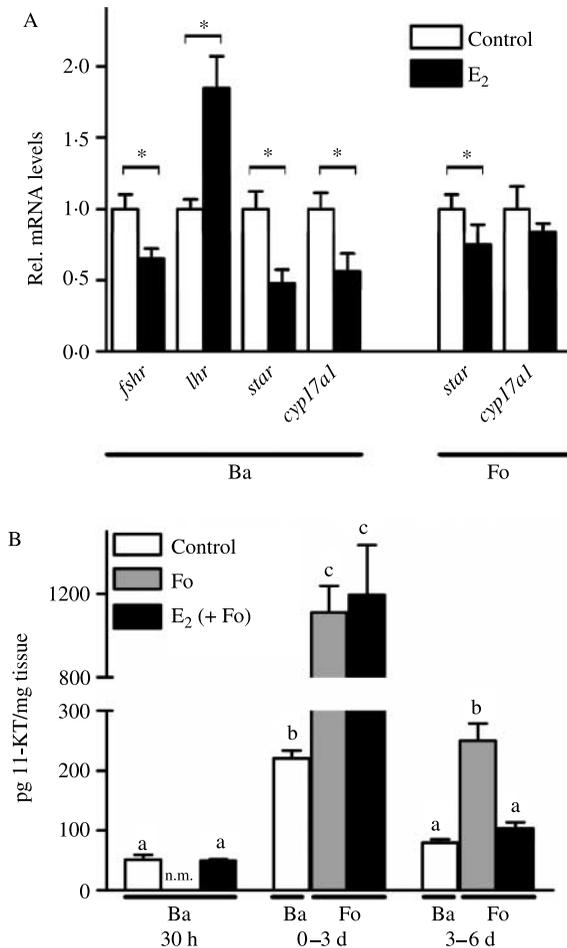


Figure 3 Steroidogenic capacity of zebrafish testis after exposure to 10 nM E₂ *ex vivo*. (A) Relative mRNA levels of *fshr*, *lhr*, *star* and *cyp17a1* after 6 days of E₂ *ex vivo* exposure either in the absence (Ba, basal) or the presence of 0.5 μM forskolin (Fo) (*n*=8 explants per group). Data are shown as relative values of respective transcript amounts measured in control fish. (B) Cumulative androgen release after *ex vivo* E₂ 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 E₂-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 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

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 G α_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 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 undulatus*), direct suppression of testicular androgen production by very high oestrogen concentrations (>36.7 μ M) 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 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 E₂ 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 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, 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 recrudescing goldfish of mixed sex with E₂ 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 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

(Kah *et al.* 1992), or the recently described Kiss1/Gpr54 system (Elizur 2009) may be influenced by estrogens in teleost fish. Therefore, E₂-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.

Funding

The present study was supported by an intramural PhD grant of the Utrecht University (The Netherlands), CAPES (Brazil), Fundación Ramón Areces (Spain), the National Institute of Health (grant number DK69711 to J.B.), and the Erasmus program of the European Union.

Acknowledgements

The authors would like to acknowledge the following people for their contribution to the work: Wytke van Dijk (Endocrinology and Metabolism) for technical assistance, Henk Schriek and Ko van Rootselaar (Aquarium Facility) for assistance during the *in vivo* experiments and maintaining the zebrafish stocks, and Roland Romijn and Wieger Hemrika (U-Protein Express BV, Utrecht, The Netherlands) for assistance in expression and purification of recombinant zebrafish Lh.

