Cyp17a1 and Cyp19a1 in the zebrafish testis are differentially affected by oestradiol

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Abstract

Oestrogens can affect expression of genes encoding steroidogenic enzymes in fish gonads. However, little information is available on their effects at the protein level. In this context, we first analysed the expression of key steroidogenic enzyme genes and proteins in zebrafish testis, paying attention also to other cell types than Leydig cells. Gene expression was analysed by quantitative PCR on fluorescence-activated cell-sorting fractions coupled or not to differential plating, while protein synthesis was studied by immunohistochemistry using specific antibodies against zebrafish Cyp17a1, Cyp19a1a and Cyp19a1b. Furthermore, we have evaluated the effect of oestrogen treatment (17β-oestradiol (E2), 10 nM) on the localization of these enzymes after 7 and 14 days of in vivo exposure in order to study how oestrogen-mediated modulation of their expression is linked to oestrogen effects on spermatogenesis. The major outcomes of this study are that Leydig cells express Cyp17a1 and Cyp19a1a, while testicular germ cells express Cyp17a1 and both, Cyp19a1a and Cyp19a1b. Furthermore, we have evaluated the effect of oestrogen treatment (17β-oestradiol (E2), 10 nM) on the localization of these enzymes after 7 and 14 days of in vivo exposure in order to study how oestrogen-mediated modulation of their expression is linked to oestrogen effects on spermatogenesis. The major outcomes of this study are that Leydig cells express Cyp17a1 and Cyp19a1a, while testicular germ cells express Cyp17a1 and both, Cyp19a1a and Cyp19a1b. As regards Cyp17a1, both protein and mRNA seem to be quantitatively dominating in Leydig cells. Moreover, E2 exposure specifically affects only Leydig cell Cyp17a1 synthesis, preceding the disruption of spermatogenesis. The oestrogen-induced suppression of the androgen production capacity in Leydig cells is a major event in altering spermatogenesis, while germ cell steroidogenesis may have to be fuelled by precursors from Leydig cells. Further studies are needed to elucidate the functionality of steroidogenic enzymes in germ cells and their potential role in testicular physiology.

Key Words
- steroidogenic enzymes
- zebrafish
- testis
- FACS
- immunolocalization
- oestradiol

Introduction

Steroid hormones are key regulators of growth, development and reproduction in vertebrates. Sex steroid hormones are mainly produced in the gonads and their plasma concentrations change greatly during the process of gonad maturation. In fish, testis tissue produces all three major types of sex steroid hormones, progestagens, androgens and oestrogens, each having distinct functions in the regulation of spermatogenesis, from...
spermatogonial stem cell self-renewal to sperm maturation, while exerting regulatory effects either directly on the testis or indirectly via feedback effects on the brain and/or pituitary (Schulz et al. 2010). However, clearly androgens are the quantitatively dominating steroid product of the testis (Schulz et al. 2010).

The synthesis of steroid hormones from a common precursor, cholesterol, is supported by numerous enzymes including several P450 cytochromes (Baroiller et al. 1999). Among these P450 cytochromes, the 17-α-hydroxylase, 17,20 lyase (Cyp17), catalyses the hydroxylation of pregnenolone to 17-α-hydroxypregnenolone or of progesterone to 17-α-hydroxyprogesterone and the cleavage of the C17,20 bond to convert 17-α-hydroxypregnenolone and 17-α-hydroxyprogesterone to dehydroepiandrosterone and androstenedione respectively. An in silico study revealed the existence of two cyp17 genes (cyp17a1 and cyp17a2) in the zebrafish genome (Zhou et al. 2007). Aromatases (aromatase A (Cyp19a1a) encoded by the cyp19a1a gene and aromatase B (Cyp19a1b) encoded by the cyp19a1b gene) catalyse the conversion of androgens into oestrogens. Similar to mammals, both Cyp17 and Cyp19a1 proteins are strongly expressed in the Leydig cells of the testes of several species of fish (Dalla Valle et al. 2002a, Halm et al. 2003, Yu et al. 2003, Blazquez & Piferrer 2004, Wang & Orban 2007) including zebrafish (Danio rerio; De Waal et al. 2009, Hinfray et al. 2011).

