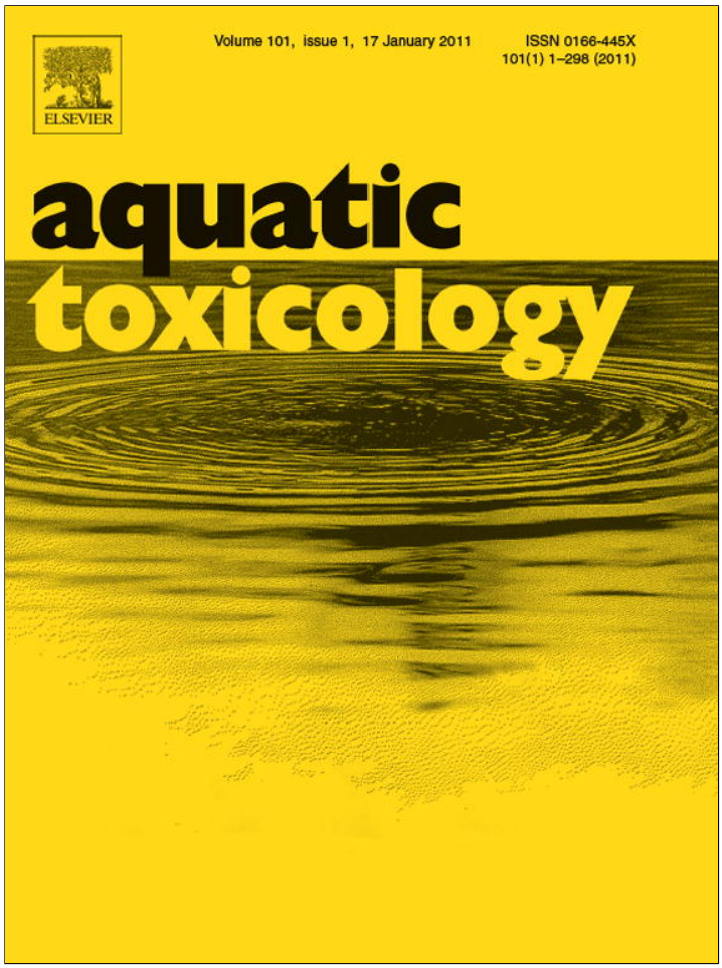

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Bezafibrate, a lipid-lowering pharmaceutical, as a potential endocrine disruptor in male zebrafish (*Danio rerio*)

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ABSTRACT

Fibrates are pharmaceuticals commonly used to control hypercholesterolemia in humans and they are frequently detected in the freshwater environment. Since cholesterol is the precursor of all steroid hormones, it is suspected that low cholesterol levels will impact steroidogenesis. However, the effect of fibrates on fish reproductive endocrinology is not clear; therefore the aim of the present study was to evaluate the effect of bezafibrate (BZF) on gonadal steroidogenesis and spermatogenesis of zebrafish (*Danio rerio*). For this purpose, adult males were exposed orally to 1.7, 33 and 70 mg BZF/g food for 21 days. Blood and gonads were collected after 48 h, 7 days and 21 days to evaluate plasma cholesterol and plasma 11-ketotestosterone (11-KT). The expression of gonadal genes involved in the steroidogenesis was quantified to determine a potential mechanism of action, likewise the effect on spermatogenesis was evaluated by examining gonadal histopathology. A time dependent monotonic decrease in the plasma cholesterol concentration was observed in fish exposed to BZF. Plasma 11-KT decreased significantly after 21 days of exposure in fish exposed to the high concentration of BZF. Different gene expression patterns were observed: down-regulation in *ppara* and *pparg* mRNA levels was observed in fish exposed to the higher concentrations after 48 h; however, the expression of *pparg* increased after 21 days. After 21 days an increase in the *star* and *cyp17a1* mRNA expression was observed in fish exposed to 70 mg BZF/g food. Sampling time and bezafibrate concentration explained 52.4% and 20%, respectively, of the gene expression variability. Gonadal histology revealed the presence of germ cell syncytia in the tubular lumen of fish exposed to bezafibrate and also an increased number of cysts containing spermatocytes, which indicate testicular degeneration. The study shows that bezafibrate exerts a hypocholesterolemic effect in adult male zebrafish and its potential as an endocrine disruptor due to its effect on the gonadal steroidogenesis and spermatogenesis.

1. Introduction

During the latest decades concern about the effects of pharmaceuticals on aquatic organisms and their possibility to interact with the endocrine systems in those organisms has been increasing (Enick and Moore, 2007; Isidori et al., 2009). However, to date only three studies have investigated the potential endocrine effects of lipid regulators on fish (Mimeault et al., 2005; Runnalls et al., 2007; Weston et al., 2009).

Epidemiological studies have shown an increased incidence of hypercholesterolemia and hypertriglyceridemia closely related to the risk of cardiovascular diseases like atherosclerosis and myocardial infarction in humans (Faergeman, 2000; Steiner, 2007) with hypercholesterolemia being the most important factor associated with these diseases (Yusuf et al., 2004). Considering this high incidence, several pharmacological treatments have been developed to reduce the levels of plasma cholesterol and triglycerides (Fruchart and Duriez, 2006; Stein, 1994). Fibrates are the oldest lipid regulators (Stein, 1994) altering mainly the apolipoprotein content in low density liproproteins (LDL); therefore they effectively reduce the levels of plasma triglycerides and LDL cholesterol (Rudney and Sexton, 1986). Fibrates act through one superfamily of nuclear receptors namely peroxisome proliferator activated receptors (PPARs), the heterodimer of which binds to retinoid X receptor (RXR) having peroxisome proliferator response elements (PPREs) as a target and subsequently altering the transcription rate of target genes.

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Fibrates can be detected in the environment and the concentrations vary from country to country. A maximum concentration of 0.79 μg/L of gemfibrozil was detected in a survey of 139 streams in U.S. with 3.6% frequency (Kolpin et al., 2002). In two United Kingdom rivers, bezafibrate was found in concentrations up to 0.066 μg/L with a frequency of 82% (Kasprzyk-Hordern et al., 2008). Bezafibrate concentrations up to 4.6 μg/L have been found in German effluents and with concentrations in rivers and streams up to 3.1 μg/L, bezafibrate was the most frequently detected in a survey of seven fibrates (Ternes, 1998).

It has been reported that fibrates, including fenofibrates and clofibric acids, reduce the level of plasma cholesterol mainly in the LDL-cholesterol form in herbivorous grass carp (*Ctenopharyngodon idella*) and fathead minnow (*Pimephales promelas*) (Du et al., 2008; Runnalls et al., 2007) supporting a hypocholesterolemic effect in fish. Since cholesterol is one of the most important sterols in the cell and all steroid hormones are synthesized from this molecule (Bose et al., 2002), it may be expected that alterations in gonadal steroid hormones may consequently occur. It has been shown that goldfish (*Carassius auratus*) exposed to the lipid regulator gemfibrozil exhibited a reduced testosterone (T) concentration after waterborne exposure to 1500 μg/L or 1.5 μg/L for 96 h or 14 days, respectively (Mimeault et al., 2005). Similarly, T levels tended to decrease in fathead minnow exposed to 0.01 and 1 mg/L clofibric acid (Runnalls et al., 2007); these authors also observed a significant decrease in the sperm count and sperm motility in exposed males in a 21 days waterborne exposure.

The steroid hormones are derived from cholesterol in a cascade involving different enzymatic reactions; therefore, the steroidogenic cells have specific requirements to maintain the normal levels of cholesterol (Eacker et al., 2008). In fish there are two main sources of productions of steroid hormones, the interrenal tissue producing cortisol, 11-deoxycorticosterone, etc. and the gonads producing estradiol, progesterone, 17, 20β -dihydroxy-4-pregne-3-one (17, 20β-P), testosterone (T) and 11-ketotestosterone (11-KT), the latter being the most relevant androgen in fish (Young et al., 2005).

Two groups of enzymes, *cyp* (cytochrome P450) and *hsd* (hydroxysteroid dehydrogenase) are involved in steroidogenesis (Payne and Hales, 2004). In fish, the genes encoding the gonadal steroidogenesis process include *star* (steroidogenic acute regulatory protein), *cyp11a1* (cytochrome P450 side chain cleavage), *cyp17a*, *cyp11b*, (11β-hydroxylase), *cyp19a1a* (aromatase). The Leydig cell is the main place of expression of *cyp11a1*, which is an important rate-limiting step in the steroidogenesis catalyzing cholesterol to pregnenolone (Payne and Hales, 2004). In fish, 3β-hydroxysteoid dehydrogenase (*hsd3b*), *hsd17b* and *hsd20b* play important roles in the synthesis of bioactive steroids (Young et al., 2005). Therefore, determination of the expression or levels of these enzymes will elucidate the most probable targets when endocrine substances are present. In addition, morphometric and histological analysis of the spermatogenic cells can be a useful tool to determine the spermatogenesis functionality (Schulz et al., 2010), and therefore to evaluate the impact of potential endocrine disruptors.

The aim of the present study was to evaluate the effect of bezafibrate as a potential endocrine disruptor in fish by evaluating its effect on the gonadal steroidogenic enzymes, cholesterol and 11-KT levels and gonadal histology during oral exposure for 21 days.

2. Materials and methods

2.1. Chemicals and kits

Bezafibrate (BZF) was purchased from Sigma-Aldrich (B7273, USA). Acetone (96% purity) was obtained from Sigma–Aldrich (Vallensbæk Stand, Denmark). Cholesterol assay and 11-KT kits were purchased from Cayman Chemical (Ann Arbour, MI, USA).

2.2. Fish

Sexually mature male zebrafish (*Danio rerio*) (500 ± 11 mg body mass) were obtained from a local supplier in Alborg, Denmark and adapted to laboratory conditions for four weeks at 27 ± 1 °C on 14 h:10 h light:dark cycle. Fish were fed with commercial flakes (TetraMin®, Melle, Germany) by an automatic feeder (F-14 Fish Mate) twice per day. The experiments were carried out following the guidelines of the Danish Law on Animal Experiments under license 2008/561-1471 from the Danish Ministry of Justice.

2.3. Food preparation

Results from a preliminary experiment in our laboratory showed that 100 mg BZF/g food was the highest concentration maintaining the food palatability and fish acceptance; therefore, the highest nominal bezafibrate concentrations was 100 mg BZF/g food. In order to establish the solubility of bezafibrate in acetone, several tests were done showing a maximum solubility of 12.5 mg BZF/mL acetone. Based on this result, for each BZF concentration a solution in acetone was produced and then added to the food in a proportion of 8 mL acetone/g food. Food offered to the control group was prepared only with acetone.

Before the experiment, all fish per aquarium were weighed and the weight average was considered to calculate the food ratio using 2% body weight twice per day. An automatic feeder was used to supply the food and immediately after the food was dispensed, the feeders were checked to guarantee that fish received the entire daily ration. The prepared food was kept at room temperature until use.