References

- Akingbemi BT, Ge R, Rosenfeld CS, Newton LG, Hardy DO, Catterall JF, Lubahn DB, Korach KS & Hardy MP 2003 Estrogen receptor- α gene deficiency enhances androgen biosynthesis in the mouse Leydig cell. *Endocrinology* **144** 84–93.
- Andersen L, Goto-Kazeto R, Trant JM, Nash JP, Korsgaard B & Bjerregaard P 2006 Short-term exposure to low concentrations of the synthetic androgen methyltestosterone affects vitellogenin and steroid levels in adult male zebrafish (*Danio rerio*). *Aquatic Toxicology* **76** 343–352.
- Arukwe A 2008 Steroidogenic acute regulatory (StAR) protein and cholesterol side-chain cleavage (P450_{sc})—regulated steroidogenesis as an organ-specific molecular and cellular target for endocrine disrupting chemicals in fish. *Cell Biology and Toxicology* **24** 527–540.
- Banerjee A & Khan IA 2008 Molecular cloning of FSH and LH β subunits and their regulation by estrogen in Atlantic croaker. *General and Comparative Endocrinology* **155** 827–837.
- Baron D, Fostier A, Breton B & Guiguen Y 2005 Androgen and estrogen treatments alter steady state messengers RNA (mRNA) levels of testicular steroidogenic enzymes in the rainbow trout, *Oncorhynchus mykiss*. *Molecular Reproduction and Development* **71** 471–479.
- Bartke A, Williams KI & Dalterio S 1977 Effects of estrogens on testicular testosterone production *in vitro*. *Biology of Reproduction* **17** 645–649.
- Van den Belt K, Berckmans P, Vangenechten C, Verheyen R & Witters H 2004 Comparative study on the *in vitro/in vivo* estrogenic potencies of 17 β -estradiol, estrone, 17 α -ethynylestradiol and nonylphenol. *Aquatic Toxicology* **66** 183–195.
- Billard R & Peter RE 1977 Gonadotropin release after implantation of anti-estrogens in the pituitary and hypothalamus of goldfish *Carassius auratus*. *General and Comparative Endocrinology* **32** 213–220.
- Blum JL, Nyagode BA, James MO & Denslow ND 2008 Effects of the pesticide methoxychlor on gene expression in the liver and testes of the male largemouth bass (*Micropterus salmoides*). *Aquatic Toxicology* **86** 459–469.
- Bouma J & Nagler JJ 2001 Estrogen receptor- α protein localization in the testis of the rainbow trout (*Oncorhynchus mykiss*) during different stages of the reproductive cycle. *Biology of Reproduction* **65** 60–65.
- Campbell B, Dickey JT & Swanson P 2003 Endocrine changes during onset of puberty in male spring Chinook salmon, *Oncorhynchus tshawytscha*. *Biology of Reproduction* **69** 2109–2117.
- Cavaco JE, Vilrocx C, Trudeau VL, Schulz RW & Goos HJ 1998 Sex steroids and the initiation of puberty in male African catfish (*Clarias gariepinus*). *American Journal of Physiology* **275** R1793–R1802.
- Chaves-Pozo E, Liarte S, Vargas-Chacoff L, Garcia-Lopez Á, Mulero V, Mesguer J, Mancera JM & Garcia-Ayala A 2007 17 β -estradiol triggers postspawning in spermatogenically active gilthead seabream (*Sparus aurata* L.) males. *Biology of Reproduction* **76** 142–148.
- Chieffi P, Colucci-D'Amato GL, Staibano S, Franco R & Tramontano D 2000 Estradiol-induced mitogen-activated protein kinase (extracellular signal-regulated kinase 1 and 2) activity in the frog (*Rana esculenta*) testis. *Journal of Endocrinology* **167** 77–84.
- Choi CY & Habibi HR 2003 Molecular cloning of estrogen receptor α and expression pattern of estrogen receptor subtypes in male and female goldfish. *Molecular and Cellular Endocrinology* **204** 169–177.
- Christianson-Heiska I, Wahteristo P, Kastilan EL, Bergelin E, Bylund G & Isomaa B 2004 Effects of the wood extractive betulinol and 17 β -oestradiol on reproduction in zebrafish, *Danio rerio* (Hamilton) – complications due to a bacterial infection. *Journal of Fish Diseases* **27** 267–276.
- Cobellis G, Pierantoni R, Minucci S, Pernas-Alonso R, Meccariello R & Fasano S 1999 *c-fos* activity in *Rana esculenta* testis: seasonal and estradiol-induced changes. *Endocrinology* **140** 3238–3244.
- Dickey JT & Swanson P 1998 Effects of sex steroids on gonadotropin (FSH and LH) regulation in coho salmon (*Oncorhynchus kisutch*). *Journal of Molecular Endocrinology* **21** 291–306.
- Eddy EM, Washburn TF, Bunch DO, Goulding EH, Gladen BC, Lubahn DB & Korach KS 1996 Targeted disruption of the estrogen receptor gene in male mice causes alteration of spermatogenesis and infertility. *Endocrinology* **137** 4796–4805.

