

1 **Effect of *in vivo* chronic exposure to clotrimazole on zebrafish testis function**

2

3 Baudiffier Damien¹, Hinfray Nathalie¹, Ravaud Catherine¹, Creusot Nicolas¹, Chadili Edith¹, Porcher Jean-
4 Marc¹, Schulz Rüdiger², Brion François^{1*}

5

6 ¹*INERIS, Direction des Risques Chroniques, Unité d'écotoxicologie in vitro et in vivo, BP2, 60550 Verneuil-en-
7 Halatte, France*

8 ²*University of Utrecht, Science Faculty, Department Biology, Division Developmental Biology,
9 Reproductive Biology Group, Padualaan 8, NL-3584 CH Utrecht, Netherlands.*

10

11

12

13 Corresponding author:

14 Dr François Brion

15 INERIS, Direction des risques chroniques, Pôle VIVA, Unité d'écotoxicologie *in vitro* et *in vivo*, Parc
16 Technologique Alata, BP2, 60550 Verneuil-en-Halatte, France

17 Phone : +33-3 44 55 65 12

18 Fax : +33-3 44 55 66 05

19 Email : francois.brion@ineris.fr

20

21

22

23

24

25

26

27

28

29

30

31 **Abstract**

32

33 Clotrimazole is an azole fungicide used as a human pharmaceutical that is known to inhibit cytochrome P450
34 (CYP) enzymatic activities, including several steroidogenic CYP. In a previous report, we showed that a 7 days
35 exposure to clotrimazole induced the expression of genes related to steroidogenesis in the testes as a
36 compensatory response, involving the activation of the Fsh/Fshr pathway. In this context, the aim of the present
37 study was to assess the effect of an *in vivo* 21-d chronic exposure to clotrimazole (30 – 197 µg/L) on zebrafish
38 testis function, i.e. spermatogenesis and androgen release. The experimental design combined (i) gene transcript
39 levels measurements along the brain-pituitary-gonad axis (ii) 11-ketotestosterone quantification in blood and (iii)
40 histology of the testes, including morphometric analysis. The chronic exposure led to an induction of
41 steroidogenesis-related genes and *fshr* in the testes as well as *fshβ* in the pituitary. Moreover, increases of the
42 gonado-somatic index and of the volume proportion of interstitial Leydig cells were observed in clotrimazole-
43 exposed fish. In accordance with these histological observations, the circulating concentration of 11-KT had
44 increased. Morphometric analysis of the testes did not show an effect of clotrimazole on meiotic (spermatocytes)
45 or postmeiotic (spermatids and spermatozoa) stages, but we observed an increase in the number of type A
46 spermatogonia, in agreement with an increase in mRNA levels of *piwill*, a specific molecular marker of type A
47 spermatogonia. Our study demonstrated that clotrimazole is able to affect testicular physiology and raised further
48 concern about the impact of clotrimazole on reproduction.

49

50 **Keywords:** clotrimazole, endocrine disruption, spermatogenesis, steroidogenesis, HPG axis, zebrafish

51

52 1. Introduction

53

54 Spermatogenesis is a cyclic and tightly regulated developmental process during which a small number of
55 spermatogonial stem cells proliferate and differentiate to form a large number of spermatozoa (Nobrega et al.
56 2009; Schulz et al. 2010). Germ cells can only survive and develop in close and continuous relationship with
57 Sertoli cells (Matta et al. 2002). Spermatogenesis is supported by testicular steroidogenesis, a multi-step process
58 involving a cascade of enzymatic reactions and producing steroid hormones (Miller 1988; Parker and Schimmer
59 1995) that control various physiological functions (Miller 1988). Spermatogenesis and steroidogenesis are under
60 control of the hypothalamus-pituitary-gonad (HPG) axis: hypothalamic gonadotropin-releasing hormone (GnRH)
61 stimulates the release of pituitary gonadotropins, luteinizing hormone (Lh) and follicle-stimulating hormone
62 (Fsh), that interact with their gonadal G protein-coupled receptors, Fsh receptor (Fshr) and Lh receptor (Lhcgr).
63 Androgenic sex steroids synthesized in Leydig cells in response to gonadotropic stimulation interact with the
64 androgen receptor (Ar) expressed by Sertoli or interstitial somatic cells in the testis (Burns and Matzuk 2002;
65 Kumar 2005; Petersen and Soder 2006; Pierce and Parsons 1981). In fish, testicular expression sites of
66 gonadotropin receptors overlap in Sertoli and Leydig cells (Garcia-Lopez et al. 2009; Ohta et al. 2007) and Fsh
67 is a potent steroidogenic hormone (Kazeto et al. 2008; Planas et al. 1993). Follicle stimulating hormone activates
68 the Fshr on Leydig and Sertoli cells to control steroidogenesis and spermatogenesis, respectively (Garcia-Lopez
69 et al. 2009; Garcia-Lopez et al. 2010; Ohta et al. 2007; Schulz et al. 2010). In male fish, Leydig cells produce 11-
70 oxygenated androgens, such as 11-ketotestosterone (11-KT), which stimulates spermatogenesis (Miura *et al.*
71 1991). Sertoli cells but not germ cells expressed functional receptors for both androgens and Fsh, so these cells
72 act as regulatory interface between the endocrine system and the germ cells (Petersen and Soder 2006).

73 Azole fungicides inhibit cytochrome P450 (CYP) 51, 14 α -lanosterol demethylase, disrupting ergosterol synthesis
74 and increasing cell permeability in fungi (Georgopapadakou 1998; Henry and Sisler 1984). Several azole
75 fungicides such as ketoconazole or prochloraz also inhibit other CYP enzyme activities and have the capacity to
76 alter gonadal steroidogenesis and reproductive function in fish (Ankley et al. 2007; Villeneuve et al. 2007a;
77 Zhang et al. 2008a). For example, several azole compounds inhibit steroidogenic cytochrome P450 17 α -
78 hydroxylase/17,20-lyase (Cyp17) and aromatase (Cyp19) in mammals and fish (Ayub and Levell 1987;
79 Heneweer et al. 2004; Hinfrey et al. 2006b; Monod et al. 1993; Vinggaard et al. 2000). Data on the occurrence
80 and fate of azole fungicides in the aquatic environment are scarce although the concentrations of several azoles
81 such as clotrimazole, propiconazole, fluconazole and tebuconazole have been reported at concentrations ranging

82 from the low ng/L to the low $\mu\text{g/L}$ range (Berenzen et al., 2005; Kahle et al., 2008; Kreuger, 1998; Peschka et
83 al., 2007; Roberts and Thomas, 2006; Thomas and Hilton, 2004). Despite their occurrence in aquatic habitat,
84 there exist very few studies on their *in vivo* endocrine-disrupting potencies in fish (Brown et al., 2011; Hinfray et
85 al., 2011).

86 In a previous short-term experiment of 7 days, we investigated the effect of clotrimazole on the pituitary-gonad
87 axis at the molecular level in adult male zebrafish. Our data showed that clotrimazole induced a biological
88 compensation as revealed by increased expression of steroidogenesis-related genes and protein *de novo* synthesis
89 in Leydig cells while the 11-KT plasma concentration was not affected. Clotrimazole exposure also induced
90 over-expression of pituitary *fsh β* and its testicular receptor *fshr*. These results suggested that Fsh/Fshr signalling
91 is involved in the clotrimazole-induced steroidogenesis (Baudiffier et al. 2012; Hinfray et al. 2011). Moreover,
92 this raised the question if also spermatogenesis, a process regulated by androgens and Fsh (McLachlan et al.
93 2002; Plant and Marshall 2001; Schulz et al. 2010), would be affected by clotrimazole.

94 Therefore, the present study aimed at assessing the effect of a prolonged *in vivo* exposure of fish to clotrimazole
95 on steroidogenesis and spermatogenesis, addressing the following questions: (i) Does biological compensation in
96 response to inhibiting enzymatic activities still occur after a prolonged 21-d exposure of male fish to
97 clotrimazole at similar concentrations as those previously used for the 7-d exposure experiment? (ii) Does a
98 prolonged exposure have a significant effect on spermatogenesis?

99

100 2. Methods

101

102 The experimental design and most of the biological analyses have been described in a previous study (Baudiffier
103 et al. 2012).

104

105 2.1. Animals and treatments

106 All experiments were approved by the ethical committee of the National Institute of Industrial Environment and
107 Risks (INERIS). Wild type adult male zebrafish (*Danio rerio*, AB strain) were obtained from our breeding units
108 (INERIS, Verneuil-en-Halatte, France). Fish were raised on a 14:10 light:dark cycle in a recirculated water
109 system (Tecniplast, France) at 25.1 ± 1 °C. Clotrimazole (CLO, purity $\geq 98\%$) was purchased from Sigma-
110 Aldrich (France) and all the stock solutions were prepared in dimethylsulfoxide (DMSO; Sigma-Aldrich).

111 Zebrafish were exposed to three concentrations of clotrimazole (30, 67 and 197 $\mu\text{g/L}$) or solvent alone (DMSO,
112 0.004 % v: v) for 21 days under semi-static conditions with a total renewal of the contaminated water every days
113 (temperature: 25 ± 0.7 °C; pH: 8.01 ± 0.37 ; conductivity: 374.5 ± 27.6 $\mu\text{S/cm}$; dissolved oxygen: 5.8 ± 0.8
114 mg/L). Each condition contained 21 fish equally distributed in three replicates 4L-glass tanks. Water samples
115 were collected from each condition at day 11 and day 18 at the time of water renewal (t= 0h) and 24h later (t=
116 24h).

117

118 2.2. Fish sampling

119 At the end of the exposure, fish were sacrificed in ice cold water and blood (2.5 or 5 μL) was collected. Liver,
120 pituitary and brain were removed and preserved in RNA laterTM (Sigma-Aldrich, France) until quantification of
121 gene expression. Testes were removed, weighted and preserved in RNA laterTM (Sigma-Aldrich, France) or fixed
122 in Bouin's fluid for histological and immunohistochemistry experiments. Testis mass was determined to
123 calculate the gonado somatic index (GSI, gonad wet mass/total body wet mass x 100).

124

125 2.3. Measurement of actual clotrimazole concentrations

126 The protocol was adapted from (Peschka et al. 2007) as described in (Baudiffier et al. 2012). Briefly,
127 clotrimazole concentrations in control and exposure tanks were determined using solid phase extraction (SPE)
128 followed by high-pressure liquid chromatography (HPLC) coupled to UV-Vis detection. An external calibration
129 was used. The limit of detection (LOD) was 0.3 µg/L, the limit of quantification was 1.1 µg/L and the recovery
130 of clotrimazole was higher than 90 % in all the experiments. The coefficient of variation, calculated from two
131 replicates per condition, range from 0.4 to 18%.