2.4. Experimental setup and sampling procedure

One week before starting the exposure, 12 fish were distributed randomly to each aquarium (8 L volume capacity) without any significant differences in the body mass between aquaria. The treatments assigned were also done randomly with four replicate aquaria per treatment with 12 fish/aquarium (48 fish/treatment in total). A flow-through test system with a water exchange of 12.5 mL/min (18 L/day) was used.

The water used during acclimatization and the experiment contains one part of tap water (ground water: pH 7.5, conductivity 85 mS/m, iron 0.013 mg/L, hardness 268 mg/L, manganese 0.005 mg/L) and three parts of deionised water. Water quality parameters in each aquarium were measured every week during the exposure using a multi-parameter display system (YSI 55 dissolved oxygen, Ohio, USA) with the following values (mean ± SEM): dissolved oxygen concentration of 5.4 ± 0.03 mg/L, oxygen saturation 67.1 ± 0.44%, temperature 26.5 ± 0.13 °C and pH 7.8 ± 0.02. Similarly, ammonium (NH_4^+), nitrite (NO_2^-) and nitrate (NO_3^-) were measured in a Lachat QuikChem® 8500 Series Ion Analyzer (Loveland, CO, USA) with the results stable throughout the experiment: 10.4 ± 4.1 μM of NH_4^+, 12.5 ± 4.9 μM of NO_2^- and 2.1 ± 0.8 μM of NO_3^-.

The three nominal concentrations of bezafibrate evaluated were 5, 50 and 100 mg BZF/g food. Bezafibrate concentrations in the water were analyzed at three different times during the day: before morning feeding, midday and late afternoon (Table 1). The experiment initiation was staggered in a 24 h interval in order to reduce the variability due to exposure time and sampling. For this purpose, the four replicates were divided in two groups in which exposure in the second group was started 24 h after the first group.

Table 1
Body mass, fork length, condition factor (K_n) and somatic indices of male adult zebrafish (*Danio rerio*) after exposure to 1.7, 33 and 70 mg bezafibrate/g food for 48 h, 7 days and 21 days. Values are expressed as mean ± SEM (n = 12).

Parameter	Sampling time											Statistic[+]
	48 h				7 days			21 days				
	0[a]	1.7[a]	33[a]	70[a]	0[a]	1.7[a]	33[a]	70[a]	0[a]	1.7[a]	33[a]	70[a]	
Body mass (mg)	522 ± 25	502 ± 25	513 ± 21	464 ± 15	529 ± 23	570 ± 32	518 ± 18	515 ± 21	582 ± 24	602 ± 25	566 ± 20	528 ± 19	$F_{8,36}$ = 2.92, p = 0.013
Fork length (mm)	36 ± 0.4	36 ± 0.6	36 ± 0.6	35 ± 0.4	37 ± 0.5	38 ± 0.8	37 ± 0.4	37 ± 0.5	37 ± 0.5	38 ± 0.7	37 ± 0.4	37 ± 0.5	$F_{8,36}$ = 2.36, p = 0.04
K_n	1.07 ± 0.03	1.04 ± 0.02	1.05 ± 0.02	1.03 ± 0.01	1.05 ± 0.02	1.01 ± 0.02	1.0 ± 0.03	1.0 ± 0.01	1.07 ± 0.02	1.09 ± 0.03	1.05 ± 0.02	1.05 ± 0.02	$F_{8,36}$ = 2.08, p = 0.06
HSI (%)[#]	0.96 ± 0.1	0.96 ± 0.09	0.99 ± 0.07	0.97 ± 0.08	1.11 ± 0.1	1.23 ± 0.1	1.15 ± 0.08	0.91 ± 0.10	1.28 ± 0.08	1.41 ± 0.08	1.28 ± 0.08	1.31 ± 0.08	$F_{8,36}$ = 2.97, p = 0.01
GSI (%)	1.49 ± 0.06	1.32 ± 0.07	1.40 ± 0.08	1.22 ± 0.08 *	1.31 ± 0.07	1.26 ± 0.13	1.27 ± 0.06	1.37 ± 0.05	1.30 ± 0.07	1.30 ± 0.04	1.27 ± 0.06	1.11 ± 0.05	$F_{8,36}$ = 1.95, p = 0.08

[a] mg BZF/g food.
[*] At each sampling time, statistical significant differences compared to control group (Dunnestt's test, $p < 0.05$).
[+] Nested ANOVA (aquarium within treatment X sampling time), $p < 0.05$ shows significant interaction.
[#] An aquarium effect was only observed in the HSI ($F_{36,96}$ = 1.96, p = 0.005).

During the exposure, fish were sampled for analysis at 48 h, 7 days and 21 days. Fish were not fed in the afternoon before sampling. At each endpoint, fish were sampled (3 fish/aquarium, n = 12 fish/treatment) for somatic indices, blood samples and gene expression. For histology analysis, 3 fish/aquarium (n = 12 fish/treatment) were sampled at 21 days. At each sampling time, the fish (n = 12 fish/treatment) were deeply anaesthetized in a buffered solution of MS-222 (100 mg/L, Sigma–Aldrich) and subsequently body mass and length were measured. Fish were euthanized by severing the spinal cord and afterwards blood was collected from a cut in the tail in a heparinised microhematocrit tube and subjected to centrifugation at 12,000 rpm for 3 min. Plasma was immediately collected, immersed in liquid nitrogen and stored at −80 °C until determination of cholesterol and 11-KT. Subsequently, liver and testis were carefully dissected using tools washed in ethanol between each sample, immediately transferred to a pre-weighed Eppendorf tube, weighed and snap-frozen in liquid nitrogen to prevent degradation of RNA and stored at −80 °C until analysis by RT-PCR.

Gonadosomatic (GSI) and hepatosomatic indices (HSI) were calculated as (tissue weight/body weight) × 100. The condition factor (K_n) was calculated as: ($K_n = W/aL^b$), where W is the body mass (g), L is the length (cm), and a (y-intercept) and b (slope) values were obtained from Velasco-Santamaría et al. (2011). At the end of the exposure, 12 fish per treatment were deeply anaesthetized in a buffered solution of MS-222 and the tail removed. The abdomen was opened and the specimen fixed in Bouin's solution for subsequently histology.

2.4.1. LC–MS/MS quantification of bezafibrate in food and water

To measure BZF in the food, 5 mL acetone was added to 100 mg food and left at room temperature for minimum 48 h. Afterwards, the acetone supernatant was taken and diluted in methanol at the ratio 1:100, 1:2000 or 1:5000 for the low, medium and high concentration of bezafibrate, respectively.

Bezafibrate was analyzed in filtered (0.45 μm PVDF syringe filter) water samples (1 mL), three times per day once per week using HPLC–MS/MS. A 1200 Series HPLC and a 6410 Triple Quad LC/MS (Agilent Technologies, 2850 Centerville Road Wilmington, DE, USA) was used. The procedure for quantification of bezafibrate is described briefly.

The sample (water or acetone) was injected in the HPLC–MS/MS with the following specifications: column Zorbax Eclipse XDB C_{18} 4.6 mm × 50 mm, 1.8 μm Rapid Resolution HT with a column temperature of 70 °C, isocratic 36:65t with ammonium acetate (10 mM) and MeOH as solvents. Flow 1.0 mL/min, stop time 1.5 min, injection 20 μL, mode ESI positive, scan type MRM, drying gas flow: 6.0 L/min, nebulizer pressure 25 psig, drying gas temperature 300 °C and capillary voltage: 3500 V. Other setups included precursor ion 362.2, quantifier ion 139.1, dwell: 200, fragmentor: 120 V and collision energy 25 V. The standard was prepared using bezafibrate (Sigma–Aldrich B7273) dissolved in 20% MeOH. Five-hundred ng/mL was used as the highest standard. The average recovery was 91.6 ± 3.2% and the detection limit was 0.00055 μg/mL.

2.5. Plasma cholesterol levels

Plasma cholesterol was quantified using a Cayman's cholesterol assay kit following the manufacturer's procedure. To evaluate the best dilution, carp and zebrafish plasma were used in the preliminary test. Plasma samples (0.5–1.0 μL) were diluted a minimum 300 fold in cholesterol assay buffer (K_3PO_4 0.1 M, NaCl 50 mM and cholic acid 5 nM, pH 7.4) and kept at 4 °C. Cholesterol was measured using a microplate fluorescence reader with Omega software v 1.01 (FLUOstar Omega BMG LABTECH) with a fluorescence of 560/590 nm (excitation/emission). The plates were incubated in darkness for

30 min at 37 °C and then read against a cholesterol standard curve (0–20 μM; $R^2 = 0.997$).

2.6. Plasma 11-KT steroid levels

Plasma was diluted to 1:100 in PBS buffer 0.1 M (pH 7.4) and then four times of ethyl acetate/hexane (1:1) were added and vigorously mixed twice for 15 s. The layers were allowed to separate by freezing the aqueous layer in a 75% ethanol/dry ice bath and decanting for a minimum of 30 min. The organic phase was carefully collected and placed into a clean TurboVap glass tube and evaporated partially in a TurboVap (TurboVap LV Caliper Life Sciences) at 30 °C for 5 min. The aqueous phase was re-extracted twice in the same way and the organic phase collected into the same tube to finally evaporate in the TurboVap at 30 °C for 15 min and stored at −20 °C until the ELISA analysis was done. The extracted sample was dissolved in EIA buffer depending on the initial volume. A Cayman 11-KT EIA kit with a detection limit (80% B/Bo) of 1.3 pg/mL was used. The assay procedure and results analysis were carried out according to Cayman instructions and the plates read at 405 nm absorbance (ThermoMax microplate reader, Molecular devices). To check the 11-KT recovery percentage, a non-radioactive spike was done with carp plasma before the extraction (106 ± 9% recovery). The intra assay variation was 5.3% and the inter assay variation was 8.1%.

2.7. Real-time qPCR

From each treatment and each sampling time, 12 testes were homogenized in 800 μL of TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and subsequently sonicated for 10 s at 50% power (Ultrasonic homogenizer Bandelin Sonoplus HD2070, Berlin, Germany). Afterwards, RNA was extracted according to the manufacturer's protocol. The RNA concentration was measured with a Nano Drop spectrophotometer at 260 nm. All samples had a ratio A_{260}/A_{280} higher than 1.9 and a ratio A_{260}/A_{230} around 2.0 confirming a low risk of protein and organic contamination, respectively. The method for reverse transcription, quantification and analysis of gene expression using real-time qPCR can be found in Velasco-Santamaría et al. (2010).

Species-specific primers were developed using the Primer3 program (Rozen and Skaletsky, 1999) and synthesized by Invitrogen (Carlsbad, CA, USA). The oligo sequence, amplicon size and the GenBank accession number for PPARα (*ppara*), PPARβ (*pparb*), PPARγ (*pparg*), StAR (*star*), P450scc (*cyp11a1*), 3βHSD (*hsd3b1*), Cyp19a1a (*cyp19a1a*), Cyp17a1 (*cyp17a1a*), Cyp11b (*cyp11b*), 17β-HSD3 (*hsd17b3*), 11β-HSD2 (*hsd11b2*) and EF1α (*elf1a*) are shown in Supplementary Table 1. Italicized gene names correspond to the fish gene nomenclature. These genes were selected based on their role in gonadal steroidogenesis or cholesterol metabolism. Elongation factor-1α (*elf1a*) was used as a normalization gene with no significant differences between treatments. To evaluate the suitable normalization gene, *elfa* and *b-actin* were evaluated in 72 samples showing that *elfa* had lower and more stable C_t values (19.2 ± 0.03, CV = 1.5%) than *b-actin* (21.5 ± 0.05, CV = 2%).

