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Chronic effects of clofibric acid in zebrafish (*Danio rerio*): A multigenerational study

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ABSTRACT

Clofibric acid (CA) is an active metabolite of the blood lipid lowering agent clofibrate, a pharmaceutical designed to work as agonist of peroxisome proliferator-activated receptor alpha (PPARa). It is the most commonly reported fibrate in aquatic environments with low degradation rate and potential environmental persistence. Previous fish exposures showed that CA may impact spermatogenesis, growth and the expression of fat binding protein genes. However, there are limited data on the effects of chronic multigenerational CA exposures.

Here, we assessed chronic multigenerational effects of CA exposure using zebrafish (*Danio rerio*) as a teleost model. Zebrafish were exposed through the diet to CA (1 and 10 mg/g) during their whole lifetime. Growth, reproduction-related parameters and embryonic development were assessed in the exposed fish (F1 generation) and their offspring (F2 generation), together with muscle triglyceride content and gonad histology. In order to study the potential underlying mechanisms, the transcription levels of genes coding for enzymes involved in lipid metabolism pathways were determined.

The results show that chronic life-cycle exposure to CA induced a significant reduction in growth of F1 generation and lowered triglyceride muscle content (10 mg/g group). Also, an impact in male gonad development was observed together with a decrease in the fecundity (10 mg/g group) and higher frequency of embryo abnormalities in the offspring of fish exposed to the lowest CA dose.

The profile of the target genes was sex- and tissue-dependent. In F1 an up-regulation of male hepatic *pparaa*, *pparb* and *acox* transcript levels was observed, suggesting an activation of the fatty acid metabolism (provided that transcript level change indicates also a protein level change). Interestingly, the F2 generation, raised with control diet, displayed a response pattern different from that observed in F1, showing an increase in weight in the descendants of CA exposed fish, in comparison with control animals, which points to a multigenerational effect.

1. Introduction

The development of modern medicine and the continuous fabrication of new drugs have increased human life expectancy across the globe but have also resulted in the release of significant amounts of pharmaceuticals into the environment. Consequently, the presence of these compounds in aquatic ecosystem is an ongoing concern (Daughton and Ternes, 1999; Fent et al., 2006; Heberer, 2002; Corcoran et al., 2010; Rodrigues et al., 2006; Santos et al., 2010; Sárria et al., 2011). Pharmaceuticals are

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designed to be biologically active, to have metabolic stability and to pass through biological membranes. When these properties are combined, their removal during wastewater treatment is limited (Fent et al., 2006), allowing these compounds to enter the aquatic environment and potentially resulting in chronic exposure of aquatic non-target organisms. Among aquatic animals, fish are a prime potential target, susceptible to both bioconcentration and biomagnification of water contaminants, since they can be exposed to municipal effluents throughout their lifetime.

Clofibric acid (CA), an active metabolite of the blood lipid lowering agent clofibrate, (Daughton and Ternes, 1999; Fent et al., 2006), was one of the first pharmaceuticals to be reported in water samples (Hignite and Azarnoff, 1977). Over the years, its presence has been detected in different countries' wastewaters, surface waters, groundwater, tap water and seawater (Buser et al., 1998; Heberer et al., 2002; Heberer and Stan, 1997; Hignite and Azarnoff, 1977; Stumpf et al., 1999; Ternes, 1998). Environmental concentrations of this compound range from 1–2 ng/L in the North Sea (Buser et al., 1998), to 270 ng/L in Berlin drinking water, and up to 4 μg/L in groundwater (Heberer and Stan, 1997). Furthermore, CA has a low degradation rate which might potentiate environmental persistence (Wu et al., 2012). Clofibrate belongs to the class of fibrates along with bezafibrate, fenofibrate, ciprofibrate and gemfibrozil (Staels et al., 1998). Fibrates are used to decrease plasma triglyceride and cholesterol levels and their mode of action involves several mechanisms, namely: induction of lipoprotein lipolysis; induction of hepatic fatty acid uptake and reduction of hepatic triglyceride production; increased removal of low-density lipoprotein (LDL) particles; reduction in neutral lipid (cholesteryl ester and triglyceride) exchange between very-low-density lipoproteins (VLDL) and high-density lipoproteins (HDL); increase in HDL production and stimulation of reverse cholesterol transport (Staels et al., 1998). Fibrates, including CA, also regulate the expression of proteins (including enzymes) involved in lipid transport and metabolism by acting as transcription activators of the nuclear receptor heterodimer peroxisome proliferator-activated receptors (PPARs)-retinoid X receptors (RXRs) (Schoonjans et al., 1996), being designed to act as agonists of PPARa (Ibabe et al., 2005b).

Considering their effects in mammalian lipid metabolism, these pharmaceuticals might also interfere with lipid homeostasis and growth of non-target exposed animals, like fish as recently shown by the regulation of the genes coding for fatty acid-binding proteins (Venkatachalam et al., 2012; Venkatachalam et al., 2013) and the enzyme fatty acyl-coenzyme-A oxidase (FAO) involved in fatty acid oxidation (Weston et al., 2009). Previous studies on the effects of CA on the growth of fish were inconclusive as both reduced growth (Owen et al., 2010; Raldua et al., 2008) or no effects at all have been observed following exposure (Owen et al., 2010). However, CA appears to have an effect on cholesterol levels, the precursor of all sex steroid hormones. In fact, exposure of the grass carp (*Ctenopharyngodon idella*), fathead minnow (*Pimephales promelas*) and zebrafish to fibrates reduced plasma cholesterol levels and consequently induced hypocholesterolemic effects (Du et al., 2008; Owen et al., 2010; Velasco-Santamaria et al., 2011). Furthermore, waterborne exposures to gemfibrozil (goldfish, *Carassius auratus*) and to CA (fathead minnow) induced a decline in testosterone concentrations (Mimeault et al., 2005; Runnalls et al., 2007). Likewise, the exposure of zebrafish to bezafibrate through the diet suppressed the levels of 11-ketotestosterone (11KT) (Velasco-Santamaria et al., 2011). Additionally, studies in fathead minnow showed that exposure to fibrates reduced both sperm count and motility, suggesting a potential impact in males (Runnalls et al., 2007) and studies of Weston et al. (2009) show that CA exposure for 21 days at a concentration of 108.91 mg/L impacts egg production. Fish can be continually exposed to these chemicals in their natural environment, through diffuse sources such as STWs, and therefore may suffer from gradual, prolonged exposure effects that may be difficult to detect. In fact, the constant, multi-generational exposure of aquatic life has unknown consequences at the population level (Sumpter, 2005).

Here we assessed chronic multigenerational effects of CA exposure in fish. Although CA is the most commonly reported and persistent fibrate in aquatic ecosystems, the risk toward teleost fish is still poorly understood and contradictory findings have previously been reported. Hence, this study aimed at (a) assessing the multigenerational impact of CA exposure in fish; (b) comparing the findings with the literature on CA effects in mammals, and (c) relating the adverse effect endpoints with the underlying mechanisms of action.