132 2.4. Gene expression analysis

133 Transcript levels of genes along the brain-pituitary-gonad axis were measured, using a combination of genes
134 involved in steroidogenesis and spermatogenesis (**table S1**). In testis tissue, expression of steroidogenic acute
135 regulatory protein (*star*); hydroxyl- Δ -5-steroid dehydrogenase, 3 β - and steroid Δ -isomerase 1 (*hsd3b1*);
136 cytochrome P450, family 17, subfamily A polypeptide 1 (*cyp17a1*); cytochrome P450, family 11, subfamily C,
137 polypeptide 1 (*cyp11c1*; previously referred to as *cyp11b2*); hydroxysteroid (11- β) dehydrogenase 3a
138 (*hsd11b3a*); cytochrome P450, family 19, subfamily A, polypeptide 1a (*cyp19a1a*); cytochrome P450, family 19,
139 subfamily B, polypeptide 1a (*cyp19a1b*); luteinizing hormone / choriogonadotropin receptor (*lhcg*); follicle
140 stimulating hormone receptor (*fshr*); were measured. The Sertoli cell markers insulin-like growth factor 3 (*igf3*),
141 anti-müllerian hormone (*amh*) as well as the androgen receptor (*ar*) and the spermatogonial cell marker piwi-like
142 1 (*piwil1*) were also quantified. In pituitary tissue, expression of beta sub-unit of luteinizing hormone /
143 choriogonadotropin (*lh β*) and follicle stimulating hormone (*fsh β*) were measured. In brain tissue, expression of
144 gonadotropin-releasing hormones 3 (*gnrh3*) and steroidogenesis-related genes were also assessed. Indeed, in
145 view of the impact of clotrimazole on testicular steroidogenesis, examination of brain steroidogenesis appears
146 interesting to consider. In addition, the expression of drug metabolizing enzyme (DME) genes, such as phase I
147 enzymes, i.e. cytochrome P450, family 1, subfamily A polypeptide 1 (*cyp1a1*), cytochrome P450, family 3,
148 subfamily A polypeptide 65 (*cyp3a65*) or phase II conjugating enzymes, i.e. glutathione S-transferase, alpha-like
149 (*gsta1*) and glutathione S-transferase *pi 1* (*gstp1*) was quantified in the liver (**table S1**). DMEs, known to be
150 involved in xenobiotic and drug metabolism, are also key enzymes of catabolism for steroid hormones (Lee *et al.*
151 2003).

152 To measure genes expression levels, total RNA was extracted from testis, liver, brain or pituitary using Trizol
153 Reagent (Life Technologies Inc., UK) following the manufacturer's instructions and complementary DNA for
154 the real-time PCR reactions was generated using a Moloney Murine Leukemia Virus reverse transcriptase (M-

155 MLV RT, Promega, France). Gene expression was analyzed using an Eppendorf realplex 4 Master cycler ep
156 gradient S (Eppendorf, France) as previously described by Baudiffier et al. (2012). Normalization to total RNA
157 in association with calibration to a gene-specific mRNA standard curve was employed in organs. For RNA
158 normalization, total RNA was previously adjusted at the same concentration using a Nanodrop ND-8000
159 spectrophotometer (Nanodrop Technologies, Wilmington, DE) for all samples before reverse transcription.
160 Standard curves were generated for each gene, using specific primers and samples amplified by classic PCR. The
161 concentration of these standards was measured using a Nanodrop ND-8000 spectrophotometer and adjusted to a
162 concentration of 10^{-4} $\mu\text{g}/\mu\text{l}$ of cDNA and then serially diluted from 10^{-4} $\mu\text{g}/\mu\text{l}$ to 10^{-11} $\mu\text{g}/\mu\text{l}$. They were run in
163 parallel to the samples to analyse in the rt-PCR to obtain the concentration in $\mu\text{g}/\mu\text{l}$ for each sample from the
164 corresponding Cycle Treshold (Ct) value. This method, excluding housekeeping genes, was described in the
165 literature. Then, a fold change in mRNA was obtained for each gene of clotrimazole-treated fish relatively to the
166 mean value of the control group for the corresponding gene (Huggett et al. 2005; Villeneuve et al. 2007a).

167

168 *2.5. Measurement of 11-KT concentrations*

169 11-Ketotestosterone was quantified in blood samples by means of a competitive ELISA, following the
170 manufacturer's instructions (11-KT EIA Kit, Cayman Chemical Company, U.S.A.). The mean $\text{EC}_{50} \pm$ standard
171 deviation was 8.6 ± 0.7 pg/ml ($n = 6$ independent experiments). The calculated coefficient of variation between
172 assays was 8 %, and the detection limit was 2.8 pg/ml ± 0.3 pg/ml.

173 *2.6. Fluorescent immunohistochemistry and histology*

174 After fixation in Bouin's fluid, testis were dehydrated in ethanol, cleared in toluene and embedded in paraffin,
175 according to conventional procedures. Samples were sectioned at 5 μm (longitudinal sections). Sections were
176 stained with Haematoxylin-Eosin for histology or mounted on gelatine-coated slides and processed for
177 immunohistochemistry.

178 Fluorescent immunohistochemistry of Cyp17a1 and Cyp11c1 proteins in zebrafish testes has been performed as
179 previously described (Baudiffier et al. 2012; Hinfrey et al. 2011). Briefly, sections were dewaxed and
180 rehydrated, and antigens were unmasked. Tissue sections were then incubated for 1 h in a saturation PBS
181 solution containing 0.2% Triton X-100 and 1% milk powder. Incubation with the primary antibody was
182 performed overnight (1:300 with 0.5% milk powder in PBS) at room temperature. After rinsing, sections were

183 incubated for 1h30 with a goat anti-rabbit antibody coupled to Alexa fluor 594 (1:200 with 0.5% milk powder in
184 PBS).

185

186 For morphometric analysis, the protocol was adapted from (Feitsma et al. 2007). The mass of germ cells and
187 other components (i.e. , somatic cells including Leydig cells, blood and lymphatic vessels, connective tissue and
188 empty spaces) were determined by light microscopy using a 352-intersection grid. Eight fields chosen randomly
189 (3016 points or 8 fields of 352-intersection grid) were scored for each animal. Intersection points over tissue
190 components were classified as being over one of the following cell types: type A spermatogonia, type B
191 spermatogonia, spermatocytes, spermatids, spermatozoa or 'others', as defined above (i.e. Leydig cells, blood
192 vessels etc.). A detailed morphological description allowing the identification of the different germ cell types in
193 zebrafish has been given previously (Leal et al., 2009); cell type identification is mainly based on the changes in
194 the size of the nuclei, the amount of heterochromatin, visibility of nucleoli, and number of cells within a
195 spermatogenic cyst. The scores were first expressed as the volume fraction per testis tissue component. The mass
196 (mg) of each testis component was then determined as the product of the testis mass (mg) x volume fraction of
197 spermatogenic parenchyma (~0.8) x volume fraction of specific tissue component. The volume fraction of
198 spermatogenic parenchyma was used as a correction factor in order to exclude the mass of the testes capsule, the
199 efferent ducts, and connective tissue associated with testes.

200 The Leydig cell volume fraction was determined by light microscopy using a 713-intersection grid. Eight fields
201 chosen randomly (5704 points or 8 fields of 713-intersection grid) were scored for each animal. Analysis was
202 performed for 5 fish of each condition. Intersection points were classified as being over Leydig cells or not.
203 Results were expressed in percentage of Leydig cells compared to the total number of cells. The mass (mg) of
204 Leydig cells was then determined as the product of the testis mass (mg) x volume fraction of spermatogenic
205 parenchyma (~0.8) x volume fraction of Leydig cells component. The volume fraction of spermatogenic
206 parenchyma was used as a correction factor in order to exclude the mass of the testes capsule, the efferent ducts,
207 and connective tissue associated with testes.

208

209 *2.7. Data analysis*

210 One way analysis of variance (ANOVA) was performed to test for differences between treatments. Then,
211 differences among treatments were determined using a post hoc test (Tukey Honestly Significant Difference).
212 Normality of the data was previously assessed using a Shapiro test and homogeneity of variance was also
213 verified using the Bartlett test. Non-normally distributed data were log-transformed prior to analysis and a
214 nonparametric Kruskal Wallis test, followed by a Multiple Comparison test, was used when data did not meet
215 parametric assumptions. For gene expression analysis in the pituitary, a Mann and Whitney U-test was employed
216 to compare gene expression levels between the highest clotrimazole-exposed fish and the control fish only.

217 Levels of gene expressions were expressed as fold changes relative to the average value of the control. Statistical
218 analyses, including principal component analysis (PCA), were conducted using RTM (R 2.13.1, software, R
219 development Core Team). All data are presented as mean \pm SEM. Significance level (p) was fixed at 0.05
220 (p<0.05 *; p<0.01 **; p<0.001 ***).

221

222 3. Results

223

224 3.1. Water chemistry

225 Concentrations of clotrimazole measured in water just after adding clotrimazole (t= 0h) were 30, 67 and 197
226 µg/L for the low, medium, and high contaminations, respectively (**table 1**). Concentrations declined by 32, 17
227 and 22%, respectively, after 24 h (t= 24h). For each condition, two measurements were performed (at day 11 and
228 day 18). The results showed that the concentrations were stable over time and concentrations of exposure.
229 Control water samples were all below the limit of detection, i.e. 0.3 µg/L.

230

231 3.2. Biometric parameters

232 No mortality or any abnormal behavior was recorded in control and clotrimazole-exposed groups during the
233 study. Furthermore, no significant differences were measured among groups for body mass or length (**table 2**).
234 In fish exposed to the highest concentration of clotrimazole, a strong (30%) and significant increase of the GSI
235 was measured as compared to control fish (**table 2**).

236

237 3.3. Expression of genes involved in testicular steroidogenesis

238 The mRNA levels of steroidogenesis-related genes in testes, i.s. gonadotropin receptors, *star* and several genes
239 coding for steroidogenic enzymes were analysed in control and clotrimazole-exposed fish (**Fig.1**). Among nine
240 genes assayed by Q-PCR, an increase in transcript levels was observed for five genes after 21 days of exposure
241 to the highest concentration of clotrimazole.

242 The expression of *fshr* but not of *lhcr* was significantly 1.69-fold up regulated at the highest concentration of
243 clotrimazole (p = 0.0013). The levels of *star* mRNA increased 1.77-fold relative to the controls (p = 0.0094).
244 Still at the highest concentration, the transcript levels of *cyp17a1*, *cyp11c1* and *hsd11b3a* were up-regulated
245 2.18-, 2.13- and 1.41 fold, respectively (p = 0.00001, p = 0.0015, p = 0.0013). The expression of *fshr* was
246 significantly correlated with these steroidogenic-related genes: *star* (Spearman 's rank correlation coefficient r =
247 0.59; p < 0.001), *cyp17a1* (r = 0.43; p < 0.001), *cyp11c1* (r = 0.53; p < 0.01) and *hsd11b3a* (r=0.63, p<0.001).
248 Besides, these steroidogenic-related genes were significantly correlated with each other (data not shown)

249 suggesting that Fsh coordinates the stimulatory effects of these genes. Interestingly, *cyp19a1a* gene expression
250 was down-regulated at 30 and 67 µg/L, and statistical significance was reached in the 67 µg/L clotrimazole-
251 exposed group. However, no effect was observed at the highest concentration.

252

253 3.4. Gene expression in brain and pituitary

254 In brain tissue, transcript levels of genes encoding brain steroidogenic enzymes, oestrogen and androgen
255 receptors as well as *gnrh3* were compared between control and clotrimazole-exposed groups (**table 3**). Clear and
256 statistically significant changes in transcript levels were not found for any of the genes at all concentrations of
257 clotrimazole. While a two-fold induction of *cyp19a1a* expression was measured in fish exposed to 30 and 197
258 µg/L of clotrimazole, the high variability observed between individuals, may contribute to the lack of statistical
259 significance.

260 In the pituitary, transcript levels of *fshβ* and *lhβ* were assessed for the 197 µg/L clotrimazole-exposed group and
261 compared to untreated fish. We only assessed gonadotropin gene expression at the highest concentration because
262 we observed a strong and significant induction of the GSI, of several steroidogenesis-related genes, and of *fshr*
263 transcript levels in this group. The results showed that *fshβ* was increased by 3.6-fold, but expression of *lhβ* was
264 unchanged (**Fig.2**).

265

266 3.5. Cyp17a1 and Cyp11c1 immunostaining in the testes

267 Using specific polyclonal antibodies, fluorescent immunohistochemistry was performed in testis sections after 21
268 days of exposure to assess the effect of clotrimazole on Cyp17a1 and Cyp11c1 protein occurrence (**Fig.3**). The
269 two proteins were localized in interstitial Leydig cells and increased immunostaining was observed for both
270 proteins from 67 µg/L of clotrimazole. This increase became very marked at the highest level of clotrimazole
271 treatment (197 µg/L) especially for the Cyp11c1 protein (**Fig.3A and .3B**). These results are in accordance with
272 the data on *cyp17a1* and *cyp11c1* genes expression.

