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A critical role of follicle-stimulating hormone (Fsh) in mediating the effect of clotrimazole on testicular steroidogenesis in adult zebrafish

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ABSTRACT

Clotrimazole is a pharmaceutical fungicide known to inhibit several cytochrome P450 enzyme activities, including several steroidogenic enzymes. This study aimed to assess short-term *in vivo* effects of clotrimazole exposure on blood 11-ketotestosterone (11-KT) levels and on the transcriptional activity of genes in pituitary and testis tissue that are functionally relevant for androgen production with the view to further characterize the mode of action of clotrimazole on the hypothalamus-pituitary-gonad axis in zebrafish, a model vertebrate in toxicology. Adult male zebrafish were exposed to measured concentrations in water of 71, 159 and 258 μg/L of clotrimazole for 7 days. Expression of pituitary gonadotropins β subunit (*lhb*, *fshb*), testicular gonadotropins receptors (*lhcgr*, *fshr*) and testicular steroidogenesis-related genes (*e.g.*, *star*, *cyp17a1*, *cyp11c1*) were assessed. Blood concentrations of 11-KT were measured. Short-term exposure to clotrimazole induced a concentration-dependent increase of *star*, *cyp17a1*, and *cyp11c1* gene expression and Cyp17a1 and Cy11c1 protein synthesis in Leydig cells, but androgen levels in blood remained unchanged. *fshb*, but not *lhb* mRNA levels in the pituitary tended to increase in clotrimazole-exposed zebrafish. Testicular expression of the Fsh receptor gene was significantly up-regulated following exposure, when expression of this receptor was significantly correlated to the expression of steroidogenesis-related genes. Moreover, the Fsh-regulated insulin-like growth factor 3 (*igf3*) gene, a fish-specific Igf peptide expressed in Sertoli cells, was induced in testes. By using a network of genes functioning in pituitary and testis tissue, our study demonstrated that clotrimazole induced a cascade of molecular and cellular events which are in agreement with a role for Fsh (1) in stimulating Leydig cell steroidogenesis to compensate the inhibitory action of clotrimazole on 11-KT synthesis and (2) in inducing the expression of Fsh-regulated *igf3* in Sertoli cells.

1. Introduction

Steroidogenesis is a multi-step process consisting of a series of enzymatic reactions that convert cholesterol to biologically active steroid hormones (Miller, 1988; Parker and Schimmer, 1995) that control a large array of important physiological functions (Miller, 1988). In vertebrates, gonadal steroidogenesis is under the control of the hypothalamus-pituitary-gonad (HPG) axis. The hypothalamus secretes gonadotropin-releasing hormones (GnRHs) that interact with their pituitary receptors (GnRH-Rs) to control synthesis and release of gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (Burns and Matzuk, 2002). Gonadotropins control steroidogenesis and gametogenesis through interaction with their gonadal G protein-coupled receptors, FSH receptor (FSHR) and LH receptor (LHR). Androgen production in the testis takes place in the steroidogenic Leydig cells that are situated between the seminiferous tubules close to blood vessels in the interstitial tissue of the testis. The regulation of Leydig cell androgen production is a typical domain of LH in all vertebrates. However, in fish, Leydig cells are also expressing the *fshr* gene and piscine Fshs have been shown to be potent steroidogenic hormones in several species (Garcia-Lopez et al., 2009, 2010; Ohta et al., 2007; Zmora et al., 2007). Hence, in fish Fsh is involved in regulating both, spermato- and steroidogenesis *via* the Fshr-expressing Sertoli and Leydig cells, respectively (Garcia-Lopez et al., 2009, 2010; Ohta et al., 2007; Schulz et al., 2010). Steroid hormones, in turn, exert positive or negative feedback on neuroendocrine circuits (Dopamine, GABA and GnRH neurons) that control the synthesis and release of gonadatropins (Zohar et al., 2010).

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Several chemical compounds belonging to the azole family alter gonadal steroidogenesis in fish leading to reproductive impairments (Ankley et al., 2007; Villeneuve et al., 2007a; Zhang et al., 2008). These compounds were designed to inhibit a cytochrome P450 (CYP) enzyme involved in ergosterol synthesis of fungi (Henry and Sisler, 1984). However, they can also inhibit other CYP enzyme activities, including steroidogenic cytochrome P450 17α-hydroxylase/17,20-lyase (CYP17) and aromatase (CYP19) in mammals and fish (Ayub and Levell, 1987a,b; Heneweer et al., 2004; Hinfray et al., 2006; Monod et al., 1993; Vinggaard et al., 2000). In addition, azole compounds have been shown to interact with different cytoplasmic or nuclear receptors such as aryl hydrocarbon receptor (Navas et al., 2004), pregnane X receptor (Lemaire et al., 2004; Moore and Kliewer, 2000) or androgen receptor (Eil, 1992; Lemaire et al., 2004; Moore and Kliewer, 2000; Navas et al., 2004; Vinggaard et al., 2002) and therefore can interfere with a broad range of physiological processes.

In vivo exposure of fish to prochloraz, ketoconazole or clotrimazole increased expression of steroidogenic genes in gonads (Ankley et al., 2007; Villeneuve et al., 2007a; Zhang et al., 2008). Recently, we have reported that in adult zebrafish clotrimazole affected testicular steroidogenesis differently *in vivo* as compared to an *ex vivo* testis tissue explant culture system (Hinfray et al., 2011). The data suggested that clotrimazole-induced 17α-hydroxylase/17,20 lyase (*cyp17a1*) expression was not due to a direct action on the testes to regulate *cyp17a1* transcription but could involve indirect actions at the pituitary level that may then alter the regulation of testicular steroidogenesis. Azoles are known to cause, directly and/or indirectly, endocrine disruption thereby affecting gene expression along the hypothalamic-pituitary-gonadal (HPG) axis (*e.g.*, Liu et al., 2011; Skolness et al., 2011; Zhang et al., 2008). However, the precise mechanism responsible for stimulating gonadal steroidogenesis has not been fully established yet.

In the present study we used zebrafish as a relevant model vertebrate for toxicology (Hill et al., 2005; Sipes et al., 2011) to investigate clotrimazole mode of action. Indeed, in addition to practicalities (small size, low cost, high fecundity, *etc.*), zebrafish physiology, genetics, and development under "normal conditions" have been studied in great detail and the species has also been used to investigate several types of toxicity from vascular toxicity to endocrine disruption (Sipes et al., 2011). For toxicology, advances in zebrafish genetics and genomics provide useful tools to investigate molecular mechanisms of toxicity of chemicals. Furthermore, conserved cell signaling pathways and development process between zebrafish and mammals, and global concordance between zebrafish and mammalian toxicity studies showed that the zebrafish model could be representative also for higher vertebrates (Sipes et al., 2011).