Template cDNA was combined with 250 nM forward and reverse primer. No amplification was detected in no-template controls (NTC). The size of each amplicon was verified using high resolution agarose gel electrophoresis (Methaphor Agarose, Cambrex Bio Science, Rockland, ME, USA). To estimate the amplification efficiency of the genes analyzed, a standard curve was constructed by graphing C_t-values from a dilution series of cDNA versus cDNA concentration. The slopes and amplification efficiency from these curves are shown in Supplementary Table 2. The relative copy numbers (RC) for each target gene were calculated using the equation $RC = 10(C_t/\text{slope})$, where C_t is the threshold cycle number and the slope is the data obtained from the efficiency curve. Two qPCR runs were done for each gene due to the high number of samples (144 samples). To correct for possible run-to-run variation, an inter-run calibration was done as recommended by Derveaux et al. (2010). The mRNA expression was calculated as copy number of each target gene/*elfa* copy numbers considering the correction factor.

2.8. Gonadal histology

After a maximum of 24 h of Bouin's fixation, each specimen was transferred to 70% ethanol and stored at 4 °C. Specimens from control group and fish exposed to the high concentration of bezafibrate were dehydrated with increasing ethanol concentrations (70–99.9%), cleared in tissue clear and then embedded in paraffin wax. The trunk was cut longitudinally through the entire gonadal region at 5 μm thickness; seven slides from each trunk were subsequently stained with Mayer haematoxylin and eosin Y (H&E) and only the uneven slides were examined using light microscopy. Two sections per slide were analyzed, each one 15–20 μm apart ($n = 8$ sections/per fish).

The testis was analyzed following the OECD guidance document No. 123 (OECD, 2010). Briefly, the histopathological alterations were classified in primary and secondary alterations. For the primary alterations (*e.g.* germ cell syncytia), five different severity scores were used: not remarkable, minimal (grade 1, less than 20% alterations), mild (grade 2, between 20 and 50% alterations), moderate (grade 3, between 50 and 80% alterations) and severe (grade 4, more than 80% alterations observed). Secondary alterations (*e.g.* altered proportion of spermatocytes) were categorically scored using present/absent classification.

2.9. Statistics

All values are expressed as mean ± SEM. The data normality distribution and homogeneity of variance were evaluated through the Kolmogorov–Smirnov and Levene's tests, respectively. In addition, the data were analyzed considering the plot residual distributions. Data transformation was carried out when data did not fulfil the normality or homogeneity of variance. After transformation, the data were suitable for carrying out the subsequent test. To determine the potential effect of the aquarium in all variables, a one level nested Model III ANOVA was used where the interaction treatment and sampling time was treated as a fixed factor and aquarium as a random factor (aquarium nested within treatment × sampling time). If in the statistical model the measured variables were significantly affected by either the Nested-ANOVA analysis (MS group/MS subgroup) or the treatment, a Dunnett's test was used as post-hoc analysis to compare all BZF treatments versus control within each time-group. Finally, a correlation analysis between GSI, plasma 11-KT, plasma cholesterol and the mRNA gene expression was carried out. A PCA plot was done to visualize the covariance of all genes evaluated between the two factors involved (BZF concentration and sampling time) using Primer6 (version 6.1.10 – 2007). The chi-square (χ^2) test was used to evaluate overall significant differences in the testis histopathological alterations between control and treatment group. In all cases, a rejection level of $p < 0.05$ was used and all statistical analysis was conducted using SAS system for Windows software version 9.2 (2002–2008 by SAS Institute, Cary, NC, USA).

3. Results

3.1. Somatic indices and growth parameters

There was no treatment-related mortality in any treatment. Fish in all treatment groups showed positive growth during the experiment with no statistical difference in the body mass (534 ± 7 mg), fork length (37 ± 0.2 cm) and condition factor (K_n) (1.04 ± 0.006).

Table 2
Bezafibrate (BZF) concentrations in food ($n = 4$) and water ($n = 3$), and the calculated intake of BZF/g fish. The measurements were done every week by HPLC–MS/MS. Values showed as mean ± SEM.

Food BZF concentration (mg BZF/g food)		Water BZF concentration (μg BZF/L)	BZF intake (μg BZF/g fish)
Nominal	Measured		
Control	0	0	0
5	1.7 ± 0.1	8.8 ± 2.4	35 ± 0.03
50	33 ± 2.3	80 ± 18	667 ± 0.7
100	70 ± 2.6	201 ± 81	1428 ± 2.0

A positive linear relationship between food and water concentration was observed ($r^2 = 0.71$, $p < 0.0001$).

The body mass, fork length and K_n for each sampling time and concentration are shown in Table 1 (interaction Nested ANOVA, df = 8,36, $p < 0.05$).

The hepatosomatic index and the gonadosomatic index for each sampling time and concentration are shown in Table 1. In terms of the relative size of the gonads, no statistical interaction between treatment versus sampling time (Nested ANOVA, $F_{8,36} = 1.95$, $p = 0.083$) neither treatment effect were observed ($F_{3,96} = 1.55$, $p = 0.207$); however, at 48 h the GSI was smaller in fish exposed to 70 mg BZF/g food when compared to control (Dunnett's test, df = 12, $p = 0.028$) and also a trend to decrease in the GSI at 21 days was observed (Dunnett's test, df = 12, $p = 0.079$). Although significant interaction between treatment versus time was observed in the HSI (Nested ANOVA, $F_{8,36} = 2.97$, $p = 0.011$), an aquarium (replica) effect was also observed ($F_{36,96} = 1.96$, $p = 0.005$); therefore a post-test was not performed.

3.2. Bezafibrate concentrations

Bezafibrate was kept stable in the food along the 21 days of exposure. The measured BZF concentrations in food were relatively close to the nominal concentrations as shown in Table 2. The measured concentration in water samples had a positive linear correlation with the food concentration as shown in Table 2 ($r^2 = 0.71$, $p < 0.0001$).

3.3. Plasma cholesterol

A time dependent monotonic decrease in the plasma cholesterol concentration was observed in fish exposed to BZF in food (Fig. 1). A significant interaction between treatment and sampling time in the nested model was observed (Nested ANOVA, $F_{8,36} = 2.54$, $p = 0.026$) with the aquarium having no effect ($F_{36,95} = 0.55$, $p = 0.977$). After the first 48 h the levels of cholesterol were similar in all treatments (Dunnett's test, df = 12, $p > 0.05$); however, after 7 days of exposure fish exposed to 33 and 70 mg BZF/g food had 25 and 48%, respectively, less cholesterol than control fish (Dunnett's test, df = 12, $p = 0.04$ and $p < 0.001$, respectively). This pattern was observed after 21 days in which all fish exposed orally to BZF had a significant decrease in the cholesterol concentration (more than 30%, Dunnett's test, df = 12, $p = 0.046$, $p = 0.032$ and $p = 0.009$, for the low, medium and high concentration) (Fig. 1).

3.4. Plasma 11-KT

An aquarium (replica) effect was observed in the 11-KT concentration ($F_{36,95} = 2.17$, $p = 0.0015$) and no significant interaction between treatment and sampling time in the nested model was observed (Nested ANOVA, $F_{8,36} = 1.52$, $p = 0.185$); however, a treatment effect was observed ($F_{3,96} = 3.54$, $p = 0.017$). The post-hoc test revealed no significant effect on the plasma 11-KT level after the first 48 h and 7 days of exposure (Dunnett's test, df = 12, $p > 0.05$); however, after 21 days of exposure a significant decrease in the plasma 11-KT was observed in fish exposed to the high concentration of bezafibrate (6187 ± 824 pg/mL) when compared to control fish (13,042 ± 1584 pg/mL) (Dunnett's test, df = 12, $p = 0.011$, Fig. 2).

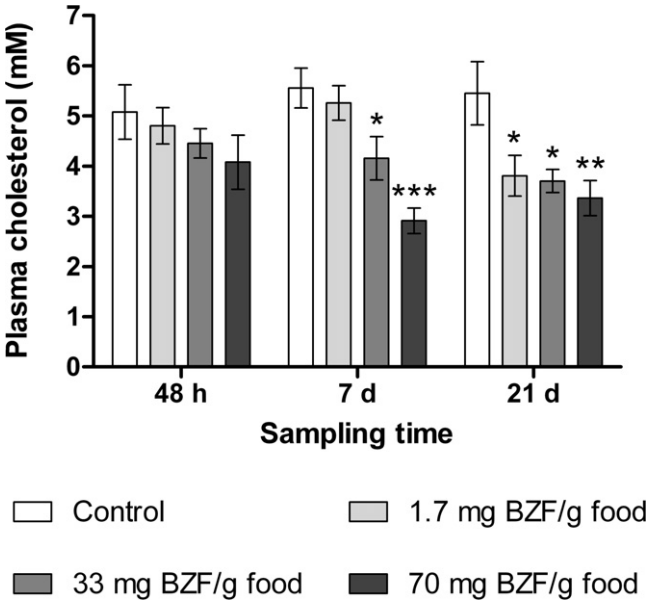

Fig. 1. Plasma cholesterol concentration (mM) in male adult zebrafish (*Danio rerio*) after exposure to 1.7, 33 and 70 mg bezafibrate/g food for 48 h, 7 days and 21 days. Bars indicate the mean and standard error for four replicate tanks per treatment ($n = 12$ fish/treatment); each fish was analyzed in duplicate. At each sampling time, asterisk (*) shows statistically significant differences compared to the control group (Dunnett's test, df = 12, $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). A significant interaction between treatment and sampling time in the nested model was observed (Nested ANOVA, $F_{8,36} = 2.54$, $p = 0.026$).

3.5. Expression of ppar isoform mRNA in testis

The three *ppar* isoforms analyzed showed different pattern of expression in the testis of zebrafish exposed to bezafibrate. No significant effect was observed in the mRNA expression of *ppara*

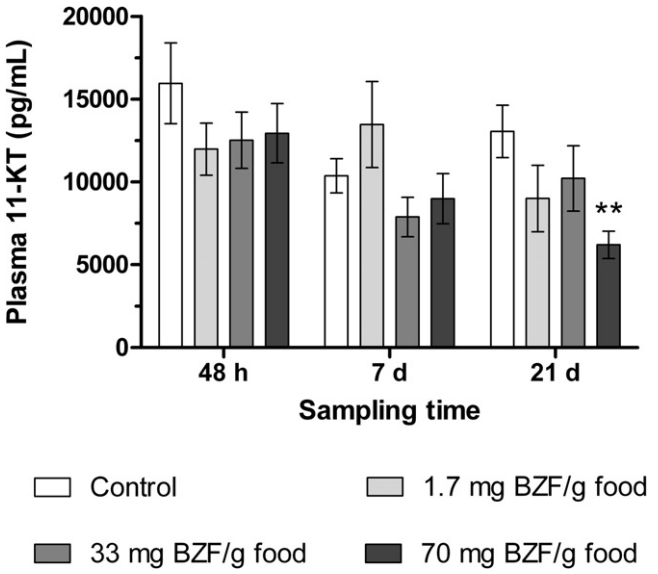

Fig. 2. Plasma 11-KT level (pg/mL) in male adult zebrafish (*Danio rerio*) after exposure to 1.7, 33 and 70 mg bezafibrate/g food for 48 h, 7 days and 21 days. Bars indicate the mean and standard error for four replicate tanks per treatment ($n = 12$ fish/treatment); each fish was analyzed in duplicate. At each sampling time, asterisk (*) shows statistically significant differences compared to the control group (Dunnett's test, df = 12, $^{**}p < 0.01$).