Initially, Cyp17 was thought to be confined to classic steroidogenic tissues but mRNA, protein and activity for this enzyme were also detected in other tissues, such as brain (Hojo et al. 2004), liver (Katagiri et al. 1998) and stomach, duodenum and kidney for rodents (Dalla Valle et al. 2002b). A wide distribution of this enzyme in different tissues also in other species, like zebrafish, has been demonstrated (Wang & Ge 2004). Recently, studies on mice detected Cyp17 in germ cells: spermatogonia, pachytene spermatocytes, spermatids and sperm (Qiang et al. 2003, Liu et al. 2005), and the loss of this enzyme is associated with sperm abnormalities and infertility (Liu et al. 2005). This suggests that in addition to steroidogenesis, Cyp17 may have a role in sperm structure and function (Liu et al. 2005). Cyp19 (P450 aromatase) has also been found in a wide variety of tissues in addition to gonads: placenta, bone, adipose tissue, blood vessels, skin, endometrium and brain (see review in Chumsri et al. (2011)). In the different mammalian species studied so far, P450 aromatase expression has been detected in several testicular somatic cells (e.g. Leydig cells) as well as in gonocytes, spermatogonia, spermatocytes (preleptotene/pachytene), spermatids and spermatozoa (see review in Chumsri et al. (2011)). However, there is little information available in fish with respect to the question whether other testicular cell types express these enzymes as well.

Testicular steroidogenesis is very sensitive to exogenous steroids. Effects following in vivo exposure of African catfish to testosterone include a loss of the 17,20 lyase but not of the 17-α-hydroxylase activity and a reduction to 50% of the number of mitochondria and of the cytoplasmic area in Leydig cells (Cavaco et al. 1999, Schulz et al. 2008). In adult zebrafish, exposure to 17-β-oestradiol (E2) in vivo led to an interruption of spermatogenesis following a down-regulation of testicular androgen synthesis, presumably via feedback inhibition of gonadotropin release (De Waal et al. 2009). Several other studies have also documented the inhibitory effect of oestrogens on the testicular expression of steroidogenic enzymes such as cyp17, P450 side-chain cleavage and 11-β-hydroxylase in fish (Goverouin et al. 2001, Baron et al. 2005, Filby et al. 2006, Brion et al. 2008, De Waal et al. 2009), which could explain the deleterious effects of these compounds on fish reproduction (Brion et al. 2004).

In this context, the aim of this study was to first localize the cellular sites of synthesis of key steroidogenic enzymes (Cyp17, aromatase A and aromatase B) involved in the production of androgens and oestrogens in zebrafish testis, paying attention also to other cell types than Leydig cells. Enzyme protein localization was studied immunohistochemically, using specific antibodies against zebrafish Cyp17a1, Cyp19a1a and Cyp19a1b. Furthermore, we have evaluated the effect of oestrogen treatment on the localization of these enzymes in order to study how deregulation of their synthesis is linked to E2-disrupted spermatogenesis. The major outcomes of this study are that Leydig cells express Cyp17a1 and Cyp19a1a, while testicular germ cells express Cyp17a1 and both Cyp19a1a and Cyp19a1b. Moreover, E2 exposure specifically affects only Leydig cell Cyp17a1 synthesis, preceding the disruption of spermatogenesis.

**Materials and methods**

**Zebrafish maintenance**

Adult (>90 days post-fertilization) wild-type zebrafish (AB strain) were bred in a laboratory facility at Institut National de l’ Environnement Industriel et des Risques (INERIS, Verneuil-en-Halatte, France). They were maintained in 3.5 L aquarium in a recirculation system (Zebtec, Tecniplast, Buguggiate, Italy) on a 14 h light:10 h darkness cycle at a temperature of 25.1 ± 1.0 °C. Animal culture,
handling and experimentation were approved by the INERIS life science ethics committee.

Zebrafish exposure

Adult male zebrafish were exposed to 10 nM E2 (Sigma–Aldrich) or to solvent alone (dimethyl sulfoxide (DMSO), 0.00001%; Sigma–Aldrich) during 7 and 14 days. Fish were exposed under semi-static conditions in 41 tanks with 100% water renewal every day (temperature: 28 ± 1 °C; pH: 8.2 ± 0.2; dissolved oxygen: 6.4 ± 0.2 mg/l; conductivity: 269 ± 37 μS/cm). Exposure was performed in one tank per condition, each containing six male fish. At the end of exposure, zebrafish were killed in ice-cold water, weighed and measured. Testes were removed and weighed to determine the gonadosomatic index using the formula (GSI = testis wet weight/total fish wet weight × 100).

