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1 **Chronic environmentally relevant levels of Simvastatin disrupt embryonic development, and**
2 **biochemical and molecular responses in Zebrafish (*Danio rerio*)**

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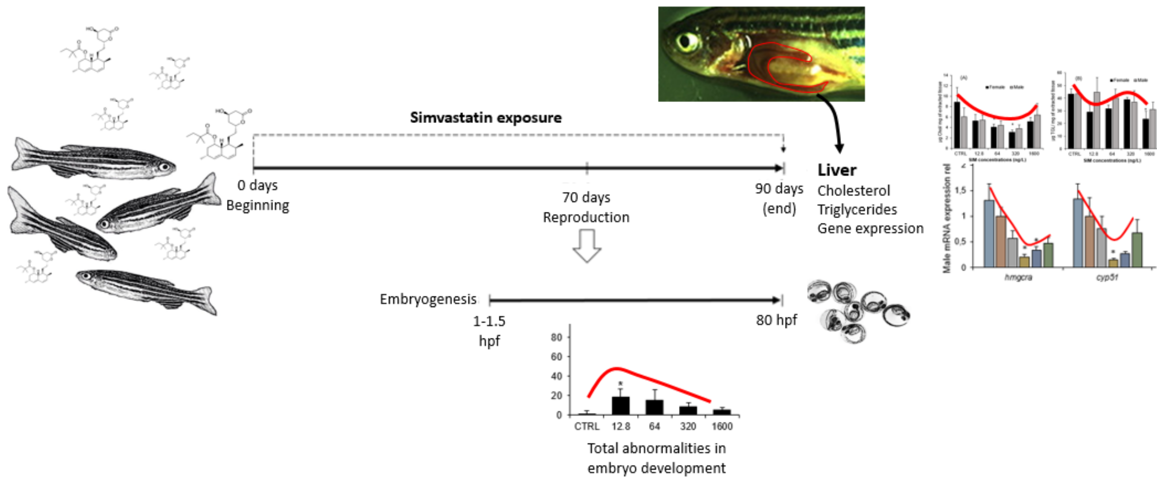
Graphical abstract



Highlights

- Several uncertainties exist regarding simvastatin mode of action in non-target organisms
- This work integrates *Danio rerio* multi-parametric responses after chronic, low level exposure to simvastatin
- Simvastatin reduced cholesterol/triglyceride levels and induced changes in the transcription levels of key genes involved in cholesterol biosynthesis.
- Parental exposure to simvastatin induced embryonic malformations of offspring.
- Embryonic abnormalities, biochemical and molecular data did not follow a concentration-response curve.

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Abstract

Simvastatin (SIM), a hypocholesterolaemic compound, is among the most prescribed pharmaceuticals for cardiovascular disease prevention worldwide. Several studies have shown that acute exposure to SIM causes multiple adverse effects in aquatic organisms. However, uncertainties still remain regarding the chronic effects of SIM in aquatic ecosystems. Therefore, the present study aimed to investigate the effects of SIM in the model freshwater teleost zebrafish (*Danio rerio*) following a chronic exposure (90 days) to environmentally relevant concentrations ranging from 8 ng/L to 1000 ng/L. This study used a multi-parameter approach integrating distinct ecologically-relevant endpoints, i.e. survival, growth, reproduction and embryonic development, with biochemical markers (cholesterol and triglycerides). Real Time PCR was used to analyse the transcription levels of key genes involved in the mevalonate pathway (*hmgcra*, *cyp51*, and *dhcr7*). Globally, SIM induced several effects that did not follow a concentration-response relationship; embryonic development, biochemical and molecular markers, were significantly impacted in the lower concentrations, 8 ng/L, 40 ng/L and/or 200 ng/L, whereas no effects were recorded for the highest tested SIM levels (1000 ng/L). Taken together, these findings expand our understanding of statin effects in teleosts, demonstrating significant impacts at environmentally relevant concentrations and highlight the importance of addressing the effects of chemicals under chronic low-level concentrations.