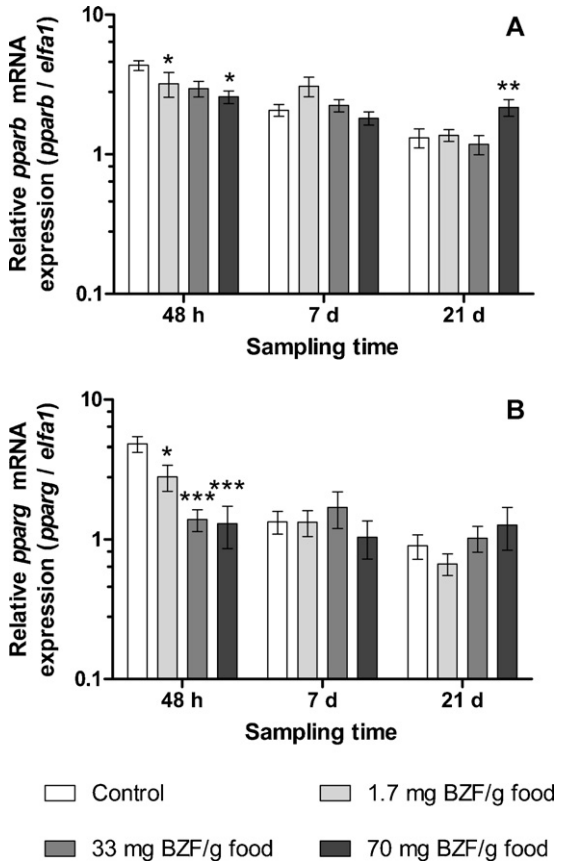

Fig. 3. Transcript mRNA abundances of *pparb* (A) and *pparg* (B) in testis of male adult zebrafish (*Danio rerio*) after exposure to 1.7, 33 and 70 mg bezafibrate/g food for 48 h, 7 days and 21 days. Bars indicate the mean and standard error for four replicate tanks per treatment ($n = 12$ fish/treatment) and are expressed on a logarithmic scale as a relative expression normalised to *elfa1* transcript abundance in the same sample. At each sampling time, asterisk (*) shows statistically significant differences compared to the control group (Dunnett's test, df = 12, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). For *pparb* and *pparg* mRNA expression, an aquarium effect was observed (df = 36,95; $p < 0.005$) with a significant interaction between treatment and sampling time in the nested model for both *pparb* (Nested ANOVA, $F_{8,36} = 6.69$, $p < 0.0001$) and *pparg* (Nested ANOVA, $F_{8,35} = 3.01$, $p = 0.011$).

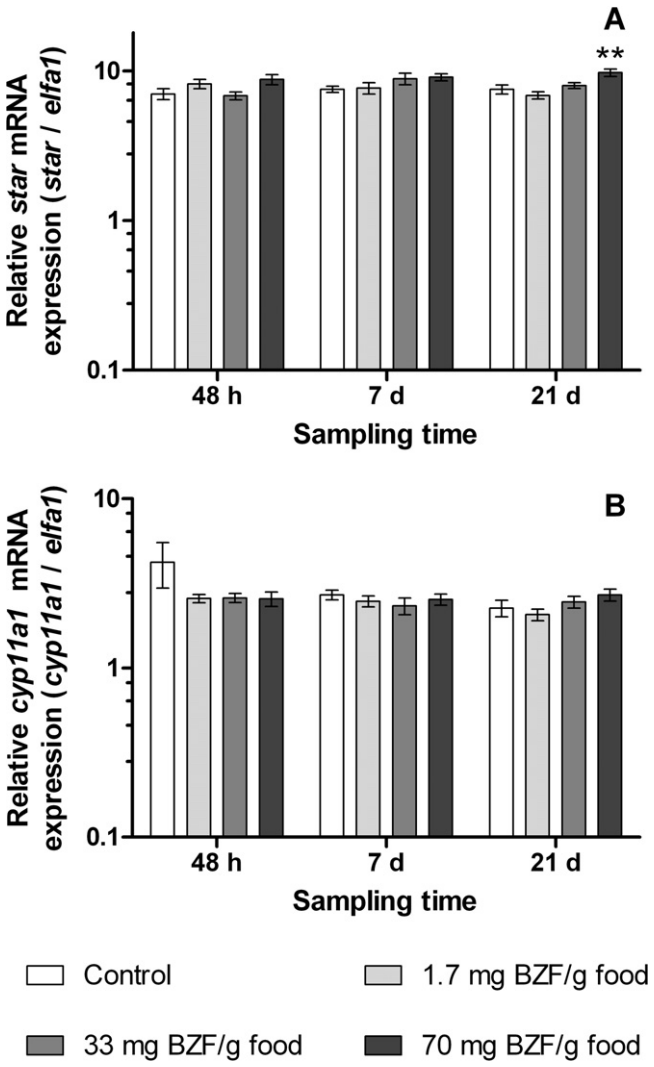

Fig. 4. Transcript mRNA abundances of *star* (A) and *cyp11a1* (*p450scc*) (B) in testis of male adult zebrafish (*Danio rerio*) after exposure to 1.7, 33 and 70 mg bezafibrate/g food for 48 h, 7 days and 21 days. Bars indicate the mean and standard error for four replicate tanks per treatment ($n = 12$ fish/treatment) and expressed in a logarithmic scale as a relative expression normalised to *elfa1* transcript abundance in the same sample. At each sampling time, asterisk (*) shows statistically significant differences compared to the control group (Dunnett's test, df = 12, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). A significant interaction between treatment and sampling time was observed in the *star* mRNA expression (Nested ANOVA, $F_{8,36} = 1.34$, $p = 0.257$).

neither in the interaction (Nested ANOVA, $F_{8,36} = 0.31$, $p = 0.955$, Supplementary Fig. 1) nor in the treatment ($F_{3,95} = 0.59$, $p = 0.624$), with the aquarium having no effect ($F_{36,95} = 1.09$, $p = 0.357$). For *pparb* and *pparg* mRNA expression, an aquarium effect was observed (df = 36,95; $p < 0.005$) with a significant interaction between treatment and sampling time in the nested model for both *pparb* (Nested ANOVA, $F_{8,36} = 6.69$, $p < 0.0001$) and *pparg* (Nested ANOVA, $F_{8,35} = 3.01$, $p = 0.011$). A significant down-regulation in the mRNA expression of *pparb* was observed in fish exposed to the low (Dunnett's test, df = 12, $p = 0.024$) and high (Dunnett's test, df = 12, $p = 0.022$) concentration of bezafibrate compared to the control fish after 48 h (Fig. 3A). This effect was not observed after 7 days of exposure, and in contrast after 21 day of exposure an up-regulation in fish exposed to 70 mg BZF/g food was evident (Dunnett's test, df = 12, $p = 0.009$, Fig. 3A). A similar pattern was observed for the mRNA expression of *pparg* which was down-regulated after 48 h in fish exposed to all bezafibrate concentrations compared to the control (Dunnett's test, df = 12, $p < 0.05$, Fig. 3B); however, throughout the rest of the experiment no significant effect was observed.

3.6. Expression of star and cyp11a1 (p450scc) mRNA in testis

No significant interaction between treatment and sampling time was observed in the *star* mRNA expression (Nested ANOVA, $F_{8,36} = 1.34$, $p = 0.257$); however, a treatment effect was observed ($F_{3,95} = 7.01$, $p = 0.0003$) with the aquarium having no effect ($F_{36,95} = 1.15$, $p = 0.29$). A significant up-regulation in *star* mRNA expression was observed in fish exposed to the high concentration of bezafibrate after 21 days (Dunnett's test, df = 12, $p = 0.007$, Fig. 4A). An aquarium (replica) effect was observed in the *cyp11a1* mRNA expression ($F_{36,95} = 1.71$, $p = 0.020$) and no significant interaction between treatment and sampling time (Nested ANOVA, $F_{8,36} = 1.20$, $p = 0.324$, Fig. 4B) neither a treatment effect ($F_{3,95} = 1.14$, $p = 0.337$) were observed.

3.7. Expression of hydroxysteroid dehydrogenase (hsd) mRNA in testis

A significant interaction between treatment and sampling time in the nested model was observed in the mRNA expression of *hsd3b* (Nested ANOVA, $F_{8,36} = 2.92$, $p = 0.013$, Fig. 5A) and *hsd17b3* (Nested ANOVA, $F_{8,36} = 3.14$, $p = 0.008$, Fig. 5B), with the aquarium having

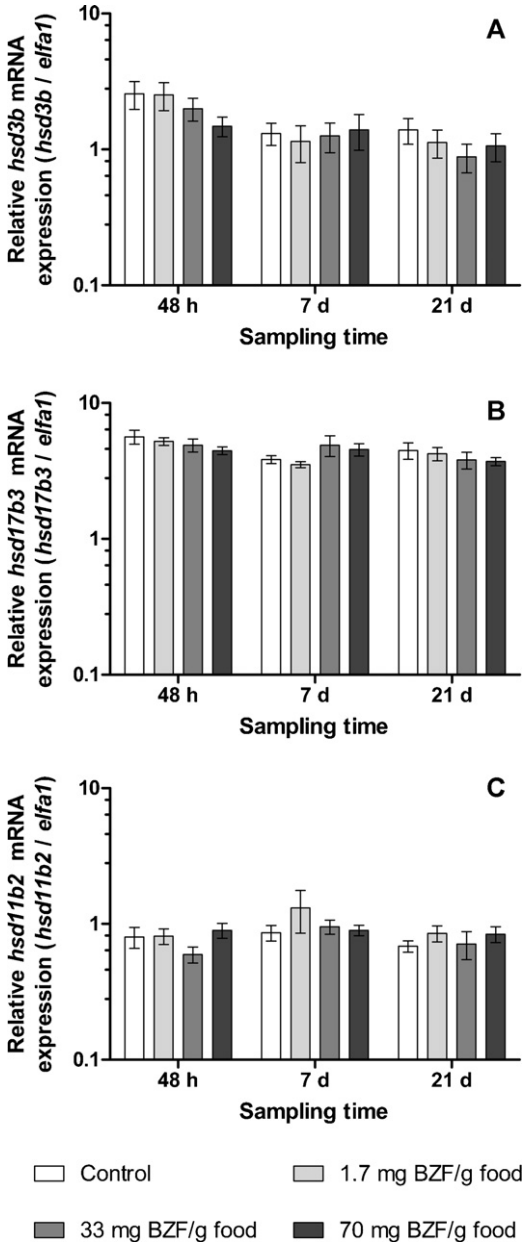

Fig. 5. Transcript mRNA abundances of *hsd3b* (A), *hsd17b3* (B), and *hsd11b2* (C) in testis of male adult zebrafish (*Danio rerio*) after exposure to 1.7, 33 and 70 mg bezafibrate/g food for 48 h, 7 days and 21 days. Bars indicate the mean and standard error for four replicate tanks per treatment ($n = 12$ fish/treatment) and expressed in a logarithmic scale as a relative expression normalised to *elfa1* transcript abundance in the same sample. Asterisk (*) shows significant effect when compared to control at 48 h ($p < 0.05$). At each sampling time, asterisk (*) shows statistically significant differences compared to the control group (Dunnett's test, df = 12, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). A significant interaction between treatment and sampling time in the nested model was observed in the mRNA expression of *hsd3b* (Nested ANOVA, $F_{8,36} = 2.92$, $p = 0.013$) and *hsd17b3* (Nested ANOVA, $F_{8,36} = 3.14$, $p = 0.008$).