- Elizur A 2009 The KiSS1/GPR54 system in fish. *Peptides* **30** 164–170.
- Filby AL & Tyler CR 2005 Molecular characterization of estrogen receptors 1, 2a, and 2b and their tissue and ontogenic expression profiles in fathead minnow (*Pimephales promelas*). *Biology of Reproduction* **73** 648–662.
- Filby AL, Thorpe KL & Tyler CR 2006 Multiple molecular effect pathways of an environmental oestrogen in fish. *Journal of Molecular Endocrinology* **37** 121–134.
- França LR, Parreira GG, Gates RJ & Russell LD 1998 Hormonal regulation of spermatogenesis in the hypophysectomized rat: quantitation of germ-cell population and effect of elimination of residual testosterone after long-term hypophysectomy. *Journal of Andrology* **19** 335–340.
- De la Fuente R, Parra MT, Viera A, Calvente A, Gomez R, Suja JA, Rufas JS & Page J 2007 Meiotic pairing and segregation of achiasmatic sex chromosomes in eutherian mammals: the role of SYCP3 protein. *PLoS Genetics* **3** e198.
- Fukuzawa NH, Ohsako S, Wu Q, Sakaue M, Fujii-Kuriyama Y, Baba T & Tohyama C 2004 Testicular cytochrome P450_{scc} and LHR as possible targets of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in the mouse. *Molecular and Cellular Endocrinology* **221** 87–96.
- De Gendt K, Swinnen JW, Saunders PT, Schoonjans L, Dewerchin M, Devos A, Tan K, Atanassova N, Claessens F, Lecureuil C *et al.* 2004 A Sertoli cell-selective knockout of the androgen receptor causes spermatogenic arrest in meiosis. *PNAS* **101** 1327–1332.
- Gould ML, Hurst PR & Nicholson HD 2007 The effects of oestrogen receptors α and β on testicular cell number and steroidogenesis in mice. *Reproduction* **134** 271–279.
- Govoroun M, McMeel OM, Mecherouki H, Smith TJ & Guiguen Y 2001 17 β -estradiol treatment decreases steroidogenic enzyme messenger ribonucleic acid levels in the rainbow trout testis. *Endocrinology* **142** 1841–1848.
- Halm S, Martinez-Rodríguez G, Rodríguez L, Prat F, Mylonas CC, Carrillo M & Zanuy S 2004 Cloning, characterization, and expression of three oestrogen receptors (ER α , ER β 1 and ER β 2) in the European sea bass, *Dicentrarchus labrax*. *Molecular and Cellular Endocrinology* **223** 63–75.
- Handelsman DJ, Spaliviero JA, Simpson JM, Allan CM & Singh J 1999 Spermatogenesis without gonadotropins: maintenance has a lower testosterone threshold than initiation. *Endocrinology* **140** 3938–3946.
- Hawkins MB, Thornton JW, Crews D, Skipper JK, Dotte A & Thomas P 2000 Identification of a third distinct estrogen receptor and reclassification of estrogen receptors in teleosts. *PNAS* **97** 10751–10756.
- Hess RA, Bunick D, Lubahn DB, Zhou Q & Bouma J 2000 Morphologic changes in efferent ductules and epididymis in estrogen receptor- α knockout mice. *Journal of Andrology* **21** 107–121.
- Huggard-Nelson DL, Nathwani PS, Kermouni A & Habibi HR 2002 Molecular characterization of LH- β and FSH- β subunits and their regulation by estrogen in the goldfish pituitary. *Molecular and Cellular Endocrinology* **188** 171–193.
- Ito K, Mochida K & Fujii K 2007 Molecular cloning of two estrogen receptors expressed in the testis of the Japanese common goby, *Acanthogobius flavimanus*. *Zoological Science* **24** 986–996.
- Jong FH, Uilenbroek J & Molen HJ 1975 Oestradiol-17 β , testosterone and gonadotrophins in oestradiol-17 β -treated intact adult male rats. *Journal of Endocrinology* **65** 281–282.
- Jukosky JA, Watzin MC & Leiter JC 2008 The effects of environmentally relevant mixtures of estrogens on Japanese medaka (*Oryzias latipes*) reproduction. *Aquatic Toxicology* **86** 323–331.
- Kah O, Trudeau VL, Sloley BD, Chang JP, Dubourg P, Yu KL & Peter RE 1992 Influence of GABA on gonadotrophin release in the goldfish. *Neuroendocrinology* **55** 396–404.
- Kinnberg K & Toft G 2003 Effects of estrogenic and antiandrogenic compounds on the testis structure of the adult guppy (*Poecilia reticulata*). *Ecotoxicology and Environmental Safety* **54** 16–24.
- Kobayashi M, Sohn YC, Yoshiura Y & Aida K 2001 Effects of sex steroids on the mRNA levels of gonadotropin subunits in juvenile and ovariectomized goldfish *Carassius auratus*. *Fisheries Science* **66** 223–231.
