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

3

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27

28 **Short title: Cyp17 testicular expression and perturbation in the zebrafish**

1 **Abstract**

2

3 The aim of the present study was to characterize P450 17 α -hydroxylase/17,20-lyase
4 (*cyp17a1*) expression in zebrafish and to assess the effect of the pharmaceutical clotrimazole,
5 a known inhibitor of various cytochrome P450 enzyme activities , on testicular gene and
6 protein expression of this enzyme as well as on the testicular release of 11-ketotestosterone
7 (11-KT), a potent androgen in fish. We first showed that *cyp17a1* is predominantly expressed
8 in gonads of zebrafish, notably in male. *In vivo*, clotrimazole induced a concentration-
9 dependent increase of *cyp17a1* gene expression and Cyp17-I protein synthesis in zebrafish
10 testis. Using zebrafish testicular explants, we further showed that clotrimazole did not directly
11 affect *cyp17a1* expression but that it did inhibit 11-KT release. These novel data deserve
12 further studies on the effect of azole fungicides on gonadal steroidogenesis.

13

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

16

1 **1. Introduction**

2
3 Endocrine-disrupting chemicals (EDCs) represent a wide range of environmental
4 contaminants that interfere with the endocrine system through multiple modes of action.
5 During the last decades, many of synthetic chemicals present in the aquatic environment have
6 been shown to interact as agonists with the estrogen receptor (ER) and to elicit biological
7 responses similar to estradiol [38]. However, other mechanisms can account for endocrine
8 disruption in aquatic organisms. Cytochromes P450 involved in the synthesis of steroid
9 hormones are considered as important EDCs targets in vertebrates [8, 20]. Among them,
10 cytochrome P450 17 α -hydroxylase,17,20-lyase (Cyp17) is a key steroidogenic enzyme
11 essential for cortisol production in adrenal tissues and for the production of sex steroids in
12 gonadal tissues. The Cyp17 enzyme possesses both the 17 α -hydroxylase and 17,20-lyase
13 activities. The 17 α -hydroxylase activity of Cyp17 converts pregnenolone to 17 α -
14 hydroxypregnenolone and progesterone to 17 α -hydroxyprogesterone. The 17,20-lyase activity
15 is required for the production of sex steroids since it cleaves the C17,20 bond to convert 17 α -
16 hydroxypregnenolone to dehydroepiandrosterone or 17 α -hydroxyprogesterone to
17 androstenedione, which is an important precursor for the production of estrogens and 11-
18 oxygenated androgens. In rice field eel gonads, the *cyp17* gene generates four isoforms of
19 Cyp17 protein by alternative splicing and polyadenylation processes [44]. In two fish species,
20 tilapia and medaka, two Cyp17 protein isoforms (Cyp17-I and Cyp17-II) encoded by two
21 different genes (*cyp17a1* and *cyp17a2*) were identified [45, 46]. The Cyp17-I isoform showed
22 both the hydroxylase and lyase activities while the Cyp17-II isoform showed only the lyase
23 activity [45]. An *in silico* search revealed the existence of two different *cyp17* genes in the
24 genomes of fugu, stickleback, tetraodon, and zebrafish [45]. In catfish and zebrafish, full
25 length cDNA encoding Cyp17 from ovary were cloned corresponding to the *cyp17a1* gene
26 [36, 43]. In these fish species, *cyp17a1* was expressed predominantly in gonadal and adrenal

1 tissues but expression was also found in brain, liver, kidney, gills, heart, muscle, and intestine
2 [36, 43].

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

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

25

1 2. Materials and methods

2

3 2.1. Fish origin and maintenance

4

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

16

17 2.2. Exposures of adult zebrafish

18

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

1 100, 250 and 500 µg/L respectively) on testis *cyp17a1* gene expression and Cyp17-I protein
2 synthesis was determined. Two replicated tanks, each containing 10 male zebrafish were used
3 for each concentration.

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

9

10 2.3. Determination of *cyp17a1*, *cyp19a1a* and *cyp19a1b* mRNA levels

11

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

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

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

7

8 2.4. Production of zebrafish Cyp17-I antiserum

9

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

22

23 2.5. Histological analysis of the testis

24

1 30, 40,60 and 180-dpf old zebrafish were euthanized in ice cold water. Samples were
2 fixed in Bouin's fluid for 48 h at 4°C. After fixation, samples were dehydrated in ethanol and
3 embedded in paraffin, according to conventional procedures. Samples were sectioned at 5 µm
4 (longitudinal sections for juvenile fish and transversal sections for adult testis) and stained
5 with Hemalun-Eosin or processed for fluorescent immunohistochemistry as described below.
6 For immunohistochemistry, sections were mounted on gelatin coated slides.