The aim of this work was therefore to assess the *in vivo* effect of short-term exposure to clotrimazole on androgen levels in blood and on the transcriptional activity of genes in pituitary and testes that are functionally relevant for androgen production (i) to characterize the mode of action of clotrimazole on the HPG axis and (ii) to investigate a potential role of gonadotropins in mediating the effect of clotrimazole on testicular steroidogenesis. For that purpose, male fish were exposed for 7 days to clotrimazole (71–258 μg/L). The rational for choosing these concentrations was based on previous experiments that showed up-regulation of *cyp17a1* gene expression in zebrafish testis after 7 days of exposure to clotrimazole (Hinfray et al., 2011). These are higher concentrations than those recently reported in waste water treatment plants (*e.g.*, Garcia-Valcarcel and Tadeo, 2011; Lacey et al., 2012). In the pituitary, changes in *lhb* and *fshb* transcript levels were measured. In testis tissue, expression of steroidogenic acute regulatory protein (*star*); hydroxyl-Δ-5-steroid dehydrogenase, 3β- and steroid Δ-isomerase 1 (*hsd3b1*); cytochrome P450, family 17, subfamily A polypeptide 1 (*cyp17a1*); cytochrome P450, family 11, subfamily C, polypeptide 1 showing a 11β-hydroxylase activity (*cyp11c1*; previously referred to as *cyp11b2*); hydroxysteroid (11-β) dehydrogenase 3a (*hsd11b3a*); cytochrome P450, family 19, subfamily A, polypeptide 1a (*cyp19a1a*); luteinizing hormone/choriogonadotropin receptor (*lhcgr*); follicle stimulating hormone receptor (*fshr*); were quantified. The Sertoli cell markers insulin-like growth factor 3 (*igf3*) and anti-müllerian hormone (*amh*) as well as the spermatogonial marker piwi-like 1 (Drosophila) (*piwil1*, previously referred to as *ziwi*) were also assessed.

2. Methods

2.1. Animals and treatments

The ethical committee of the National Institute of Industrial Environment and Risks (INERIS) approved all experiments. Mature wild type male zebrafish (*Danio rerio*, AB strain) were obtained from our breeding unit. Fish were raised under controlled photoperiod (14 h light/10 h dark cycle) in a recirculated water system (Techniplast, France) at 25 ± 1 °C. They were fed with TetraMin Pro® twice a day and live brine shrimp (*Artemia* spp.; Ocean Nutrition). Clotrimazole (CLO) was purchased from Sigma–Aldrich (St Quentin Fallavier, France) and all the stock solutions were prepared in dimethylsulfoxide (DMSO; Sigma–Aldrich).

Adult male zebrafish were exposed to three concentrations of clotrimazole or solvent alone (DMSO, 0.004% v:v) for 7 days under semi-static conditions with a total renewal of the water every 24 h. Each group contained 20 fish equally distributed in two replicate 4 L-glass tanks. Water samples were collected from each condition at day 5 at $t = 0$ h and $t = 24$ h before renewal of water.

2.2. Fish sampling

At the end of the exposure, fish were sacrificed in ice-cold water, measured and weighed. Blood (2.5 or 5 μL) was collected by cardiac puncture for each fish with a heparinized syringe (1000 U of heparin per ml) and transferred into Eppendorf tubes (held on ice) containing enzyme immunoassay (EIA) buffer supplemented with proteinase inhibitor phenylmethanesulfonylfluoride (PMSF, 1 mM) and heparin (1000 U/ml). Blood was diluted 1:10 (v:v) in this solution. Then, blood was vortexed, centrifuged (3000 g, 15 min, 4 °C) and stored at −20 °C until analysis. Liver and pituitary were removed and preserved in RNA later™ (Sigma-Aldrich, France) at −20 °C until analysis. Testes were removed and preserved in RNA later™ (Sigma-Aldrich, France) at −20 °C until analysis or fixed in Bouin's fluid. Testis weight was determined to calculate the gonado-somatic index (GSI, gonad wet weight/total body wet weight × 100).

2.3. Analytical chemistry for clotrimazole actual concentrations determination

Clotrimazole concentrations in exposure tanks were determined using solid phase extraction (SPE) followed by high-pressure liquid chromatography (HPLC) coupled to UV–Vis detection (λ = 194 nm). The extraction protocol was adapted from (Peschka et al., 2007). Briefly, water samples were collected from tanks (250 mL per condition) and adjusted to pH of 2. Then, samples were filtered under vacuum through SPE cartridges (Waters® OASIS-HLB, 6cc, 200 mg). Prior to extraction, the cartridges were conditioned with 6 mL of *n*-heptane, 6 mL of methanol, and 5 mL of ultrapure water (pH 2). After drying under vacuum for 90 min, clotrimazole was eluted with 3 × 1.5 mL of acetone. Finally, acetone extracts were evaporated to dryness under nitrogen gas and residues were redissolved in 1 mL of acetonitrile. Samples were analyzed using a Varian® HPLC system (Prostar 230 ternary pump, Prostar 420 autosampler, Prostar 9050 UV-Vis detector) based on a C18 column (Poursuit C18, 5 μM, 250 × 4.6 d.i., Varian®). Actual clotrimazole concentrations in exposure tanks have been determinated using external calibration based on four points calibration curve (0 to 100 μg/L). The limit of detection (LOD), obtained with signal/noise ratios equal to 3, was 1.5 μg/L. For each experiment, a 20 μg/L clotrimazole-spiked has been processed as quality control of the extraction procedure. The recovery of clotrimazole was higher than 90% in all the experiment.