Expression of cyp17a1 and cyp19a1 genes in sorted testicular cell fractions

To generate data on the cellular localization of testicular cyp17a1 and cyp19a1 expression with an independent approach, we used testes from vasa::egfp zebrafish (Krovel & Olsen 2002) as tissue donors. Testis tissue from transgenic fish was digested with 0.2% collagenase and 0.12% dispase as described previously (Nóbrega et al. 2010). The resulting cell suspension was immediately submitted to fluorescence-activated cell sorting (FACS) using an in Flux cell sorter (BD Bioscience, San Jose, CA, USA, www.bdbiosciences.com). In some cases, the testicular cell suspension was also submitted to a differential plating method (Luo et al. 2006) before FACS. Using this method, somatic cells adhere to the bottom of the plate while germ cells either remain in suspension after 2–3 days of culture or are only loosely associated with the firmly adhering somatic cells (Fig. 1). Then, FACS settings were adjusted to sort Egfp-positive and -negative cells from total vasa::egfp testicular cell suspension and also from floating/loosely associated vasa::egfp cells after differential plating (Fig. 2). Autofluorescence was eliminated through the FACS dot plot profile generated with a testicular cell suspension from wild-type males (Fig. 2A). Then, Egfp-positive and -negative cells were collected into tubes, centrifuged in PBS (pH 7.4) for 10 min at 100 g and immediately submitted to RNA extraction using RNAquous – Micro Kit (Ambion, Austin, TX, USA, http://www.ambion.com) as described earlier (Nóbrega et al. 2010). After cDNA synthesis, the threshold cycle (Ct) values of two endogenous reference genes (elongation factor

Figure 1
Differential plating method. (A) Scheme showing the differential plating method adapted for zebrafish testicular cell suspension from Luo et al. (2006). A total cell suspension (fraction 1) was obtained from zebrafish testes and harvested in cell culture medium (L-15). After 2 days of culture, only somatic cells with adhesive properties (Sertoli cells, blue triangular symbols; Leydig cells, yellow oval symbols) adhere to the bottom of the plate (fraction 2), while germ cells (non-adherent cells; green symbols reflecting their transgenic vasa::egfp state) remain floating or attach loosely to the firmly attached Sertoli cells. After an extensive washing step, it is possible to remove floating germ cells and also those germ cells weakly attached to the somatic cells, leaving the adherent somatic cells (Sertoli cells, Leydig cells, fibroblast and others – fraction 3) at the bottom of the plate. This method is called differential plating due to the different adhesive properties of the different testicular cell types. (B and C fraction 1) Floating cells: adherent and non-adherent cells that can be isolated or in groups. GC, germ cell. (D and E fraction 2) Somatic cells (SC) with adhesive properties at the bottom of the plate. Arrow shows interaction between somatic cells and germ cells (GC). (F and G fraction 3) After extensive washing, somatic adherent cells (SC) remain attached to the bottom of the plate, while the floating and the weakly attached germ cells were removed.
18S rRNA) and of the target genes were determined by qPCR. Primers used for the quantification of 18S rRNA were provided by the Eukaryotic 18S rRNA TaqMan Assay (20X mix of two unlabeled PCR primers and a FAM dye-labeled 18S TaqMan MGB probe; Applied Biosystems). The target genes were cytochrome P450, family 17, subfamily A, polypeptide 1 (cyp17a1; Garcia-Lopez et al. 2010); cytochrome P450, family 19, subfamily A, polypeptide 1 (cyp19a1a; Chiang et al. 2001); star (Garcia-Lopez et al. 2010); insulin-like 3 (insl3) (Leydig cell-specific gene) (Good-Avila et al. 2009); gonadal soma-derived growth factor (gsdf) (Sertoli cell-specific gene) (Gautier et al. 2011) and piwi-like 2 (piwil2) (germ cell-specific gene) (Houwing et al. 2008). All qPCRs were

Figure 2
(A) FACS dot plot profile obtained from wild-type (left) and vasa::egfp transgenic zebrafish (right) testicular cell suspension. Each dot represents one single event (one cell), x-axis represents the intensity of Egfp (more to the right = more Egfp, while more to the left less or no Egfp), y-axis represents another filter that was used to evaluate autofluorescence. The percentage of Egfp-positive events in the total testicular cell suspension for wild-type (left; 0.07%) and vasa::egfp transgenic (right; 71.53%) zebrafish is indicated in the selected area. The selected area in the right graph shows the Egfp-positive population that was harvested by FACS; the rest consists of the Egfp-negative fraction. (B and C) Testicular cell suspension from vasa::egfp transgenic animals before FACS analysis, showing Egfp-positive and -negative cells. (B) DIC microscopy. (C) Fluorescence microscopy. (D) Relative mRNA expression of several testicular genes (gsdf, insl3, cyp17a1, cyp19a1a, star and piwil2) in the different cell fractions: Egfp+ (dark green; immediately sorted after cell suspension; n=4), Egfp− (grey; immediately sorted after cell suspension; n=2) and Egfp+ sorted after differential plating (after 2 days of culture; light green; n=2) to reduce somatic cell numbers and to enrich germ cells. Data are expressed as mean ± s.e.m. The level of statistical significance between the reference group (Egfp+ ) and the other groups (Egfp− and EGFP+ sorted after differential plating) are indicated by *P < 0.05, **P < 0.01, ***P < 0.005 or NS (not significant).
performed in 20 µl reactions, containing 10 µl Power SYBR Green PCR Master Mix (Applied Biosystems), 0.9 µM forward and 0.9 µM reverse primers (Supplementary Table 1, see section on supplementary data given at the end of this article) and 5 µl cDNA for each gene. For the endogenous reference genes (ef1a; 18S rRNA), qPCRs were done in 20 µl reactions, containing 10 µl TaqMan Universal PCR Master Mix (Applied Biosystems), 0.3 µM forward and 0.3 µM reverse primers (Supplementary Table 1) and 5 µl cDNA. For 18S rRNA, cDNA was first diluted to 1:200 in RNAse-free water. Relative mRNA levels were determined using the 7900HT Fast Real-Time PCR System (Applied Biosystems), using default settings. Ct values of the target genes (cyp17a1, cyp19a1a, star, insl3, gsdf and piwil2) were normalized with the Ct values of the most stable reference gene (ef1a) (Supplementary Figure 1) and calibrated with the mean of delta Ct (dCt) of all samples according to the ΔΔCt value method.

