Characterization of testicular expression of P450 17alpha-hydroxylase, 17,20-lyase in zebrafish and its perturbation by the pharmaceutical fungicide clotrimazole

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Full title: **Characterization of testicular expression of P450 17α-hydroxylase, 17,20-lyase in zebrafish and its perturbation by the pharmaceutical fungicide clotrimazole.**

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Short title: Cyp17 testicular expression and perturbation in the zebrafish

Abstract

The aim of the present study was to characterize P450 17α-hydroxylase/17,20-lyase (*cyp17a1*) expression in zebrafish and to assess the effect of the pharmaceutical clotrimazole, a known inhibitor of various cytochrome P450 enzyme activities , on testicular gene and protein expression of this enzyme as well as on the testicular release of 11-ketotestosterone (11-KT), a potent androgen in fish. We first showed that *cyp17a1* is predominantly expressed in gonads of zebrafish, notably in male. *In vivo*, clotrimazole induced a concentration-dependent increase of *cyp17a1* gene expression and Cyp17-I protein synthesis in zebrafish testis. Using zebrafish testicular explants, we further showed that clotrimazole did not directly affect *cyp17a1* expression but that it did inhibit 11-KT release. These novel data deserve further studies on the effect of azole fungicides on gonadal steroidogenesis.

Key words: zebrafish, steroidogenesis, *cyp17a1*, pharmaceutical, clotrimazole, testis culture

1. Introduction

Endocrine-disrupting chemicals (EDCs) represent a wide range of environmental contaminants that interfere with the endocrine system through multiple modes of action. During the last decades, many of synthetic chemicals present in the aquatic environment have been shown to interact as agonists with the estrogen receptor (ER) and to elicit biological responses similar to estradiol [38]. However, other mechanisms can account for endocrine disruption in aquatic organisms. Cytochromes P450 involved in the synthesis of steroid hormones are considered as important EDCs targets in vertebrates [8, 20]. Among them, cytochrome P450 17α-hydroxylase,17,20-lyase (Cyp17) is a key steroidogenic enzyme essential for cortisol production in adrenal tissues and for the production of sex steroids in gonadal tissues. The Cyp17 enzyme possesses both the 17α-hydroxylase and 17,20-lyase activities. The 17α-hydroxylase activity of Cyp17 converts pregnenolone to 17α-hydroxypregnenolone and progesterone to 17α-hydroxyprogesterone. The 17,20-lyase activity is required for the production of sex steroids since it cleaves the C17,20 bond to convert 17α-hydroxypregnenolone to dehydroepiandrosterone or 17α-hydroxyprogesterone to androstenedione, which is an important precursor for the production of estrogens and 11-oxygenated androgens. In rice field eel gonads, the *cyp17* gene generates four isoforms of Cyp17 protein by alternative splicing and polyadenylation processes [44]. In two fish species, tilapia and medaka, two Cyp17 protein isoforms (Cyp17-I and Cyp17-II) encoded by two different genes (*cyp17a1* and *cyp17a2*) were identified [45, 46]. The Cyp17-I isoform showed both the hydroxylase and lyase activities while the Cyp17-II isoform showed only the lyase activity [45]. An *in silico* search revealed the existence of two different *cyp17* genes in the genomes of fugu, stickleback, tetraodon, and zebrafish [45]. In catfish and zebrafish, full length cDNA encoding Cyp17 from ovary were cloned corresponding to the *cyp17a1* gene [36, 43]. In these fish species, *cyp17a1* was expressed predominantly in gonadal and adrenal

tissues but expression was also found in brain, liver, kidney, gills, heart, muscle, and intestine [36, 43].