273

274 3.6. 11-ketotestosterone concentrations in blood

275 Clotrimazole significantly affected blood 11-KT concentrations as compared to the control group (**Fig.4**). A
276 concentration-dependent increase was measured with a significant effect at the highest concentration of
277 clotrimazole. Moreover, we noticed a higher inter-individual variation in clotrimazole-exposed groups compared
278 to the solvent control group.

279

280 3.7. Expression of spermatogenesis-related genes

281 Transcription of genes involved in spermatogenesis was assessed in testes of control and clotrimazole-exposed
282 fish after 21 days of exposure (**Fig.5**).

283 The androgen receptor *ar* that is expressed in Sertoli and interstitial cells but not in germ cells was significantly
284 up-regulated for all clotrimazole treatments with a similar fold induction whatever the exposure concentration.
285 Transcriptional levels of the two Sertoli cells markers *amh* and *igf3* were also examined. Anti-müllerian
286 hormone *amh* expression was unchanged whereas *igf3* expression was induced in the 67 µg/L clotrimazole-
287 exposed group ($p= 0.02$) but not at the highest clotrimazole concentration. Finally, transcript level of the
288 spermatogonial marker *piwill* was significantly up-regulated at the highest clotrimazole concentration (1.38-fold
289 for 197 µg/L).

290

291 3.8. Expression of genes involved in the catabolism of steroid hormones

292 Transcript levels of different genes encoding for drug metabolizing enzymes (DMEs) were assessed after 21
293 days of exposure in testis and in liver (**Fig.S1, Fig.S2**). In the liver, transcriptional levels of phase I DMEs, i.e.
294 *cyp1a* and *cyp3a65*, and of phase II conjugating enzymes, i.e. *gstal* and *gstp1*, remained unchanged in
295 clotrimazole-exposed groups compared to the control group (**Fig.S1**). However, in testis, *cyp3a65* expression
296 was significantly up-regulated (**Fig.S2**).

297

298 3.9. Principal Component analysis (PCA)

299 A PCA was performed in order to have an overview of correlations between the different endpoints measured in
300 the testis and in the blood (**Fig.6**). The factor 1 explained 34.02% of the data variability whereas the factor 2

301 explained 16 % of the data variability. A plot with respect to this two principal components revealed that
302 expression of all endpoints influenced variation in the negative direction along factor 1. Along factor 2, a strong
303 association between several steroidogenesis-related genes involved in androgen synthesis (*star*, *cyp17a1* and
304 *cyp11c1*) was observed. This group of genes was closely associated with *igf3*, a Sertoli cell marker and *piwill*, a
305 spermatogonia type A marker (**Fig.6A**). A close association between 11-KT and GSI was also found. These two
306 parameters were significantly affected in the highest clotrimazole-exposed group and increase GSI might be a
307 consequence of elevated 11-KT production. Interestingly, genes related to estrogens biosynthesis (i.e., *cyp19a1a*
308 and *cyp19a1b*) and ER-signalling pathway (i.e., *esr1*, *esr2a*, *esr2b*) were closely associated and located at the
309 opposite side of those related to androgen biosynthesis. The genes involved in the metabolism of xenobiotic and
310 endogenous hormones (*cyp1a* and *cyp3a65*) were closely associated and localized at the opposite from the
311 biological parameters related to androgen synthesis. Although clotrimazole differently affected the expression of
312 genes encoding for gonadotropin receptors, they were closely associated together with androgen receptor and
313 *amh*, another Sertoli cell marker. A second plot, representing condition of exposures, showed a strong difference
314 between the 197 µg/L clotrimazole-exposed group that influenced variation along factor 1 compared to the other
315 groups including control and the two other clotrimazole-exposed groups (**Fig.6B**) showing that all the selected
316 genes allowed to discriminate the different treatments to clotrimazole.

317 3.10. Histology and morphometric analysis of the testes

318 Histological analysis showed an enlargement of the interstitial space in fish exposed to clotrimazole, as indicated
319 by white arrows (**Fig.7A**, control; **Fig.7B**, 197 µg/L clotrimazole). Interestingly, morphometric analysis revealed
320 a significant 2-fold increase of Leydig cell mass (**Fig.7C**). Finally, quantitative morphometric analysis of testis
321 tissue components was realized in the group exposed to 197 µg/L clotrimazole and the control group (seven
322 males for each group). We focused on the highest clotrimazole concentration since it evoked the most clear
323 effects regarding Leydig cells, target gene expression and GSI. In control individuals, the mass of germ cells, i.e.
324 spermatogonia, spermatocytes, spermatids and spermatozoa, agrees with previously reported data in adult male
325 zebrafish (de Waal et al. 2009). In exposed-fish, a significant 2-fold increase of type A spermatogonia (but not
326 type B) was quantified (**Fig.7D**).

327

328 4. Discussion

329

330 The present work investigated the chronic effects of clotrimazole on two related process, testicular
331 steroidogenesis and spermatogenesis, after 21 days of exposure of zebrafish. A biological compensation of the
332 steroidogenic enzymes inhibition by clotrimazole was revealed, with an up-regulation of genes coding for
333 steroidogenic enzymes, pituitary *fsh β* , and its testicular receptor *fshr*. This underlines the critical role of the
334 Fsh/Fshr signalling pathway in mediating the effects of clotrimazole on testicular steroidogenesis. In accordance
335 with inducing Leydig cell steroidogenesis and stimulating the Fsh/Fshr pathway, transcript levels of the Sertoli
336 cell genes *igf3* and *ar* were increased, as well as the 11-KT circulating levels. Furthermore, morphometric
337 analysis of the testes demonstrates that spermatogenesis was impacted by clotrimazole as shown by the increase
338 of the mass of type A spermatogonia in clotrimazole-exposed fish, in accordance with the up-regulation of a
339 marker gene for this germ cell type, *piwill*.

340

341 4.1. Chronic exposure to clotrimazole led to activation of Fsh/Fshr signalling and over- compensation of the 342 steroidogenic parameters

343 In the present study, we investigated the effects of clotrimazole on the brain-pituitary-gonadal axis, in particular
344 the impact on testicular steroidogenesis after 21 days of exposure. We showed that clotrimazole up-regulated the
345 expression of steroidogenesis-related genes (*star*, *cyp17a1*, *cyp11c1*). Principal Component Analysis (PCA)
346 revealed that these endpoints were correlated. Cyp17a1 and Cyp11c1 protein expression was also increased in
347 Leydig cells and 11-KT blood levels were elevated. Induction of steroidogenesis-related genes agrees with recent
348 work on adult male fathead minnow or medaka exposed 21 days to azoles, i.e. ketoconazole or prochloraz
349 (Ankley et al. 2007; Villeneuve et al. 2007a). Clotrimazole is known to inhibit several CYP enzyme activities in
350 fish and mammals; including Cyp19 and Cyp17 (Ayub and Levell 1987; Hinfray et al. 2006b; Monod et al.
351 1993; Schuster 1985). This inhibition of enzymatic activities in testis tissue can lead to reduced 11-KT release,
352 as noticed in an *in vitro* zebrafish testicular explant culture system (Hinfray et al. 2011). For that reason,
353 stimulation of the steroidogenic system *in vivo* was interpreted as a biological compensation in response to
354 enzyme inhibition (Ankley et al. 2007; Baudiffier et al. 2012; Hinfray et al. 2006b; Villeneuve et al. 2007b;
355 Zhang et al. 2008a). However, in the present study, we observed an increase of circulating 11-KT concentrations
356 surmounting control levels, suggesting that an exposure to clotrimazole for 21 days led to an over-compensation.

357 We postulate that clotrimazole initially depressed plasma androgen levels, provoking a compensatory response
358 via Fsh, including an increased *de novo* synthesis of steroidogenic enzymes, such that 11-KT blood levels were
359 normal after 7 days of exposure (Baudiffier et al. 2012), but exceeded normal levels after 21 days of exposure
360 (present study). In recent works on adult male fathead minnows exposed for 21 days to ketoconazole or
361 fadrozole, plasma concentration of 11-KT was not affected (Ankley et al. 2007) or induced (Ankley et al. 2002),
362 respectively. In mammals, plasma and intra-testicular concentrations of testosterone were increased following
363 long-term exposure to azoles (**table 4**). Taken together, these studies and our study suggest that the relationship
364 between azole exposures and final androgen plasma concentrations cannot be predicted easily. This is probably
365 due to the balance between a direct inhibition of enzyme activities and a compensatory response that stimulates
366 testicular steroidogenic gene expression by gonadotropin. The impact of azole on plasma 11-KT may also
367 depend on the biological model, the tested azole, or the exposure conditions (concentration and duration).
368 Furthermore, the 11-KT concentration is a result of its production, degradation, and excretion. So, an important
369 point to consider is steroid hormone degradation (Lou et al. 2004) by DME, expressed in the liver but also in the
370 gonads. For example, in the present study, we observed an induction of *cyp3a65* mRNA transcript level in testes
371 of the group exposed to the highest clotrimazole concentration. This increased gene expression could lead to
372 Cyp3a65 enzyme production and influence circulating 11-KT concentrations. In addition, we noticed an
373 inhibition of *cyp19a1* aromatase in the testes following clotrimazole contamination whereas expression of other
374 enzymes involved in androgen production was induced, such as *cyp17a1* and *cyp11c1*. Reduced aromatisation of
375 androgens could contribute to increasing substrate availability for 11-KT synthesis.

376 The increase in testicular *fshr* and pituitary *fsh β* expression is consistent with a compensatory response
377 supporting the role of Fsh/Fshr-signalling in the clotrimazole-induced steroidogenesis, as demonstrated in our
378 previous 7-days experiment (Baudiffier et al. 2012), if the assumed initial decrease of plasma 11-KT
379 concentration would stimulate pituitary Fsh synthesis and release. However, a change in *gnrh3* expression, an
380 important regulator of gonadotropin release in teleost species possessing two Gnrh variants (Kobayashi et al.
381 1997; Steven et al. 2003), was not demonstrated in our study.

382 As clotrimazole can directly inhibit CYP activities in testes (Ayub and Levell 1987; Hinfray et al. 2006a; Monod
383 et al. 1993), it was also interesting to investigate the effects of clotrimazole on brain steroidogenesis. Indeed, in
384 teleost fish, the brain possesses high aromatase activity (Pasmanik and Callard 1985) due to the strong
385 expression of the *cyp19alb* gene coding for aromatase B protein in radial glial cells (Forlano et al. 2001; Menuet
386 et al. 2005). Furthermore, a recent study suggested that radial glial cells express the whole set of key

387 steroidogenic enzymes necessary to produce active steroids from cholesterol (Diotel et al. 2011). Our data
388 showed that clotrimazole had no effect on expression of steroidogenesis-related genes in brain tissue. However,
389 considering that these genes are expressed in specific area of the brain, we cannot exclude a dilution effect has
390 masked possible, more subtle changes, since mRNA levels were measured in the whole brain. This reasoning
391 also applies to GnRH-producing neurones, so that mRNA quantification in specific areas of the brain may provide
392 more relevant data in future experiments.