no effect (df = 36,95, $p > 0.05$). In contrast, an aquarium (replica) effect was observed in the *hsd11b2* mRNA expression ($F_{36,95} = 2.02$, $p = 0.0035$) with neither significant treatment and time interaction (Nested ANOVA, $F_{8,36} = 0.83$, $p = 0.581$, Fig. 5C) or treatment effect ($F_{3,95} = 2.39$, $p = 0.074$). A trend of down-regulation in the *hsd3b* was observed after 48 h in fish exposed to the high BZF concentration compared to control fish (Dunnett's test, df = 12, $p = 0.096$) (Fig. 5A).

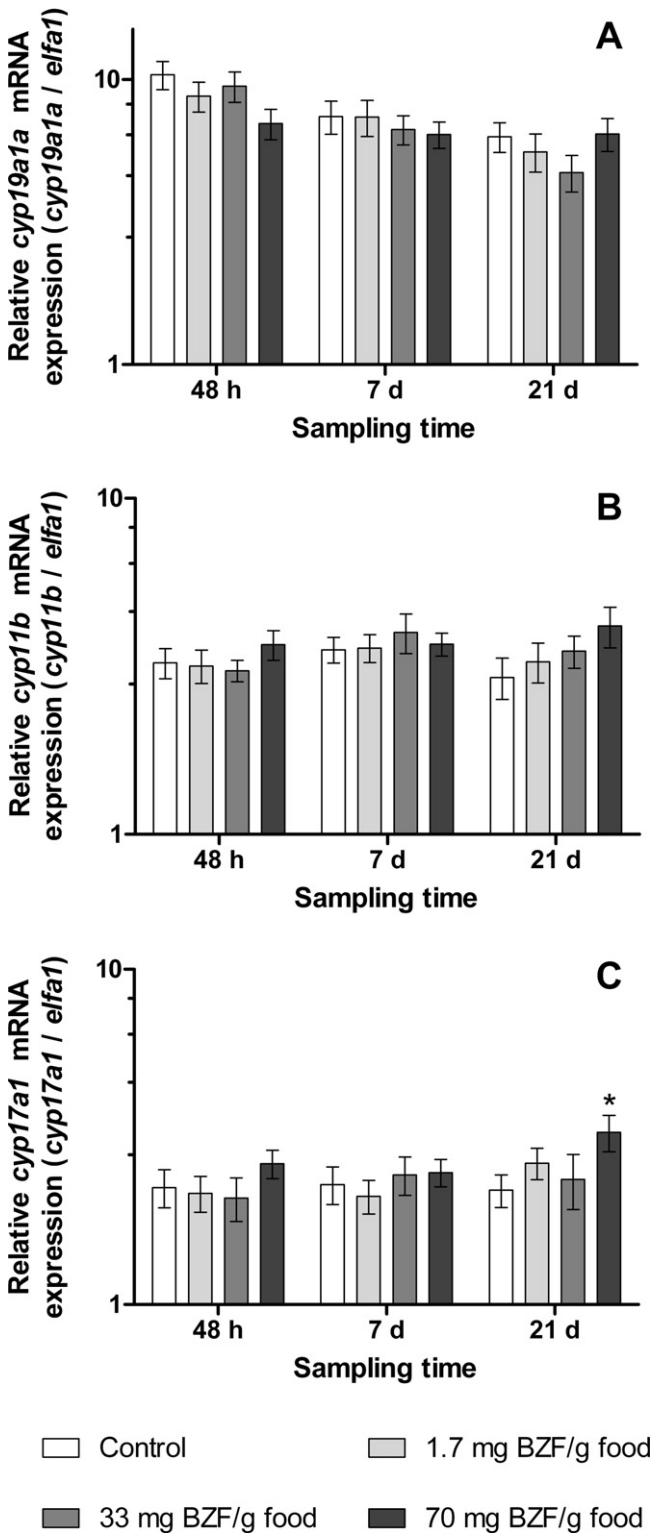

Fig. 6. Transcript mRNA abundances of *cyp19a1a* (A), *cyp17a1* (B) and *cyp11b* (C) in testis of male adult zebrafish (*Danio rerio*) after exposure to 1.7, 33 and 70 mg bezafibrate/g food for 48 h, 7 days and 21 days. Bars indicate the mean and standard error for four replicate tanks per treatment ($n = 12$ fish/treatment) and expressed in a logarithmic scale as a relative expression normalised to *elfa1* transcript abundance in the same sample. Asterisk (*) shows significant effect when compared to control at 48 h ($p < 0.05$). At each sampling time, asterisk (*) shows statistically significant differences compared to the control group (Dunnett's test, df = 12, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). A significant interaction between treatment and sampling time in the nested model was only observed in the *cyp19a1a* mRNA expression (Nested ANOVA, $F_{8,36} = 4.0$, $p = 0.002$).

Table 3
Pearson correlation coefficients between mRNA gene expression, 11-KT, cholesterol and GSI from male zebrafish unexposed and exposed to bezafibrate during 21 days (n = 124–144).

	ppara	*pparb*	*pparg*	*star*	*cyp11a1*	*cyp11b*	*cyp17a1*	*cyp19a1a*	*hsd11b2*	*hsd3b1*	*hsd17b3*
11-KT	**0.168**	**0.196**	0.158	0.015	0.029	−0.023	−0.082	0.025	0.083	0.113	0.102
Chol	−0.025	0.088	0.163	**−0.196**	**0.210**	−0.066	−0.138	**0.167**	−0.092	0.159	0.020
GSI	0.071	0.153	0.096	***−0.444***	0.009	***0.301***	**−0.246**	**0.211**	−0.099	***0.280***	**0.219**
ppara		**0.226**	0.515	0.052	0.129	−0.010	−0.035	**0.169**	−0.040	**0.174**	**0.235**
pparb			**0.235**	−0.072	0.083	−0.036	−0.068	***0.374***	0.142	**0.201**	**0.235**
pparg				−0.025	0.082	−0.126	0.0815	0.045	0.057	**0.254**	0.098
star					**0.213**	***0.533***	***0.502***	−0.002	***0.273***	**−0.218**	−0.041
cyp11a1						***0.306***	0.041	**0.249**	−0.078	***0.306***	0.090
cyp11b							**0.241**	0.054	0.095	−0.043	***−0.291***
cyp17a1								−0.066	***0.362***	−0.009	−0.010
cyp19a1a									−0.066	***0.344***	0.134
hsd11b2										−0.056	**0.214**
hsd3b1											**0.266**

Pearson correlation coefficient indicates statistical significant correlation: bold ($p < 0.05$), bold italic ($p < 0.001$).

3.8. Expression of aromatase, cyp17a1 and cyp11b mRNA in testis

Only the *cyp19a1a* mRNA expression showed a significant interaction between treatment and sampling time in the nested model (Nested ANOVA, $F_{8,36} = 4.0$, $p = 0.002$) and a treatment effect was only observed in the *cyp17a1* mRNA expression ($F_{3,95} = 3.66$, $p = 0.015$). An aquarium (replica) effect was observed in the *cyp11b* ($F_{36,95} = 1.66$, $p = 0.027$) and *cyp17a1* mRNA expression ($F_{36,95} = 2.24$, $p = 0.001$). A trend of down-regulation and up-regulation was observed in the mRNA expression of *cyp19a1a* (Dunnett's test, df = 12, $p = 0.079$, Fig. 6A) and *cyp11b* (Dunnett's test, df = 12, $p = 0.087$, Fig. 6B), respectively, in those fish exposed to 70 mg BZF/g food, after 48 h and 21 days of exposure, respectively, compared to the control. A significant up-regulation in *cyp17a1* mRNA expression was observed in fish exposed to the high concentration of bezafibrate after 21 days (Dunnett's test, df = 12, $p = 0.032$, Fig. 6C).

3.9. Principal Components Analysis (PCA) analysis and correlation between cholesterol, 11-KT and genes involved in the steroidogenesis

The PCA of gene expression in fish exposed to different concentrations of bezafibrate over 21 days is shown in Fig. 7. The first axis explains 52.4% of the time factor effect from the left to the right, and the second axis explains 20% of the concentration effect observed principally after 7 days and 21 days of exposure. There was no clear concentration effect after 48 h. In the vector overlay, it is possible to see the pattern direction of each gene and the genes are grouped in four different groups, one containing *pparb*, *pparg* and *cyp11a1*, the second group containing *hsd3b1* and *cyp19a1a*, the third group having *ppara* and *hsd17b3* and the last group containing *hsd11b2*, *cyp17a1a*, *cyp11b* and *star*.

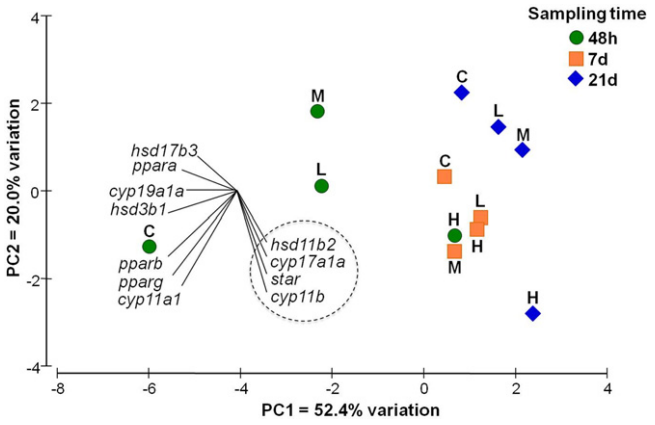

Fig. 7. PCA plot showing the covariance between bezafibrate concentration (C, control; L, 1.7; M, 33; H, 70 mg BZF/g food) and the three sampling time (48 h, circle; 7 days, square, and 21 days, diamond). Note that 72.4% of the gene expression variation is explained by the bezafibrate concentration and sampling time, with the first axis explaining 52.4% and the second axis 20%.