2.4. Gene expression analysis

Total RNA was extracted from testis or pituitary using Trizol Reagent (Life Technologies Inc., Gaithersburg, MD) following manufacturer's instructions by using a fast tissue homogenizer (Precellys 24,). The mRNA quality was verified with a 1% agarose gel electrophoresis stained with Sybr Safe (Invitrogen). For each sample, the total RNA concentration was quantified using a Nanodrop ND-8000 spectrophotometer (Nanodrop Technologies, Wilmington, DE) and the samples were diluted to 0.5 μg/μL. DNA for the real-time PCR reactions was generated using a combination of random hexamers, M-MLV reverse transcriptase, 2.5 mM dNTP and RNAsin to avoid RNA degradation. The expression of target genes was analyzed using an Eppendorff realplex4 Mastercycler epgradient S (Eppendorf, France). Standard curves from 10^{-4}

to 10^{-11} μg/μL were created for each gene to determine the PCR efficiency and to obtain concentrations in μg/μL for each sample. Dilution of standards, mRNA and cDNA samples were prepared in RNAse-free water (Sigma–Aldrich). Every 25-μL cDNA amplification reaction contained 5 μL of diluted sample or standard, 15 μL of QuantiTect Sybr Green Mix (Qiagen, France) and 2 μL of each 400 nM forward and 400 nM reverse primers. Primer sequences are presented in the Table S1. The real-time PCR program included an enzyme activation step at 95 °C (15 min) and 40 cycles of 95 °C (5 s), 60 °C (30 s) and 72 °C (30 s). Determination of transcript abundance of genes was conducted in duplicate.

Normalization to total RNA in association with calibration to a gene-specific mRNA standard curve was employed in testes and pituitaries rather than normalization to "housekeeping" genes. Indeed, when we assayed "housekeeping" genes (*gapdh* and *elf1α*), we observed significant changes between treatments (data not shown). We assume that both normalization to "housekeeping" gene and to total RNA have limitations when changes in tissue composition occur in response to an exposure (Arukwe, 2006; Huggett et al., 2005; Villeneuve et al., 2007a) and "housekeeping" genes often simply rely on normalization to total RNA (Arukwe, 2006; Huggett et al., 2005).

2.5. Fluorescent immunohistochemistry

The antiserum raised against zebrafish Cyp17a1 has been described previously (Hinfray et al., 2011). The antiserum against Cyp11c1 was a generous gift of Yann Guiguen (INRA-Scribe, Rennes, France). The antiserum was produced in rats by co-injection of two synthetic peptides of the rainbow trout Cyp11c1 sequence (EMBL accession number: Q918S6). The first peptide (PWATHRETRQHSKGV) showed 93% identity with the zebrafish Cyp11c1 protein sequence (EMBL accession number: Q0P493) while no sequence identity was found between the second rainbow trout peptide (EKDGGKEERGHSLTI) and the zebrafish Cyp11c1 amino-acids sequence.

At the end of exposure, testes were fixed in Bouin's fluid for 48 h at 4 °C. After fixation, samples were dehydrated in ethanol, cleared in toluene and embedded in paraffin, according to conventional procedures. Samples were sectioned at 5 μm (longitudinal sections) and sections were mounted on gelatin-coated slides.

Then, Cyp17a1 and Cyp11c1 labeling on zebrafish testes were performed by fluorescent immunochemistry as described below. Sections from four or five fish per condition were used in the present study. Briefly, sections were dewaxed and rehydrated, and antigens were unmasked for 3 h at 80 °C in ethylenediaminetetraacetic acid buffer (pH 8.5). Tissue sections were then incubated for 1 h in a saturation PBS solution containing 0.2% Triton X-100 and 1% milk powder. Incubations with the anti-zebrafish Cyp17a1 antibody or the anti-rainbow trout Cyp11c1 antibody were performed overnight (1:300 with 0.5% milk powder in PBS) at room temperature. After rinsing, sections were incubated for 90 min with a goat anti-rabbit antibody (for Cyp17a1) or a goat anti-rat antibody (for Cyp11c1) coupled to Alexafluor 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.

2.6. 11-KT blood level determination

11-KT was quantified in blood samples by means of a competitive ELISA, following manufacturer's instructions (11-KT EIA Kit, Cayman Chemical Company, Ann Harbor, U.S.A.). The mean EC50 ± standard deviation was 8.6 ± 0.7 pg/mL ($n = 6$ independent experiments). The calculated coefficient of variation between assays was 8%, and the detection limit was 2.8 pg/mL ± 0.3 pg/mL. For the analysis, blood samples were assayed in triplicates with differential dilutions: 1:300; 1:900; 1:2700 in EIA buffer. To sum up the principle, acetylcholinesterase (AChE)-labeled 11-KT (secondary antibody) was added to the pre-coated wells with primary antibody anti-11-KT. A competition was established between 11-KT-AChE and natural 11-KT supplied by samples. The plate was then incubated overnight (18 h, 4 °C), washed 5 times with wash buffer and Ellman's reagent was added to the wells. Finally, the plates were developed on an orbital shaker for 75 min, before reading at 420 nm using a microtiter plate reader (EL340, Bio-Tek Instrument).

2.7. Data analysis

One-way analysis of variance (ANOVA) was performed to test for differences between treatments. Then, differences among treatments were determined using a *post hoc* test (Tukey Honestly Significant Difference). Normality of the data was previously assessed using a Shapiro test and homogeneity of variance was also verified using the Bartlett test. Non-normally distributed data were log-transformed prior to analysis and a non-parametric Kruskal–Wallis test, followed by a multiple comparison test, was used when data did not meet parametric assumptions. Furthermore, non-parametric Spearman rank correlation tests were applied to test correlations among expression levels of different testicular genes for each condition.

Levels of gene expressions were expressed as fold changes relative to the average value of the control. Statistical analyses were conducted using R™ (R 2.13.1, software, R development Core Team). All data are presented as mean ± standard error to the mean (SEM) except for biometrical parameters that are presented as mean ± standard deviation (SD). Significance level (p) was fixed at 0.05 ($p < 0.05^{*}$; $p < 0.01^{**}$; $p < 0.001^{***}$).

Table 1
Clotrimazole concentrations measured in tank at $t = 0$ h and $t = 24$ h.

	SC	Low	Medium	High
Measured (μg/L) $t = 0$ h	<LOD	71	159	258
Measured (μg/L) $t = 24$ h	<LOD	55	149	229

SC: solvent control, LOD: limit of detection.

3. Results

3.1. Water chemistry

Water concentrations of clotrimazole measured at T0 (day 5) in the clotrimazole-treated tanks were 71, 159 and 258 μg/L for the low, medium, and high contaminations, respectively (Table 1). After 24 h, concentrations declined by 22%, 6% and 11% of the concentrations measured at T0 respectively. Control water samples were all below the limit of detection; *i.e.* 1.5 μg/L.

3.2. Biometrical parameters

There was no clotrimazole-induced mortality or any observation of abnormal behavior during the study. Furthermore, no significant differences were observed between clotrimazole-contaminated fishes and controls in body weight, length or GSI (Table 2).