393

394 4.2. Biological relevance of Fsh-driven clotrimazole-disrupted steroidogenesis on spermatogenesis

395 In the present study, we assessed the effect of clotrimazole on testicular physiology, i.e. steroidogenesis and
396 spermatogenic function at the histological and molecular level. Morphometric analysis of germ cells showed a
397 significant effect in the number of type A spermatogonia in the 197 µg/L clotrimazole-treated fish compared to
398 control fish. This result is in agreement with the induction of the germ cell marker *piwill* expression (formerly
399 known as *ziwi*), which is a component of a germline-specifying structure called nuage and expressed strongly
400 and exclusively in type A spermatogonia of zebrafish (Chen SX et al. *in revision*). This increase of type A
401 spermatogonia could be related to molecular changes in Sertoli cells, which are in close contact with germ cells
402 and provide physical, nutritional and regulatory support to the developing germ cells (Griswold 1995; Petersen
403 and Soder 2006). It is also of interest to notice that Sertoli cells that express the highest level of *ar* mRNA, are in
404 contact with early spermatogonia (de Waal et al. 2008). This is consistent with the notion that these Sertoli cells
405 respond to 11-KT, resulting in a stimulation of spermatogonial proliferation and differentiation (de Waal et al.
406 2008; Miura et al. 2001a; Miura et al. 1991). Furthermore, it has been demonstrated in eel testes that *fshr* is
407 expressed in Leydig cells and high Fshr immunoreactivity was found in Sertoli cells surrounding type A and B
408 spermatogonia (Ohta et al. 2007). In the present study, the observed increase in the mass of type A
409 spermatogonia in adult male zebrafish is in agreement with an induction of the *ar* and *fshr* mRNA.

410 In accordance with an Fsh/Fshr signalling activation, we also observed an induction of *igf3*, which is under the
411 control of Fsh and androgens in Sertoli cells. *Igf3* has been recently discovered in zebrafish, medaka and Nile
412 tilapia and is specifically expressed in gonadal tissue (Wang et al. 2008). Igf3 belongs to insulin growth factor
413 (IGF) family, which is known to stimulate spermatogenesis in vertebrates (Le Roy et al. 1999), including fish
414 (Loir 1999).

415 An important result of our study was that morphometric analysis clearly demonstrated an increase in the mass of
416 Leydig cells in clotrimazole-exposed fish. This is in accordance with an increase in circulating 11-KT
417 concentrations and illustrates the stimulation of the steroidogenic system triggered by the Fsh/Fshr pathway. At
418 present, it is not known why elevated Fsh-signalling and androgen levels did not result in an increase in the mass
419 of spermatogonia B, spermatids and spermatozooids. As shown in **table 4**, an increased mass of interstitial Leydig
420 cell has also been observed in fish exposed to clotrimazole and ketoconazole (Ankley et al. 2007; Brown et al.
421 2011), but the precise mechanism of action has not been described. It seems reasonable to assume that an
422 increase in Leydig cell volume can partially explain the elevation of circulating 11-KT concentrations, as these
423 cells are responsible for androgen production.

424 In conclusion, this study showed that expression of steroidogenesis-related genes in testicular tissue remained
425 induced after 21 days of exposure. Induction of *fsh β* , *fshr* and *igf3* is consistent with and activation of Fsh/Fshr
426 signalling in stimulating Leydig cells steroidogenesis. In comparison with 7 days-exposure period to
427 clotrimazole, we observed in the present study an induction of blood 11-KT concentrations after 21 days of
428 exposure. In parallel, an increase of GSI and a duplication of the mass of Leydig cells were observed. This over-
429 compensation was not followed by a drastic adjustment of spermatogenesis as the proportion of most germ cells
430 remained largely unchanged, except for an increase in the early spermatogonial generations. This study
431 highlights the relevance of studying a network of functional genes along the brain-gonad axis in parallel with
432 histological analysis at the testicular level to study the effect of clotrimazole as an endocrine disruptor. The
433 present study contributes to progress in our understanding of the molecular mechanisms of clotrimazole and its
434 impact on testis physiology.

435

436 **Acknowledgments**

437 This work was funded by a grant of the French ministry of Ecology P189-NEMO to FB, and by the European
438 Union LIFECYCLE projects no FP7-222719 to RWS. DB was supported by a doctoral fellowship from ANRT
439 and INERIS. We thank Dr. Alexis Fostier (INRA, Rennes, France) for comments on the manuscript. We thank
440 Benjamin Piccini for his technical assistance.

441

442 **References**

- 443 Ankley GT, Jensen KM, Kahl MD, Makynen EA, Blake LS, Greene KJ, Johnson RD, Villeneuve DL
444 (2007) Ketoconazole in the fathead minnow (*Pimephales promelas*): Reproductive toxicity
445 and biological compensation. *Environ Toxicol Chem* 26 (6):1214-1223
- 446 Ankley GT, Kahl MD, Jensen KM, Hornung MW, Korte JJ, Makynen EA, Leino RL (2002)
447 Evaluation of the aromatase inhibitor fadrozole in a short-term reproduction assay with the
448 fathead minnow (*Pimephales promelas*). *Toxicol Sci* 67 (1):121-130
- 449 Ayub M, Levell MJ (1987) Inhibition of Testicular 17-Alpha-Hydroxylase and 17,20-Lyase but Not 3-
450 Beta-Hydroxysteroid Dehydrogenase-Isomerase or 17-Beta-Hydroxysteroid Oxidoreductase
451 by Ketoconazole and Other Imidazole Drugs. *J Steroid Biochem Mol Biol* 28 (5):521-531.
452 doi:10.1016/0022-4731(87)90511-5
- 453 Baudiffier D, Hinfrey N, Vosges M, Creusot N, Chadili E, Porcher J-M, Schulz RW, Brion F (2012) A
454 critical role of follicle-stimulating hormone (Fsh) in mediating the effect of clotrimazole on
455 testicular steroidogenesis in adult zebrafish. *Toxicology* 298 (1-3):30-39
- 456 Berenzen N, Lentzen-Godding A, Probst M, Schulz H, Schulz R, Liess M (2005) A comparison of
457 predicted and measured levels of runoff-related pesticide concentrations in small lowland
458 streams on a landscape level, *Chemosphere* 58 (5):683-691.
- 459 Brown AR, Bickley LK, Le Page G, Hosken DJ, Paull GC, Hamilton PB, Owen SF, Robinson J,
460 Sharpe AD, Tyler CR (2011) Are Toxicological Responses in Laboratory (Inbred) Zebrafish
461 Representative of Those in Outbred (Wild) Populations? - A Case Study with an Endocrine
462 Disrupting Chemical. *Environ Sci Technol* 45 (9):4166-4172. doi:10.1021/es200122r
- 463 Burns KH, Matzuk MM (2002) Minireview: Genetic models for the study of gonadotropin actions.
464 *Endocrinology* 143 (8):2823-2835. doi:10.1210/en.143.8.2823
- 465 Chen SX, Bogerd J, Schoonen NE, Martijn J, de Waal PP, Schulz RW A progestin (17 α ,20 β -
466 dihydroxy-4-pregnen-3-one) stimulates early stages of spermatogenesis in zebrafish. *Gen
467 Comp Endocrinol* (in revision).
- 468 de Waal PP, Leal MC, Garcia-Lopez A, Liarte S, de Jonge H, Hinfrey N, Brion F, Schulz RW, Bogerd
469 J (2009) Oestrogen-induced androgen insufficiency results in a reduction of proliferation and
470 differentiation of spermatogonia in the zebrafish testis. *J Endocrinol* 202 (2):287-297.
471 doi:10.1677/joe-09-0050
- 472 de Waal PP, Wang DS, Nijenhuis WA, Schulz RW, Bogerd J (2008) Functional characterization and
473 expression analysis of the androgen receptor in zebrafish (*Danio rerio*) testis. *Reproduction*
474 136 (2):225-234. doi:10.1530/rep-08-0055
- 475 Diotel N, Do Rego JL, Anglade I, Vaillant C, Pellegrini E, Gueguen MM, Mironov S, Vaudry H, Kah
476 O (2011) Activity and expression of steroidogenic enzymes in the brain of adult zebrafish. *Eur
477 J Neurosci* 34 (1):45-56. doi:10.1111/j.1460-9568.2011.07731.x
- 478 Feitsma H, Leal MC, Moens PB, Cuppen E, Schulz RW (2007) *Mlh1* deficiency in zebrafish results in
479 male sterility and aneuploid as well as triploid progeny in females. *Genetics* 175 (4):1561-
480 1569. doi:10.1534/genetics.106.068171
- 481 Forlano PM, Deitcher DL, Myers DA, Bass AH (2001) Anatomical distribution and cellular basis for
482 high levels of aromatase activity in the brain of teleost fish: Aromatase enzyme and mRNA
483 expression identify glia as source. *J Neurosci* 21 (22):8943-8955
- 484 Garcia-Lopez A, Bogerd J, Granneman JCM, van Dijk W, Trant JM, Taranger GL, Schulz RW (2009)
485 Leydig Cells Express Follicle-Stimulating Hormone Receptors in African Catfish.
486 *Endocrinology* 150 (1):357-365. doi:10.1210/en.2008-0447
- 487 Garcia-Lopez A, de Jonge H, Nobrega RH, de Waal PP, van Dijk W, Hemrika W, Taranger GL,
488 Bogerd J, Schulz RW (2010) Studies in Zebrafish Reveal Unusual Cellular Expression
489 Patterns of Gonadotropin Receptor Messenger Ribonucleic Acids in the Testis and
490 Unexpected Functional Differentiation of the Gonadotropins. *Endocrinology* 151 (5):2349-
491 2360. doi:10.1210/en.2009-1227
- 492 Georgopapadakou NH (1998) Antifungals: mechanism of action and resistance, established and novel
493 drugs. *Curr Opin Microbiol* 1 (5):547-557. doi:10.1016/s1369-5274(98)80087-8