The correlation among the genes and also to cholesterol, 11-KT and GSI is shown in Table 2. A positive correlation between cholesterol and 11-KT was observed ($r = 0.291$, $p = 0.0004$). Plasma cholesterol was significantly correlated with *star*, *cyp11a1* and *cyp19a1a*, key enzymes involved in steroidogenesis. The gene expression of six of the 11 genes evaluated was significantly correlated with GSI ($p < 0.05$). On the other hand, 11-KT was only correlated with *ppara* and *pparb* ($p < 0.05$). *star*, *hsd3* and *hsd17b3* mRNA highly correlated with more than five genes ($p < 0.05$). Most of the genes grouped in the four groups of the PCA plot showed a significant correlation as shown in Table 3.

3.10. Gonadal histology

Evident histopathological alterations were observed in fish exposed to 70 mg BZF/g food compared to the control group (Fig. 8A) after 21 days of exposure. The presence of germ cell syncytia in the tubular lumen was associated with the bezafibrate concentration (df = 2, $\chi^2 = 8.09$, $p = 0.01$, Fig. 8B and C). The control group had 23% minimal, 11% mild and 2% moderate germ cell syncytia. On the other hand, fish exposed to bezafibrate showed 31% minimal, 14% mild and 20% moderate germ cell syncytia in the tubular lumen. The histopathological analysis also revealed a higher number of cysts containing spermatocytes in fish exposed to bezafibrate (20%) than the control group (3%) (df = 1, $\chi^2 = 36.06$, $p < 0.001$).

4. Discussion

This study clearly demonstrates the hypocholesterolemic effect of bezafibrate in zebrafish (*D. rerio*). In addition, the decreased levels of plasma 11-KT together with testicular degeneration and alterations in the testis gene expression reflect an evident effect in the gonadal steroidogenesis due to bezafibrate exposure. This study evidenced the importance to accomplish a good experimental design to guaranty better conclusions about the adverse effects of pharmaceutical. As shown, a variability in the BZF effects was observed at different sampling times which can allow us to suggest a test durations for this kind of studies. For example, when a hypolipidemic effect is evaluated, only 7 days of exposure may be enough; however, if the main goal is to evaluate the effect on the steroidogenesis, a longer exposure i.e. (21 days) must be carried out.

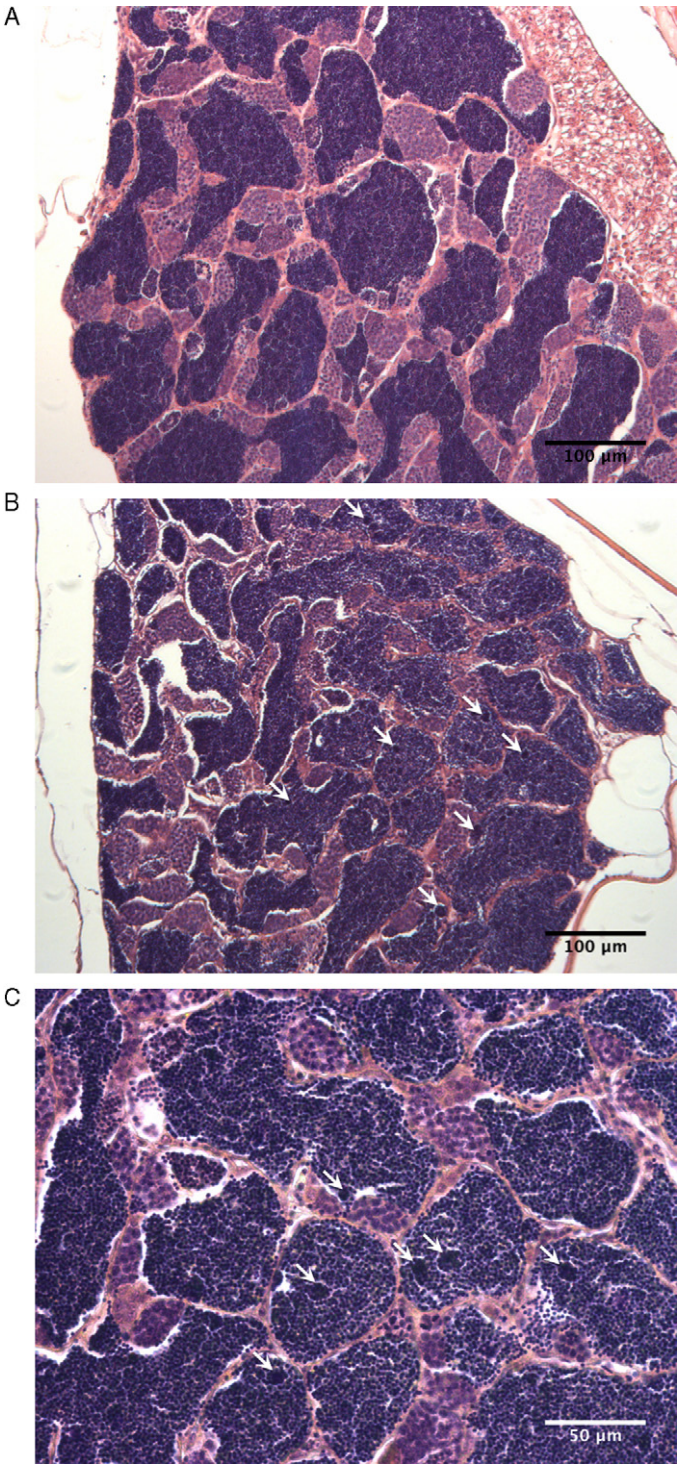

Fig. 8. Histological analysis of testis of male adult zebrafish (*Danio rerio*) from control group and after exposure to 70 mg bezafibrate/g food for 21 days. (A) Testis from control group showing active spermatogenesis, (B) testis from fish exposed to high bezafibrate concentration showing moderate germ cell syncytia in the tubular lumen (white arrows), and (C) magnification of Fig. 8B showing clear signs of testicular degeneration in fish exposed to 70 mg BZF/g food.

4.1. Effect of bezafibrate in Peroxisome Proliferator Active Receptors (ppar)

In fish, three different *ppar* genes have been identified (alpha – *a*, beta – *b* and gamma – *g*) (Bertrand et al., 2007; Ibabe et al., 2005a; Mimeault et al., 2006). In contrast to humans, where fibrates act through direct interaction with the nuclear receptor exhibiting the highest affinity to PPARα (Stocco and Clark, 1996), several lines of evidence suggest this is not the case in fish when *in vivo* exposure is carried out. In goldfish (*C. auratus*) exposed to 1.5 and 1500 μg/L gemfibrozil, no effects on the levels of hepatic *ppara* mRNA were observed. Likewise, no alterations in the expression of *ppara* mRNA were observed in fathead minnow (*P. promelas*) after waterborne exposure to 106 mg/L bezafibrate or 1 to 108 mg/L clofibric acid for 14 days (Weston et al., 2009). A similar result was observed in the present study, in which no significant alterations in the expression of *ppara* mRNA in liver (data not shown) and testis of zebrafish were observed in any of the sampling points during 21 days of exposure to bezafibrate. Recently, it has been shown that in mice the action of bezafibrate on cholesterol metabolism varied according to the dose, the low clinical doses (10 mg/kg/day) having an independent effect on the PPARα activation (Nakajima et al., 2008). Although in the present study lack of regulation in zebrafish *ppara* mRNA was observed after bezafibrate exposure, we cannot discard the possibility that this fibrate is not dependent on *ppara* to exert its effect since it may act via its receptor even if it does not affect the mRNA level; therefore, additional research at the protein level or maybe using *ppara* null-fish is required to support this hypothesis.

It has been reported that PPARα is not the exclusive receptor involved in the effects of fibrates (Stocco and Clark, 1996) and in mice this has been proved specifically with bezafibrate (Peters et al., 2003). In mammals, PPARα and -β are widely expressed in Leydig and Sertolli cells but the β-isoform has not been detected in spermatocytes despite it is ubiquitously expressed and often at higher levels than PPARα (Braissant et al., 1996; Gazouli et al., 2002). However, despite the fact that only few studies have been carried out to understand the function of PPARβ on the reproductive function and steroidogenesis in mammals (Corton and Lapinskas, 2005; Froment et al., 2006), in fish it is still unclear if the *pparb* affects these aspects. In the present study, a down-regulation in the expression of *pparb* was observed in testis after 48 h of exposure to 70 mg BZF/g food (201 μg BZF/L water); however, after 21 days of exposure an up-regulation was observed in fish exposed to the high concentration of bezafibrate which could be related to the decrease in both plasma cholesterol and 11-KT. Mimeault et al. (2006) also reported decreased transcript levels in the hepatic *pparb* mRNA expression in goldfish after 14 days exposure to 1.5 mg/L gemfibrozil.

PPARγ is another important isoform which is involved in different metabolic processes, adipocyte differentiation, inflammation and prostaglandin production (Corton et al., 2000). Recently, in mammals it has been proposed that this isoform has a potential role in the steroidogenesis, follicular development, ovulation and implantation (Froment et al., 2006; Komar, 2005). In the present study, a down-regulation in the expression of *pparg* mRNA was observed in fish exposed to the highest bezafibrate concentrations after 48 h; however no significant effect after 7 or 21 days of exposure was observed. It is possible that the low amplification efficiency (72%) obtained with this gene could to some extent explain the differences observed even in the control group. Similar to the present study, no significant effect was observed in the expression of hepatic *pparg* mRNA in goldfish exposed to gemfibrozil (Mimeault et al., 2006).

These *in vivo* results differ from the *in vitro* results obtained in Atlantic salmon hepatocytes exposed to 0.5 mM bezafibrate or clofibric acid (Ruyter et al., 1997) and in zebrafish hepatocytes exposed to 1–2 mM clofibrate (Ibabe et al., 2005b) in which increased expression of *pparg* mRNA and increased immunolabeling of *pparg* were observed, respectively. So far the differences between *in vivo* and *in vitro* results do not elucidate clearly the mechanism of fibrates on *pparg* in fish; however we do not exclude that this isoform plays an important role in the effects of fibrates.