Keywords: Zebrafish; Simvastatin; HMGCR; Chronic effects; Low-level exposures

1. Introduction

In the past, pharmaceuticals were overlooked as aquatic pollutants because exposure levels were considered to be too low to induce significant effects in non-target organisms (Arnold et al., 2014; Daughton, 2016; EEA, 1999). However, from the mid-90s, a growing attention has been devoted to this class of compounds. Indeed, the detection of pharmaceuticals in the aquatic environments has increased in the last years, not only because of the pharmaceutical industry growth, but also due to improvements of analytical methods (LaLone, et al., 2014). Most pharmaceuticals are detected in surface waters at trace levels, generally at concentrations ranging between ng/L and low µg/L levels (Arnold et al., 2014; Azzouz & Ballesteros, 2012; BIO Intelligence Service, 2013; Daughton, 2016; Fent et al., 2006). However, since they are bioactive substances, designed to produce biological effects at rather low concentrations, the scientific community is now in broad agreement that pharmaceuticals may pose a considerable environmental risk (BIO

Intelligence Service, 2013; Ferreira et al., 2009; Rodrigues et al. 2006). In fact, several pharmaceuticals have been demonstrated to induce effects at environmentally relevant concentrations in non-target organisms (Arnold et al., 2014; Fent et al., 2006; Neuparth et al., 2014). Nevertheless, the ecological risk assessment of pharmaceuticals is still in its infancy with studies dealing with this class of compounds reporting mostly acute or sub-lethal toxicity effects, with concentrations above environmental relevance (Dahl et al., 2006; Fent et al., 2006; Neuparth et al., 2014; Santos et al., 2016; Sárria et al., 2011). Hence, given that non-target aquatic organisms may be continuously exposed to low levels of this class of compounds for several generations, there is an urging need to assess the chronic effects of environmentally relevant concentrations of pharmaceuticals (Arnold et al., 2014; BIO Intelligence Service, 2013; Fent et al., 2006).

Simvastatin (SIM) is a hypolipidemic drug of the statin class used in humans as the primary treatment of hypercholesterolemia to decrease serum LDL-cholesterol levels (Burg & Espenshade, 2011; Igel, et al., 2001; Neuparth et al., 2014; Vázquez et al., 2017). Simvastatin, as well as other statins, is known to specifically inhibit the enzyme 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGCR), which is essential for the *de novo* synthesis of cholesterol in mammalian cells through the mevalonate pathway (Al-Habsi, et al., 2016; Blumenthal, 2000; Endo et al., 1976a; Endo, et al., 1976b; Fent, et al., 2006; Sehayek, et al., 1994). Simvastatin competes with 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) for the active binding site in the enzyme HMGCR, having an affinity of about three orders of magnitude greater than the natural substrate. Once bound to the enzyme, statins alter its conformation, inhibiting its function and thereby decreasing the cholesterol synthesis (Al-Habsi et al., 2016; Istvan, 2003; Moghadasian, 1999).

Statins, including SIM, have been reported to be among the most prescribed human pharmaceuticals in the western countries (Ellesat et al., 2010; Kaufman et al., 2010; Miao & Metcalfe, 2003; Neuparth et al., 2014; Walley et al., 2005). Due to the lack of mechanisms that ensure complete removal of statins in wastewater treatment plants (WWTP), significant amounts of these pharmaceuticals are discharged in the aquatic environments (Fent et al., 2006; Lapworth et al., 2012). Several authors have reported the presence of SIM in WWTPs worldwide (Kasprzyk-Hordern et al., 2009; Miao & Metcalfe, 2003; Ottmar et al., 2012; Pereira et al., 2015; Pereira et al., 2016; Sousa, 2013; Verlicchi et al., 2012). Concentrations up to 8.9 µg/L and 1.23 µg/L (influents) and 1.5 µg/L and 90 ng/L (effluents), were reported in WWTPs in Portugal and USA, respectively (Ottmar et al., 2012; Pereira et al., 2016). The predicted environmental concentrations for SIM in Norwegian and Portuguese surface waters have been estimated at 630 ng/L and 369.8 ng/L, respectively (Grung et al., 2007; Pereira et al. 2015). Considering that SIM is a common prescribed drug in western countries, and its discharge into aquatic environment has increased in the last years, many aquatic taxa might be at risk. This is particularly true given that statins were predicted