Azole fungicides are widely used in agriculture, but also as antifungal agents in human and veterinary medicine, and some of them are used in the treatment of hormone-dependant cancer. Their antifungal activity is based on their ability to inhibit cytochrome P450 14α-demethylase activity, a key enzyme in the formation of fungal membranes. They also have been shown to inhibit other cytochrome P450 activities *in vitro*, including several P450 involved in steroidogenesis such as Cyp17 [4] and P450 aromatase activities [20, 28, 40]. Although the occurrence and fate of azole fungicides in the aquatic environment are poorly documented, several azoles such as clotrimazole, propiconazole, fluconazole or tebuconazole have been measured in surface waters of rivers, lakes and estuaries in several countries at concentrations ranging from the low ng/L to the low µg/L range [5, 21, 23, 30, 33, 37]. However, little is known about their *in vivo* endocrine disrupting potency in fish [6].

The aim of this study was first to characterize *cyp17a1* gene expression and Cyp17-I protein synthesis in zebrafish, and to assess the *in vivo* effects of clotrimazole, a pharmaceutical azole fungicide, in a model fish species the zebrafish. For that purpose, we first characterized the expression of *cyp17a1* gene in male and female zebrafish brain and gonads. For comparative purpose, expression of the specific isoforms of aromatase genes, *cyp19a1a* and *cyp19a1b*, was also analysed. Using specific polyclonal antibodies against zebrafish Cyp17-I, Cyp17-I protein was analyzed in gonads at different developmental stages. Then, the effects of clotrimazole on the testicular *cyp17a1* gene expression and Cyp17-I protein amounts were assessed both *in vivo* and *ex vivo* using a zebrafish testicular explant model. In addition, the effect of clotrimazole on the release of 11-ketotestosterone (11-KT), a potent androgen in fish, was assessed both *in vivo* and *ex vivo*.

2. Materials and methods

2.1. Fish origin and maintenance

Wild type larvae and adult zebrafish (AB strain) originated from our breeding unit (INERIS, Verneuil-en-Halatte, France). Adult zebrafish were maintained in 3.5 L aquaria in a recirculation system (Zebtec, Techniplast) on a 14:10 light:dark cycle at a temperature of 25.1 ± 1.0°C. They were allowed to reproduce (2 males for 1 female) at a temperature of 27°C, and fertilized eggs were harvested. Eggs were disinfected 5 minutes in water supplemented with 0.1 % of commercial bleach (2.6 % of sodium hypochlorite). Eggs/larvae were maintained in semi-static conditions until 8 days post fertilization (dpf) and then transferred in 3.5 L aquaria of the Zebtec system (around 25 larvae per litre) to grow. Larvae were fed around 20% of their weight per day with: protogen (once at 8dpf, Europrix, France); seramicron (7 to 21 dpf, Europrix, France); tetramin baby (21 to 45 dpf, Europrix, France); tetramin junior (45 to 60 dpf, Europrix, France) and living artemia (15 to 60 dpf).

2.2. Exposures of adult zebrafish

Exposures of adult male zebrafish to clotrimazole or solvent alone (DMSO, 0.004 % v/v) were realised in 4-L tanks for 7 days under semi-static conditions with a total renewal of the water every day. Two independent experiments were performed. In the first experiment, the effect of one concentration of clotrimazole (1.45 µM = 500 µg/L) on testis *cyp17a1* gene expression was assessed and compared to a control group. Exposure was performed in one tank per condition, each containing 10 male fish. In the second experiment, the effect of graded concentrations of clotrimazole (0.145; 0.290; 0.725 and 1.45 µM equivalent to 50,

100, 250 and 500 µg/L respectively) on testis *cyp17a1* gene expression and Cyp17-I protein synthesis was determined. Two replicated tanks, each containing 10 male zebrafish were used for each concentration.

At the end of the exposure period, fish were euthanized in ice cold water, measured and weighted. 5µl of blood were sampled, diluted in 45µl of phosphate buffer saline (PBS) solution containing 10% heparin and stored at -20°C until analysis. Gonads were removed, weighed, and the gonadosomatic index (GSI) was calculated as (gonad wet weight / total fish wet weight) × 100.