2. Materials and methods

2.1. Fish maintenance and reproduction

Adult wild-type zebrafish (*Danio rerio*), obtained from local suppliers in Singapore, were used as breeding stocks. Zebrafish were maintained under controlled temperature (28 ± 1 °C) and photoperiod 14:10 h (light:dark) in dechlorinated and aerated water in a recirculation system with both mechanical and biological filters. Water parameters (i.e., dissolved oxygen, nitrates and ammonium) were monitored twice a week, whereas temperature was monitored daily. The fish were fed *ad libitum* twice a day with a fish based diet, prepared according to Carvalho et al. (2006), and every other day with a *Artemia* supplement.

In order to obtain the fish for the exposure assay (F1 generation) and for the multigenerational assays (F2 and F3 generations), in the afternoon before breeding, groups of males and females were independently housed in cages with a net bottom covered with glass marbles (Soares et al., 2009). In the following day, 1.5 h after the beginning of the light period, breeding fish were removed and the eggs were collected and cleaned. Fertilized eggs were randomly allocated (F1 generation) or allocated according to their parenthood (F2) generation. F3 generation was maintained only up to 6 days post fertilization (dpf) to evaluate embryonic development according to Soares et al. (2009).

2.2. Experimental design

In order to study the effects of clofibric acid (CA) on teleost fish, a multigenerational life-cycle study was performed with zebrafish (Fig. 1).

In F1 generation, 5 dpf eleuthero-embryos ($n = 330$) were randomly allocated to 5 L aquaria kept in a water-bath inside 30 L aquaria to keep temperature constant. At 20 dpf, the number of fish was adjusted to 100 and the fish were allocated to the corresponding 30 L aquaria. This number was further reduced to 30 fish per aquarium at 60 dpf. For the exposure experiments, seven flow-through systems were used (Soares et al., 2009) with an approximate flow rate of 40 L/day: three replicates for control fish and two replicates for each CA concentration. CA was dosed through the diet, at nominal contents of 1 and 10 mg/g of food and the exposure period was from 5 to 140 dpf. These contents were selected based on human therapeutic doses and according to previous studies that used a similar range of bezofibrate and clofibrate contents incorporated in the diet in zebrafish (Velasco-Santamaria et al., 2011; Venkatachalam et al., 2012; Venkatachalam et al., 2013). CA was incorporated in the diet using acetone as vehicle. Diet from control treatments received acetone only (Santos et al., 2006). Zebrafish were fed twice a day with the previously described diet, and the amount of food was adjusted according to fish develop-

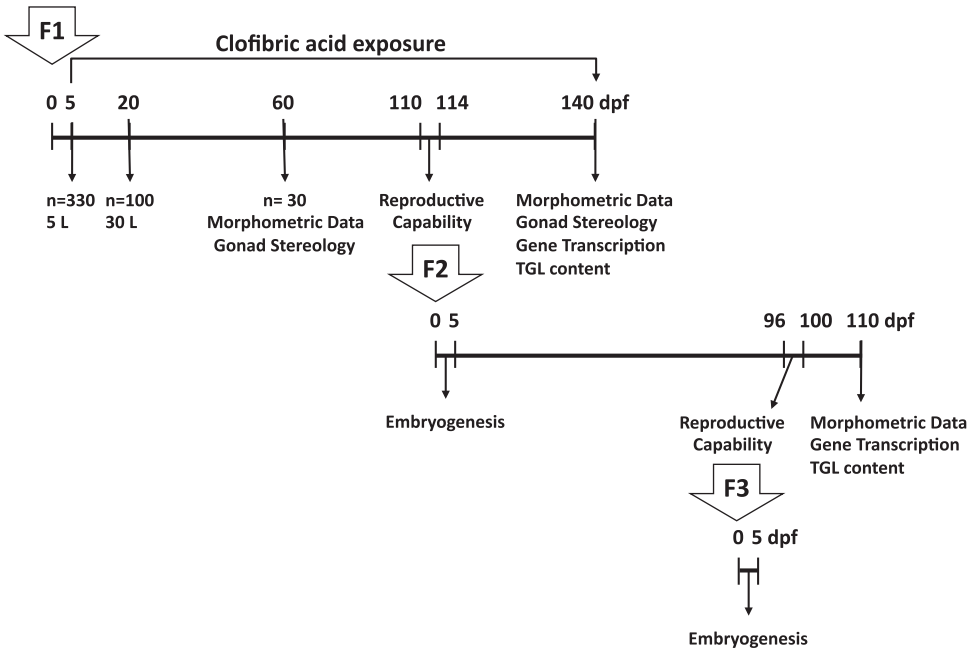

Fig. 1. Schematic representation of the experimental setup: exposure period, time points and endpoints determined in each zebrafish (*D. rerio*) generation.

ment, i.e., the amount of food ingested within 5 min after feeding (Santos et al., 2006). Every other day animals received a supplement of brine shrimp. F1 fish samplings were performed at 60 and 140 dpf and morphometric parameters, weight and length were registered and used for condition factor calculation, as follows: (K = [weight/(length)^3] × 100).

F2 generation embryos were allocated according to their parental group and fish were maintained under the same conditions as F1 but fed with control diet. F2 fish sampling was performed at 110 dpf and morphometric parameters registered as for the F1 generation. In the F1 generation, the following endpoints were determined: gonad development, fecundity, gene transcription in gonads and liver and triglyceride content in muscle tissue. In the F2 generation, the studied endpoints were embryo development, growth, reproductive capability, triglyceride content in muscle tissue and male hepatic gene transcription. In the F3 generation only embryo development was evaluated. All experiments complied with the European Directive (2010/63/EU) for the correct use of laboratory animals.

2.3. CA concentration in diet

The actual CA concentrations in the diet were confirmed by HPLC. Briefly, feed samples (200 mg) were homogenized in 10 volumes (2 mL) of methanol. After centrifugation (2000 × *g*, 15 min, 4 °C), the pellet was extracted again with 10 mL of methanol. Both supernatants were combined and diluted 10 times with methanol to be analyzed in the HPLC system.

The analysis was performed in a Hitachi LaChrom ELITE® HPLC System (VWR International, Darmstadt, Germany) equipped with a L-2130 pump, a L-2300 column oven, a L-2200 autosampler and a L-2455 diode array detector (DAD). The chromatographic column was a SUPELCOSIL™ Suplex™ pKb-100 column (Supelco, Bellefonte, PA, USA). The mobile phase (methanol: acetate buffer 430 mM pH 4.33, 90:10) was delivered isocratically at 1.4 mL/min and column oven and autosampler were maintained at 40 °C and 4 °C, respectively. Samples, 20 μL, were injected into the HPLC system and the CA peak was quantified by comparing peak area at a wavelength of 230 nm with those of external standards. The limit of detection was 800 pg (on-column). CA concentration was below the detection limit in the control diet and was 1.05 ± 0.05 mg/g in the 1 mg/g diet and 9.25 ± 0.20 mg/g in the 10 mg/g diet (n = 3).