to inhibit HMGR in a broad range of animal *taxa* (Santos et al., 2016). In fact, the main concern regarding the presence of SIM in the aquatic ecosystems is its environmental persistence, toxicity, and bioactivity, at rather very low concentrations. Furthermore, SIM has a high log K_{ow} of 4.68, which might indicate high bioaccumulation potential in aquatic organisms (Santos et al., 2016). Previous studies have reported multiple detrimental effects of SIM in aquatic organisms at several levels of biologic organization, such as impairment of embryo development (*Danio rerio* and *Parachentrotus lividus* – Ribeiro et al., 2015), decreased metabolic activity and membrane stability (*Oncorhynchus mykiss* hepatocyte - Ellesat, et al., 2010), alteration of acetylcholinesterase and lipid peroxidation levels (*Fundulus heteroclitus* - Key et al., 2009; *Palaemonetes pugio* – Key et al., 2008), disturbances in growth (*Dunaliella tertiolecta*, *Nitocra spinipes and Gammarus locusta* - Dahl et al., 2006; DeLorenzo & Fleming, 2008; Neuparth et al., 2014), severe reproductive impairments (*Gammarus locusta* – Neuparth et al., 2014), and even mortality (*Plaemonetes pugio* - Key et al., 2008). With the exception of the Neuparth et al (2014) study that reported chronic effects of SIM in the ng/L range, all the aforementioned studies have been based on acute toxicity tests, with SIM concentrations above environmental relevance.

Despite the diversity of studies regarding SIM toxicity, the underlying mechanisms of action in aquatic organisms are still not fully understood (Gee et al., 2015). Acknowledging the mode of action (MOA) of SIM in non-target aquatic organisms is important not only to establish its ecotoxicological response upon SIM exposure, but also to anticipate the effects of other compounds acting through homologous pathways. This is particularly relevant for bioactive molecules designed to produce biological effects at very low concentrations, such as pharmaceuticals. Hence, there is an urgent need to perform long-term, low level exposure assays, that integrate effects of ecologically-relevant endpoints with molecular and biochemical responses.

Therefore, the main aim of the present study was to evaluate SIM effects on zebrafish, following a chronic partial life-cycle exposure to environmentally relevant concentrations (ng/L) and early offspring embryonic development effects of parental SIM exposure. We integrate multiple key ecological endpoints (survival, growth, reproduction, and embryonic development), with biochemical markers of lipid homeostasis (cholesterol and triglycerides) and molecular analysis (expression of key genes coding for modules of the mevalonate pathway: *hmgcra*, *cyp51*, and *dhcr7*), to gain insights into the long-term adverse effects of SIM on ecologically-relevant endpoints and to address the underlying mechanism of toxicity in fish.

2. Material and Methods

2.1 Species Selection

1 Zebrafish *(Danio rerio)* is recommended as a test species in a wide range of ecotoxicological
2 test protocols (Oberemm, 2000). Its small size, robustness, multiple progeny from a single mating,
3 embryo transparency and easy maintenance under laboratory conditions are advantages for its use
4 as test organism (Fang & Miller, 2012; Lawrence, 2007; Soares et al., 2009). In addition, the close
5 phylogeny of zebrafish and mammals, with highly conserved mevalonate pathway genes, makes
6 this species ideal for performing the present study.
7
8 **2.2. Zebrafish maintenance**