- 494 Griswold MD (1995) Interactions between Germ-Cells and Sertoli Cells in the Testis. *Biol Reprod* 52
495 (2):211-216. doi:10.1095/biolreprod52.2.211
- 496 Heneweer M, van den Berg M, Sanderson JT (2004) A comparison of human H295R and rat R2C cell
497 lines as in vitro screening tools for effects on aromatase. *Toxicol Lett* 146 (2):183-194.
498 doi:10.1016/j.toxlet.2003.10.002
- 499 Henry MJ, Sisler HD (1984) Effects of Sterol Biosynthesis-Inhibiting (Sbi) Fungicides on
500 Cytochrome-P-450 Oxygenations in Fungi. *Pestic Biochem Physiol* 22 (3):262-275.
501 doi:10.1016/0048-3575(84)90019-1
- 502 Hinfray N, Baudiffier D, Leal MC, Porcher JM, Ait-Aissa S, Le Gac F, Schulz RW, Brion F (2011)
503 Characterization of testicular expression of P450 17 alpha-hydroxylase, 17,20-lyase in
504 zebrafish and its perturbation by the pharmaceutical fungicide clotrimazole. *Gen Comp*
505 *Endocrinol* 174 (3):309-317. doi:10.1016/j.ygcen.2011.09.008
- 506 Hinfray N, Palluel O, Turies C, Cousin C, Porcher JM, Brion F (2006a) Brain and gonadal aromatase
507 as potential targets of endocrine disrupting chemicals in a model species, the zebrafish (*Danio*
508 *rerio*). *Environ Toxicol* 21 (4):332-337. doi:10.1002/tox.20203
- 509 Hinfray N, Porcher JM, Brion F (2006b) Inhibition of rainbow trout (*Oncorhynchus mykiss*) P450
510 aromatase activities in brain and ovarian microsomes by various environmental substances.
511 *Comp Biochem and Physiol C-Toxicology & Pharmacology* 144 (3):252-262
- 512 Huggett J, Dheda K, Bustin S, Zumla A (2005) Real-time RT-PCR normalisation; strategies and
513 considerations. *Genes Immun* 6 (4):279-284. doi:10.1038/sj.gene.6364190
- 514 Kahle M, Buerge IJ, Hauser A, Muller MD, Poiger T (2008) Azole Fungicides: Occurrence and Fate
515 in Wastewater and Surface Waters. *Environ Sci Technol* 42 (19):7193-7200.
- 516 Kazeto Y, Kohara M, Miura T, Miura C, Yamaguchi S, Trant JM, Adachi S, Yamauchi K (2008)
517 Japanese Eel Follicle-Stimulating Hormone (Fsh) and Luteinizing Hormone (Lh): Production
518 of Biologically Active Recombinant Fsh and Lh by *Drosophila* S2 Cells and Their Differential
519 Actions on the Reproductive Biology. *Biol Reprod* 79 (5):938-946.
520 doi:10.1095/biolreprod.108.070052
- 521 Kobayashi M, Amano M, Kim MH, Yoshiura Y, Sohn YC, Suetake H, Aida K (1997) Gonadotropin-
522 releasing hormone and gonadotropin in goldfish and masu salmon. *Fish Physiol Biochem* 17
523 (1-6):1-8. doi:10.1023/a:1007764430746
- 524 Kreuger J (1998) Pesticides in stream water within an agricultural catchment in southern Sweden,
525 1990-1996. *Sci Total Environ* 216 (3): 227-251.
- 526 Kumar TR (2005) What have we learned about gonadotropin function from gonadotropin subunit and
527 receptor knockout mice? *Reproduction* 130 (3):293-302. doi:10.1530/rep.1.00660
- 528 Leal MC, Cardoso ER, Nobrega RH, Batlouni SR, Bogerd J, Franca LR, Schulz RW (2009)
529 Histological and stereological evaluation of zebrafish (*Danio rerio*) spermatogenesis with an
530 emphasis on spermatogonial generations. *Biol Reprod* 81: 177-187
- 531 Lee AJ, Cai MX, Thomas PE, Conney AH, Zhu BT (2003) Characterization of the oxidative
532 metabolites of 17 β -estradiol and estrone formed by 15 selectively expressed human
533 cytochrome p450 isoforms. *Endocrinology* 144: 3382-3398
- 534 Le Roy C, Lejeune H, Chuzel F, Saez JM, Langlois D (1999) Autocrine regulation of Leydig cell
535 differentiated functions by insulin-like growth factor I and transforming growth factor beta. *J*
536 *Steroid Biochem Mol Biol* 69 (1-6):379-384. doi:10.1016/s0960-0760(99)00075-8
- 537 Loir M (1999) Spermatogonia of rainbow trout: II. In vitro study of the influence of pituitary
538 hormones, growth factors and steroids on mitotic activity. *Mol Reprod Dev* 53 (4):434-442.
539 doi:10.1002/(sici)1098-2795(199908)53:4<434::aid-mrd9>3.0.co;2-1
- 540 Lou YR, Qiao S, Talonpoika R, Syvala H, Tuohimaa P (2004) The role of vitamin D-3 metabolism in
541 prostate cancer. *J Steroid Biochem Mol Biol* 92 (4):317-325. doi:10.1016/j.jsbmb.2004.10.007
- 542 Matta SLP, Vilela DAR, Godinho HP, Franca LR (2002) The goitrogen 6-n-propyl-2-thiouracil (PTU)
543 given during testis development increases sertoli and germ cell numbers per cyst in fish: The
544 tilapia (*Oreochromis niloticus*) model. *Endocrinology* 143 (3):970-978.
545 doi:10.1210/en.143.3.970
- 546 McLachlan RI, O'Donnell L, Stanton PG, Balourdos G, Frydenberg M, de Kretser DM, Robertson DM
547 (2002) Effects of testosterone plus medroxyprogesterone acetate on semen quality,

- 548 reproductive hormones, and germ cell populations in normal young men. *J Clin Endocrinol*
549 *Metab* 87 (2):546-556. doi:10.1210/jc.87.2.546
- 550 Menuet A, Pellegrini E, Brion F, Gueguen MM, Anglade I, Pakdel F, Kah O (2005) Expression and
551 estrogen-dependent regulation of the zebrafish brain aromatase gene. *J Comp Neurol* 485
552 (4):304-320. doi:10.1002/cne.20497
- 553 Miller WL (1988) *Molecular-Biology of Steroid-Hormone Synthesis*. *Endocr Rev* 9 (3):295-318
- 554 Miura T, Miura C, Yamauchi K (2001a) cDNA cloning of spermatogenesis relating substances and the
555 analysis of their functions in Japanese eel. In: *Perspective in Comparative Endocrinology:*
556 *Unity and Diversity*. Medimond S R L, 40128 Bologna, pp 969-976
- 557 Miura T, Yamauchi K, Takahashi H, Nagahama Y (1991) Hormonal Induction of All Stages of
558 Spermatogenesis *Invitro* in the Male Japanese Eel (*Anguilla-Japonica*). *Proc Natl Acad Sci U*
559 *S A* 88 (13):5774-5778. doi:10.1073/pnas.88.13.5774
- 560 Monod G, Demones A, Fostier A (1993) Inhibition of Ovarian Microsomal Aromatase and Follicular
561 Estradiol Secretion by Imidazole Fungicides in Rainbow-Trout. *Mar Environ Res* 35 (1-
562 2):153-157
- 563 Nobrega RH, Batlouni SR, Franca LR (2009) An overview of functional and stereological evaluation
564 of spermatogenesis and germ cell transplantation in fish. *Fish Physiol Biochem* 35 (1):197-
565 206. doi:10.1007/s10695-008-9252-z
- 566 Ohta T, Miyake H, Miura C, Kamei H, Aida K, Miura T (2007) Follicle-stimulating hormone induces
567 spermatogenesis mediated by androgen production in Japanese Eel, *Anguilla japonica*. *Biol*
568 *Reprod* 77 (6):970-977. doi:10.1095/biolreprod.107.062299
- 569 Parker KL, Schimmer BP (1995) Transcriptional regulation of the genes encoding the cytochrome P-
570 450 steroid hydroxylases. *Vitam Horm* 51:339-370
- 571 Pasmanik M, Callard GV (1985) Aromatase and 5-Alpha-Reductase in the Teleost Brain, Spinal-Cord,
572 and Pituitary-Gland. *Gen Comp Endocrinol* 60 (2):244-251. doi:10.1016/0016-
573 6480(85)90320-x
- 574 Peschka M, Roberts PH, Knepper TP (2007) Analysis, fate studies and monitoring of the antifungal
575 agent clotrimazole in the aquatic environment. *Anal Bioanal Chem* 389 (3):959-968.
576 doi:10.1007/s00216-007-1480-z
- 577 Petersen C, Soder O (2006) The Sertoli cell - A hormonal target and "Super" nurse for germ cells that
578 determines testicular size. *Horm Res* 66 (4):153-161. doi:10.1159/000094142
- 579 Pierce JG, Parsons TF (1981) Glycoprotein Hormones - Structure and Function. *Annu Rev Biochem*
580 50:465-495. doi:10.1146/annurev.bi.50.070181.002341
- 581 Planas JV, Swanson P, Dickhoff WW (1993) Regulation of Testicular-Steroid Production *in-Vitro* by
582 Gonadotropins (Gth-I and Gth-II) and Cyclic-Amp in Coho Salmon (*Oncorhynchus-Kisutch*).
583 *Gen Comp Endocrinol* 91 (1):8-24. doi:10.1006/gcen.1993.1099
- 584 Plant TM, Marshall GR (2001) The functional significance of FSH in spermatogenesis and the control
585 of its secretion in male primates. *Endocr Rev* 22 (6):764-786. doi:10.1210/er.22.6.764
- 586 Roberts PH, Thomas KV (2006) The occurrence of selected pharmaceuticals in wastewater effluent
587 and surface waters of the lower Tyne catchment. *Sci Total Environ* 356 (1-3):143-153.
- 588 Schulz RW, de Franca LR, Lareyre JJ, Legac F, Chiarini-Garcia H, Nobrega RH, Miura T (2010)
589 Spermatogenesis in fish. *Gen Comp Endocrinol* 165 (3):390-411.
590 doi:10.1016/j.ygcen.2009.02.013
- 591 Schuster I (1985) The Interaction of Representative Members from 2 Classes of Antimycotics - the
592 Azoles and the Allylamines - with Cytochromes-P-450 in Steroidogenic Tissues and Liver.
593 *Xenobiotica* 15 (6):529-546
- 594 Steven C, Lehnen N, Kight K, Ijiri S, Klenke U, Harris WA, Zohar Y (2003) Molecular
595 characterization of the GnRH system in zebrafish (*Danio rerio*): cloning of chicken GnRH-II,
596 adult brain expression patterns and pituitary content of salmon GnRH and chicken GnRH-II.
597 *Gen Comp Endocrinol* 133 (1):27-37. doi:10.1016/s0016-6480(03)00144-8
- 598 Thomas KV, Hilton MJ (2004) The occurrence of selected human pharmaceutical compounds in UK
599 estuaries. *Mar Pollut Bull* 49 (5-6):436-444.
- 600 Villeneuve DL, Blake LS, Brodin JD, Greene KJ, Knoebl I, Miracle AL, Martinovic D, Ankley GT
601 (2007a) Transcription of key genes regulating gonadal steroidogenesis in control and

- 602 ketoconazole- or vinclozolin-exposed fathead minnows. *Toxicol Sci* 98 (2):395-407.
603 doi:10.1093/toxsci/kfm124
- 604 Villeneuve DL, Miracle AL, Jensen KM, Degitz SJ, Kahl MD, Korte JJ, Greene KJ, Blake LS,
605 Linnum AL, Ankley GT (2007b) Development of quantitative real-time PCR assays for
606 fathead minnow (*Pimephales promelas*) gonadotropin beta subunit mRNAs to support
607 endocrine disruptor research. *Com Biochem and Physiol C-Toxicology & Pharmacology* 145
608 (2):171-183. doi:10.1016/j.cbpc.2006.11.003
- 609 Vinggaard AM, Hnida C, Breinholt V, Larsen JC (2000) Screening of selected pesticides for inhibition
610 of CYP19 aromatase activity in vitro. *Toxicol In Vitro* 14 (3):227-234. doi:10.1016/s0887-
611 2333(00)00018-7
- 612 Wang DS, Jiao BW, Hu CJ, Huang XG, Liu ZH, Cheng CHK (2008) Discovery of a gonad-specific
613 IGF subtype in teleost. *Biochem Biophys Res Commun* 367 (2):336-341.
614 doi:10.1016/j.bbrc.2007.12.136
- 615 Zhang XW, Hecker M, Jones PD, Newsted J, Au D, Kong R, Wu RSS, Giesy JP (2008a) Responses of
616 the medaka HPG axis PCR array and reproduction to prochloraz and ketoconazole. *Environ*
617 *Sci Technol* 42 (17):6762-6769. doi:10.1021/es800591t
- 618

Figure caption

Fig.1: Expression of gonadotropin receptors and of genes involved in testis steroidogenesis of adult male zebrafish following a 21 days exposure to clotrimazole. Data are presented as the fold change in gene expression relative to the control (mean \pm SEM; n = 9 - 15 fish for each condition). An asterisk indicates a significant difference compared to control group (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$).