2.4. Fecundity

To evaluate the effects of CA on fecundity of F1 and F2 generations, reproduction trials were performed between 110 and 114 dpf (F1) and 96–100 (F2) dpf. The methodology used was previously described in Soares et al. (2009). Briefly, the 30 L aquaria were divided in two by polypropylene plates, maintaining the sex ratio in each duplicate and allowing 4 replicates for each treatment. On the day before the reproduction trial, fish were placed in spawning nets suspended in each division and the bottom was covered with marbles. During 5 consecutive days, 1–1:30 h after the beginning of the light period, eggs were collected. Non-fertilized eggs were counted and discarded, while fertilized eggs were placed in ethanol (96%) for quantification and determination of fecundity (number of eggs/female/day).

2.5. Embryogenesis studies of F2 and F3 generations

To assess multigenerational effects of CA during zebrafish early phases, embryos obtained from F1 and F2 generations were raised in clean water for development endpoint evaluation according to Soares et al. (2009), following minor modifications. Briefly, 40 eggs that had passed the 4 cell stage (1 h post fertilization – hpf), were randomly distributed to 100 mL beakers (four beakers per experimental condition) with daily replacement of water. The beakers were maintained in a water bath at 26.5 ± 0.5 °C, under the same photoperiod as the adults (Lammer at al., 2009; Soares et al., 2009). Embryos were allowed to develop up to 120 hpf. At this development stage, 15 embryos from each beaker were randomly placed in a 12-well microplate for observation with a stereomicroscope (SMZ1000, Nikon,) and morphological anomalies were recorded by digital photography (Nikon Coolpix 5400). The following endpoints were determined: daily mortality, hatching, head and tail differentiation, spontaneous movements, malformations of the circulatory system, edema and melanophore distribution, expression of pig-

Table 1
Gene, NCBI accession number, sequence of primers used for real-time PCR (5′–3′), annealing temperature (°C) and product size (bp).

Gene accession no.	Forward sequence	Reverse sequence	Annealing temperature	Size
actb2[a] NM_181601.3	ACTGTATTGTCTGGTGGTAC	TACTCCTGCTTGCTAATCC	56	197
*apoa1*a NM_131128.1	ACCAAGCAGCTGCGCGAGAG	ATGTGCTTCTGCAGCGCGGT	58	101
rxraa NM_001161551.1	ATTCAATGGCATCTCCTG	GCGGCTTAATATCCTCTG	55	120
pparaa[b] NM_001161333.1	CATCTTGCCTTGCAGACATT	CACGCTCACTTTTCATTTCAC	54	81
pparb1[b] AF342937.1	GCGTAAGCTAGTCGCAGGTC	TGCACCAGAGAGTCCATGTC	56	204
pparg[b] NM_131467.1	GGTTTCATTACGGCGTTCAC	TGGTTCACGTCACTGGAGAA	56	250
acox1[d] BC097101.1	GGGGCACGGTACACACATCCG	GCAGGCCATGACACTTGCCCT	56	162
star NM_131663.1	TCAAATTGTGTGCTGGCATT	CCAAGTGCTAGCTCCAGGTC	56	90
vtg1[c] NM_170767	CTTACGACACAGGATTCAG	GTCTTCATAGGTCTCAATGG	59	200

[a] Soares et al., 2012.
[b] Velasco-Santamaria et al., 2011.
[c] Soares et al., 2009.
[d] Alsop and Vijayan, 2008.

mentation, and finfold lesions. Dead embryos were removed from all beakers to assure that no water quality decay occurred.

2.6. Histology and stereological analysis

To evaluate gonad development of the F1 generation fish, whole animals at 60 dpf ($n = 20$), and ovaries and testis at 140 dpf ($n = 8$ of each sex), were fixed in Bouin's solution (Panreac) for 48 h, paraffin-embedded, sectioned into 3 μm sections. Glass slides were stained with hematoxylin–eosin and mounted with Entellan®. Identification of gonad cell populations was conducted according to Weber et al. (2003) and as described by Soares et al. (2009). Five fish from each sex and five sections per fish were randomly chosen and analyzed using a point count system (Ferre, 2004; Freere and Weibel, 1967; Soares et al., 2009).

2.7. Gene transcription evaluation

Taking into account the role of fibrates in human therapeutics as blood lipid lowering agents, a set of genes were selected to study the impact of CA on fish. This set included: pparaa, pparb1, pparg and rxraa, as CA acts as transcription activator of the nuclear receptor PPAR-RXR; *apoa1a*, as APOA1 is involved in fat mobilization from tissues and its transport through plasma; *acox1*, as acyl-Coenzyme A oxidase 1 enzyme catalyzes the first step in fatty acid beta-oxidation; *star*, as the steroidogenic acute regulatory protein is a limiting enzyme in the sex steroid synthesis pathway responsible for the cholesterol import into mitochondria and vtg1, as vitellogenin expression is a biomarker associated with reproduction.

For the evaluation of gene transcription, F1 gonads and livers and F2 male livers (according to F1 results) were preserved in RNAlater® and kept at −80 °C until use ($n = 5$–8).

Total RNA was isolated using the "illustra RNAspin Mini kit" (GE Healthcare kit) according to the manufacturer's protocol, with on column DNase digestion during the extraction procedure. After column elution with 30 μL of RNase-free water, RNA concentration was determined by fluorescence (Fluoroskan Ascent, Labsystems) using the "Quant-iT™ RiboGreen® RNA Assay Kit" (Invitrogen) and RNA integrity and DNA contamination were checked by electrophoresis in a 1% agarose gel. All RNA samples were stored at −80 °C until further use. For cDNA synthesis, the "iScript cDNA synthesis kit" (Bio-Rad) was used with 150–250 ng of total RNA.

Primers used for qPCR (Table 1) had been previously published or were designed for the present study in a region outflanking an intron using Beacons DesignTM software (Premier Biosoft International) and synthesized by STABVida (Portugal). The identity of the PCR products was confirmed by cloning and subsequent sequencing (STABVida, Portugal).

The qPCR reactions were prepared to a final volume of 20 μL with final concentration of primers 200 nM, using 1 μL of cDNA and "PerfeCTa® SYBR® Green SuperMix UNG, for iQ™" (Quanta Biosciences). The qPCR was initiated at 95 °C for 5 min. Thereafter, 40 cycles of amplification were carried out with denaturation at 95 °C for 10 s, annealing temperature (Table 1) for 30 s and extension at 72 °C for 30 s (data collection), followed by a melting curve analysis to determine the specificity of the reactions. Standard curves consisted of five 5-fold serial dilutions of positive control cDNA prepared from a total RNA mixture isolated from animals from all groups and used as reference sample. The PCR efficiency for target genes ranged from 80 to 105%. To evaluate the relative transcript levels, the $2^{-\Delta\Delta CT}$ method was used (Livak and Schmittgen, 2001) with β-actin as housekeeping gene. For each gene and organ, the transcript expression is presented as arbitrary units, using the control group transcription level as the unit (control sample average equals 1 unit).