3.3. 11-KT concentrations in blood

Circulating concentrations of 11-KT were quantified in control and exposed-fish. In control fish, the mean measured concentration was 9.6 ± 5.5 ng/mL, which agrees with previously reported data in adult male zebrafish (Brown et al., 2011; Hinfray et al., 2011). In controls, 11-KT concentrations ranged between 6.4 and 21.8 ng/mL. Although there was no significant difference between control and clotrimazole-exposed fish (Fig. 1), we noted an increased inter-individual variation in the group exposed to the highest concentration of clotrimazole: 2 of 20 fish had concentrations of 0.36 and 1 ng/mL and 5 of 20 fish had elevated concentrations of 11-KT between 25.1 and 33.7 ng/mL.

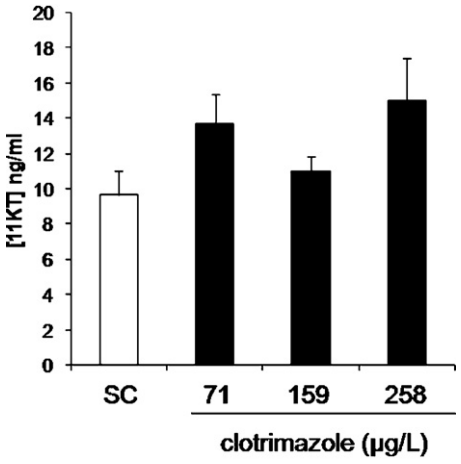

Fig. 1. *In vivo* effect of clotrimazole on circulating 11-KT concentration following a 7 days exposure. Data are represented as mean ± SEM and expressed in pg/ml ($N = 16$–20 fish per condition). Each blood sample was analyzed in triplicate. 11-KT = 11-ketotestosterone.

Table 2
Fish biometrical parameters after the 7 days exposure.

Group	*N*	Body weight (mg)	Gonad weight (mg)	Body length (cm)	GSI (%)
Solvent control	20	475 ± 69	8.1 ± 2.6	3.8 ± 0.2	1.7 ± 0.4
Clotrimazole low	20	416 ± 87	6.3 ± 2.8	3.8 ± 0.2	1.6 ± 0.3
Clotrimazole medium	18	448 ± 81	7.0 ± 2.8	3.8 ± 0.2	1.5 ± 0.4
Clotrimazole high	20	492 ± 86	7.9 ± 2.7	3.9 ± 0.2	1.7 ± 0.3

GSI = Gonado somatic index.

3.4. Expression of steroidogenesis-related genes

Transcriptional levels of genes involved in gonadal steroidogenesis were determined by Q-PCR and compared in control and clotrimazole-exposed groups.

4 of the 6 steroidogenic genes measured were up-regulated in a concentration-dependent manner (Fig. 2). A 1.7-fold induction was observed for *star* transcript levels at 159 μg/L ($p < 0.01$) and 258 μg/L ($p < 0.05$). The 3 other over-expressed genes belonged to the CYP family. Among them, *cyp17a1* was induced most clearly, with a significant up-regulation at the lowest concentration ($p < 0.001$) and a 3-fold induction at 159 ($p < 0.001$) and 258 μg/L ($p < 0.001$). For *cyp11c1*, a significant 2-fold increase was measured at 159 μg/L and 258 μg/L ($p < 0.001$, $p < 0.001$ respectively). *Star*, *cyp17a1* and *cyp11c1* showed similar patterns of expression and their expression was significantly correlated in control and clotrimazole-exposed groups (Table S2). Interestingly, the correlation between steroidogenic genes was stronger in all clotrimazole-exposed groups than in the control group (Table S2). *Cyp19a1a* was also significantly (2-fold) up-regulated ($p < 0.05$) but only at the highest concentration of clotrimazole.

3.5. Cyp17a1 and Cyp11c1 immunostaining in testes

The effect of clotrimazole on Cyp17a1 and Cyp11c1 protein expression was examined by fluorescent immunohistochemistry in testis sections using specific polyclonal antibodies (Fig. 3). We observed a strong increase of Cyp17a1 and Cyp11c1 protein expressions that both were localized in Leydig cells. Pictures presented in Fig. 3 are representative of all individuals for each condition. Cyp17a1 immunostaining was induced from 71 μg/L to 258 μg/L (Fig. 3A) and Cyp11c1 was clearly induced at the highest concentration of clotrimazole (Fig. 3B).

3.6. Expression of pituitary gonadotropins and their gonadal receptors

An 8- and 6-fold up-regulation of pituitary *fshb* was observed at 159 μg/L and 258 μg/L, respectively (Fig. 4A). Still, statistical significance was not reached, probably due to the high variability of transcript levels, in particular in the clotrimazole-exposed groups.

In testes, *fshr* was up-regulated 1.65-fold in the 159 μg/L clotrimazole-exposed group ($p < 0.01$), whereas no effect was observed for *lhcgr* transcripts levels (Fig. 4B). Interestingly, significant and strong correlations were found between *fshr* expression and either *star*, *cyp17a1* or *cyp11c1* in clotrimazole-exposed groups, whereas such correlations were not found in the control group (Fig. 5, Table S2).

3.7. Expression of insulin-like growth factor 3 (igf3) and anti-müllerian hormone (amh)

The data presented above showed that Leydig cell genes related to steroidogenesis were affected by clotrimazole. To determine whether genes expressed in Sertoli cells and known to be regulated by Fsh respond to clotrimazole exposure *in vivo*, we quantified *amh* and *igf3* mRNA levels. While the exposure had no effect on *amh* expression, significantly increased *igf3* mRNA levels (3-fold induction) were found at the intermediate clotrimazole concentration of 159 μg/L ($p < 0.05$, Fig. 6).

4. Discussion

In this study, we investigated the effects of clotrimazole on the expression and function of genes operating at crucial sites within the pituitary-gonad axis in adult male zebrafish. Our data demonstrate that clotrimazole induced the expression of steroidogenic enzymes in Leydig cells (mRNA and protein level) that are required for the production of the 11-oxygenated androgens typically found in fish (like 11-KT), while circulating androgen concentrations were not altered. Measurement of transcript levels of pituitary *fshb*, testicular *fshr* and the significant correlations between *fshr* and expression of steroidogenesis-related genes suggest that gonadotropins, in particular Fsh, are involved in the clotrimazole-induced changes in testicular physiology. Additional evidence for an increased signaling *via* Fsh/Fshr is provided by the induction of *igf3*, a gene expressed by Sertoli cells and up-regulated by Fsh (Nobrega, Morais, de Waal, Bogerd and Schulz; unpublished data). This data set highlights the importance of studying expression and functioning of key genes of the pituitary-gonad axis to start elucidating the mode of action of clotrimazole on the endocrine system of fish.