2.3. Determination of *cyp17a1*, *cyp19a1a* and *cyp19a1b* mRNA levels

After dissection, tissues were immediately stored at 4°C in RNA*later*™ (Sigma-Aldrich, France) (10mg of tissue / 300 µl) to stabilise and protect cellular RNA by immediate RNase inactivation. Samples were kept at 4°C overnight and stored at -20°C until mRNA level measurements.

Cyp17a1, *cyp19a1a* and *cyp19a1b* mRNA levels were measured by specific branched DNA assay (QuantiGene, Genospectra, Fremont, CA, USA) as previously described by [19]. Briefly, tissues were lysed and incubated in a 96-well plate coated with synthetic oligonucleotides in the presence of a specific probe set designed according to the *cyp17a1*, *cyp19a1a,* or *cyp19a1b* mRNA sequences (gene bank accession number AY281362.1, AF183906 and AF183908 respectively). Capture probe allowed capture of the target mRNA to the synthetic oligonucleotide. Blocking probe linearized the target mRNA and a labeled probe hybridized to the target mRNA and to a branched DNA (bDNA) coupled with alkaline-phosphatase-bound probes. Addition of a chemiluminescence substrate (dioxetan) yields a luminescence signal that is proportional to the amount of mRNA present in the sample.

Quantification of luminescence was made on a microplate luminometer (Wallac Victor2, Perkin Elmer, Courteboeuf, France). *Cyp17a1*, *cyp19a1a* and *cyp19a1b* expression values were normalized to a housekeeping gene, zf β-actin1 (gene bank accession number NM_131031), which has been shown to be stably expressed in zebrafish following chemical treatments [26]. Measurements of target and housekeeping genes were realized in triplicate for each gene and each sample.

2.4. Production of zebrafish Cyp17-I antiserum

Due to the lack of specific anti-zebrafish Cyp17 antibodies available, an antiserum was produced in rabbits. The antiserum was directed to the synthetic peptides AFADYSSTWKFHRK and KVRADWEKSPLMQHC coupled to keyhole limpet hemocyanin, corresponding to the amino acids 126-139 and 505-519 respectively of the zebrafish Cyp17-I sequence (AAP41821). Two rabbits were immunized by intradermic injection of 500µg purified synthetic peptides emulsified in Freund's complete adjuvant. Two booster injections of purified synthetic peptides in Freud's incomplete adjuvant were given three and six week followed by a subcutaneous injection eight weeks after the first injection. The rabbits were bled through the ear vein ten days after the last dose injection. The resulting antiserum was purified by affinity chromatography. The specificity of the zf-Cyp17-I antibody has been confirmed in western-blotting and immunohistochemistry experiments as recently reported by de Waal *et al*. [10].

2.5. Histological analysis of the testis

30, 40,60 and 180-dpf old zebrafish were euthanized in ice cold water. Samples were fixed in Bouin's fluid for 48 h at 4°C. After fixation, samples were dehydrated in ethanol and embedded in paraffin, according to conventional procedures. Samples were sectioned at 5 µm (longitudinal sections for juvenile fish and transversal sections for adult testis) and stained with Hemalun-Eosin or processed for fluorescent immunohistochemistry as described below. For immunohistochemistry, sections were mounted on gelatin coated slides.

2.6. Fluorescent immunohistochemistry

Cyp17-I labeling on zebrafish larvae were performed by fluorescent immunohistochemistry. Sections were dewaxed and rehydrated, and antigens were unmasked for 3 hours at 80°C in ethylenediaminetetraacetic acid buffer (pH 8.5). Tissue sections were then incubated for 1 hour in a saturation PBS solution containing 0.2% Triton X-100 and 1% milk powder. Incubation with the anti-zf Cyp17-I antibodies was performed overnight (1:300 with 0.5% milk powder in PBS) at room temperature. After rinsing, sections were incubated for 1h30 with a goat anti-rabbit antibody coupled to Alexa fluor 594 (1:200 with 0.5% milk powder in PBS). The specificity of the staining was controlled by processing adjacent sections without primary antibody, with the pre immune serum or with the antibody pre-absorbed with the synthetic peptides.