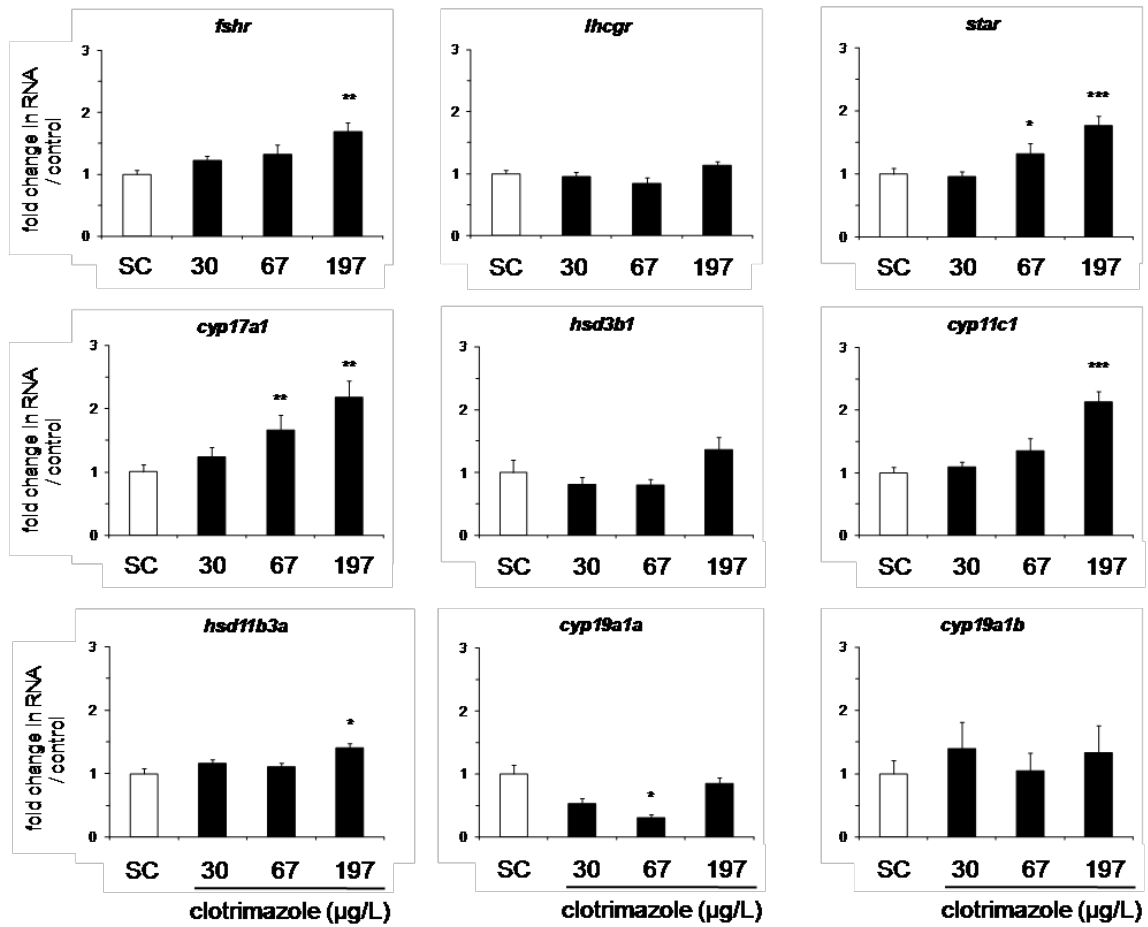


Fig.2: Expression of gonadotropins beta sub-unit genes (*fsh β* and *lh β*) in pituitary of adult male zebrafish following a 21 days exposure to clotrimazole (197 $\mu\text{g/L}$). Data are presented as the fold change in gene expression relative to the control (mean \pm SEM; n = 14 - 18 fish for each condition). An asterisk indicates a significant difference compared to control group (* $p \leq 0.05$, ** $p \leq 0.01$).

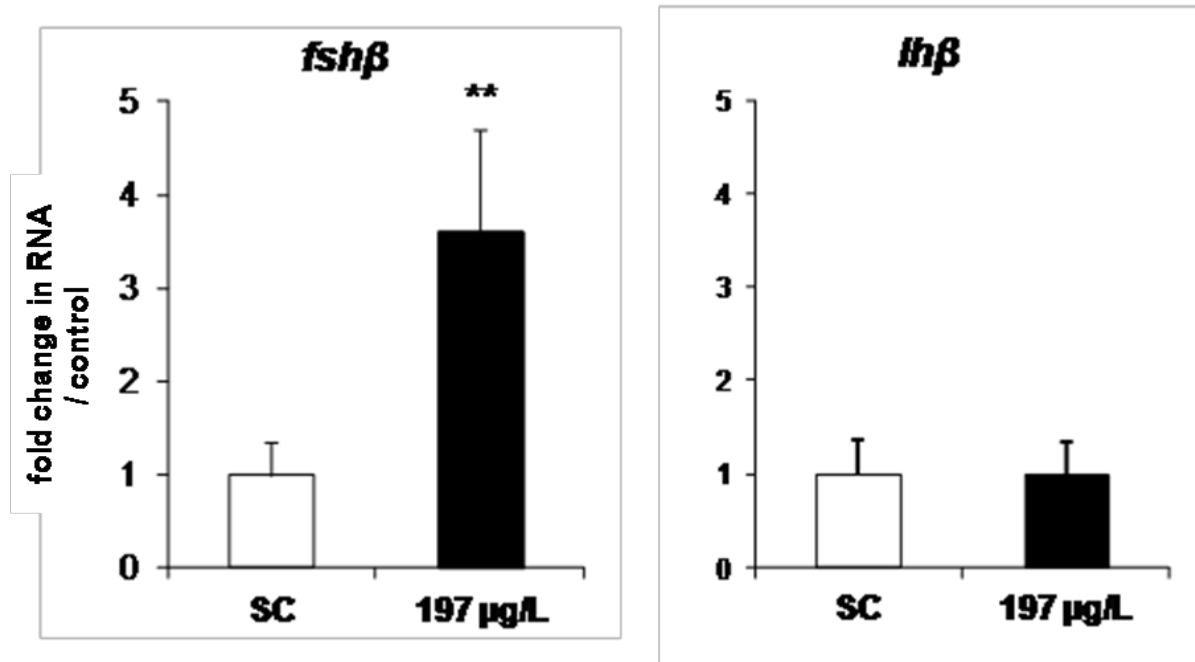


Fig.3: Change in (A) Cyp17a1 and (B) Cyp11c1 protein labelling in zebrafish testes by fluorescent immunohistochemistry after a 21 days exposure to clotrimazole. Immunoreactivity was localized in Leydig cells and observed in all groups. Red: Cyp17a1 or Cyp11c1 immunostaining, blue: Hoechst staining. N = 6 fish /condition. Pictures are representatives of the 6 individuals analyzed as regards the staining patterns. Scale Bars = 50 μ m (white).

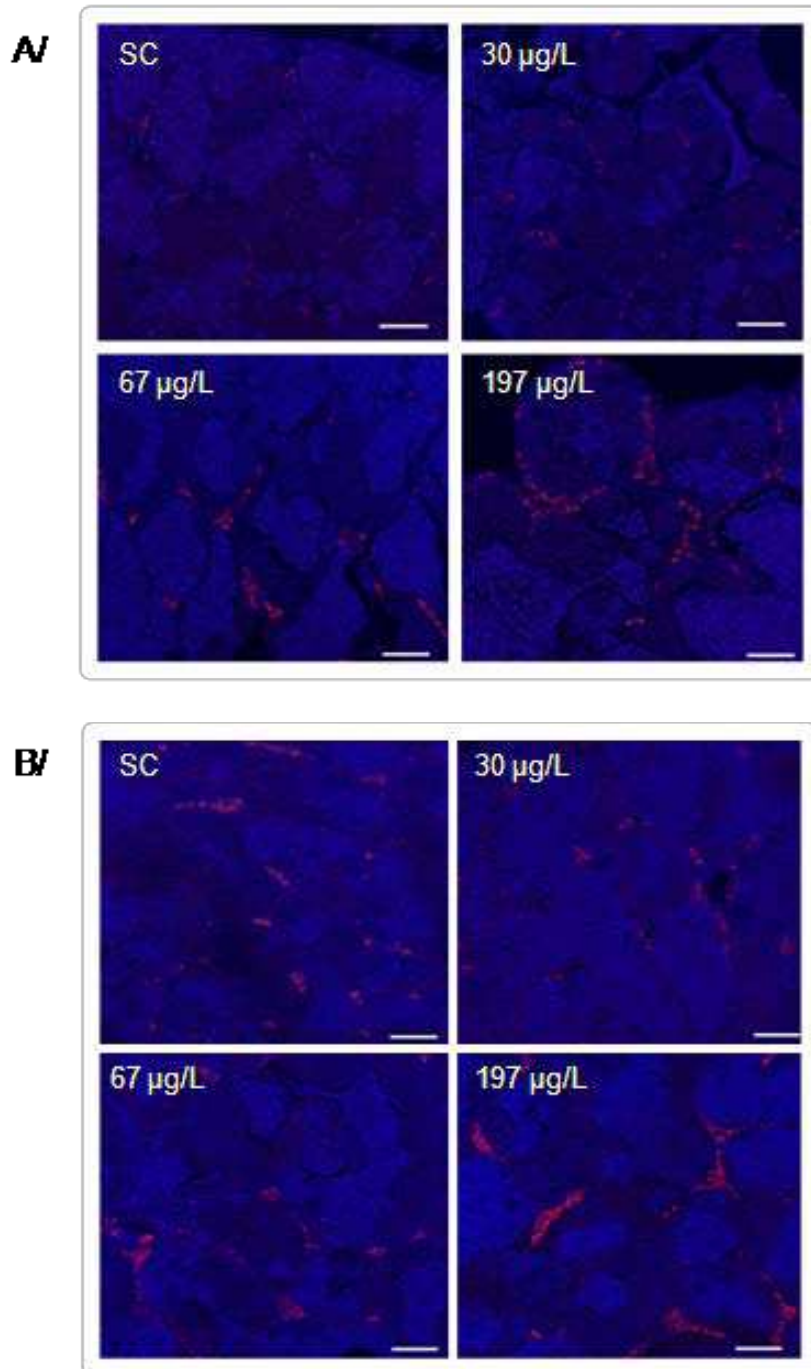


Fig.4: Plasma concentrations of 11-KT in adult male zebrafish following a 21 days exposure to clotrimazole. Data are expressed in pg/ml (mean \pm SEM; N = 15 - 21 fish /condition). 11-KT= 11 ketotestosterone (* $p \leq 0.05$).

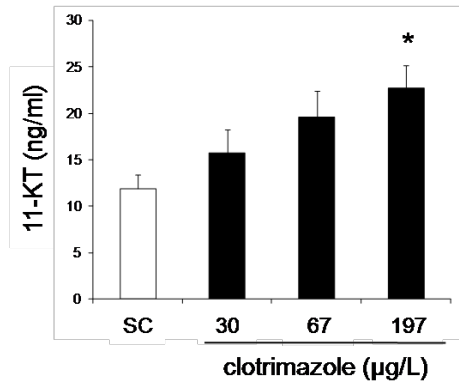


Fig. 5: Expression of Sertoli and germ cells localized-genes in testes of adult male zebrafish following a 21 days exposure to clotrimazole. Data are presented as the fold change in gene expression relative to the control (mean \pm SEM, n = 11 - 15 fish for each condition). An asterisk indicates a significant difference compared to control group (* $p \leq 0.05$, ** $p \leq 0.01$).