4.1. Clotrimazole-induced expression of testicular genes related to steroidogenesis

In the present study, short-term *in vivo* exposure of zebrafish to clotrimazole led to over-expression in zebrafish testis of *star* and steroidogenic enzymes crucial for fish androgen production, *i.e. cyp17a1* and *cyp11c1*. Interestingly, expression of these genes was strongly correlated in clotrimazole-treated groups, suggesting an orchestrated response to clotrimazole (Fig. 2, Table S2). By means of immunohistochemistry, we further showed an increased of Cyp17a1 and Cyp11c1 protein levels in Leydig cells, suggesting *de novo* synthesis of these enzymes. This is in agreement with previously published data on the effect of clotrimazole on *cyp17a1* expression in zebrafish (Hinfray et al., 2011) and extends the findings to another steroidogenic enzyme. Even though clotrimazole concentrations used to trigger *in vivo* changes in testicular steroidogenesis-related genes are higher than concentrations found in the aquatic environment (Garcia-Valcarcel and Tadeo, 2011; Lacey et al., 2012), it should be stressed that clotrimazole can potentially bioaccumulated in fish given its lipophilic nature (OSPAR, 2005; Schulz et al., 2012).

Induction of testicular steroidogenic gene expression following exposure to other azole fungicides (ketoconazole and prochloraz) has been reported in fathead minnow and medaka (Ankley et al., 2007; Zhang et al., 2008). Interestingly, in fathead minnow exposed to flutamide, an androgen receptor antagonist, *cyp17a1* and *hsd11b3a* were induced in testes (Filby et al., 2007). Induction of steroidogenic genes in testes of flutamide-exposed fathead minnow has been interpreted as an inhibitory action on endogenous

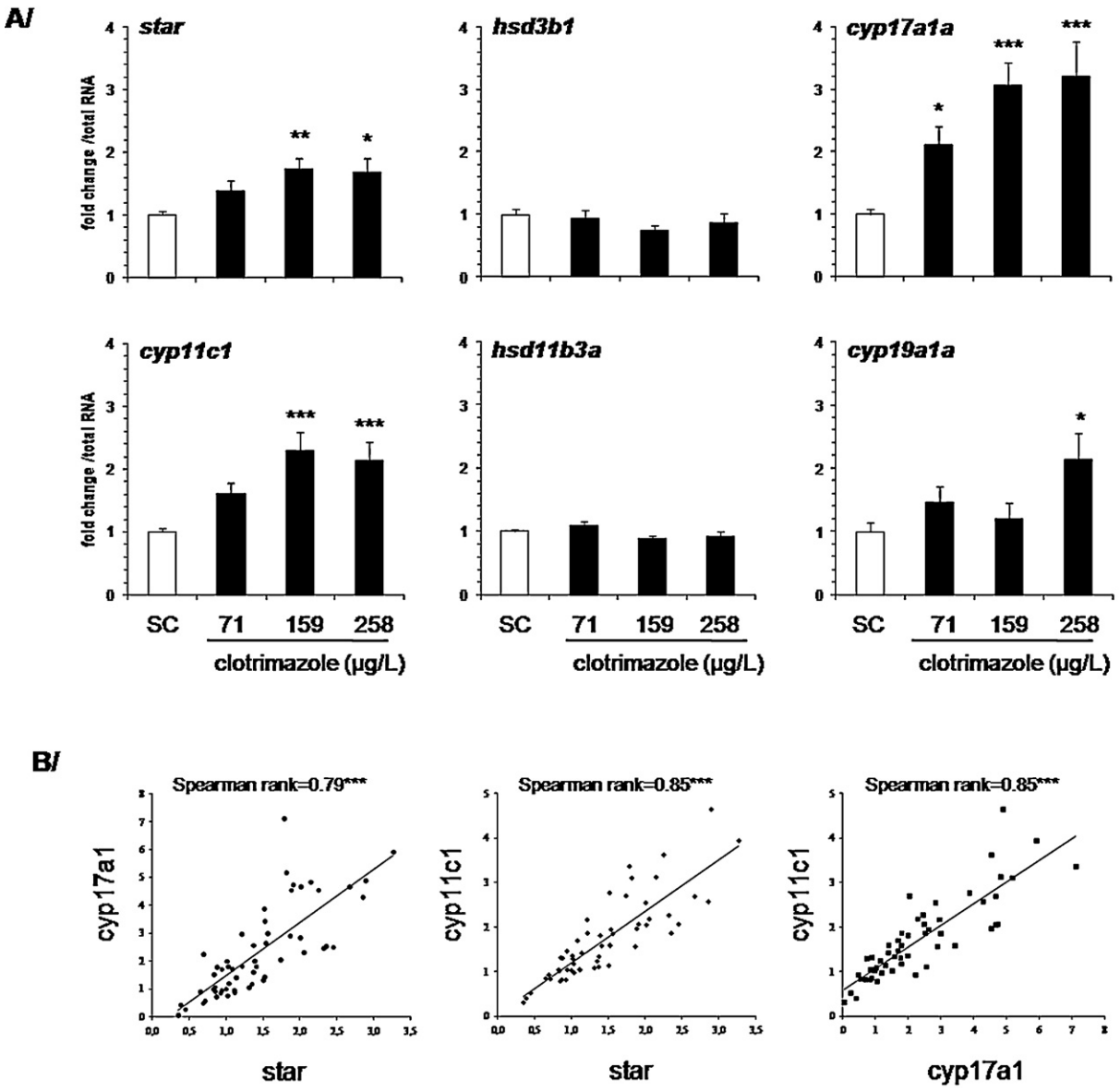

Fig. 2. (A) *In vivo* effect of clotrimazole on key testicular gene expressions following a 7 days exposure. Data are represented as mean ± SEM and expressed as fold change in mRNA expression from the control. Relative mRNA expression was determined as the ratio of target gene mRNA/2 µg total RNA (N = 12–19 fish per condition). Each sample was analyzed in duplicate. (B) Correlation between *star*, *cyp17a1* and *cyp11c1* expressions in control group and clotrimazole-exposed groups. Spearman rank number is indicated. Asterisks indicated a significant difference compared to control group ($^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$).