2.7. Colorimetric immunohistochemistry

Cyp17-I labelling on adult zebrafish testis of the second *in vivo* exposure was performed by colorimetric immunohistochemistry. Samples were fixed in PBS (pH 7.4) containing 4% of paraformaldehyde for 48 h at 4°C, and entirely processed for frozen sections

(12µm). Immunohistochemistry was performed as described previously [27] with some minor modifications. Briefly, endogenous peroxidase activity was blocked in 0.3% H_2O_2 in PBS for 45 minutes. Tissue sections were then incubated for 1 hour in a saturation PBS solution containing 0.2% Triton X-100 and 0.5% milk powder. Incubation with the zf-Cyp17-I antibody was performed overnight (1:300 with 0.5% milk powder in PBS). After rinsing, sections were incubated for 1h30 with a goat anti-rabbit IgG conjugated to horseradish peroxidase (1:1500). Cyp17-I immunoreactivity was revealed by using 3,3' diaminobenzydine as peroxidase substrate. As for fluorescent immunohistochemistry, all the specificity controls were included.

2.8. Primary culture of zebrafish testis

Zebrafish testes were cultured as previously described by Leal *et al.* [24] with some minor modifications. Briefly, male zebrafish were anaesthetized in ice-cold water and decapitated. The testes were removed, rinsed in PBS buffer (D-PBS+1mM $CaCl_2$ and $MgCl_2$, Invitrogen Ltd., CA, USA), then immersed for 2 minutes in PBS buffer supplemented with 0.5% of commercial bleach (2.6 % of sodium hypochlorite) and finally rinsed in PBS buffer for 2 minutes. The two testes of a zebrafish were incubated in parallel, one serving as control for the contra-lateral one. During the culture, testis explants were placed on a nitrocellulose membrane, itself resting on a 750 µl cylindrical agarose bloc (1.5% w/v prepared into Ringer's solution: 153.6mM NaCl, 3.08mM KCl, 5.04mM $CaCl_2$, 4mM $MgCl_2$, 10mM Hepes, 0.1% glucose, adjusted to pH 7.4) placed in 1 ml of culture medium in 24-well flat bottom culture plates (Corning Inc., New-York, USA). The culture medium (pH 7.4) consisted of Leibowitz' L-15 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10 mM Hepes (Merck,Darmstadt, Germany), 0.3 µg/ml amphotericine B (Fungizone, Invitrogen), 100 U/ml

antibiotics (penicillin/streptomycin, Invitrogen), 0.5% w/v Bovine Serum Albumin (fraction V) (Sigma-Aldrich, France), 10 nM retinoic acid (Sigma-Aldrich,St. Louis, MO, USA) and 50 ng/ml red sea bream IGF-1 (ProSpec-Tany TechnoGene Ltd, Israel). For *in vitro* exposures to contaminants, forskolin (FSK, 1 µM final concentration), clotrimazole (Clo; 0.2; 1 and 5 µM final concentration) or DMSO alone (0.017% final concentration) were added to the culture medium. Incubations were performed for 6 days at 25°C in a humidified air atmosphere and the medium was renewed once after 3 days. At the end of the exposure period, testes were transferred into RNAlater® solution (Sigma-Aldrich, France) for mRNA analysis and culture medium were stocked at -80°C until 11-ketotestosterone (11-KT) concentrations analysis.

2.9. 11-ketotestosterone analysis

The 11-KT concentrations were measured in blood samples and in the *ex vivo* culture medium by using a 11-KT Enzyme Immuno Assay Kit (Cayman Chemical Company, USA) performed according to the manufacturer's protocol. The specificity of the 11-KT EIA antiserum given by the manufacturer is as follow: 11-KT testosterone (100%), Adrenosterone (2.9%), 4-Androsten-11β,17β-diol-3-one (0.01%), 5α-Androstan-17β-ol-3-one (<0.01%), 5α-Androsten-3.beta,17.beta-diol (<0.01%) and Testosterone (<0.01%).