8 2.6. Fluorescent immunohistochemistry

9
10 Cyp17-I labeling on zebrafish larvae were performed by fluorescent
11 immunohistochemistry. Sections were dewaxed and rehydrated, and antigens were unmasked
12 for 3 hours at 80°C in ethylenediaminetetraacetic acid buffer (pH 8.5). Tissue sections were
13 then incubated for 1 hour in a saturation PBS solution containing 0.2% Triton X-100 and 1%
14 milk powder. Incubation with the anti-zf Cyp17-I antibodies was performed overnight (1:300
15 with 0.5% milk powder in PBS) at room temperature. After rinsing, sections were incubated
16 for 1h30 with a goat anti-rabbit antibody coupled to Alexa fluor 594 (1:200 with 0.5% milk
17 powder in PBS). The specificity of the staining was controlled by processing adjacent sections
18 without primary antibody, with the pre immune serum or with the antibody pre-absorbed with
19 the synthetic peptides.

21 2.7. Colorimetric immunohistochemistry

22
23 Cyp17-I labelling on adult zebrafish testis of the second *in vivo* exposure was
24 performed by colorimetric immunohistochemistry. Samples were fixed in PBS (pH 7.4)
25 containing 4% of paraformaldehyde for 48 h at 4°C, and entirely processed for frozen sections

1 (12 μ m). Immunohistochemistry was performed as described previously [27] with some minor
2 modifications. Briefly, endogenous peroxidase activity was blocked in 0.3% H₂O₂ in PBS for
3 45 minutes. Tissue sections were then incubated for 1 hour in a saturation PBS solution
4 containing 0.2% Triton X-100 and 0.5% milk powder. Incubation with the zf-Cyp17-I
5 antibody was performed overnight (1:300 with 0.5% milk powder in PBS). After rinsing,
6 sections were incubated for 1h30 with a goat anti-rabbit IgG conjugated to horseradish
7 peroxidase (1:1500). Cyp17-I immunoreactivity was revealed by using 3,3' diaminobenzidine
8 as peroxidase substrate. As for fluorescent immunohistochemistry, all the specificity controls
9 were included.

10

11 2.8. Primary culture of zebrafish testis

12

13 Zebrafish testes were cultured as previously described by Leal *et al.* [24] with some
14 minor modifications. Briefly, male zebrafish were anaesthetized in ice-cold water and
15 decapitated. The testes were removed, rinsed in PBS buffer (D-PBS+1mM CaCl₂ and MgCl₂,
16 Invitrogen Ltd., CA, USA), then immersed for 2 minutes in PBS buffer supplemented with
17 0.5% of commercial bleach (2.6 % of sodium hypochlorite) and finally rinsed in PBS buffer
18 for 2 minutes. The two testes of a zebrafish were incubated in parallel, one serving as control
19 for the contra-lateral one. During the culture, testis explants were placed on a nitrocellulose
20 membrane, itself resting on a 750 μ l cylindrical agarose bloc (1.5% w/v prepared into
21 Ringer's solution: 153.6mM NaCl, 3.08mM KCl, 5.04mM CaCl₂, 4mM MgCl₂, 10mM Hepes,
22 0.1% glucose, adjusted to pH 7.4) placed in 1 ml of culture medium in 24-well flat bottom
23 culture plates (Corning Inc., New-York, USA). The culture medium (pH 7.4) consisted of
24 Leibowitz' L-15 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10 mM Hepes
25 (Merck,Darmstadt, Germany), 0.3 μ g/ml amphotericine B (Fungizone, Invitrogen), 100 U/ml

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

10

11 2.9. 11-ketotestosterone analysis

12

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

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

23

24 2.10. Data analysis and statistics

25

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

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

11

12

1 3. Results

2

3 3.1. Zebrafish *cyp17a1* gene expression in gonads and brain of adult zebrafish

4

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

12

13 3.2. Immunolocalization of cells expressing Cyp17-I in the gonads

14

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

24

1 3.3. *In vivo* effect of clotrimazole on testicular *cyp17a1* gene expression, Cyp17-I protein
2 synthesis and on circulating 11-KT concentrations

3
4 After examining *cyp17a1* gene expression in untreated zebrafish, we assessed the *in*
5 *vivo* effect of clotrimazole on its testicular expression in adult zebrafish. *In vivo* exposure to
6 clotrimazole for 7 days had no significant effect on body weight, gonad weight, total length or
7 GSI (Table 1). In a first experiment, zebrafish were exposed to a single dose of clotrimazole
8 (1.45 μ M). The results showed the ability of this compound to induce markedly *cyp17a1* gene
9 expression by a factor 7 as compared to control group (Figure 3 A). In a second independent
10 experiment, exposing zebrafish to graded concentrations of clotrimazole for 7 days, we
11 measured a concentration-dependent increase in *cyp17a1* mRNA levels with a significant
12 effect from the lowest concentration of clotrimazole tested (Figure 3 B), and with an induction
13 in response to 1.45 μ M clotrimazole that was similar to the one found in the first experiment.