4.2. Effect of bezafibrate on cholesterol and 11-KT levels

The decreased plasma cholesterol levels observed in zebrafish exposed to bezafibrate after 7 days of exposure confirm the well-known hypolipidemic effect of fibrates. In general, in humans and rodents, fibrates enhance the lipoprotein lipase activity leading to a reduced level of serum lipoproteins and concomitantly a reduced level of cholesterol. Secondly, fibrates increase the affinity of LDL to their receptor and then induce a faster LDL catabolism leading to reduced levels of plasma cholesterol. In addition, induction of uptake of fatty acids by increased β-oxidation of fatty acids via PPAR is reported (Staels et al., 1998). In contrast to the studies in mammals, few studies have been carried out in fish trying to explore the effect of fibrates on lipid and fatty acid metabolism. Recently, Du et al. (2008) reported a significant decrease in triglycerides, cholesterol, LDL-cholesterol and Apo A-1 in juvenile grass carp (*C. idella*) fed with fenofibrate (100 mg/kg BW day) for 14 days after 6 weeks of high-fat diets confirming the hypolipidemic effect of fibrates in fish. This hypocholesterolemic effect is in agreement with the present study in that decreased plasma cholesterol was observed in zebrafish after 7 days of exposure to bezafibrate. To understand the effect of fibrates on fatty acid metabolism, some exposures of fathead minnow and goldfish to waterborne bezafibrate and gemfibrozil, respectively, have been carried out; however, no significant effects were reported in the activity of fatty acyl-coenzyme-A oxidase (FAO) (Mimeault et al., 2006; Weston et al., 2009), an important enzyme in the β-oxidation of long chain-fatty acids. Nevertheless, fathead minnow males exhibited a significant increase in FAO activity at 108.9 mg/L clofibric acid (Weston et al., 2009) although this high concentration could lead also to a general toxicity effect rather than a lipid metabolic effect.

Since cholesterol is the essential precursor of all steroid hormones, it is expected that alterations in its concentration will affect the steroidogenesis (Eacker et al., 2008). For that reason one of the objectives of this study was to determine to what extent the hypocholesterolemia induced by bezafibrate could lead to alterations in the 11-KT concentrations in zebrafish. This steroid was measured since it is a unique, potent androgen found in fish; however, testosterone and estradiol also play important roles in the testicular steroidogenesis (Schulz et al., 2010). In this study, no significant alterations at 48 h of exposure were observed in plasma 11-KT which could be associated to the normal levels of plasma cholesterol at this time. It has been proposed that the Leydig cells meet their cholesterol requirements using two pathways, one using the *de novo* synthesis of cholesterol from acetate within the cell, and one through an uptake of extracellular cholesterol (Eacker et al., 2008; Stocco and Clark, 1996). Since the reduced levels of plasma cholesterol in fish exposed to bezafibrate after 7 days of exposure were not paralleled with changes in 11-KT, we suggest that zebrafish exposed to bezafibrate could obtain the cholesterol necessary for steroid production using the *de novo* synthesis of cholesterol in the Leydig cells. Therefore, we hypothesize that the high dose of bezafibrate induced a progressive depletion in the *de novo* cholesterol synthesis and together with the low plasma cholesterol, the fish did not have enough substrate to maintain the normal synthesis of 11-KT, thus decreasing significantly this steroid in fish exposed to bezafibrate after 21 days of exposure. However, to evaluate this hypothesis it will be important to know the intracellular cholesterol levels.

Similarly, goldfish exposed to 1.5 μg/L or even higher concentrations of gemfibrozil (1.5 mg/L) exhibited significant decreases in the levels of plasma testosterone associated with a down-regulation in the levels of *star* mRNA in testis (Mimeault et al., 2005). Contrarily, no significant effect on testosterone or 11-KT concentration was observed in fathead minnow exposed to up to 1 mg/L clofibric acid, although the authors noted that 11-KT appeared to be lower in exposed fish (Runnalls et al., 2007). Interestingly, cholesterol levels correlated with the expression of the most important genes involved in the steroidogenesis, confirming our hypothesis that lower cholesterol levels could impact the gonadal steroid pathway. Specifically, a negative correlation of cholesterol between *star* and *cyp17a1a* supports the findings observed at 21 days in fish exposed to bezafibrate, suggesting the important role of cholesterol on the *star* and *cyp17a1a* transcription and emphasising the relevance to always evaluate these two genes when gonadal steroidogenesis is studied in zebrafish.

4.3. Gonadal steroidogenesis and spermatogenesis

Steroid hormones exert an important feedback in the regulation of gonadotropins in the hypothalamus and pituitary. In several fish species it has been reported that in males, 11-KT exerts a negative feedback on GnRH neurons and subsequently modulates gonadotrophs to decrease the production of FSH and in some cases LH (Schulz et al., 2001; Yamaguchi et al., 2003, 2005). Based on the results of the present study, it is possible that the decreased levels of 11-KT stimulated the production of gonadotrophic hormones and then through cAMP and protein kinase A activated the cascade of acute production of steroid hormones by the steroidogenic acute regulatory protein (StAR) (Stocco and Clark, 1996). One of the first limiting steps in the steroidogenesis is the transport of cholesterol from the outer mitochondrial membrane (OMM) to the inner mitochondrial membrane by StAR (Stocco and Clark, 1996; Young et al., 2005). In the present study, an up-regulation of *ca.* 30% of *star* mRNA in testis of zebrafish after 21 days of exposure to 70 mg BZF/g food (201 μg BZF/L water) might enable the steroidogenic cells to regulate its steroid hormone levels and facilitates the use of cholesterol for steroid synthesis. This is supported by the study of Bose et al. (2002) who concluded that StAR exerts its activity through a temporal location in the OMM facilitating in this way a rapid regulation of the steroid hormone levels in the case that low steroid concentration are present. In addition, the increase in the *star* mRNA expression could be due to an increased activity of the Leydig cells to stimulate the uptake and use of cholesterol for steroid synthesis (Eacker et al., 2008). Since *star* mRNA up-regulation was observed at the end of the experiment, we do not discard the possibility that by longer exposure an attempt to stabilize the 11-KT levels could be observed due to the fast responses in the *star*. However, if this situation occurred it would be at the expense of the reserves of lipid droplets in the Leydig cells and the cholesterol metabolism with the subsequent physiological consequences.

To our knowledge, this is the first study to determine simultaneously the mRNA expression levels of 11 genes involved in the steroidogenesis of zebrafish to understand the effect of fibrates at this level. Since androgens strongly stimulate testicular gene expression (Schulz et al., 2010), we expected that the reduced plasma 11-KT levels would affect these genes to some extent. The steroidogenesis in mammals or fish involves an enzymatic cascade with mainly two major classes of proteins, a cytochrome P450 (CYP450, *cyp450*) and the hydroxysteroid dehydrogenases (HSD, *hsd*) having as intermediary and final products different steroid hormones (Payne and Hales, 2004; Young et al., 2005). Briefly, trophic hormone stimulation leads to *star* induction which facilitates the transport of cholesterol to the inner mitochondrial membrane and then by the *cyp11a* (P450scc) catalyzes the conversion of cholesterol to pregnenolone, a C21 steroid from which all of the steroid hormones are synthesized. From this point two different pathways are present, one is the production of progesterone by *hsd3b*, and one is the production of 17α-hydroxyprogesterone and androstenedione by *cyp17a1* which was significantly up-regulated after 21 days of exposure in the present study. Subsequently several steps involving enzymes like *hsd17b*, *hsd11b*, *cyp11b* lead to the syn-

thesis of testosterone and 11-oxygenated androgens (11-KT, OHT, OHA) which are the most important androgens in teleost fish with 11-KT as the most predominant. However, in some fish species this steroid is not the principal androgen (for more detail see review by Borg, 1994). Despite the fact that we did not observe any significant alterations in the mRNA expression of most of these individual enzymes, some interesting trends were observed. This occurred both at the individual level such as down-regulation of *hsd3b1* and *cyp19a1* and also up-regulation of *cyp11b* expression or when a multivariate analysis (PCA) was carried out (*hsd11b2*, *cyp17a1a*, *star* and *cyp11b*), in both cases the trends were strongly associated to the decreased 11-KT levels. Therefore, we cannot exclude an effect of this fibrate on the testis steroidogenesis. This fact together with the significant correlation between cholesterol and enzymes involved in the steroidogenesis support the idea. However, additional studies involving protein expression (IHC and proteomics), steroid levels measurements like testosterone and estradiol, longer *in vivo* exposure and even *in vitro* experiments are necessary in order to make a definitive conclusion.

The PCA analysis revealed four groups of genes with similar patterns suggesting the possibility that for future experiments the selection of one or two genes from each group will allow us to observe similar patterns in zebrafish exposed to bezafibrate, which will be less time-consuming and cheaper. The fact that low levels of 11-KT and the majority of alterations in the gene expression were observed at the end of the exposure, allows us to suggest that 21 days could be a suitable test duration when the aim is to evaluate an effect on the gonadal steroidogenesis. It was interesting to observe that 80% of the genes correlated significantly with GSI, although no clear statistical treatment effect was observed.

The reduced level of 11-KT and the alteration pattern in some genes in exposed zebrafish could affect the spermatogenesis and therefore testis histology. The high incidence of germ cell syncytia in the tubular lumen and the trend to increased number of spermatocytes in fish exposed to the high concentration of bezafibrate correspond to some of the criteria defined by the OECD to determine gonadal alterations after exposure to potential endocrine disruptors (OECD, 2010). Since 11-KT plays an important role in the spermatogonial proliferation, decreased steroid levels lead to a possible arrest of meiosis and germ cell maturation reflecting in a high number of cyst containing spermatocytes (Schulz et al., 2010). This arrest of germinal cells could lead to degeneration of spermatozoa revealed with the high presence of germ cell syncytia which forms part of the alterations observed in fish testicular degeneration (OECD, 2010). Similar histological findings were observed in fathead minnow in which more severe alterations were observed after 14 days of waterborne exposure to 17β-estradiol (Miles-Richardson et al., 1999). Similarly, Uren-Webster et al. (2010) reported that zebrafish injected with 50 and 5000 mg/kg of di(2-ethylhexyl) phthalate (DEHP) exhibited alterations in the proportion of germ cells *e.g.* increased proportion of spermatocytes as a consequence of disruption in the spermatogenesis probably via *PPAR* signaling pathways. Despite the fact that some studies have been carried out trying to understand the mechanism of action of fibrates in fish, to our knowledge this is the first study to evaluate the gonadal histopathological alteration caused by fibrates in fish. Even though no evident alterations were observed in the amount or appearance of Leydig cell in fish exposed to bezafibrate, we do not discard that detailed analysis using electron microscopy might reveal some type of alterations since these cells are the primary site of gonadal steroid synthesis. Further histopathological studies are needed in order to determine if histopathological alterations are irreversible or not when bezafibrate exposure is discontinued.

This study clearly demonstrates that bezafibrate (up to 200 μg/L) exerts a physiological effect on male zebrafish characterized by decreased levels of plasma cholesterol and plasma 11-KT. Likewise, it was observed that bezafibrate altered the expression patterns of genes involved in the gonadal steroidogenesis and also led to alterations in the spermatogenesis. These results support the evidence that lipid regulator compounds like fibrates are potential endocrine disruptors in fish.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.aquatox.2011.05.018.

References

Bertrand, S., Thisse, B., Tavares, R., Sachs, L., Chaumot, A., Bardet, P.L., Escrivà, H., Duffraisse, M., Marchand, O., Safi, R., Thisse, C., Laudet, V., 2007. Unexpected novel relational links uncovered by extensive developmental profiling of nuclear receptor expression. PLoS Genet. 3, e188.

Borg, B., 1994. Androgens in teleost fishes. Comp. Biochem. Physiol., C: Pharmacol. Toxicol. Endocrinol. 109, 219–245.

Bose, H.S., Lingappa, V.R., Miller, W.L., 2002. Rapid regulation of steroidogenesis by mitochondrial protein import. Nature 417, 87–91.