- Leal MC, Skaar KS, França LR & Schulz RW 2006 The effects of IGF-I on zebrafish testis in tissue culture. *Animal Reproduction: Proceedings of the International Symposium on Animal Biology of Reproduction, Nov. 15–18, 2006, Belo Horizonte, MG, Brazil* **3** 181.
- Leal MC, de Waal PP, García-López Á, Chen SX, Bogerd J & Schulz RW 2009a Zebrafish primary testis tissue culture: an approach to study testis function *ex vivo*. *General and Comparative Endocrinology* **162** 134–138.
- Leal MC, Cardoso ER, Nóbrega RH, Batlouni SR, Bogerd J, França LR & Schulz RW 2009b Histological and stereological evaluation of zebrafish (*Danio rerio*) spermatogenesis with an emphasis on spermatogonial generations. *Biology of Reproduction* (in press: DOI 10.1095/biolreprod.109.076299).
- Lin SW & Ge W 2009 Differential regulation of gonadotropins (FSH and LH) and growth hormone (GH) by neuroendocrine, endocrine, and paracrine factors in the zebrafish – an *in vitro* approach. *General and Comparative Endocrinology* **160** 183–193.
- Lindzey J, Wetzel WC, Couse JF, Stoker T, Cooper R & Korach KS 1998 Effects of castration and chronic steroid treatments on hypothalamic gonadotropin-releasing hormone content and pituitary gonadotropins in male wild-type and estrogen receptor- α knockout mice. *Endocrinology* **139** 4092–4101.
- Liu X, Zhu P, Sham KW, Yuen JM, Xie C, Zhang Y, Liu Y, Li S, Huang X, Cheng CH *et al.* 2009 Identification of a membrane estrogen receptor in zebrafish with homology to mammalian GPR30 and its high expression in early germ cells of the testis. *Biology of Reproduction* (in press: DOI 10.1095/biolreprod.108.070250).
- Loomis AK & Thomas P 2000 Effects of estrogens and xenoestrogens on androgen production by Atlantic croaker testes *in vitro*: evidence for a nongenomic action mediated by an estrogen membrane receptor. *Biology of Reproduction* **62** 995–1004.
- Manna PR, Wang XJ & Stocco DM 2003 Involvement of multiple transcription factors in the regulation of steroidogenic acute regulatory protein gene expression. *Steroids* **68** 1125–1134.
- Meier S, Andersen TE, Norberg B, Thorsen A, Taranger GL, Kjesbu OS, Dale R, Morton HC, Klungsoyr J & Svardal A 2007 Effects of alkylphenols on the reproductive system of Atlantic cod (*Gadus morhua*). *Aquatic Toxicology* **81** 207–218.
- Menuet A, Pellegrini E, Anglade I, Blaise O, Laudet V, Kah O & Pakdel F 2002 Molecular characterization of three estrogen receptor forms in zebrafish: binding characteristics, transactivation properties, and tissue distributions. *Biology of Reproduction* **66** 1881–1892.
- Minucci S, Di Matteo L, Chieffi P, Pierantoni R & Fasano S 1997 17 β -estradiol effects on mast cell number and spermatogonial mitotic index in the testis of the frog, *Rana esculenta*. *Journal of Experimental Zoology* **278** 93–100.
- Miura T, Yamauchi K, Takahashi H & Nagahama Y 1991 Hormonal induction of all stages of spermatogenesis *in vitro* in the male Japanese eel (*Anguilla japonica*). *PNAS* **88** 5774–5778.
- Miura T, Miura C, Ohta T, Nader MR, Todo T & Yamauchi K 1999 Estradiol-17 β stimulates the renewal of spermatogonial stem cells in males. *Biochemical and Biophysical Research Communications* **264** 230–234.
- Miura T, Ohta T, Miura CI & Yamauchi K 2003 Complementary deoxyribonucleic acid cloning of spermatogonial stem cell renewal factor. *Endocrinology* **144** 5504–5510.
- Nielsen L & Baatrup E 2006 Quantitative studies on the effects of environmental estrogens on the testis of the guppy, *Poecilia reticulata*. *Aquatic Toxicology* **80** 140–148.
- O'Donnell L, McLachlan RI, Wreford NG & Robertson DM 1994 Testosterone promotes the conversion of round spermatids between stages VII and VIII of the rat spermatogenic cycle. *Endocrinology* **135** 2608–2614.
- Pawlowski S, van Aerle R, Tyler CR & Braunbeck T 2004 Effects of 17 α -ethinylestradiol in a fathead minnow (*Pimephales promelas*) gonadal recrudescence assay. *Ecotoxicology and Environmental Safety* **57** 330–345.
- Pierantoni R, Varriale B, Minucci S, Di Matteo L, Fasano S, D'Antonio M & Chieffi G 1986 Regulation of androgen production by frog (*Rana esculenta*) testis: an *in vitro* study on the effects exerted by estradiol, 5 α -dihydrotestosterone, testosterone, melatonin, and serotonin. *General and Comparative Endocrinology* **64** 405–410.