Dilution factors were comprised between 2 and 2700 depending on sample type (blood or culture medium) and treatment. Figure 1S demonstrates parallelism between the competition curves for blood and culture medium samples and the 11-KT standard curve, allowing accurate quantification of 11-KT concentrations in blood and culture medium.

2.10. Data analysis and statistics

For *in vivo* data, differences between groups were analysed for statistical significance with a Kruskall-Wallis test and a Mann-Whitney U test. For *ex vivo* data, differences between controls and exposed testes were analysed using a paired t-test. Results were expressed as mean ± standard deviation (SD), and differences between groups were considered to be significant if $p < 0.05$.

To document results of immunohistochemistry experiments, all micrographs were taken with a Zeiss Apotome Upright microscope with the Axiovision software. No alterations were made on the micrographs after taking the picture. All micrographs were analysed with image J software. For immunolabeling of Cyp17-I in the adult testis, the analysis reports the ratio between the labeled surface and the total surface of the testis.

3. Results

3.1. Zebrafish *cyp17a1* gene expression in gonads and brain of adult zebrafish

Analysis of the *cyp17a1, cyp19a1a and cyp19a1b* mRNA levels in the brain and the gonads of male and female were achieved by using specific bDNA assays. A low but significant expression of *cyp17a1* was found in the brain of adult male and female zebrafish without any obvious sexual dimorphism (Figure 1A). *cyp17a1* gene expression in the brain of male and female was at least 50-fold lower than *cyp19a1b*. In the ovary and testis, a strong expression of *cyp17a1* gene was measured (Figure 1B). As compared to *cyp19* genes, *cyp17a1* was predominantly expressed in the ovary and testis.

3.2. Immunolocalization of cells expressing Cyp17-I in the gonads

By means of immunohistochemistry, we further investigated the cellular localization of Cyp17-I protein within the gonads of zebrafish at various stages of their development, from histologically undifferentiated gonads to mature testis and ovary. As shown in Figure 2, no immunoreactive cell was observed in histologically undifferentiated gonads of 30 dpf juvenile zebrafish. At 40 dpf, only a few immunoreactive cells were found in early-differentiated ovaries while more immunoreactive cells were found in presumptive testis. In well-differentiated ovaries of 60 dpf zebrafish, we found expression of the Cyp17-I protein in follicular cells surrounding the oocytes. In 60 dpf zebrafish testes, strong immunolabelling was observed in interstitial cells.

3.3. *In vivo* effect of clotrimazole on testicular *cyp17a1* gene expression, Cyp17-I protein synthesis and on circulating 11-KT concentrations

After examining *cyp17a1* gene expression in untreated zebrafish, we assessed the *in vivo* effect of clotrimazole on its testicular expression in adult zebrafish. *In vivo* exposure to clotrimazole for 7 days had no significant effect on body weight, gonad weight, total length or GSI (Table 1). In a first experiment, zebrafish were exposed to a single dose of clotrimazole (1.45 µM). The results showed the ability of this compound to induce markedly *cyp17a1* gene expression by a factor 7 as compared to control group (Figure 3 A). In a second independent experiment, exposing zebrafish to graded concentrations of clotrimazole for 7 days, we measured a concentration-dependent increase in *cyp17a1* mRNA levels with a significant effect from the lowest concentration of clotrimazole tested (Figure 3 B), and with an induction in response to 1.45 µM clotrimazole that was similar to the one found in the first experiment.

We then determined the effect of clotrimazole on the Cyp17-I protein expression in testes. The analysis revealed that clotrimazole led to a strong increase of Cyp17-I immunoreactivity in interstitial cells (Figure 4). Image analysis of surfaces labeled by the Cyp17-I antibody normalized to the total surface of testis demonstrated that the increased expression of the Cyp17-I within the testicular tissue was dependent of the concentration of clotrimazole (Figure 5). At the histological level, spermatogonia type A were observed more frequently in two third of fish exposed to 1.45µM of clotrimazole (Figure 2S). However, future work has to be done to quantify this effect.