androgen negative feedback pathways (Filby et al., 2007). While prochloraz and ketoconazole behaved also as androgen receptor agonists (Eil, 1992; Vinggaard et al., 2002), clotrimazole did not bind to AR in mammals (Ayub and Levell, 1989; Eil, 1992); information as regards the possible binding to piscine ARs is not available. Therefore, it is unlikely that induction of steroidogenic genes in zebrafish exposed to clotrimazole involve an inhibitory action of endogenous androgens. On the contrary, the primary mode of action of clotrimazole on testicular steroidogenesis is through the inhibition of cytochrome P-450 dependent steroidogenic enzyme activities. Indeed, previous studies reported that clotrimazole inhibited ovarian P450 aromatase and testicular P450 Cyp17 17α-hydroxylase and 17,20 lyase enzyme activities in *in vitro* microsomal assays at concentrations ranging from 0.01 µM to 1.7 µM depending on the model used and the enzyme activity assessed (Ayub and Levell, 1987a,b; Hinfray et al., 2006; Monod et al., 1993; Schuster, 1985). We assume that this inhibitory action on cytochrome P-450 dependent steroidogenic enzymes explains, at least partially, the inhibition of 11-KT production that we measured previously in the culture medium of zebrafish testicular explants after a 6-days *in vitro* exposure to 1 µM (equivalent to 344.8 µg/L) clotrimazole (Hinfray et al., 2011). These data, demonstrating the *in vitro* inhibitory action of clotrimazole on 11-KT production, seemingly contrast with *in vivo* results showing that 11-KT blood concentrations were not affected in males after a comparable time (7 days) and concentration (258 µg/L) of exposure to clotrimazole, as reported herein and in our previous work (Hinfray et al., 2011). The *in vivo* over-expression of steroidogenic genes has been interpreted as a compensatory response to inhibiting enzyme activities (Ankley et al., 2007; Hinfray et al., 2011; Villeneuve et al., 2007b; Zhang et al., 2008) and would also explain that no significant effect of clotrimazole on 11-KT blood concentrations was measured (this study, (Hinfray et al., 2011). In this setting we assume that clotrimazole initially induced a decrease of testicular androgen output and hence a depression of circulating androgen levels, which triggered the compensatory response, resulting in an

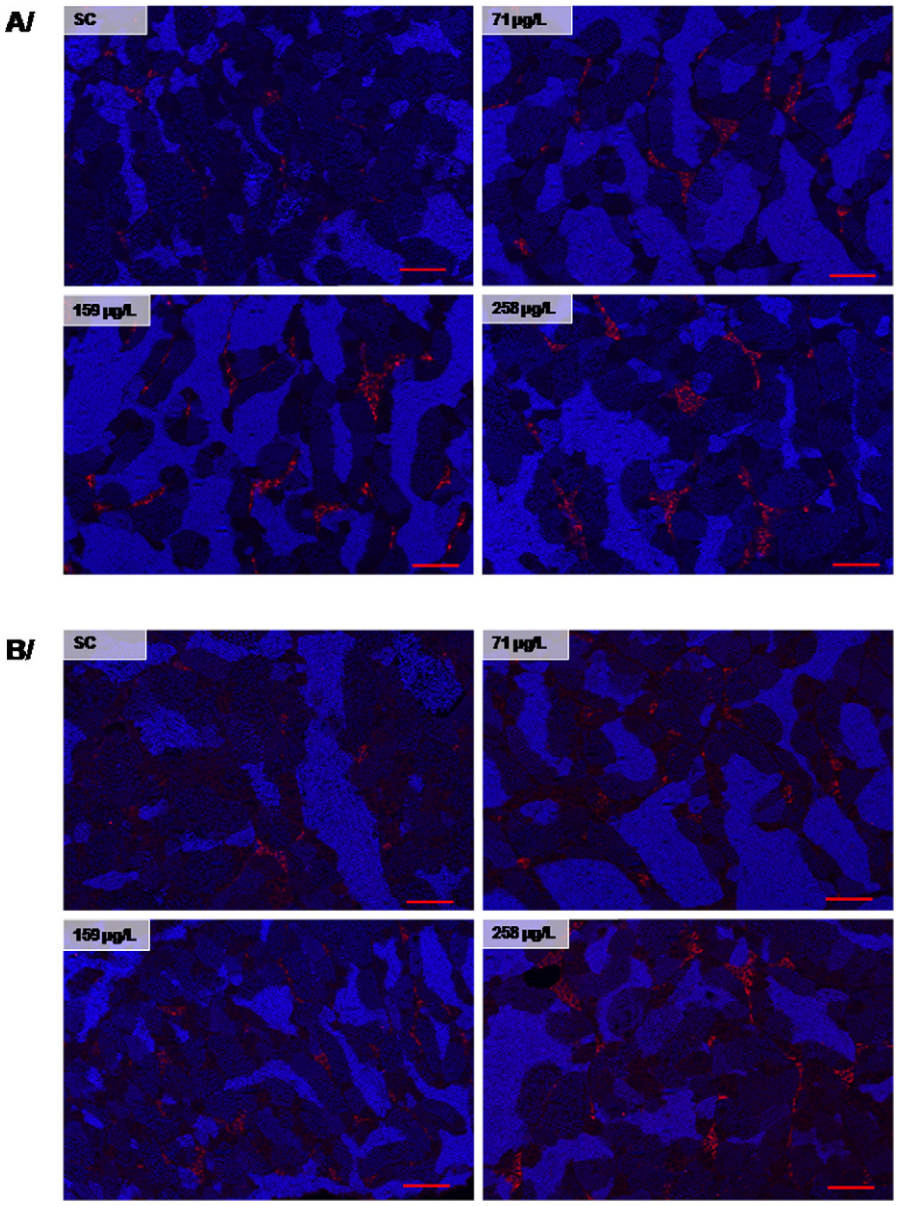

Fig. 3. (A) Cyp17-I and (B) Cyp11c1 labeling on zebrafish testes by fluorescent immunohistochemistry following a 7 days exposure to clotrimazole. Immunostaining was observed in all individuals and localized in Leydig cells ($N = 4–5$ fish per condition). The present pictures are representative of all individuals. Red: P450c17 and P450c11B immunostaining, blue: Hoechst. Scale bars represent 50 μm (red). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

increased *de novo* synthesis of steroidogenic enzymes, allowing to attain normal circulating androgen levels. Such transient depression of circulating 11-KT was recently reported after 1 and 4 days of exposure of fish to 30 and 300 μg/L of ketoconazole while 11-KT concentrations return to a normal level after 8 days (Ankley et al., 2012). Physiologically, a transient decrease in circulating androgen levels seems a signal similar to reduced signaling *via* the AR, which may explain the similarity of the response to the AR antagonist flutamide and to clotrimazole. However, it should be noted that high inter-individual variability was observed in the group of fish exposed to the highest concentration of clotrimazole, perhaps reflecting that the physiological limits of compensation have been reached. For instance, some fish had very high levels of 11-KT (overcompensated), while others had very low levels. In the latter, up-regulation of enzyme expression may be insufficient to balance the pharmaceutical's inhibitory effect on enzyme activity.