- Schulz RW, van der Corput L, Janssen-Dommerholt C & Goos HJT 1994 Sexual steroids during puberty in male African catfish (*Clarias gariepinus*): serum levels and gonadotropin-stimulated testicular secretion *in vitro*. *Journal of Comparative Physiology. B, Biochemical, Systemic, and Environmental Physiology* **164** 195–205.
- Schwartz JR & Roy SK 2000 Expression of P450 side-chain cleavage (CYP11A1) and P450 17 α -hydroxylase-17/20 lyase (CYP17) messenger ribonucleic acid in hamster primary interstitial cells *in vitro*: differential regulation of steroidogenesis by cyclic adenosine monophosphate. *Biology of Reproduction* **63** 503–507.
- Sohn YC, Yoshiura Y, Kobayashi M & Aida K 1998 Effect of sex steroids on the mRNA levels of gonadotropin I and II subunits in the goldfish *Carassius auratus*. *Fisheries Science* **64** 715–721.
- Song M & Gutzeit HO 2003 Effect of 17- α -ethynylestradiol on germ cell proliferation in organ and primary culture of medaka (*Oryzias latipes*) testis. *Development, Growth and Differentiation* **45** 327–337.
- Tsai PS, Kessler AE, Jones JT & Wahr KB 2005 Alteration of the hypothalamic–pituitary–gonadal axis in estrogen- and androgen-treated adult male leopard frog, *Rana pipiens*. *Reproductive Biology and Endocrinology* **3** 2.
- Turner KJ, Morley M, Atanassova N, Swanston ID & Sharpe RM 2000 Effect of chronic administration of an aromatase inhibitor to adult male rats on pituitary and testicular function and fertility. *Journal of Endocrinology* **164** 225–238.
- Van der Ven LT, Wester PW & Vos JG 2003 Histopathology as a tool for the evaluation of endocrine disruption in zebrafish (*Danio rerio*). *Environmental Toxicology and Chemistry* **22** 908–913.
- Van der Ven LT, van den Brandhof EJ, Vos JH & Wester PW 2007 Effects of the estrogen agonist 17 β -estradiol and antagonist tamoxifen in a partial life-cycle assay with zebrafish (*Danio rerio*). *Environmental Toxicology and Chemistry* **26** 92–99.
- De Waal PP, Wang DS, Nijenhuis WA, Schulz RW & Bogerd J 2008 Functional characterization and expression analysis of the androgen receptor in zebrafish (*Danio rerio*) testis. *Reproduction* **136** 225–234.
- Weber LP, Balch GC, Metcalfe CD & Janz DM 2004 Increased kidney, liver, and testicular cell death after chronic exposure to 17 α -ethynylestradiol in medaka (*Oryzias latipes*). *Environmental Toxicology and Chemistry* **23** 792–797.
- Westerfield M 2000 *The Zebrafish Book. A Guide for the Laboratory use of Zebrafish (Danio rerio)*. 4th edn, Eugene: Univ. of Oregon Press.
- Wu C, Patino R, Davis KB & Chang X 2001 Localization of estrogen receptor α and β RNA in germinal and nongerminal epithelia of the channel catfish testis. *General and Comparative Endocrinology* **124** 12–20.
- Zhang X, Hecker M, Park JW, Tompsett AR, Newsted J, Nakayama K, Jones PD, Au D, Kong R, Wu RS *et al.* 2008 Real-time PCR array to study effects of chemicals on the hypothalamic–pituitary–gonadal axis of the Japanese medaka. *Aquatic Toxicology* **88** 173–182.
- Zhou Q, Nie R, Prins GS, Saunders PT, Katzenellenbogen BS & Hess RA 2002 Localization of androgen and estrogen receptors in adult male mouse reproductive tract. *Journal of Andrology* **23** 870–881.

Received in final form 23 April 2009

Accepted 5 May 2009

Made available online as an Accepted Preprint

5 May 2009