Finally, we assessed the effect of clotrimazole on the circulating 11-KT concentrations. In control fish, the mean concentration of circulating 11-KT was 9.9 ± 4.8 ng/ml (Table 1), which is in accordance with the level measured in zebrafish by Christianson-

Heiska et al. [9]. The 7 days exposure to clotrimazole revealed no significant effect on circulating concentration of 11-KT whatever the concentration (Table 1).

3.4. *Ex- vivo* effect of clotrimazole on *cyp17a1* expression and 11-KT synthesis in a primary testis culture system for zebrafish

To study possible direct effects of clotrimazole on zebrafish testicular *cyp17a1* expression and on the release of 11-KT, an *ex vivo* organ culture system for zebrafish testis was used [24]. In testicular tissue explants exposed *ex vivo* to 1 µM of the adenylate cyclase activator FSK (used as a positive control) for 6 days, a significant 3-fold increase of *cyp17a1* mRNA levels has been measured (Figure 6A). In contrast, clotrimazole (0.2, 1, 5 µM) had no effect on *cyp17a1* basal and FSK-induced gene expression in the testis explants culture system (Figure 6B and C). We showed that 1µM FSK clearly elevated the release of 11-KT into the culture medium from 58 ± 48 pg/ml in controls to 6415 ± 5842 pg/ml in FSK-exposed testes (Figure 7A). In contrast, a significant inhibition of the 11-KT release *ex vivo* was measured at 5 µM of clotrimazole (Figure 7B). At 1µM, clotrimazole had no effect on the basal 11-KT release (Figure 7B) while it significantly inhibited the FSK-induced 11-KT release (Figure 7C).

4. Discussion

4.1. *Cyp17a1 is predominantly expressed in the gonads of male and female zebrafish at various stages of development*

In this study, *cyp17a1* transcripts, as measured by the bDNA assay, were detected in both brain and gonad tissue of male and female zebrafish. These results are in agreement with previously reported *cyp17a1* gene expression measured in brain and gonads by RT-PCR in zebrafish [11, 43], fathead minnow [17], catfish [36] as well as in half-smooth tongue sole [7]. In the brain of adult zebrafish, we found that genes encoding the steroidogenic enzymes P450 17α-hydroxylase,17,20-lyase and P450 aromatase were expressed in both genders, without sexual dimorphism. Marked sex differences in expression levels were noticed with much higher levels of *cyp19a1b* as compared to *cyp17a1*. In contrast to mammals, the brain of teleosts is well known for its exceptional capacity to synthesise neuroestrogens that are produced locally in radial glial cells by the P450aromatase B (encoded by *cyp19a1b*), which is not only highly expressed during embryonic development but persists into adulthood [12, 42]. Apart from aromatase, little information is available as regards to the expression and activity of other steroidogenic enzymes in the central nervous system (CNS) of fish (for a review see [13]). Nonetheless, the findings of Cyp11a1 (P450 scc), 3β-hydroxysteroid dehydrogenase, 11β-hydroxysteroid dehydrogenase, 11β-hydroxylase and *cyp17* genes expression found in brain of fish [3, 7, 11, 12, 17, 36, 43, this study] argue for a local synthesis of steroids within the CNS, which is further supported by the finding that zebrafish brain has the ability to convert [3H]-pregnenolone into a variety of radiolabeled steroids [12]. Consistent with previous studies in zebrafish [43] and fathead minnow [17], a predominant expression of the *cyp17a1* gene was found in ovary and testis of adult zebrafish, suggesting that gonads are major expression sites of *cyp17a1*. In the present study, we further identified the localization of the cellular sites of Cyp17-I protein expression in ovary and testis and