4.2. The effect of clotrimazole on testicular steroidogenesis is likely regulated by pituitary follicle-stimulating hormone

To support the hypothesis that the increased activity of the steroidogenic system may be due to a compensatory response of feedback loops within the HPG-axis to overcome the fungicide-mediated inhibition of enzyme activities, we studied the expression of genes encoding for gonadotropin β-subunits as well as the testicular expression of gonadotropin receptors. At the pituitary level, induction of genes encoding for the β-subunit of Fsh, but not of Lh, was observed in clotrimazole-exposed fish. However, probably due to the variability of expression between individuals within groups, these effects did not reach statistical significance. Concomitantly, a significant up-regulation of the *fshr*, but not the *lhcgr*, gene expression was measured in testes of clotrimazole-exposed fish. Moreover, *fshr* expression was strongly correlated with the expression of testicular genes related to steroidogenesis in

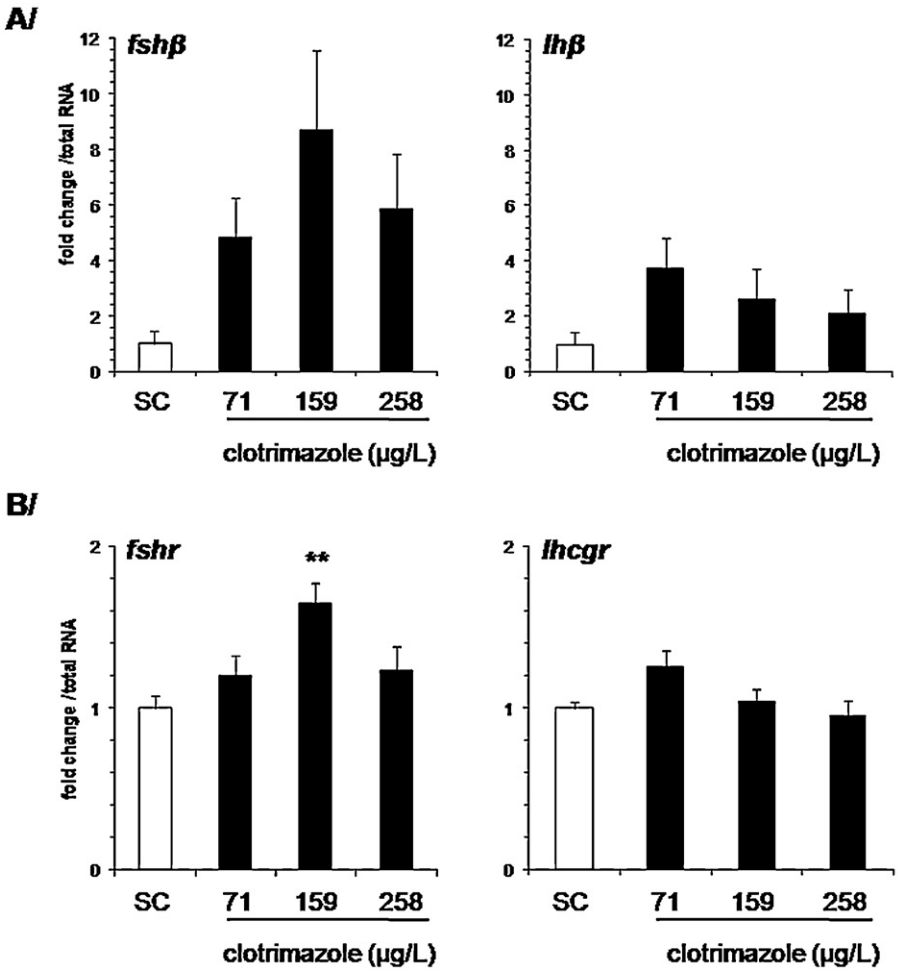

Fig. 4. *In vivo* effect of clotrimazole on (A) pituitary gonadotropins β subunit and (B) testicular gonadotropin receptors gene expressions following a 7 days exposure. Data are represented as mean ± SEM and expressed as fold change in mRNA expression from the control ($N = 11–19$ fish per condition). Relative mRNA expression was determined as the ratio of target gene mRNA/2 µg RNA. Each sample was analyzed in duplicate. Asterisks indicated a significant difference compared to control group ($^{*}p \leq 0.05$, $^{**}p \leq 0.01$).

clotrimazole-exposed groups (Figure 5, Table S2). As regards *lhcgr* mRNA levels, no clear correlation with steroidogenic genes was found. In agreement with these findings, it has been shown that Fsh is a potent steroidogenic hormone in fish, acting through the Fshr, which in fish is also expressed by Leydig cells. In Japanese eel and zebrafish, recombinant Fsh, but not Lh, induced *star* and *cyp17a1* gene expressions in testicular explants system (Garcia-Lopez et al., 2010; Kazeto et al., 2008). Activation of Fsh-/Fshr signaling pathway in response to a decrease in androgen production is further illustrated by a recent work in which unilateral gonadectomy in male African catfish lead to induction of *fshr* gene expression and steroid production capacity of the remaining testis. In consequence, the final concentration of androgens was unchanged (Schulz et al., 2012).

Taken together, these data suggest that Fsh-/Fshr-signaling played an important role in mediating the clotrimazole-induced compensatory response of the steroidogenic Leydig cells. Interestingly in mammals, treatment of male with azole compounds such as anastrozole and letrozole led to increase concentrations of circulating gonadatropins and testosterone (de Boer et al., 2005; Goetz et al., 2009; Turner et al., 2000). This supports the view that disruption of the HPG axis by azoles is well-conserved among vertebrates with the notable difference that in fish Fsh seems to play a predominant role in stimulating steroidogenesis.

In addition to affecting steroidogenesis in Leydig cells, clotrimazole exposure up-regulated the levels of *igf3* mRNA. Igf3 is a recently discovered member of the Igf family that is only present in fish and is expressed specifically in gonadal tissue (Wang et al., 2008). In zebrafish, *igf3* expression was localized in Sertoli cells and testicular expression of *igf3* is up-regulated by zebrafish Fsh, but not by Lh (Nobrega, Morais, de Waal, Bogerd and Schulz; unpublished data). Our results, showing an up-regulation of *igf3* expression in testes of clotrimazole-exposed fish, are thus also consistent with activating Fsh-/Fshr-signaling.