2.8. Lipid extraction and triglyceride determination

Lipids were extracted from the muscle of sacrificed female and male animals ($n = 5$ per sex) as described by Schwartz and Wolins, (2007), with modifications. Muscle was homogenised (approximately 100 mg/mL) in PBS, 10 mM pH 7.4, containing 10 mM EDTA, using ceramic bead technology on a Precellys 24 homogenizer. Subsequently, 200 μL of the homogenate were transferred, in duplicates, into test glass tubes containing 2 mL of isopropanol/hexane (4:1) solution, vortexed for 1 min and incubated at room temperature in the dark with constant shaking for 1 h. The solution containing the lipids was then washed with 0.8 mL of petroleum ether/hexane (1:1) by vortexing for 1 min and the tubes were left to stand at room temperature in the dark for 10 min. The phases were then separated by adding a third of the total volume of Milli-Q H_2O (1 mL), vortexed for 1 min, incubated at room temperature in the dark with constant shaking for 20 min and centrifuged at 1000 × *g* for 10 min. The upper phase containing the lipids was collected and evaporated to dryness under nitrogen current.

Table 2
Sex ratio(%), weight (mg), length (mm), condition factor (CF) and muscle triglyceride content (μg/mg) of F1 (60 and 140 dpf) and F2 (110 dpf) zebrafish (*D. rerio*), in 0, 1 and 10 mg/g clofibric acid groups.

Generation		F1			F2	
dpf		60	140		110	
Sex			Females	Males	Females	Males
Sex ratio (%)	0 mg/g	ND	52	48	54	46
	1 mg/g	ND	39*	61*	50	50
	10 mg/g	ND	49	51	46	54
Weight (mg)	0 mg/g	144.7 ± 66	830.5 ± 154a	598.2 ± 97a	543.3 ± 84a	422.6 ± 51a
	1 mg/g	134.8 ± 49	798.0 ± 195a	621.6 ± 67a	623.8 ± 105b	436.7 ± 52ab
	10 mg/g	123.5 ± 47	593.9 ± 144b	479.6 ± 76b	607.4 ± 112b	448.7 ± 55b
	r (p)	0.14 (0.076)	0.53 (0.000)	0.41 (0.000)	0.03 (0.747)	0.07 (0.518)
Length (mm)	0 mg/g	23.3 ± 3.4	41.2 ± 2.3a	39.1 ± 2.7[a]	36.9 ± 2.0[a]	36.1 ± 1.7
	1 mg/g	22.9 ± 2.9	40.5 ± 2.9a	39.4 ± 1.9[a]	39.2 ± 1.9[b]	36.6 ± 1.5
	10 mg/g	22.2 ± 3.1	37.3 ± 3.1b	36.7 ± 2.6[b]	37.8 ± 2.3[a]	36.4 ± 1.7
	r (p)	0.14 (0.092)	0.54 (0.000)	0.57 (0.000)	0.16 (0.107)	0.20 (0.057)
CF	0 mg/g	1.06 ± 0.14	1.18 ± 0.12[a]	1.00 ± 0.10[a]	1.08 ± 0.12[ab]	0.9 ± 0.10
	1 mg/g	1.08 ± 0.20	1.18 ± 0.11[ab]	1.01 ± 0.08[a]	1.03 ± 0.08[b]	0.89 ± 0.06
	10 mg/g	1.10 ± 0.24	1.12 ± 0.02[b]	0.96±0.09[b]	1.11 ± 0.10[a]	0.93 ± 0.07
	r (p)	0.08 (0.338)	0.22 (0.030)	0.20 (0.039)	0.20 (0.034)	0.16 (0.131)
TGL (μg/mg)	0 mg/g	ND	24.6 ± 3.6ab	20.7 ± 14a	30.0 ± 10	12.9 ± 8.1
	1 mg/g	ND	31.4 ± 5.7a	14.7 ± 8.2ab	26.2 ± 5.3	13.0 ± 4.7
	10 mg/g	ND	16.2 ± 3.2b	6.7 ± 3.7b	35.1 ± 14	16.1 ± 7.1

The sex ratio (% of females and males) tested by χ^2 test. Different lower case letters indicate significant differences between groups within each time and sex. Values presented as mean ± standard deviation; $p < 0.05$, one-way ANOVA, followed by Fisher LSD multiple comparison test. *r* (p) – Pearson correlation coefficient (significance level). ND – not determined. (morphometric endpoints and sex ratio $n = 55–58$; TGL $n = 5$).
* $p < 0.05$.

Dry extracts containing the lipids were resuspended in 200 μL isopropanol and determination of triglycerides was performed by an enzymatic and colorimetric assay using the commercial "Infinity Triglycerides Liquid Stable Reagent" (Thermo Scientific, Biognóstica, Portugal) following the manufacture's protocol. Standard curves using triolein dissolved in isopropanol were performed in every run. The coefficient of variance (CV) for intra-assay variability using a pooled sample was 8.7% ($n = 5$).

2.9. Statistical analysis

Data were analyzed using Statistica 6.0 software. After testing for ANOVA assumptions (homogeneity of variances and normality of data), statistical differences between groups were evaluated by one-way factorial ANOVA, followed by Fisher LSD multiple comparison test with a 95% confidence interval ($p < 0.05$). Data failing ANOVA assumptions were square root or log transformed before ANOVA analysis. Sex ratio differences were tested by the chi square test, using as expected values the control group ($p < 0.05$). Correlation coefficients were calculated using Pearson correlation ($p < 0.05$).

3. Results

3.1. Morphometric parameters

Weight, length and condition factor (Table 2) were determined in the F1 generation individuals at 60 dpf and 140 dpf and in the F2 generation at day 110 dpf. In the F1 generation, a significant ($p < 0.001$) decrease in both weight and length was observed in fish exposed to 10 mg/g of CA at 140 dpf. Conversely, the male and female descendants (F2) from this group presented higher weight ($p = 0.040$ and 0.008, respectively) than descendants of the control group. Regarding the condition factor, the F1 females exposed to 10 mg/g of CA ($p = 0.042$) and the F2 females descendants of the group exposed to 1 mg/g of CA ($p = 0.048$), presented lower values than their control groups.

3.2. Sex ratio

In the F1 generation, exposure to 1 mg/g of CA led to a significant bias in the sex ratio toward males, in comparison to the control group ($p < 0.05$, chi square test). In the F2 generation, all groups presented a similar proportion between males and females (Table 2).

3.3. Fecundity

To assess CA action on reproduction of exposed zebrafish and their descendants, fecundity (number of eggs per female per day) and fertilization rates of F1 and F2 generations were determined (Fig. 2). In the F1 generation, CA exposure induced a decrease in the number of deposited eggs ($p < 0.001$, $r = 0.85$), with a significant reduction at the highest exposure level ($p < 0.001$). In the F2 generation, the descendants of the highest CA concentration group displayed higher mean fecundity level, but did not differ significantly from control females ($p = 0.052$). In either generation, the fertilization rates were not affected by CA exposure (data not shown).