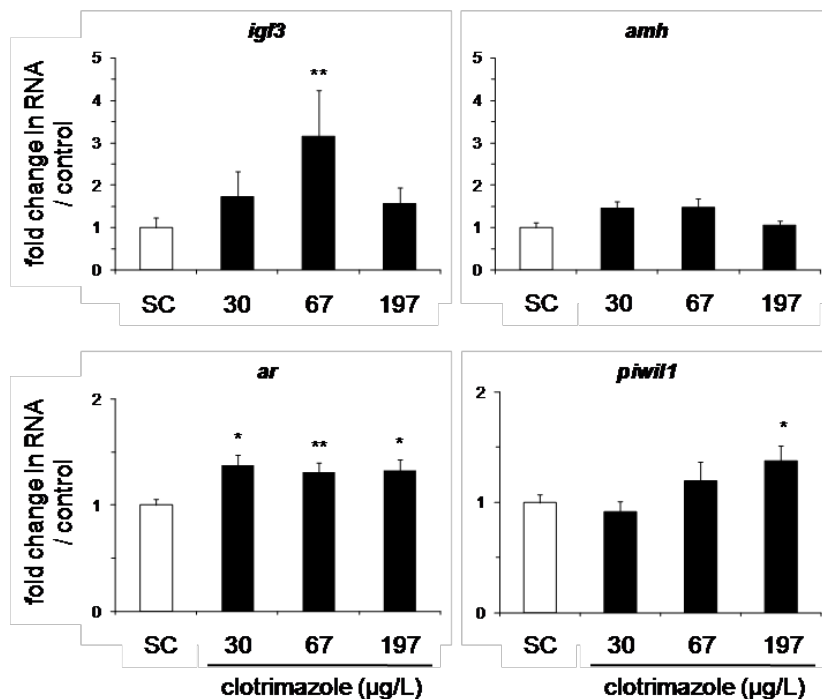


Fig.6: Principal Component Analysis (PCA) of testicular gene expressions data as well as GSI and plasma concentration of 11-KT in adult male zebrafish for control fish and clotrimazole-exposed fish: figure 6A is a plot of the two dimensional PCA and figure 6B is a trajectory plot representing each treatment groups. The 95% confidence ellipses were drawn around each group (green: solvent control, yellow: clotrimazole 28 µg/L, brown: clotrimazole 67 µg/L, red: clotrimazole 197 µg/L).

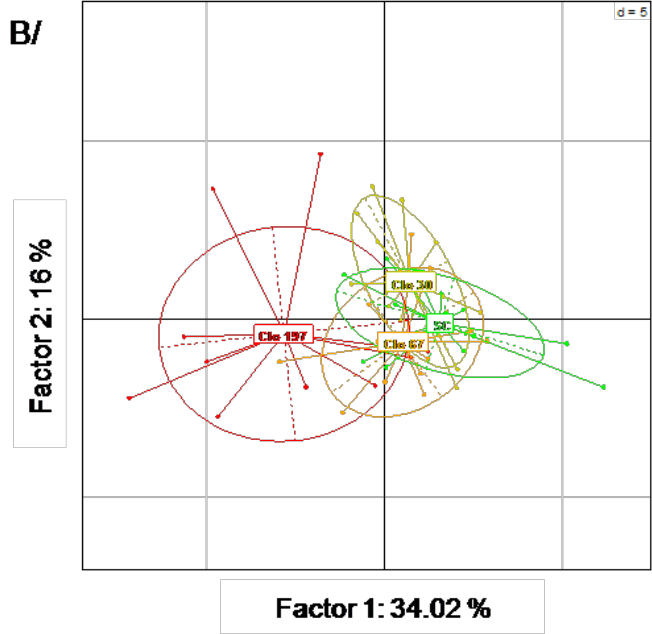
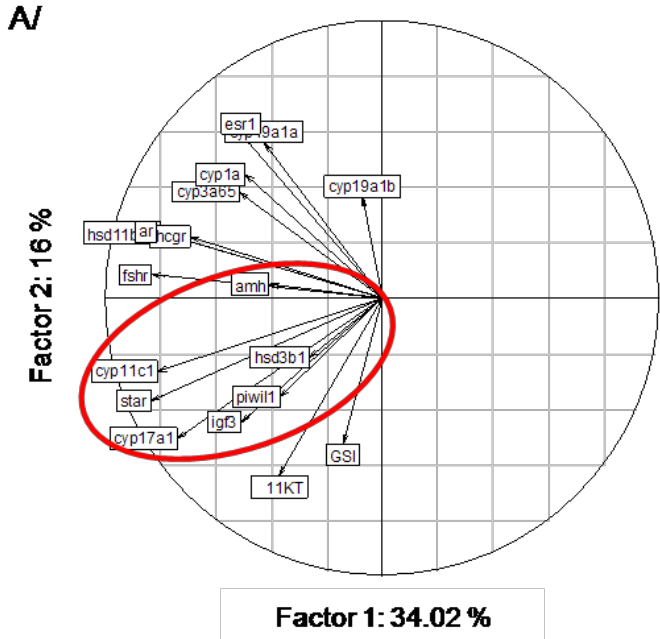


Fig.7: Zebrafish testicular structure following a 21 days exposure to 197 $\mu\text{g/L}$ clotrimazole: zebrafish testis sections from control fish (A), or from clotrimazole-exposed fish (B). % of Leydig cells in testicular tissue from control fish or clotrimazole-exposed fish (n=3-5) (C). Morphometric analysis (D) of zebrafish testis sections from control group (empty white circle, n = 5) or from the 197 $\mu\text{g/L}$ clotrimazole-exposed group (full dark circle, n = 6) following a 21 days exposure. Data are represented as mass (mg) of testicular cell types: SPGA: spermatogonia A, SPGB: spermatogonia B, SPC: spermatocytes, SPT: spermatids, SPZ: spermatozoa and others.

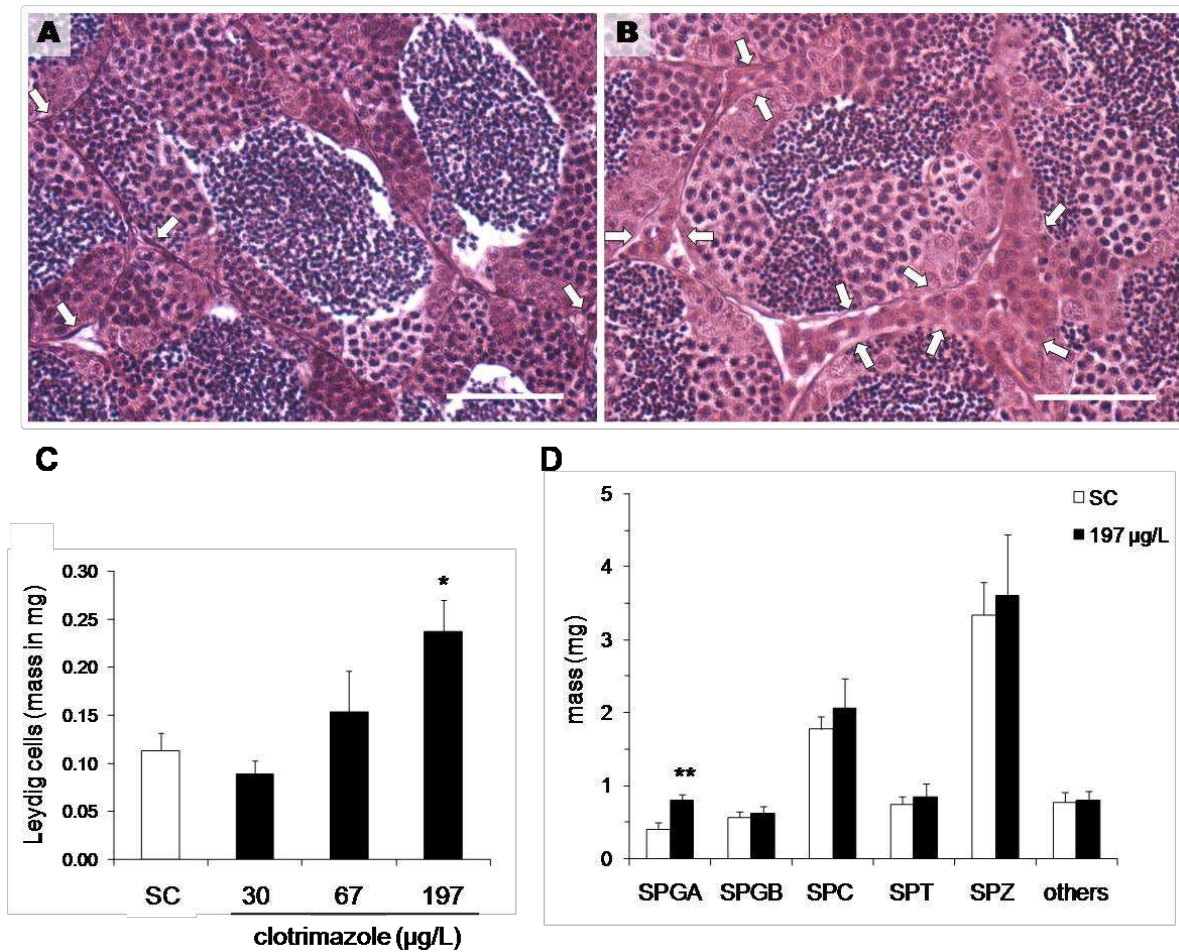


Fig.S1: Expression of phase I drug metabolizing enzymes (DMEs) and phase II conjugating enzymes genes as well as steroid receptors in the liver of adult male zebrafish following a 21 days exposure to clotrimazole. Data are presented as the fold change in gene expression relative to the control (mean \pm SEM; n = 3 - 9 fish for each condition). An asterisk indicates a significant difference compared to control group.

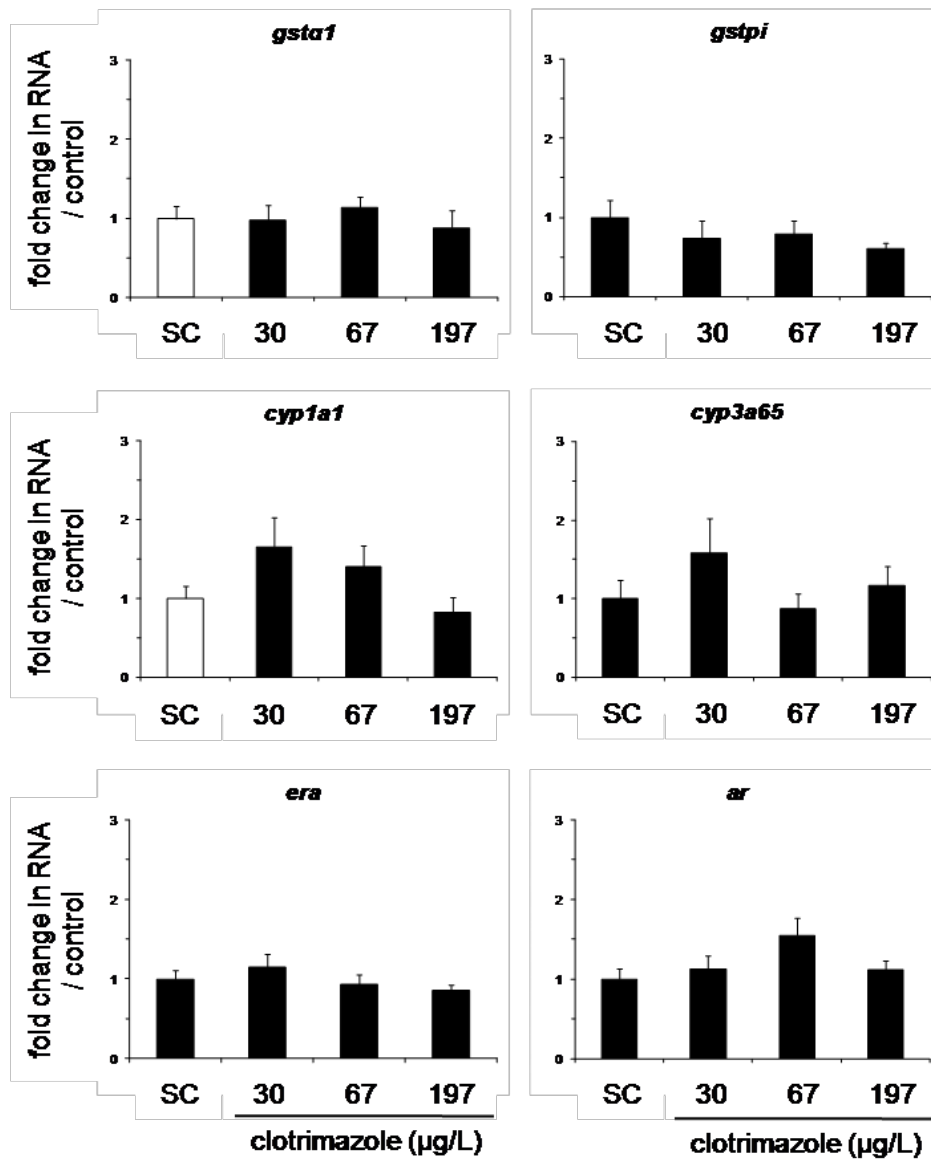


Fig.S2: Expression of drug metabolizing enzymes (DMEs) genes in testes of adult male zebrafish following a 21 days exposure to clotrimazole. Data are represented as the fold change in gene expression relative to the control (mean \pm SEM; n = 9 - 10 fish for each condition). An asterisk indicates a significant difference compared to control group (* $p \leq 0.05$).