In addition to these molecular and cellular changes, we previously reported an increased proportion of spermatogonia type A in clotrimazole-exposed fish under similar conditions of exposure (Hinfray et al., 2011). However, in this study no significant difference as regards *piwil1* gene expression, a spermatogonial marker (Leal et al., 2009), was seen between treatment groups (Fig. S1). Nonetheless, considering the role of Fsh-stimulated androgen production and the role of androgens in stimulating the first steps of spermatogenesis (Campbell et al., 2003), as well as the stimulation of germ cell mitosis by other Igf peptides (Loir, 1994), we hypothesize that the effects seen at the histological level on spermatogonial proliferation in our previous study (Hinfray et al., 2011) may have been a direct consequence of the Fsh-mediated effect on the testes of zebrafish exposed to clotrimazole.

In conclusion, this study provides further evidence of the endocrine disrupting potency of clotrimazole in fish as demonstrated by the elevated expression of steroidogenesis-related genes in testicular tissue. By studying a functional network of genes

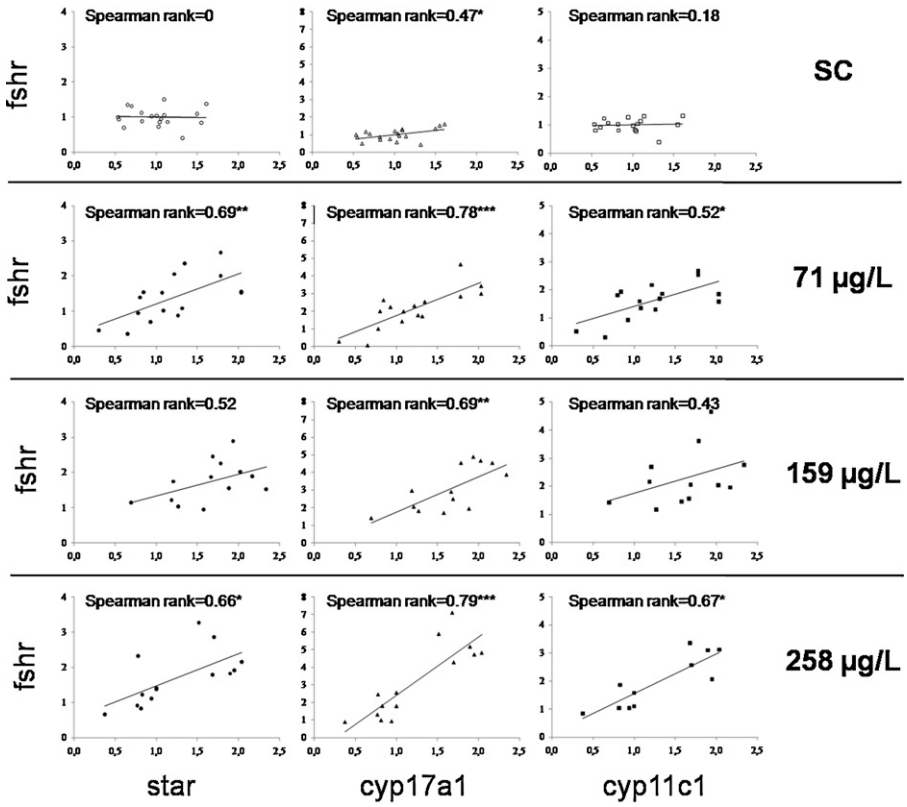

Fig. 5. Correlation between *fshr* expression and *star*, *cyp17a1* and *cyp11c1* expressions in control group and clotrimazole-exposed-groups. Spearman rank number is indicated Asterisks indicated a significant correlation ($^{*}p \leq 0.05$, $^{**}p \leq 0.01$).

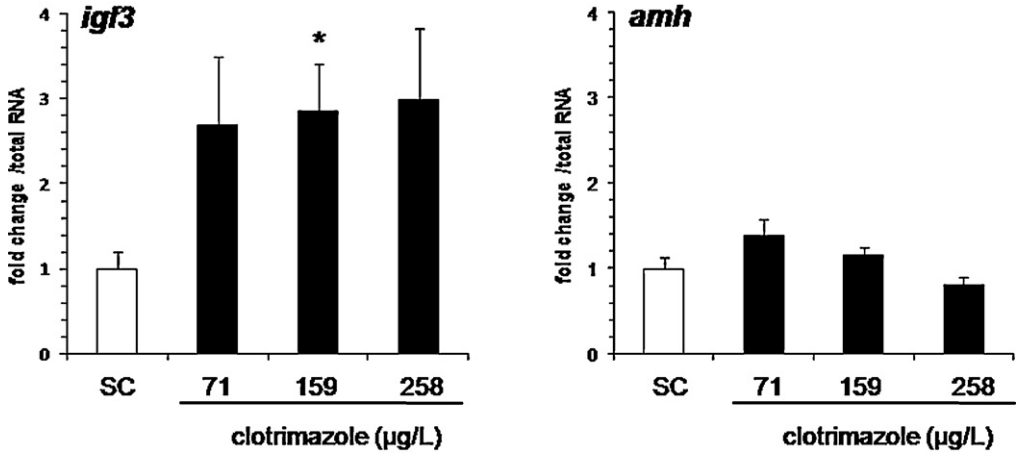

Fig. 6. *In vivo* effect of clotrimazole on Sertoli cell marker expressions following a 7 days exposure. Data are represented as mean ± SEM and expressed as fold change in mRNA expression from the control. Relative mRNA expression was determined as the ratio of target gene mRNA/2 µg total RNA ($N = 12–19$ fish per condition). Asterisks indicated a significant difference compared to control group ($^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$).

operating in the pituitary-gonad axis, we show that clotrimazole induced a cascade of molecular and cellular events at the pituitary and testicular level that is compatible with assuming an important role for Fsh (1) in stimulating Leydig cell steroidogenesis to compensate the inhibitory action of clotrimazole on 11-KT synthesis and (2) in inducing the expression of the Fsh-regulated *igf3* gene in Sertoli cells. Studying this gene network along the pituitary-gonad axis appears useful and relevant to elucidate further the mode of action of clotrimazole and it demonstrated that this chemical triggered in a model vertebrate compensatory responses of the HPG axis. This approach could be extended to other azole compounds acting as inhibitor of P450-steroidogenic enzymes in *in vitro* assays (Hecker et al., 2006; Heneweer et al., 2004; Laville et al., 2006; Vinggaard et al., 2000).

Conflict of interest statement

None.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tox.2012.04.012.

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