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

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

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

3

4 3.4. *Ex- vivo* effect of clotrimazole on *cyp17a1* expression and 11-KT synthesis in a primary
5 testis culture system for zebrafish

6

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

19

1 **4. Discussion**

2 **4.1. *Cyp17a1* is predominantly expressed in the gonads of male and female zebrafish at** 3 ***various stages of development***

4 In this study, *cyp17a1* transcripts, as measured by the bDNA assay, were detected in both
5 brain and gonad tissue of male and female zebrafish. These results are in agreement with
6 previously reported *cyp17a1* gene expression measured in brain and gonads by RT-PCR in
7 zebrafish [11, 43], fathead minnow [17], catfish [36] as well as in half-smooth tongue sole [7].
8 In the brain of adult zebrafish, we found that genes encoding the steroidogenic enzymes P450
9 17α -hydroxylase, $17,20$ -lyase and P450 aromatase were expressed in both genders, without
10 sexual dimorphism. Marked sex differences in expression levels were noticed with much
11 higher levels of *cyp19a1b* as compared to *cyp17a1*. In contrast to mammals, the brain of
12 teleosts is well known for its exceptional capacity to synthesise neuroestrogens that are
13 produced locally in radial glial cells by the P450aromatase B (encoded by *cyp19a1b*), which
14 is not only highly expressed during embryonic development but persists into adulthood [12,
15 42]. Apart from aromatase, little information is available as regards to the expression and
16 activity of other steroidogenic enzymes in the central nervous system (CNS) of fish (for a
17 review see [13]). Nonetheless, the findings of Cyp11a1 (P450 scc), 3β -hydroxysteroid
18 dehydrogenase, 11β -hydroxysteroid dehydrogenase, 11β -hydroxylase and *cyp17* genes
19 expression found in brain of fish [3, 7, 11, 12, 17, 36, 43, this study] argue for a local
20 synthesis of steroids within the CNS, which is further supported by the finding that zebrafish
21 brain has the ability to convert [3H]-pregnenolone into a variety of radiolabeled steroids [12].
22 Consistent with previous studies in zebrafish [43] and fathead minnow [17], a predominant
23 expression of the *cyp17a1* gene was found in ovary and testis of adult zebrafish, suggesting
24 that gonads are major expression sites of *cyp17a1*. In the present study, we further identified
25 the localization of the cellular sites of Cyp17-I protein expression in ovary and testis and

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

19

20 **4.2. Clotrimazole affects differently the zebrafish testicular steroidogenesis *in vivo* and *ex*** 21 ***vivo***

22

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

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

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

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

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

19 In addition, bioavailability and/or biotransformation of clotrimazole may vary *in vivo*
20 and *ex vivo*, thus influencing its effect on target tissues. To our knowledge, no information is
21 available on clotrimazole metabolism in fish but in rat, it has been shown that radiolabeled
22 clotrimazole is rapidly metabolized and eliminated [14]. Cross-species comparisons of several
23 conazole fungicide hepatic metabolites indicate a high degree of conservation among species
24 [25]. Antifungal azoles are known to modulate expression and activities of various hepatic
25 phase I (Cyp1A, CYP3A) and phase II (glutathione S-transferase) biotransformation enzymes

1 in mammals [4, 16, 35] and fish [18, 29]. In full-grown post-vitellogenic ovarian follicles of
2 rainbow trout incubated *in vitro* with prochloraz, both *cyp11a* and *cyp3a* expressions were up-
3 regulated [32]. Thus, it would be of interest to better characterize the xenobiotic
4 biotransformation capacity of testicular tissue explants and also to compare metabolic
5 transformation pathways of clotrimazole among *in vivo* and *ex vivo* bio-assays in order to
6 determine to which extent it can explain the pattern of responses observed on testicular
7 steroidogenesis in the model used.

8

9 **5. Conclusion**

10 Our study provides new and relevant data on the expression of *cyp17a1* in the
11 zebrafish, notably by characterizing the spatio-temporal expression of Cyp17-I protein during
12 gonad development. Further, we demonstrated for the first time the *in vivo* perturbation of
13 transcription and protein synthesis of *cyp17a1* in testicular tissue of zebrafish exposed to
14 clotrimazole. The marked differences observed between *in vivo* and *ex vivo* experiments
15 suggest that clotrimazole does not act directly on testes to regulate *cyp17a1* transcription and
16 protein synthesis, thus raising the need to conduct further work to explore the mechanisms of
17 action of clotrimazole on steroidogenesis. In any case, using the testis tissue explant model,
18 we did demonstrate a direct action of clotrimazole on the gonad resulting in an inhibition of
19 11-KT release. All together, these novel data deserve further studies on the mechanisms of
20 action and effects of azole fungicides on gonadal steroidogenesis and their consequences on
21 the reproductive physiology of fish to assess their ecotoxicological risks.

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23 **6. Conflict of interest**

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25 The authors have no conflict of interest.

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