3.4. Embryogenesis studies

The impact of CA exposure on embryonic and early eleuthero-embryos development was screened in F2 and F3 generations at 144 hpf (Table 3). The overall mortality rate at 144 hpf was not affected in both generations, although in the F3 generation, the offspring from parental 1 mg/g of CA exposure showed a higher ($p = 0.016$) embryonic mortality rate than the offspring from the 10 mg/g of CA. In the F2 generation, parental exposure to the lowest CA level induced a significant ($p = 0.020$) increase in the percentage of abnormal eleuthero-embryos. Abnormal eleuthero-embryos presented a similar phenotype, with massive edema in the head region and body curvature. The proportion of abnormal eleuthero-embryos at the 10 mg/g of CA treatment did not differ significantly from the control group ($p > 0.05$). Embryonic development was also evalu-

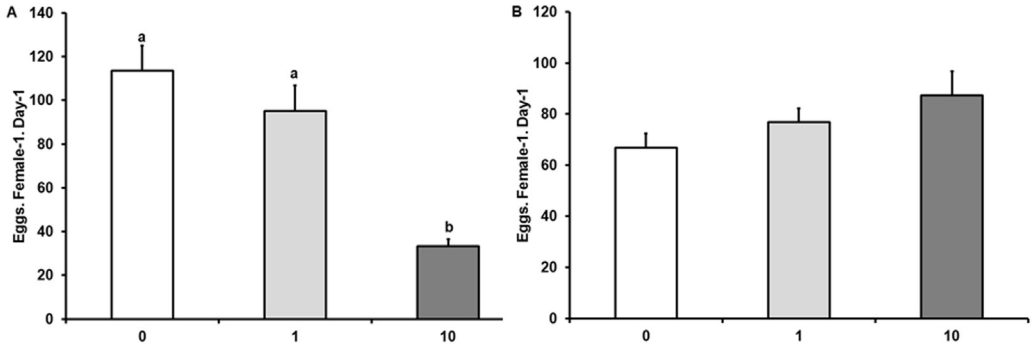

Fig. 2. Zebrafish (*D. rerio*) fecundity (number of eggs per female per day) in 0, 1 and 10 mg/g clofibric acid groups. A-F1 generation. B-F2 generation. Different lower case letters indicate significant differences between groups. Values presented as mean ± standard deviation; $p < 0.05$, one-way ANOVA, followed by Fisher LSD multiple comparison test.

Table 3
Zebrafish (*D. rerio*) embryo mortality rate (%) and abnormal larvae (%) at 144 hpf in the F2 and F3 generations in 0, 1 and 10 mg/g clofibric acid descendents groups.

	Mortality			Abnormal larvae	
Generation		F2	F3	F2	F3
Group	0 mg/g	8.3 ± 4.9	18.8 ± 8.2ab	0.8 ± 2.0a	4.2 ± 4.0
	1 mg/g	4.4 ± 4.3	26.3 ± 11.3a	17.5 ± 13.2b	5.1 ± 4.4
	10 mg/g	14.0 ± 11.5	9.5 ± 4.0b	6.3 ± 4.8ab	5.5 ± 4.9

Different lower case letters (a, b) indicate significant differences between groups within each generation. Values presented as mean ± standard deviation; $p < 0.05$, one-way ANOVA, followed by Fisher LSD multiple comparison test ($n = 4$ independent reproduction trials; 60 analyzed embryos per treatment).

ated in the offspring's of the F2 generation raised on control diet, but no effects were recorded.

3.5. Stereological analysis

A stereological analysis of ovaries and testis was used to identify possible alterations in gonad development induced by the CA exposure in the F1 generation. Two time-points were selected for screening the impact of CA on gonads: the end of the differentiation period (60 dpf) and adulthood (140 dpf). The statistical analysis of the data from the relative volume of each oocyte stage showed a significantly higher ($p = 0.024$) relative volume of previtellogenic oocytes, at 60 dpf, in females exposed to 10 mg/g of CA (Fig. 3A). No differences were observed for the relative volume of vitellogenic and preovulatory oocytes at this time-point (Fig. 3A). At 140 dpf, no differences were observed between exposure groups for the relative volume of all oocyte types (Fig. 3B).

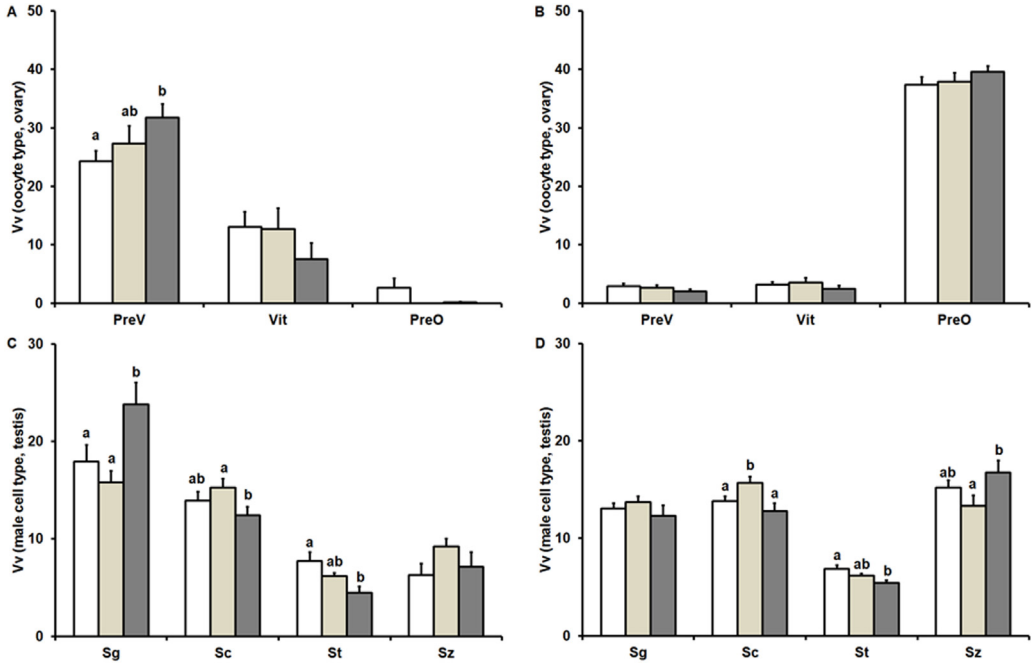

Fig. 3. Relative volume of germ line cells of F1 zebrafish (*D. rerio*) gonad (ovary-A and B; testis-C and D) at 60 dpf (A and C) and 140 dpf (B and D), in 0, 1 and 10 mg/g clofibric acid groups -previtellogenic follicles (PreV), -vitellogenic follicles (Vit), preovolatory follicles (PreO), spermatogonia (Sg), spermatocytes (Sc), spermatids (St) and spermatozoa (Sz). Different lower case letters indicate significant differences between groups. Values presented as mean ± standard deviation; $p < 0.05$, one-way ANOVA, followed by Fisher LSD multiple comparison test.

Table 4
F1 zebrafish (*D. rerio*) ovary, female liver and testis relative transcription levels (fold induction in comparison with the control group) in 0, 1 and 10 mg/g clofibric acid groups.