Braissant, O., Foufelle, F., Scotto, C., Dauca, M., Wahli, W., 1996. Differential expression of peroxisome proliferator-activated receptors (PPARs): tissue distribution of PPAR-alpha, -beta, and -gamma in the adult rat. Endocrinology 137, 354–366.

Corton, J.C., Anderson, S.P., Stauber, A., 2000. Central role of peroxisome proliferator-activated receptors in the actions of peroxisome proliferators. Annu. Rev. Pharmacol. Toxicol. 40, 491–518.

Corton, J.C., Lapinskas, P.J., 2005. Peroxisome proliferator-activated receptors: mediators of phthalate ester-induced effects in the male reproductive tract? Toxicol. Sci. 83, 4–17.

Derveaux, S., Vandesompele, J., Hellemans, J., 2010. How to do successful gene expression analysis using real-time PCR. Methods 50, 227–230.

Du, Z.-Y., Clouet, P., Degrace, P., Zheng, W.-H., Frøyland, L., Tian, L.-X., Liu, Y.-J., 2008. Hypolipidaemic effects of fenofibrate and fasting in the herbivorous grass carp (*Ctenopharyngodon idella*) fed a high-fat diet. Br. J. Nutr. 100, 1200–1212.

Eacker, S.M., Agrawal, N., Qian, K., Dichek, H.L., Gong, E.-Y., Lee, K., Braun, R.E., 2008. Hormonal regulation of testicular steroid and cholesterol homeostasis. Mol. Endocrinol. 22, 623–635.

Enick, O.V., Moore, M.M., 2007. Assessing the assessments: pharmaceuticals in the environment. Environ. Impact Assess. Rev. 27, 707–729.

Faergeman, O., 2000. Hypertriglyceridemia and the fibrate trials. Curr. Opin. Lipidol. 11, 609–614.

Froment, P., Gizard, F., Defever, D., Staels, B., Dupont, J., Monget, P., 2006. Peroxisome proliferator-activated receptors in reproductive tissues: from gametogenesis to parturition. J. Endocrinol. 189, 199–209.

Fruchart, J.C., Duriez, P., 2006. Mode of action of fibrates in the regulation of triglyceride and HDL-cholesterol metabolism. Drugs Today 42, 39–64.

Gazouli, M., Yao, Z.-X., Boujrad, N., Corton, J.C., Culty, M., Papadopoulos, V., 2002. Effect of peroxisome proliferators on Leydig cell peripheral-type benzodiazepine receptor gene expression, hormone-stimulated cholesterol transport, and steroidogenesis: role of the peroxisome proliferator-activator receptor α. Endocrinology 143, 2571–2583.

Ibabe, A., Bilbao, E., Cajaraville, M.P., 2005a. Expression of peroxisome proliferator-activated receptors in zebrafish (*Danio rerio*) depending on gender and developmental stage. Histochem. Cell Biol. 123, 75–87.

Ibabe, A., Herrero, A., Cajaraville, M.P., 2005b. Modulation of peroxisome proliferator-activated receptors (PPARs) by PPARα- and PPARγ-specific ligands and by 17β-estradiol in isolated zebrafish hepatocytes. Toxicol. In Vitro 19, 725–735.

Isidori, M., Bellotta, M., Cangiano, M., Parrella, A., 2009. Estrogenic activity of pharmaceuticals in the aquatic environment. Environ. Int. 35, 826–829.

Kasprzyk-Hordern, B., Dinsdale, R.M., Guwy, A.J., 2008. The occurrence of pharmaceuticals, personal care products, endocrine disruptors and illicit drugs in surface water in South Wales, UK. Water Res. 42, 3498–3518.

Kolpin, D.W., Furlong, E.T., Meyer, M.T., Thurman, E.M., Zaugg, S.D., Barber, L.B., Buxton, H.T., 2002. Pharmaceuticals, hormones, and other organic wastewater contaminants in U.S. streams, 1999–2000: a national reconnaissance. Environ. Sci. Technol. 36, 1202–1211.

Komar, C., 2005. Peroxisome proliferator-activated receptors (PPARs) and ovarian function – implications for regulating steroidogenesis, differentiation, and tissue remodeling. Reprod. Biol. Endocrinol. 3, 41–55.

Miles-Richardson, S.R., Kramer, V.J., Fitzgerald, S.D., Render, J.A., Yamini, B., Barbee, S.J., Giesy, J.P., 1999. Effects of waterborne exposure of 17β-estradiol on secondary sex characteristics and gonads of fathead minnows (*Pimephales promelas*). Aquat. Toxicol. 47, 129–145.

Mimeault, C., Trudeau, V.L., Moon, T.W., 2006. Waterborne gemfibrozil challenges the hepatic antioxidant defense system and down-regulates peroxisome proliferator-activated receptor beta (PPARβ) mRNA levels in male goldfish (*Carassius auratus*). Toxicology 228, 140–150.

Mimeault, C., Woodhouse, A.J., Miao, X.S., Metcalfe, C.D., Moon, T.W., Trudeau, V.L., 2005. The human lipid regulator, gemfibrozil bioconcentrates and reduces testosterone in the goldfish, *Carassius auratus*. Aquat. Toxicol. 73, 44–54.

Nakajima, T., Tanaka, N., Sugiyama, E., Kamijo, Y., Hara, A., Hu, R., Li, G., Li, Y., Nakamura, K., Gonzalez, F.J., Aoyama, T., 2008. Cholesterol-lowering effect of bezafibrate is independent of peroxisome proliferator-activated receptor activation in mice. Biochem. Pharmacol. 76, 108–119.

OECD, 2010. Guidance Document on the Diagnosis of Endocrine-Related Histopathology in Fish Gonads. Organisation for Economic Co-operation and Development (OECD), Paris, p. 144.

Payne, A.H., Hales, D.B., 2004. Overview of steroidogenic enzymes in the pathway from cholesterol to active steroid hormones. Endocr. Rev. 25, 947–970.

Peters, J.M., Aoyama, T., Burns, A.M., Gonzalez, F.J., 2003. Bezafibrate is a dual ligand for PPARα and PPARβ: studies using null mice. Biochim. Biophys. Acta, Mol. Cell Biol. Lipids 1632, 80–89.

Rozen, S., Skaletsky, H., 1999. Primer3 on the WWW for General Users and for Biologist Programmers, Bioinformatics Methods and Protocols, pp. 365–386.

Rudney, H., Sexton, R.C., 1986. Regulation of cholesterol biosynthesis. Annu. Rev. Nutr. 6, 245–272.

Runnalls, T.J., Hala, D.N., Sumpter, J.P., 2007. Preliminary studies into the effects of the human pharmaceutical clofibric acid on sperm parameters in adult Fathead minnow. Aquat. Toxicol. 84, 111–118.

Ruyter, B., Andersen, Ø., Dehli, A., Östlund Farrants, A.-K., Gjøen, T., Thomassen, M.S., 1997. Peroxisome proliferator activated receptors in Atlantic salmon (*Salmo salar*): effects on PPAR transcription and acyl-CoA oxidase activity in hepatocytes by peroxisome proliferators and fatty acids. Biochim. Biophys. Acta, Lipids Lipid Metabol. 1348, 331–338.

Schulz, R.W., de França, L.R., Lareyre, J.J., LeGac, F., Chiarini-Garcia, H., Nobrega, R.H., Miura, T., 2010. Spermatogenesis in fish. Gen. Comp. Endocrinol. 165, 390–411.

Schulz, R.W., Vischer, H.F., Cavaco, J.E.B., Santos, E.M., Tyler, C.R., Goos, H.J.T., Bogerd, J., 2001. Gonadotropins, their receptors, and the regulation of testicular functions in fish. Comp. Biochem. Physiol. B: Biochem. Mol. Biol. 129, 407–417.

Staels, B., Dallongeville, J., Auwerx, J., Schoonjans, K., Leitersdorf, E., Fruchart, J.-C., 1998. Mechanism of action of fibrates on lipid and lipoprotein metabolism. Circulation 98, 2088–2093.

Stein, E.A., 1994. Drug and alternative therapies for hyperlipidemia. Atherosclerosis 108, S105–S116.

Steiner, G., 2007. Atherosclerosis in type 2 diabetes: a role for fibrate therapy? Diab. Vasc. Dis. Res. 4, 368–374.

Stocco, D.M., Clark, B.J., 1996. Regulation of the acute production of steroids in steroidogenic cells. Endocr. Rev. 17, 221–244.

Ternes, T.A., 1998. Occurrence of drugs in German sewage treatment plants and rivers. Water Res. 32, 3245–3260.

Uren-Webster, T.M., Lewis, C., Filby, A.L., Paull, G.C., Santos, E.M., 2010. Mechanisms of toxicity of di(2-ethylhexyl) phthalate on the reproductive health of male zebrafish. Aquat. Toxicol. 99, 360–369.

Velasco-Santamaría, Y.M., Handy, R.D., Sloman, K.A., 2011. Endosulfan affects health variables in adult zebrafish (*Danio rerio*) and induces alterations in larvae development. Comp. Biochem. Physiol., C: Toxicol. Pharmacol. 153, 372–380.

Velasco-Santamaría, Y.M., Madsen, S.S., Bjerregaard, P., Korsgaard, B., 2010. Effects of 17β-trenbolone in male eelpout *Zoarces viviparus* exposed to ethinylestradiol. Anal. Bioanal. Chem. 396, 631–640.

Weston, A., Caminada, D., Galicia, H., Fent, K., 2009. Effects of lipid-lowering pharmaceuticals bezafibrate and clofibric acid on lipid metabolism in fathead minnow (*Pimephales promelas*). Environ. Toxicol. Chem. 28, 2648–2655.

Yamaguchi, S., Gen, K., Okuzawa, K., Kumakura, N., Kagawa, H., Matsuyama, M., 2003. Regulation of gonadotropin subunit genes expression by 11-ketotestosterone during early spermatogenesis in male red seabream, *Pagrus major*. Fish Physiol. Biochem. 28, 111–112.

Yamaguchi, S., Gen, K., Okuzawa, K., Kumakura, N., Matsuyama, M., Kagawa, H., 2005. Effects of 11-ketotestosterone and gonadotropin-releasing hormone on follicle-stimulating hormone and luteinizing hormone gene expression in castrated and sham-operated male red seabream *Pagrus major*. Fish. Sci. 71, 1049–1058.

Young, G., Kusakabe, M., Nakamura, I., Lokma, P.M., Goetz, F.W., 2005. Gonadal steroidogenesis in teleost fish. In: Melamed, P., Sherwood, N. (Eds.), Hormones and Their Receptors in Fish Reproduction. World Scientific Publishing Company, New Jersey, USA, p. 308.

Yusuf, S., Hawken, S., Ôunpuu, S., Dans, T., Avezum, A., Lanas, F., McQueen, M., Budaj, A., Pais, P., Varigos, J., Lisheng, L., 2004. Effect of potentially modifiable risk factors associated with myocardial infarction in 52 countries (the INTERHEART study): case–control study. Lancet 364, 937–952.