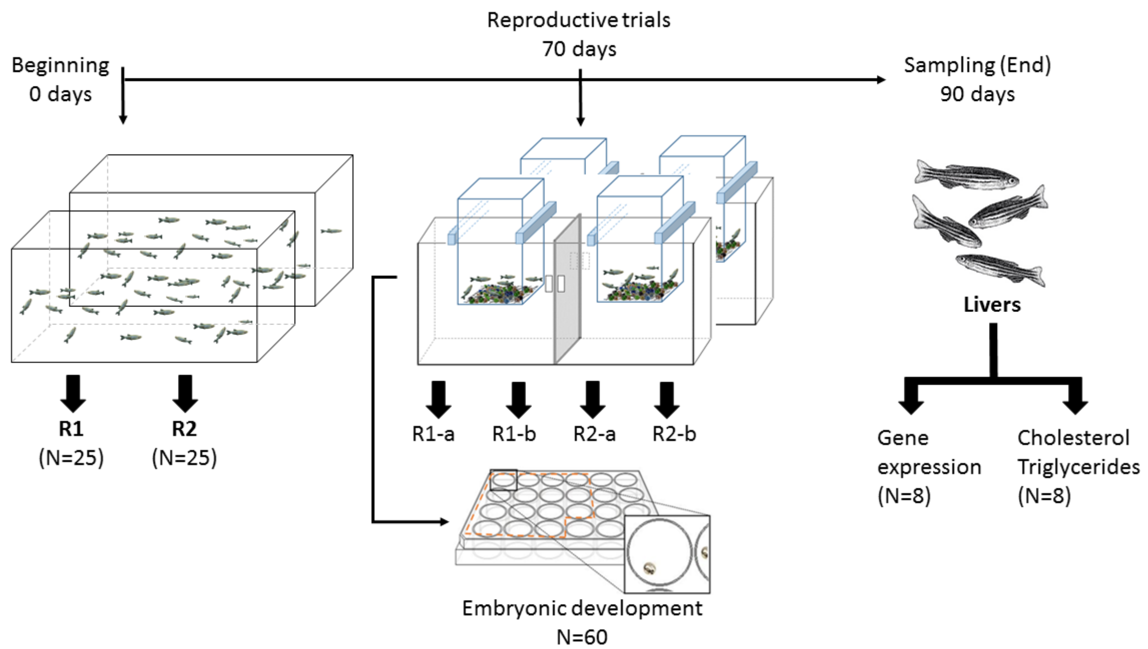
9 Wild-type zebrafish, 50-day old, were obtained from Orniex, Portugal (purchased from local
10 suppliers in Singapore). Animals were acclimated to controlled laboratory conditions, in 250 L
11 aquarium with dechlorinated filtered and aerated water. During this period, fish were kept at 28 ±
12 1°C, under a photoperiod of 14:10 h (light:dark) and fed, *ad libitum*, three times per day with
13 commercial fish diet Tetramin (Tetra, Melle, Germany). These conditions were maintained for 15
14 days until the beginning of the chronic bioassay.

15

16 **2.3. Chronic toxicity bioassay**

17 The chronic bioassay was carried out at "Biotério de organismos aquáticos" (BOGA)
18 located at Interdisciplinary Centre of Marine and Environmental Research (CIIMAR), Matosinhos,
19 Portugal. The experiment was subject to a previous ethical review process carried out by CIIMAR
20 animal welfare body (ORBEA, 2010/63/EU Directive). The bioassay was performed in compliance
21 with the European Directive 2010/63/EU, on the protection of animals used for scientific purposes,
22 and the Portuguese "Decreto Lei" 113/2013.
23 A partial life-cycle bioassay was performed for 90 days (Figure 1). The experiment was
24 started by randomly allocating 25 sub-adult zebrafish in 30 L aquaria (two per treatment), under a
25 flow-through system. The water flow was maintained at 1.08 L per hour by means of a peristaltic
26 pump (ISM 444, ISMATEC) supplied with dechlorinated, heated and charcoal filtered tap water.
27 Each aquarium was maintained with a water temperature of 28 ± 1°C, 14:10 h (light:dark)
28 photoperiod, pH 7.5 ±0.2 and a mean ammonia concentration of 0.08 ± 0.04 mg/L. Fish were fed
29 twice a day with a commercial fish diet Tetramin (Tetra, Melle, Germany), supplemented with 48-
30 hour-old live brine shrimp (*Artemia* spp) from one week before the onset of reproduction until the
31 end of the experiment. During the bioassay, the amount of food delivered was adjusted according
32 to fish development and size. The amount of food provided was equal for all aquaria.

1 The experiment consisted of six treatments in duplicate: a control (dechlorinated water); a
2 solvent control (0.0002% acetone, ACET), and four SIM treatments at the following concentrations:
3 8, 40, 200 and 1000 ng/L. The selection of SIM treatments was based on previous studies (Neuparth
4 et al., 2014), taking into consideration environmentally relevant concentrations. SIM (CAS no.
5 79902-63-9; Sigma Aldrich) was diluted in ACET to obtain a stock solution of 4 mg/mL. From this
6 solution, working solutions were prepared by serial dilutions of the stock solution and aliquots were
7 stored in the dark at -20°C until use. All solutions were prepared in order to have a final ACET
8 concentration of 0.0002%. Based on preliminary tests and in Lyssimachou et al. (2015), in order to
9 maintain exposure concentrations, the working solutions were manually dosed directly into the
10 water of aquaria twice a day; in the morning (T_{0h}) and in the afternoon (T_{8h}), in a volume that was
11 equivalent to the water renewal during that period.