		Group	*apoa1a*	*rxraa*	*pparaa*	*pparb1*	*star*	*pparg*	*acox1*	*vtg1*
Female	Ovary	0 mg/g	1.0 ± 0.4	1.0 ± 0.3	1.0 ± 0.5a	1.0 ± 0.3ab	1.0 ± 0.5	ND	ND	ND
		1 mg/g	1.0 ± 0.3	1.1 ± 0.3	0.04 ± 0.03b	0.3 ± 0.2a	0.3 ± 0.1	ND	ND	ND
		10 mg/g	1.3 ± 0.3	1.1 ± 0.3	0.2 ± 0.1a	5.8 ± 2.3b	0.5 ± 0.2	ND	ND	ND
	Liver	0 mg/g	1.0 ± 0.3	1.0 ± 0.3	1.0 ± 0.8	1.0 ± 0.4ab	ND	1.0 ± 0.4	1.0 ± 0.5	1.0 ± 0.2
		1 mg/g	2.5 ± 1.4	0.5 ± 0.2	1.2 ± 0.7	2.1 ± 0.9a	ND	0.5 ± 0.2	0.6 ± 0.5	1.1 ± 0.3
		10 mg/g	1.5 ± 0.6	0.8 ± 0.3	1.4 ± 0.8	0.4 ± 0.1b	ND	0.4 ± 0.1	4.0 ± 2.3	1.1 ± 0.5
Male	Testis	0 mg/g	1.0 ± 0.3	1.0 ± 0.2	1.0 ± 0.6	1.0 ± 0.2	1.0 ± 0.6	ND	ND	ND
		1 mg/g	1.9 ± 1.3	0.7 ± 0.2	1.5 ± 1.1	0.7 ± 0.3	0.6 ± 0.3	ND	ND	ND
		10 mg/g	4.1 ± 2.7	0.5 ± 0.2	1.3 ± 0.7	0.8 ± 0.4	0.3 ± 0.1	ND	ND	ND

Different lower case letters (a, b) indicate significant differences between groups. Values presented as mean ± standard deviation; $p < 0.05$, one-way ANOVA, followed by Fisher LSD multiple comparison test. ND – not determined. $n = 5–8$ samples per group.

The testicular relative volume occupied by the different spermatogenic stages, at 60 dpf (Fig. 3C), indicated that the group exposed to 10 mg/g of CA had more immature testis than the other groups, as shown by the significantly higher ($p = 0.025$) relative volume of spermatogonia and similar or lower relative volumes of the other cell types. At 140 dpf (Fig. 3D), the relative volume of spermatozoa was higher in the group exposed to 10 mg/g of CA when compared to the lower CA concentration ($p = 0.027$) and the relative volume of spermatids was lower in the group exposed to 10 mg/g of CA when compared to the relative volumes presented by the control group ($p = 0.007$).

3.6. Gene transcription

The ovary transcription levels (Table 4) of *apoa1a*, *rxraa* and the gene encoding the steroidogenic acute regulatory protein, *star*, were not affected by the exposure to CA; *pparaa* transcription levels were found to be lower in fish exposed to 1 mg/g of CA when

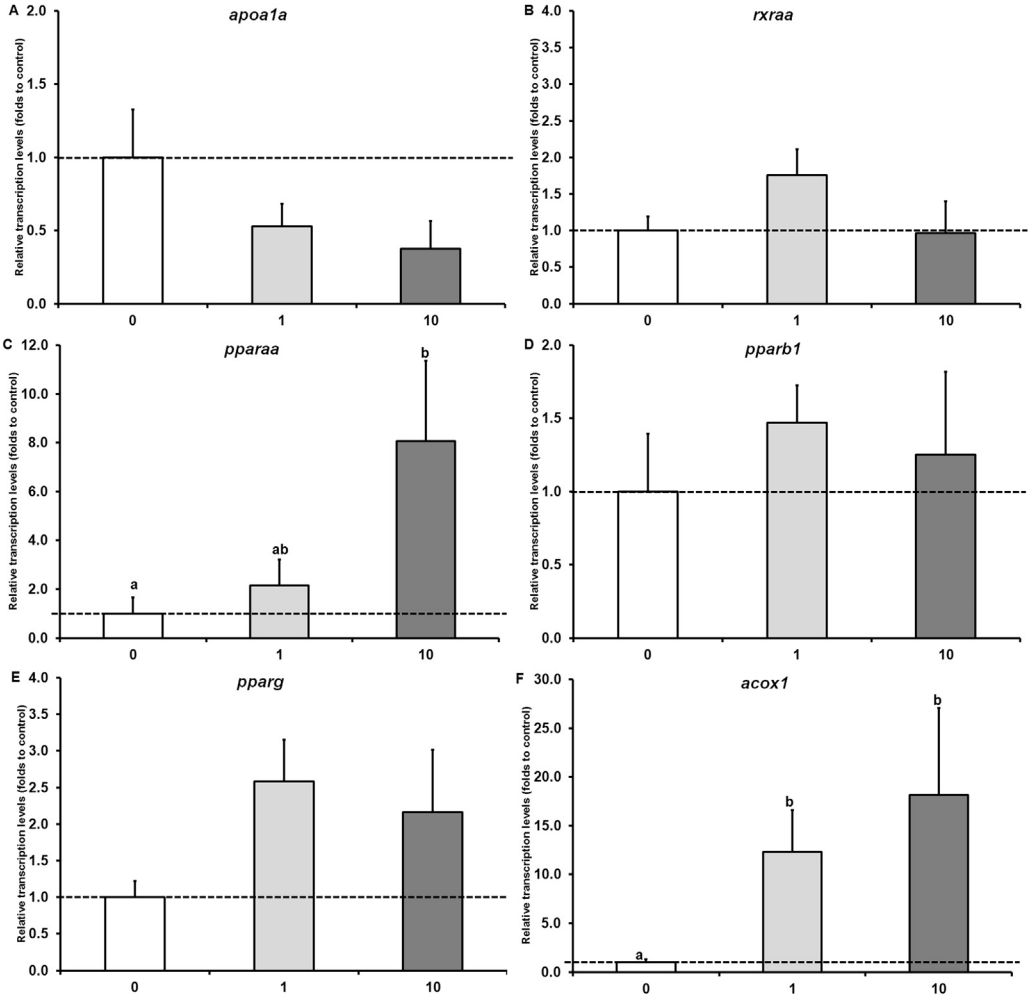

Fig. 4. Relative transcription levels (folds to control) of F1 zebrafish (*D. rerio*) male liver in 0, 1 and 10 mg/g clofibric acid groups. A-*apoa1a*, B-*rxraa*, C-*pparaa*, D-*pparb1*, E-*pparg*, F-*acox1*. Different lower case letters indicate significant differences between groups. Values presented as mean ± standard deviation; $p < 0.05$, one-way ANOVA, followed by Fisher LSD multiple comparison test. ($n = 5–8$).