Antibodies

The immunopurified anti-Cyp19a1a antibody was purchased from AnaSpec (Fremont, CA, USA distributed in France by Tebu-bio; reference number: 55474s). According to the manufacturer, this antibody was raised in rabbit against a synthetic peptide derived from the C-terminal region of the zebrafish Cyp19a1a protein (GenBank accession no. NP_571229). The manufacturer provided specificity data in zebrafish by western blots: only one band was detected and this band disappeared when blocked by the peptide used for immunization. The immunopurified anti-Cyp19a1b antibody used in this study was raised in rabbit against the peptide CNSNGETADNRRTSKE, corresponding to the last 15 residues (amino acids 497–511) of the zebrafish Cyp19a1b sequence (AF183908). The immunopurified anti-Cyp17a1 antibody was also raised in rabbit against two synthetic peptides (AFADYSSTWKFRK and KVRADWKE6PLMQCH) corresponding to the amino acids 126–139 and 505–519 respectively of the zebrafish Cyp17a1 sequence (AAP41821). These two antibodies have been shown to be specific for the Cyp19a1b protein in zebrafish brain (Menuet et al. 2005, Vosges et al. 2010) and for the Cyp17a1 protein in zebrafish testis (De Waal et al. 2009, Hinfray et al. 2011). In addition, the specificity of the Cyp19a1b antibody in zebrafish testis was assessed by western blotting as described previously for Cyp17a1 (De Waal et al. 2009). The Cyp19a1b antibody specifically recognized a single band of about 50 kDa. The observed size of protein is in agreement with those reported in the literature for zebrafish (Menuet et al. 2005) and with the deduced molecular weight based on the amino acid sequence (Supplementary Figure 2, see section on supplementary data given at the end of this article).

Histology

Testes were fixed in Bouin’s fluid for 48 h. After fixation, samples were dehydrated in ethanol and embedded in paraffin, according to conventional procedures. Testis were longitudinaly sectioned at 5 µm and stained with haematoxylin/eosin or processed in immunohistochemistry as described below.

Morphometric analysis of testis of control and E2-exposed fish was conducted according to Feitsma et al. (2007) with some modifications. Volume fractions of various testicular tissue components were determined as the area of cysts containing cells normalized by the total area of testis analysed to obtain a percentage of total tissue for each component. These volume fractions were determined for each of the following testicular components: type A spermatogonia, type B spermatogonia, spermatocytes, spermatids, spermatozoa and others (somatic cells, blood and lymphatic vessels, connective tissue and empty spaces). Four testicular sections (~80 µm apart from each other) were analysed for each fish (n=6 per condition). The weight (mg) of each testis component was determined as the product of the volume fraction (%) and the testis weight. These quantifications were blindly performed without a priori knowledge of the specimen identity.

Immunohistochemistry

Sections were dewaxed and rehydrated, and antigens were unmasked for 3 h at 80 °C in EDTA buffer (1 mM, pH 8.5). Tissue sections were then incubated for 1 h in a blocking solution (PBS containing 0.2% Triton X-100 and 1% milk powder). Incubation with primary antibodies (anti-Cyp19a1a, Cyp19a1b or anti-Cyp17a1) was performed overnight (1:300 (anti-Cyp19a1b or anti-Cyp17a1) or 1:600 (anti-Cyp19a1a) with 0.5% milk powder in PBS) at ambient temperature. After rinsing, sections were incubated for 1 h 30 min with a goat anti-rabbit antibody coupled to AlexaFluor 594 (Invitrogen) 1:200 with 0.5% milk powder in PBS. The specificity of the staining was controlled by processing adjacent sections without primary antibody or with primary antibody pre-adsorbed with the peptides (100 µg/ml) for Cyp19a1b and Cyp17a1. In these negative controls, no cross-reactivity was seen for...
any of the three antibodies (data not shown). For immunolocalization experiments in non-exposed fish from our breeding unit, and of DMSO- and E₂-exposed fish, six adult male zebrafish were used.