characterized its expression at different stages of gonad development, using a specific anti-zebrafish Cyp17-I antiserum. In histologically undifferentiated gonads, Cyp17-I protein was not detected. We observed the onset of Cyp17-I expression in 40-dpf old zebrafish, when gonads start differentiating into ovary and presumptive testis. Then, the Cyp17-I protein expression increased markedly between 40 and 60 dpf, notably in testis. At adult stages, Cyp17-I expression was found in follicular cells around oocytes and in interstitial cells in the testis. On the one hand, the localization of expression sites of the Cyp17-I protein in ovary and testis is in agreement with that one reported in zebrafish and rainbow trout by means of immunohistochemistry [10, 22], and in medaka and tilapia by means of *in situ* hybridisation [45, 46]. On the other hand, the expression pattern of Cyp17-I contrasts markedly with that of aromatase (c*yp19a1a*) during gonad development of zebrafish. Indeed, it has been clearly demonstrated that the expression of *cyp19a1a* occurred when the gonads were undifferentiated, and that this expression persisted in the ovary but not in the testis [34]. While down-regulation of *cyp19a1a* appears crucial for testis differentiation in zebrafish [34], our results on Cyp17-I do not support a key role of this enzyme in testis differentiation. Interestingly, in the gonochoristic fish species rainbow trout, it has been found that the *cyp17a1* expression was not sexually dimorphic during early gonad differentiation, in contrast to *cyp19a1a* [41].

4.2. *Clotrimazole affects differently the zebrafish testicular steroidogenesis in vivo and ex vivo*

One major objective of our study was to assess the effect of clotrimazole on the testicular expression of the *cyp17a1*. To our knowledge, our results show for the first time that clotrimazole disrupts the expression of a key steroidogenic enzyme in the testes *in vivo* and the release of 11-KT, a specific potent 11-oxygenated androgen in fish, *ex vivo*. These effects

measured occurred at nominal concentrations of clotrimazole that are high as compared to the concentrations found in aquatic systems.

In vivo exposure to clotrimazole for 7 days caused a strong concentration-dependent up-regulation of expression of the *cyp17a1* gene and Cyp17-I protein in the testis. This suggests that clotrimazole treatment leads to a modulation of *cyp17a1* gene transcription and to *de novo* synthesis of the protein in interstitial cells of the testis. Immunohistochemistry experiments indicated that Cyp17-I increase is related, at least in part, to an increase of the immunolabeled surface and may indicate a hypertrophy or hyperplasia of Cyp17-I synthesizing cells as previously stated by Ankley *et al.* [2].

In search of a mechanism explaining the clotrimazole-induced effects at the testicular level, we studied a possible direct action of clotrimazole on testicular *cyp17a1* expression, using a recently developed tissue culture system for zebrafish testis explants [24]. In this *ex vivo* system, forskolin (1µM) up-regulated *cyp17a1* gene expression and the release of 11-KT in the culture medium, likely as a consequence of up-regulation of *cyp17a1* (and maybe other steroidogenesis-related genes) through the cAMP/PKA pathway [15, 24]. In contrast, clotrimazole had no effect on transcription of *cyp17a1*, after 6 days of *ex vivo* exposure. However, it inhibited both basal and FSK-induced biosynthesis of 11-KT, a potent androgen in fish. Previous studies have shown the ability of clotrimazole to inhibit *in vitro* several testicular steroidogenic P450 enzyme activities including 17α-hydroxylase and 17,20-lyase activities [4, 35]. Therefore, it is possible that inhibition of 11-KT release *ex vivo* reflects, at least partially, an inhibition of the Cyp17-I enzyme activity by clotrimazole as well as of other enzymes involved in steroid biosynthesis. The measurements of enzymatic activities of cytochromes involved in the biosynthesis of 11-KT would help to clarify the precise mode of action of clotrimazole.