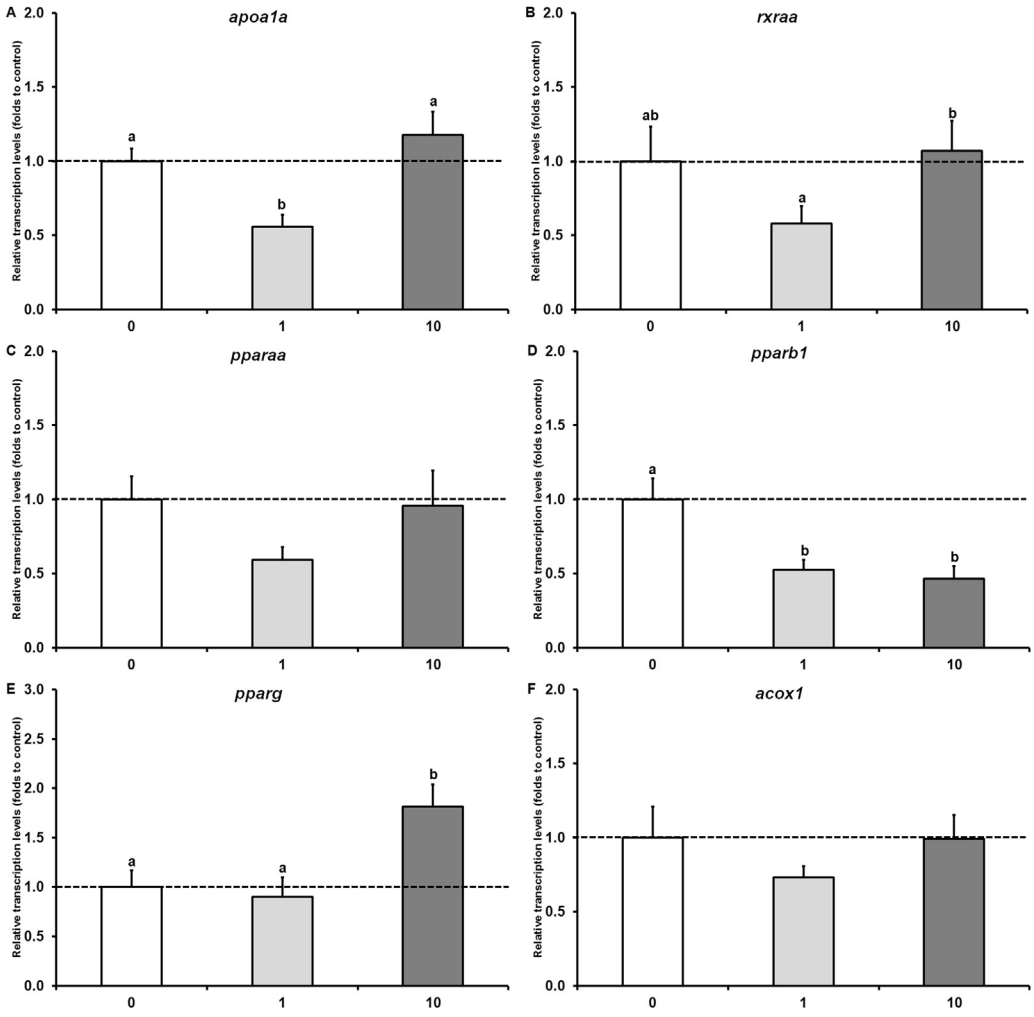

Fig. 5. Relative transcription levels (folds to control) of F2 zebrafish (*D. rerio*) male liver in 0, 1 and 10 mg/g clofibric acid groups. A-*apoa1a*, B-*rxraa*, C-*pparaa*, D-*pparb1*, E-*pparg*, F-*acox1*. Different lower case letters indicate significant differences between groups. Values presented as mean ± standard deviation; $p < 0.05$, one-way ANOVA, followed by Fisher LSD multiple comparison test. ($n = 5–8$).

compared to control ($p = 0.009$) and fish exposed to 10 mg/g of CA ($p = 0.042$). Likewise, *pparb1* expression levels in fish exposed to 1 mg/g of CA were also found to be lower when compared to 10 mg/g of CA exposed fish ($p = 0.005$), this decline on the transcript expression was also observed when compared to the control, although the decline was not significant ($p = 0.072$). In testis, the transcription levels of all studied genes were not significantly altered by CA exposure (Table 4).

In female zebrafish liver (Table 4), CA did not affect gene transcription in comparison with the control group. Statistical differences were only observed between the two CA exposed fish groups for the transcription of *pparb1* ($p = 0.040$).

Male liver transcription levels of *pparaa* (Fig. 4C) seemed to be up-regulated by the exposure to 10 mg/g of CA, although this effect was not statistically significant for a 95% confidence interval ($p = 0.098$, 8-fold induction). However, *acox1* transcription (Fig. 4F) was up-regulated in the groups exposed to CA (1 mg/g $p = 0.017$, 13-fold induction; 10 mg/g $p = 0.019$, 17-fold induction). Transcription levels of the remaining studied genes were not significantly affected by CA exposure.

Based on the findings for the F1 generation, male liver was selected to screen the transcription levels of target genes in the F2 generation (Fig. 5). Similar to F1 generation, the *rxraa* expression levels did not differ among treatments (Fig. 5B). In contrast to what was observed in F1, liver *acox1* (Fig. 5F) transcription levels did not show differences among groups, while all of the other studied genes in F2 male livers presented transcription level differences among groups (except for *pparaa*). In F2 males, *apoa1a* transcription (Fig. 5A) was lower in the descendants of F1 exposed to 1 mg/g of CA when compared to control ($p = 0.015$) and 10 mg/g of CA ($p = 0.001$) descendants. In addition, F2 descendants of both CA exposed groups had lower *pparb1* (Fig. 5D) transcription levels in comparison to control descendants (1 mg/g $p = 0.005$; 10 mg/g $p = 0.002$). Conversely, *pparg* (Fig. 5E) transcription levels in the descendants of 10 mg/g of CA exposed zebrafish were significantly higher than the ones presented by control ($p = 0.008$) and 1 mg/g of CA ($p = 0.004$) descendants.

3.7. Triglyceride content

In F1 females, muscle triglyceride content (Table 2) in fish exposed to 10 mg/g of CA was lower ($p = 0.010$) when compared to fish exposed to 1 mg/g of CA. In males from the F1 generation, a decline in muscle triglyceride content was observed ($p = 0.047$, $r = -0.52$), with males exposed to 10 mg/g of CA showing significantly lower levels when compared to non-exposed fish ($p = 0.027$). Differences among muscle triglyceride content in F2 generation were not observed.

4. Discussion

Clofibric acid is a well-known persistent pharmaceutical in the aquatic environment; nonetheless, chronic data on its effects in fish are very limited and mainly focused on male exposures during the adult stage. Nowadays, it is well established that if we aim at improving risk assessment of bioactive compounds, full-life cycle exposures and early development studies are essential. Therefore, the present work addresses the chronic multigenerational effects of CA exposure using zebrafish as animal model. In particular, we addressed here several key questions; does chronic CA exposure lead to changes on ecologically-relevant endpoints in teleost fish? Are these changes observed concomitantly with perturbation on lipid homeostasis? Does chronic CA exposure induce multigenerational effects?