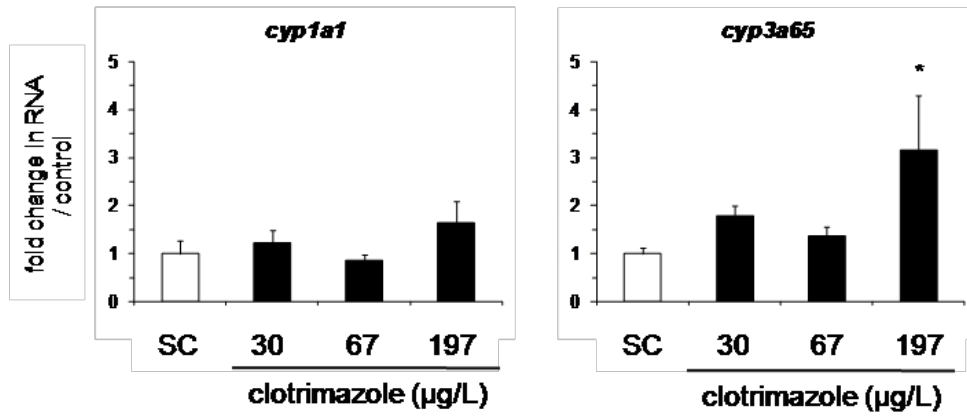


Table 1: Clotrimazole concentrations ($\mu\text{g/L}$) measured in water just after contamination ($t= 0\text{h}$) and 24h later ($t= 24\text{h}$)

	Solvent control	contamination		
		low	medium	high
t0h	< LOD	30.2 ± 1.6	66.8 ± 0.3	197.3 ± 6.4
t24h	< LOD	20.5 ± 0.8	55.5 ± 7.1	153.6 ± 3.0

LOD = Limit of detection

Table 2: Biometric parameters of fish after 21 days of chronic treatment with various concentrations of clotrimazole

Group		N	body weight (mg)	gonad weight (mg)	body lenght (cm)	GSI (%)
control	solvent	21	486 ± 103	8.3 ± 4.1	3.70 ± 0.27	1.6 ± 0.5
clotrimazole	low (29 µg/L) ¹	20	513 ± 133	8.9 ± 4.0	3.82 ± 0.27	1.7 ± 0.6
	medium (67 µg/L) ¹	21	454 ± 133	8.0 ± 4.7	3.71 ± 0.27	1.6 ± 0.8
	high (197 µg/L) ¹	21	523 ± 119	10.3 ± 3.9	3.83 ± 0.24	2.0 ± 0.9*

¹ Measured concentrations just after adding clotrimazole in water

* significant difference compared to control group (ANOVA paramétrique p ≤ 0.05)

GSI = Gonado somatic index; N = number of fish

Table 3: Effect of a 21 days exposure to clotrimazole on brain gene expression. Results are presented as ratio of clotrimazole-treated to control fish

Experimental groups	<i>genes</i>											
	<i>star</i>	<i>hsd3b1</i>	<i>cyp17a1</i>	<i>cyp11c1</i>	<i>hsd11b3a</i>	<i>cyp19a1a</i>	<i>cyp19a1b</i>	<i>gnrh3</i>	<i>ar</i>	<i>esr1</i>	<i>esr2b</i>	<i>esr2a</i>
Solvent control	1 ± 0.20	1 ± 0.25	1 ± 0.20	1 ± 0.28	1 ± 0.23	1 ± 0.29	1 ± 0.22	1 ± 0.27	1 ± 0.10	1 ± 0.11	1 ± 0.14	1 ± 0.11
Clotrimazole 30 µg/L	0.99 ± 0.17	0.83 ± 0.16	0.71 ± 0.06	0.80 ± 0.06	1.32 ± 0.23	2.34 ± 0.28	1.42 ± 0.41	0.72 ± 0.15	0.84 ± 0.06	1.35 ± 0.07	0.92 ± 0.08	0.85 ± 0.06
Clotrimazole 67 µg/L	1.05 ± 0.31	0.72 ± 0.19	0.64 ± 0.10	0.58 ± 0.15	1.02 ± 0.32	1.26 ± 0.33	0.86 ± 0.40	0.38 ± 0.11	0.86 ± 0.13	0.91 ± 0.13	0.84 ± 0.12	0.74 ± 0.11
Clotrimazole 197 µg/L	1.28 ± 0.06	1.20 ± 0.27	1.05 ± 0.08	0.84 ± 0.06	1.07 ± 0.17	1.97 ± 0.47	0.73 ± 0.21	0.58 ± 0.13	0.99 ± 0.06	1.18 ± 0.21	1.02 ± 0.14	1.17 ± 0.15

Table 4 : Morphometric analysis of the testes following several azole or triazole exposures and impact on GSI

reference	species	azole	time of exposure	concentration / duration	steroidogenesis	T /11-KT	GSI and morphometric analysis of the gonads
Brown - 2011	zebrafish (<i>Danio rerio</i>)	clotrimazole (CYPs inhibitor)	juvenile to adult (37 - 133 dph)	2.9 and 43.7 µg/L (96 days)	<i>hsd17b3, cyp19a1a</i> (-)	11-KT reduced	- increase in the GSI - increase in the proliferation of Leydig cells - germ cell development was significantly more advanced based on the proportions of spermatids and spermatozoa compared with spermatocytes and spermatogonia
Hinfray - 2011	zebrafish (<i>Danio rerio</i>)	clotrimazole (CYPs inhibitor)	adult	50 - 500 µg/L (7 days)	<i>cyp17a1</i> (++)	11-KT unchanged	- spermatogonia type A were observed more frequently
Baudiffier - 2012	zebrafish (<i>Danio rerio</i>)	clotrimazole (CYPs inhibitor)	adult	71 - 258 µg/L (7 days)	<i>star, cyp17a1, cyp11c1</i> (++)	11-KT unchanged	
Ankley - 2007	fathead minnow (<i>Pimephales promelas</i>)	ketoconazole (CYPs inhibitor)	adult	6 - 400 µg/L (21 days)	<i>cyp11a</i> and <i>cyp17a1</i> (++)	T unchanged	- increase in the GSI - marked proliferation of interstitial (Leydig) cells - interstitial cells have more rounded nuclei and more cytoplasm
Ankley - 2002	fathead minnow (<i>Pimephales promelas</i>)	fadrozole (aromatase inhibitor)	adult	2 - 50 µg/L (21 days)	not measured	T / 11-KT induced	- increase in the GSI - enlarged seminiferous tubule, accumulation of sperm in the testes - lack of germinal epithelium
Panter - 2004	fathead minnow (<i>Pimephales promelas</i>)	fadrozole (aromatase inhibitor)	adult	51.7 - 95.5 µg/L (21 days)	not measured	not measured	- increase in the GSI
Navarro-Martin - 2009	sea bass (<i>Dicentrarchus labrax</i>)	fadrozole (aromatase inhibitor)	sexually undifferentiated (90–150 dph)	100 mg.kg ⁻¹ food twice a day (60 days)	not measured	not measured	- increase in the GSI
Goetz - 2009	rat	triadimefon (CYPs inhibitor)	adult	126 mg/kg body weight/day (30 days)	not measured	T induced (plasma and intra-testicular)	- no change in the gonad weight
Turner - 2000	rat	anastrozole	adult	200 mg/L	not measured	T induced	- increase in the GSI

(aromatase
inhibitor)

(63 days)

(plasma and intra-
testicular)

- spermatogenesis was grossly normal
- 10% of rats had testes that appeared to contain only
Sertoli cells

GSI: Gonado Somatic Index; *dph*: days post hatch

Table S1: primer sequences of target genes

<i>Name</i>	forward primer sequence (5' --> 3')	reverse primer sequence (5' --> 3')	accession number
<i>star</i>	ACCTGTTTTCTGGCTGGGATG	GGGTCCATTCTCAGCCCTTAC	NM_131663
<i>hsd3b1</i>	GCAACTCTGGTTTTCCACACTG	CAGCAGGAGCCGTGTAGCTT	NM_212797.1
<i>cyp17a1</i>	GGGAGGCCACGGACTGTTA	CCATGTGGAAGTGTAGTCAGCAA	NM_212806.3
<i>cyp11c1</i>	GCTCATGCACATTCTGAGGA	TGTGCTGAAGGTGATTCTCG	DQ650710.1
<i>hsd11b3a</i>	TGGTGAAGTATGCCATCGAA	AGTAGCCATCGTGTGTGCTG	AY578180
<i>cyp19a1a</i>	CTGAAAGGGCTCAGGACAA	TGGTCGATGGTGTCTGATG	AF_226620
<i>cyp19a1b</i>	ACTAAGCAAGTCTCCGCTGTGTACC	TTTAAACATACCTATGCATTGCAGACC	NM_131642.1
<i>lhβ</i>	GCAGAGACACTTACAACAGCC	AAAACCAAGCTCTGAGCAGCC	NM_205622.2
<i>fshβ</i>	CAGATGAGGATGCGTGTGC	ACCCCTGCAGGACAGCC	NM_205624.1
<i>lhcgrr</i>	ATCACTCACGCTCTCCGACT	GCTGCTGACGCCTATTAAGG	NM_205625.1
<i>fshr</i>	GAGGATTCCCAGTAATGCTTTCCT	TCTATCTCACGAATCCCGTTCTTC	NM_001001812.1
<i>ar</i>	ACGTGCCTGGCGTGAAAA	CAAACCTGCCATCCGTGAAC	NM_001083123.1
<i>esr1</i>	GGAGATGCTGGACGCTCA	GCTGCAGCTCCTCCTTGG	NM_152959.1
<i>esr2b</i>	TGATCCTGCTCAACTCTAATAAC	TCCAGCAGATTCAGCACCTTCCC	NM_174862.3
<i>esr2a</i>	TGATCCTCCTGAACTCCAACA	TCCAGCAGACACAGCAGCTTGGA	NM_180966.2
<i>amh</i>	CTCTGACCTTGATGAGCCTCATT	GGATGTCCCTTAAGAACTTTTGCA	NM_001007779.1
<i>igf3</i>	GTGCTGCGTTCTCATCCT	TGTTGAGGAGTTTGGGT	NM_001115050
<i>piwil1</i>	CAAAATGGGAGGAGAGCTGTG	CAAAGAATCGGGAGCTGATGC	NM_183338.1
<i>cyp1a1</i>	GACAGGCGCTCCTAAAACAG	CTGAACGCCAGACTCTTCC	NM_131879.1
<i>cyp3a65</i>	CGGTGCGTACAGTATGGATG	AGAGAGGGTTCAGCAGGTCA	NM_001037438.1
<i>gstal</i>	CGCAGGAAAATACAACCTCTATG	AGCTTCCAGAAGATGAACATCAG	NM_213394.1
<i>gstp1</i>	CAGTTGCCTAAATTTGAAGATGG	AGCTTCCAGAAGATGAACATCAG	NM_131734.3
<i>gnrh3</i>	AAATGGAGGCAACATTCAGG	CCTTCAGCATCCACCTCATT	NM_182887.2