Overall, this study demonstrates marked differential effect of clotrimazole on *cyp17a1* expression and biosynthesis of 11-KT depending on the biological model used. Such differences suggest that the clotrimazole-induced *cyp17a1* gene expression and protein synthesis are not due to a direct action of clotrimazole on the testes to regulate *cyp17a1* transcription and Cyp17-I protein synthesis. Similar to our study, ketoconazole exposure of fathead minnow increased testicular expression of several steroidogenesis-relevant genes, including *cyp17a1* [2, 39]. This increased activity of the steroidogenic system has been interpreted as a compensatory response of the feedback loop to the hypothalamus-pituitary level [1, 2] to the fungicide-mediated inhibition of the enzyme activities. However, a direct mechanistic link has not been established yet [39]. Such compensatory response might explain the absence of effect on circulating concentrations of 11-KT after *in vivo* exposure to clotrimazole that we observed in this study. Circulating levels of 11-KT result from the ability of clotrimazole to modulate cytochrome P450 enzymatic activity, including those involved in steroid synthesis [4, 28, 35] as well as those involved in steroid catabolism in fish [31]. Therefore, clotrimazole is expected to strongly affect several key steps of the metabolism of steroid hormones (biosynthesis, excretion and elimination) along the hypothalamus-pituitary-gonad axis, hence making difficult to predict the *in vivo* effect of this compound on circulating concentrations of 11-KT.

In addition, bioavailability and/or biotransformation of clotrimazole may vary *in vivo* and *ex vivo*, thus influencing its effect on target tissues. To our knowledge, no information is available on clotrimazole metabolism in fish but in rat, it has been shown that radiolabeled clotrimazole is rapidly metabolized and eliminated [14]. Cross-species comparisons of several conazole fungicide hepatic metabolites indicate a high degree of conservation among species [25]. Antifungal azoles are known to modulate expression and activities of various hepatic phase I (Cyp1A, CYP3A) and phase II (glutathione S-transferase) biotransformation enzymes

in mammals [4, 16, 35] and fish [18, 29]. In full-grown post-vitellogenic ovarian follicles of rainbow trout incubated *in vitro* with prochloraz, both *cyp1a* and *cyp3a* expressions were up-regulated [32]. Thus, it would be of interest to better characterize the xenobiotic biotransformation capacity of testicular tissue explants and also to compare metabolic transformation pathways of clotrimazole among *in vivo* and *ex vivo* bio-assays in order to determine to which extent it can explain the pattern of responses observed on testicular steroidogenesis in the model used.

5. Conclusion

Our study provides new and relevant data on the expression of *cyp17a1* in the zebrafish, notably by characterizing the spatio-temporal expression of Cyp17-I protein during gonad development. Further, we demonstrated for the first time the *in vivo* perturbation of transcription and protein synthesis of *cyp17a1* in testicular tissue of zebrafish exposed to clotrimazole. The marked differences observed between *in vivo* and *ex vivo* experiments suggest that clotrimazole does not act directly on testes to regulate *cyp17a1* transcription and protein synthesis, thus raising the need to conduct further work to explore the mechanisms of action of clotrimazole on steroidogenesis. In any case, using the testis tissue explant model, we did demonstrate a direct action of clotrimazole on the gonad resulting in an inhibition of 11-KT release. All together, these novel data deserve further studies on the mechanisms of action and effects of azole fungicides on gonadal steroidogenesis and their consequences on the reproductive physiology of fish to assess their ecotoxicological risks.

6. Conflict of interest

The authors have no conflict of interest.

7. Acknowledgements

The authors would like to express their acknowledgments to Dr. Alexis Fostier (INRA-Scribe, Rennes, France) for his critical reading and comments on the manuscript. We thank Emmanuelle Maillot-Maréchal and Benjamin Piccini (INERIS) for their excellent technical help in conducting laboratory exposures. This study was supported by the post-Grenelle P189-NEMO and Grant P189-DRC01-07 of the French Ministry of Ecology and Sustainable Development.

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