In mammals, CA acts by activation of PPARa and its downstream regulated pathways, leading to the increase in size and number of peroxisomes, organelles mainly containing enzymes involved in lipid homeostasis. Given the sequence conservation between human and fish PPARs, it is plausible to hypothesize that the observed effects of CA in mammals, i.e., decreased total plasma lipid content, in addition to side effects such as impact on spermatogenesis (Cook et al., 1999; Liu et al., 1996), could also occur in fish. In previous studies with fathead minnow and rainbow trout, contradictory findings were reported (Runnalls et al., 2007; Owen et al., 2010). Fathead minnow juvenile exposure to CA for 28 days resulted in a reduction of growth and liver size, whereas a study involving rainbow trout, with more replicate aquaria, did not observe any impact of CA in these endpoints (Owen et al., 2010). In mosquitofish (*Gambusia holbrooki*) males, short-term exposure to CA showed a tendency for weight reduction while this reduction was not observed in females (Nunes et al., 2004). The results of the present study show that chronic life-cycle exposure to CA induces a significant decrease in weight and growth (F1) suggesting alterations of the lipid metabolism. This integrates well with the transcriptional up-regulations of *pparb1* in the ovary and *pparaa* and *acox1* in male liver. Provided that transcriptional up-regulation is reflected in a similar change in the activity of encoded protein, it indicates a possible activation of the fatty acid oxidation pathways (Schoonjan*set al.*, 1996). Our findings integrate well with previous studies reporting an induction of zebrafish liver transcription of *acox*, when clofibrate was administrated through the diet (5 mg/g) (Venkatachalam et al., 2012), and increase in *ppara* and *pparb* transcripts, when injected (ip 50 mg/kg) to *C. idella* (He et al., 2012). Also, fibrate injection induced an up-regulation in the transcription of *acox* in *Oncorhynchus mykiss, Oryzias latipes* (Scarano et al., 1994) and *Salmo salar* (Ruyter et al., 1997), though with no significant gender-related differences observed, as reported elsewhere (Ibabe et al., 2005a). Overall, taking together our findings and previous studies, compiled evidence points to an impact of fibrates in lipid homeostasis in fish.

In contrast to F1 observations, the descendants of CA-exposed fish (F2) presented higher weight in comparison with control animals, although this was not reflected in the condition factor, the muscle triglyceride content or male length. Interestingly, we found in F2 male liver a transcriptional up-regulation of *pparg* (descendants of 10 mg/g diet group), a gene encoding a transcription factor that has a central role in lipid homeostasis, stimulating lipid storage pathways, and a transcriptional down regulation of *pparb1*, which encodes a protein that has been reported to play a role in the stimulation of lipid mobilization. We hypothesize that this pattern of gene transcription might be related with the increased weight of males from the parental CA exposure groups. In mammals it is well established that the nutritional environment during certain life-development stages, particularly early-life stages, has a critical role in development and weight later in life (Janesick and Blumberg, 2011; Boekelheide et al., 2012). In humans and mammalian animal models it has been observed that malnutrition early in life leads to an adaptive response that prepares the organism for the postnatal environment (Hanson et al., 2011). This explains the reported increased incidence of overweight observed during adulthood in mammals that go through malnutrition during embryonic development. Similarly, exposure to certain chemicals such as nicotine, perfluorooctanoic acid and tributyltin during fetal development has been associated with overweight later in life (Boekelheide et al., 2012). Hence, we hypothesize that the observed increase in weight in the animals from the F2 generation, and the concomitant changes on the gene transcription profile in male liver, could represent an adaptive response to an environment where lipid mobilization was stimulated under CA exposure during F1 generation and possible during F2 embryonic development. the mechanisms underlying these observations are still, in part, unclear, but some endocrine disrupting chemicals such as vinclozolin (Anway et al., 2005) and tributyltin (Chamorro-García et al., 2013) have been shown to determine a disease phenotype in the following generations. The most plausible explanation relates to epigenetic modifications that regulate gene expression. Given the present findings, and considering the extensive use of fibrates in human therapies and their ubiquitous presence in aquatic ecosystems, future studies should evaluate if CA exposure induces epigenetic changes both in mammalian and teleost species.

Considering the central role of lipid homeostasis in a key ecologically-relevant endpoint such as reproduction, we then addressed the impact of CA on fertility, fecundity, gonad stereology of F1 generation and the embryonic development of their offspring. Although the fertility rate was not affected, CA (10 mg/g) led to a decrease in the fecundity rates. This is consistent with the impact on morphometric parameters and triglyceride content since it is well known that larger females normally display higher fecundity (Uusi-Heikkilä et al., 2010). CA exposure did not impact the relative volumes of ovarian cells at 140 dpf and was not associated with an interference with vitellogenesis, as has been observed in fathead minnow (Weston et al., 2009). However, in male zebrafish, we observed a decline in the relative volume of spermatids at the highest CA exposure, while no differences were observed in the relative volume of spermatozoa. A previous study with fathead minnow reported a decline in spermatozoa number and viability under CA exposure (Runnalls et al., 2007), while zebrafish testicular morphological alterations were observed after bezafibrate administration (Velasco-Santamaria et al., 2011).

Given the findings of the F1 generation, we then focused on the multigenerational impacts of CA in reproduction and embryonic development. Although the 10 mg/g CA exposure impaired fecundity rates in F1, in the F2 generation this group displayed an opposite pattern although fecundity did not differ significantly from control females. This might be related with the increase weight of F2 females from parental CA exposure, further supporting the hypothesis of multigenerational effects. Interestingly, F1 offsprings from the 1 mg/g CA treatment, but not those from the 10 mg/g CA diet, presented a significant increase in the percentage of embryo malformations, displaying a phenotype different from that earlier reported under clofibrate and gemfibrozil exposure (Raldua et al., 2008). Additionally, the descendents of the 1 mg/g CA groups (F3 embryos), presented higher mortality in comparison with the 10 mg/g group. Hence, our results further support the hypothesis that maternal fibrate exposure might affect embryo development compromising eleuthero-embryo survival. These results suggest that the exposure of one generation to CA may impact the reproductive success and recruitment of individuals in, at least, two future generations as shown by the F2 and F3 embryos descendents of the group exposed to 1 mg/g of CA that, respectively, showed higher incidence of abnormalities and mortality.

In summary, taken together the findings of the present study and previous reports, compiled evidence points to an impact of CA in fish lipid metabolism, with some similarities to those reported in mammals. The present study shows that chronic CA exposure impacts triglyceride accumulation in muscle and fish growth, and affects fecundity, gonad development, sex ratio and embryo development. All these effects occur concomitantly with changes in the transcription levels of key genes involved in lipid homeostasis, both in the exposed fish and in their progeny suggesting the trigger of epigenetic mechanisms, which gives further support to a multigenerational disruption of lipid signaling pathways. Given the scarcity of data on actual accumulation of CA in the food chain, the environmental relevance of the current findings requires additional research.

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