Fluorescence analysis

Immunofluorescence was observed with a Zeiss AxioImager.Z1 fluorescence microscope equipped with an AxioCam Mrm camera and combined with an ApoTome (Zeiss GmbH, Göttingen, Germany). Micrographs were taken using the Axiovision Imaging software. All micrographs presented were series of optical sections along the z-axis that were acquired at 0.25–0.45 μm intervals with the 20× and 40× objectives and projected in a single image (maximum intensity projection). Fluorescence pictures of testes of DMSO- or E₂-exposed fish were series of 20 optical sections acquired with the same time of exposure; however, the time of exposure was adjusted for each antibody used.

Quantification of fluorescence of DMSO- or E₂-exposed fish testes was performed on micrographs taken with the same magnification (20× objective) and time of exposure for a given antibody for all individuals. Fluorescence was measured as integrated density (IntDen) software, i.e. the sum of the grey values of all the pixels in the region of interest. For Cyp19a1, the IntDen data were then normalized to the total area of testis tissue on the micrograph. For Cyp17α1, the IntDen data were normalized to the area occupied by interstitial cells (including Leydig cells) or germ cells. All results were expressed as percentage of their respective controls. For each antibody, four micrographs per individual and four individuals per treatment were analysed. As no statistically significant difference of fluorescence quantification was observed between DMSO-exposed fish at 7 and 14 days for any of the three antibodies, all the DMSO-exposed fish (7 and 14 days) were pooled.

Statistical analysis

All data presented are mean ± S.D. or S.E.M. as indicated in the legend of each figure. Data of the morphometric analysis of spermatogenesis and of the quantification of fluorescence were statistically analysed using the Kruskal–Wallis non-parametric test followed by the Mann–Whitney U test (SPSS software, IBM Incorporation, Armonk, NY, USA). The qPCR data were analysed by one-way ANOVA followed by the Dunnett’s multiple comparison test, in which the Egfp-positive cells served as reference group, which was compared to either the Egfp-negative cells or the Egfp-positive cells after differential plating. Differences between groups were considered to be significant if P<0.05.

Results

Immunolocalization of Cyp19α1 and Cyp17α1 proteins in mature zebrafish testis

Using our specific anti-Cyp19α1 and anti-Cyp17α1 zebrafish antibodies, we localized for the first time the cellular sites of expression of the Cyp19α1a and Cyp19α1b enzymes in the zebrafish testis and confirmed and detailed the cellular sites of expression of the Cyp17α1 protein previously described (De Waal et al. 2009, Hinfray et al. 2011).

Until now, information on the identity of aromatase-producing cells in the fish testis was scarce. In our study, Cyp19α1a protein was detected in the cytoplasm of Leydig cells (Fig. 3A and B) and in all types of germ cells of zebrafish testis (Fig. 3A, C, D, E and F). Cyp19α1b protein was detected in germ cells of zebrafish testis but was absent from interstitial cells (Fig. 4). Immunoreactivity was found in the cytoplasm of spermatagonia (Fig. 4A and B), spermatocytes (Fig. 4A and C) and spermatids (Fig. 4A and D) while spermatozoa remained unlabeled (Fig. 4A and E).

Androgens also play an important role in spermatogenesis and Cyp17α1 is a key enzyme for androgen synthesis. Cyp17α1 protein was detected in both germ cells and interstitial cells (Fig. 5). Labelling representing Cyp17α1 protein was observed in the cytoplasm of Leydig cells (Fig. 5A and B), spermatagonia (Fig. 5A and C), spermatocytes (Fig. 5A and D), spermatids (Fig. 5A and E) and spermatozoa (Fig. 5A and F).

Expression of cyp19α1 and cyp17α1 genes in testicular germ cell-enriched fractions

Only germ cells express Egfp in vasa::egfp transgenic animals. Still, the Egfp-positive cell fraction isolated by FACS directly after preparing the cell suspension clearly contained Sertoli cells as shown by the high levels of gsdf expression (Fig. 2D). We hypothesized that this observation reflected a re-association of germ with Sertoli cells after preparing the cell suspension and until the start of the FACS procedure. We speculated that differential plating, in order to deplete somatic cells from the suspension, may reduce the contamination. Indeed,
2 days after differential plating, when FACS-harvesting Egfp-positive cells that floated freely or were only loosely attached, Sertoli cell marker gene expression was clearly reduced in the Egfp-positive fraction (Figs 1 and 2D). Further gene expression analysis showed that zebrafish cyp19a1a expression was equally found in the somatic (Egfp-negative) and germ cell-enriched fractions (Egfp-positive, floating or loosely attached cells sorted after differential plating) (Fig. 2D). A slight increase in cyp19a1a mRNA levels after reducing Sertoli cell contamination, similar to the change seen as regards piwil2 mRNA levels, supports the view that cyp19a1a is indeed expressed by germ cells also. Moreover, a clear signal for cyp19a1a expression in the Egfp-negative fraction (immediately sorted after preparing the cell suspension) and hence a pattern different from the Sertoli cell gene gsdf suggests that cyp19a1a expression in the germ cell-free fraction is largely attributable to Leydig cells.

cyp17a1 expression was detected in both somatic (Egfp-negative) and germ cell-enriched cell fractions (Egfp-positive sorted after differential plating; Fig. 2D). However, somatic cyp17a1 mRNA seems to dominate quantitatively over germ cell-associated expression and appears to be associated rather with Leydig than with Sertoli cells, as its expression pattern was similar to Leydig cell genes (insl3 and star) in the Egfp-negative fraction.

**Figure 3**
Immunolocalization of Cyp19a1a protein in testis of adult zebrafish (*Danio rerio*). (A) Representative micrograph (from n = 6 fish) of Cyp19a1a immunoreactive cells (red). Arrows indicate Leydig cells. Higher magnifications show that immunoreactivity was localized in the cytoplasm of Leydig cells (B), spermatogonia (C), spermatocytes (D), spermatids (E) and spermatozoa (F). sc, spermatocytes; sg, spermatogonia; st, spermatids; sz, spermatozoa. White: Hoechst staining. Scale bars = 50 μm (A) and 10 μm (B, C, D, E and F).

**Figure 4**
Immunolocalization of Cyp19a1b protein in testis of adult zebrafish (*Danio rerio*). (A) Representative micrograph (from n = 6 fish) of Cyp19a1b immunoreactive cells (red). Higher magnifications show that immunoreactivity was localized in the cytoplasm of spermatogonia (B), spermatocytes (C), spermatids (D) but not spermatozoa (E). White arrows show the localization of spermatogonia nucleus. *Sectioning artefacts filled with air bubbles. sc, spermatocytes; sg, spermatogonia; st, spermatids; sz, spermatozoa. White: Hoechst staining. Scale bars = 50 μm (A) and 10 μm (B, C, D and E).
Moreover, when reducing Sertoli cell numbers by differential plating, cyp17a1 mRNA levels increased in the germ cell-enriched fraction. The latter observation also shows that cyp17a1 mRNA is present in germ cells as well.

Effect of E2 exposure on spermatogenesis and Cyp19a1 and Cyp17a1 protein synthesis in mature zebrafish testis

In vivo exposure to 10 nM E2 for either 7 or 14 days had no effect on the body weight of exposed fish (data not shown), while a decrease in gonad weight and GSI was observed. The gonad weight significantly decreased from 9.4 ± 2.2 to 3.3 ± 0.3 mg after 14 days of E2 exposure and the GSI was significantly reduced from 1.8 ± 0.3 to 1.4 ± 0.1% after 7 days and from 2.2 ± 0.4 to 1.0 ± 0.0% after 14 days.

Quantitative morphometric analysis of zebrafish testis tissue samples collected after 7 and 14 days of E2 exposure revealed clear, statistically significant shifts in the relative proportion of the different germ cell types (Fig. 6A and B). After 7 days, a decrease in spermatocytes to 60% of the control levels was observed (Fig. 6A). At 14 days of E2 exposure, the main changes in the germ cell populations revealed by morphometric analysis were the increase in the mass of type A spermatogonia (w 175% of the control level) and the decrease in the mass of type B spermatogonia (w 32% of the control level), spermatocytes (w 11% of the control level), spermatids (w 25% of the control level) and spermatozoa (w 43% of the control level) (Fig. 6B).

We then examined the effect of E2 exposure on the testicular synthesis of Cyp19a1a and b and Cyp17a1 proteins. Immunohistochemistry experiments with anti-Cyp19a1 antibodies revealed that in control and E2-exposed fish testis (7 and 14 days), Cyp19a1a immunoreactivity was observed in Leydig cells (B), spermatogonia (C), spermatocytes (D), spermatids (E) and spermatozoa (F). sc, spermatocytes; sg, spermatogonia; st, spermatids; sz, spermatozoa. White: Hoechst staining. Scale bars = 50 μm (A) and 10 μm (B, C, D, E and F).
Moreover, evaluation of Cyp19a1a and Cyp19a1b fluorescence showed no statistically significant difference between control and E2-exposed fish whatever the duration of exposure (7 and 14 days) (Figs 7B and 8B).

Contrary to aromatases, E2 exposure (7 and 14 days) led to a strong inhibition of Cyp17a1 protein immunolabelling in zebrafish testis (Fig. 9A and B). Interestingly, our study revealed that this inhibition occurred only in Leydig cells and not in germ cells. Indeed, after 7 and 14 days of exposure, Cyp17a1 fluorescence decreased drastically in interstitial cells including Leydig cells from testis of E2-exposed fish (Fig. 9A and B).

**Discussion**

**Localization of Cyp19a1 and Cyp17a1 proteins in mature zebrafish testis**

In zebrafish, the two cyp19a1 genes were known to be expressed in the mature testis (Sawyer et al. 2006, Hinfray et al. 2011). Using immunohistochemistry experiment, we have localized here the cellular sites of expression of Cyp19a1 enzymes in the zebrafish testis. Cyp19a1a is expressed in both Leydig and germ cells, as shown by two independent techniques, i.e. immunocytochemistry and analysis of gene expression in FACS-sorted testicular cell fractions. Using immunohistochemistry, Cyp19a1b, on the other hand, was detected only in germ cells. In the serially sex changing gobiid fish, Trimma okinawae, expression of the cyp19a1a and cyp19a1b genes was detected in the testis by RT-PCR; in situ hybridization detected cyp19a1a mRNA in interstitial cells of the testis whereas cyp19a1b mRNA was not detected (Kobayashi et al. 2004). In sea bass, RT-PCR experiments revealed the presence of cyp19a1a in testicular germ cells (Vinas & Piferrer 2008). In rainbow trout, while some reported no aromatase immunoreactivity in the testis (Kobayashi et al. 1998), others showed aromatase immunolabelling in germ, Leydig and Sertoli cells, although the use of an antibody against human placental aromatase did not allow differentiating the two aromatase isoforms (Kotula-Balak et al. 2008). In different mammalian species, aromatase protein and enzymatic activity was
Effect of E₂ exposure on spermatogenesis and Cyp19a1 and Cyp17a1 protein synthesis in zebrafish testis

In this study, exposure of adult male zebrafish for 7 and 14 days to 10 nM E₂ led to alterations in spermatogenesis. Indeed, fish exposed to E₂ for 14 days presented testis with more type A spermatogonia and less type B spermatogonia, spermatocytes, spermatids and spermatozoa. The effects on the accumulation of spermatogonia type A and the reduced mass of spermatogonia type B, spermatocytes and spermatids were similar to findings in a previous study where zebrafish were exposed for 21 days to the same concentration of E₂ (De Waal et al. 2009). However, in the latter study, we did not observe a decrease in spermatozoa or others (i.e. somatic cells, blood vessels, hydration or in providing the proper environment for sperm maturation/conservation has been hypothesized in fish (Rolland et al. 2009).

Like Cyp19a1a, Cyp17a1 protein and gene expression was localized both in Leydig and germ cells of zebrafish testis, with a quantitative prevalence in Leydig cells. Other studies previously reported that Cyp17a1 is expressed in Leydig cells (Kobayashi et al. 1998, Zhou et al. 2007, De Waal et al. 2009, Hinfray et al. 2011). Recent studies also showed that the enzymes required for the production of the 11-oxygenated androgens typically found in fish (Cyp17 and Cyp11b) are present in male germ cells (Vinas & Piferrer 2008, Sreenivasulu & Senthilkumaran 2009, Zhang et al. 2010). Androgens are required for spermatogenesis ex vivo (Miura et al. 1991) or in vivo (De Waal et al. 2009), and recently, a role for the androgens also in mit
connective tissue and empty space). At present, we do not have an explanation for these differences but it cannot be excluded that certain differences in the histological analysis techniques (e.g. glutaraldehyde vs Bouin’s fixative; plastic vs paraffin embedding or area of cysts vs intersection grid for the calculation of volume fractions) may have contributed to the slight differences.

Immunohistochemistry experiments with anti-Cyp19a1 antibodies showed no evidence for a difference between control and E2-exposed fish whatever the duration of exposure (7 and 14 days). Short-term exposures of fathead minnows and medaka to oestrogens from 5 to 500 ng/l during either 7 or 14 days had no effect on cyp19a1a gene expression in the testis (Filby et al. 2006, Zhang et al. 2008). In zebrafish, an exposure for 7 days to 10 nM E2 had no effect on testicular cyp19a1a gene expression (Hinfray N & Brion F 2011, unpublished observations) whereas it strikingly inhibited ovarian cyp19a1a gene expression (Hinfray et al. 2006). For aromatase B, some studies showed that testicular cyp19a1b gene expression in fathead minnow increased following a 14-day-long exposure to oestrogens (Halm et al. 2002, Filby et al. 2006). In zebrafish, no effect was observed on testicular cyp19a1b gene expression after 5 days of exposure to 1 nM E2 (Sawyer et al. 2006), or after 7 days of exposure to 10 nM E2 (Hinfray N & Brion F 2011, unpublished observations). Neural cyp19a1b gene expression, on the other hand, is oestrogen sensitive in zebrafish, and it was only in glial or neuro-glial cells that stimulation of the zebrafish cyp19a1b gene promoter by oestrogens could be demonstrated (Diotel et al. 2010). Altogether, these reports are in agreement with the absence of effect of E2 on testicular aromatases in our study.

Contrary to aromatases, E2 exposure (7 and 14 days) led to a strong inhibition of Cyp17a1 protein immunolabelling in zebrafish testis. While this observation is consistent with the oestrogen-induced decrease in cyp17a1 mRNA levels reported in fish testis (Govoroun et al. 2001, Baron et al. 2005, Brion et al. 2008, Zhang et al. 2008, De Waal et al. 2009), our study moreover revealed that this inhibition occurred only in Leydig cells but not in germ cells. Indeed, after 7 and 14 days of exposure, a drastic inhibition of Cyp17a1 fluorescence was observed in Leydig cells from testis of E2-exposed fish. In this study, perturbations of spermatogenesis started to become evident after 7 days and were very clear after 14 days of E2 exposure. It was previously stated that oestrogen treatment induced an androgen insufficiency in zebrafish testis resulting in an interruption of spermatogenesis (De Waal et al. 2009). Indeed, in zebrafish, exposures to oestrogens for 7 days led to inhibition of cyp17a1 expression (Brion et al. 2008, De Waal et al. 2009) and a decrease in testicular 11-ketotestosterone production (De Waal et al. 2009) probably related to the disrupted spermatogenesis observed after 14 days of E2 exposure. The present results demonstrate that down-regulation of Cyp17a1 and hence of androgen synthesis occurs specifically in Leydig cells, thereby becoming a major molecular and cellular event in the oestrogen-induced perturbation of zebrafish spermatogenesis. Our findings also raise questions as regards the regulation of expression of steroidogenic enzymes in germ cells. It is unclear at present why Cyp17a1 synthesis was not down-regulated by E2 in germ cells, in contrast to Leydig cells. It can be hypothesized that only in Leydig cells there is an appropriate cellular context for transcriptional inhibition by E2. Another hypothesis is that E2 exerts a negative feedback on the brain/pituitary system, resulting in a reduced gonadotropin release and consequently a fading stimulation of cyp17a1 gene expression and Cyp17a1 synthesis in Leydig cells that, in contrast to germ cells, express receptors for both Fsh and Lh in fish (Garcia-Lopez et al. 2010).

Our study also raises questions as regards the functionality of the steroidogenic enzymes in germ cells. Steroid metabolizing enzyme activity in spermatozoa has been demonstrated in different vertebrates, ranging from mammals (for review, see Carreau (2001) and Carreau et al. (2006)) to fish (Sakai et al. 1989, Asahina et al. 1990, 1994). In zebrafish, the expression of both aromatases and Cyp17a1 in germ cells suggests that these cells can produce locally androgens and oestrogens de novo. However, we do not know whether germ cells possess all the steroidogenic enzymes allowing the entire steroidogenic process to occur locally or whether germ cell steroid production depends on the supply of steroidal substrates from other (probably Leydig) cells for androgen, and possibly also for the subsequent oestrogen production.

In summary, our study provides new data on the cellular localization and expression of Cyp19a1 and Cyp17a1 in the zebrafish testis. Cyp19a1b is synthesized only by germ cells (spermatogonia, spermatocytes and spermatids) while Cyp19a1a and Cyp17a1 are synthesized by Leydig cells and all types of germ cells. As regards Cyp17a1, both protein and mRNA seem to be quantitatively dominating in Leydig cells. Further, we demonstrate that E2 exposure had no effect on the synthesis of these proteins in germ cells but suppressed Cyp17a1 synthesis in Leydig cells after 7 and 14 days of exposure. As oestrogen-induced androgen insufficiency is associated with a Leydig cell-specific down-regulation of Cyp17a1,
the latter can be identified as a major event in altering testicular steroidogenesis and consequently spermatogenesis. The physiological predominance of Leydig cell expression of steroidogenic enzymes moreover suggests that germ cell steroid production may have to be fuelled by precursors from Leydig cells. Further studies are needed to elucidate the functionality of steroidogenic enzymes in germ cells and their potential role in the testicular physiology.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/JOE-12-0509.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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