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13 **Figure 1.** Schematic representation of the experimental setup. Each treatment had two aquaria
14 (R1 and R2), with 25 animals each. Aquaria were then subdivided in two (a and b) for the
15 reproductive trials and embryonic studies at day 70. Embryonic development was analyzed using
16 60 embryos (15 each in four replicates).The experiment finished at day 90 and all animals were
17 sacrificed, measured, weighted, and livers from both males and females were collected for gene
18 expression analysis (N=8) and for cholesterol/triglyceride quantification (N=8 for males and N=8
19 for females).

20

21 **2.4. Reproductive capability**

22 Reproductive capability studies were performed 70 days after the beginning of the chronic
23 exposure for all the tested groups (120-day old fish). Reproductive success was assessed through

the evaluation of two endpoints: fecundity (number of eggs per female per day) and percentage of fertilization (% of fertilized eggs per female per day). In the afternoon, before the beginning of the trials, each aquarium was divided in two separate sections in which a suspended cage with a net bottom covered with marbles was fixed. Males and females were equally distributed through the cages in a manner that matched the sex ratio of the respective treatment (four reproductive units per treatment) (Figure 1). The sex ratio was determined by visual inspection of each animal. However, given the small size of the animals, for some fish sex determination was not totally clear. Therefore, at the end of the bioassay the sex ratio of each aquarium was confirmed by stereomicroscope observation of gonads. The fecundity and % of fertilization were then determined considering the total number of females in each replicate based on the gonad observation. During five consecutive days, 1-1.5 hour after the beginning of the light period, breeding fish were removed and the eggs collected, cleaned and conserved in 70% ethanol, for posterior counting and determination of the percentage of fertilized eggs.

2.5. Embryogenesis studies

Embryogenesis studies were carried out with slight modification of OECD Fish Embryo Acute Toxicity (FET) Test 236 (OECD, 2013; Torres et al. 2016). During the reproductive trials, 15 embryos from each treatment sub-replicate, with 1-1.5 hpf, were kept and randomly placed in 24-well plates (one egg per well) with 2 ml of clean dechlorinated water, free of SIM (Figure 1). The 24-well plates were randomly maintained on a water bath at 26.5 ± 0.5°C for 80 h. Embryos were checked, every day for mortality, and at the end of the assay (80 hpf), under a stereomicroscope in a acclimatized room at 26 ± 1°C, for embryo development analysis. Morphological abnormalities on eyes, head, tail or yolk-sac; pericardial oedema; abnormal cell growth and developmental arrest were recorded as present or absent (Torres et al., 2016). Heart rate, i.e. the cardiac frequency, was evaluated in four embryos per reproductive unit (16 embryos per treatment) during 15 s using a stop-watch, restarting the counting if the embryo moved.

2.6. Sampling

At end of the assay, the animals were sacrificed with an anaesthetic overdose of 300 mg/L tricane methanesulfonate (MS-222), to which sodium bicarbonate was added to prevent the acidification of the solution. All animals were measured and weighed, and Fulton's condition factor (K) was determined ($K=(weight / lenght^3)\times100$) – Nash et al., 2006). Livers from 16 males and 16 females of each treatment were individually collected and i) preserved separately in RNA Later for

gene expression analysis (8 males and 8 females), and ii) frozen in liquid nitrogen and stored individually at -80 °C for cholesterol and triglyceride quantification (8 males and 8 females). .

2.7. Lipid extraction and cholesterol and triglyceride quantification

Lipids were individually extracted from the livers of 8 males and 8 females using a low toxicity solvent extraction protocol, adapted from Schwartz and Wolins (2007). The tissues were homogenized in 10 mM PBS buffer pH 7.4 containing 10 mM EDTA (10 mg of tissue per 1 mL buffer with two ceramic beads) on a Precellys 24 homogenizer. 500 µl of homogenates were then transferred, in duplicate, to test glass vials containing 5 mL of isopropanol/hexane solution (4:1). The samples were vortexed for 1 min and incubated at room temperature in the dark with constant shaking for 2 h. In order to avoid lipid peroxidation, vials were submitted to N_2 stream before closing. After the incubation period, lipids were dissolved in 2 mL of petroleum ether/hexane solution (1:1). Vials were again vortexed for 1 min and left in the dark at room temperature for 10 min. The phases were then separated by adding 1 mL of Milli-Q water, vortexed for 1 min, incubated in the dark at room temperature, with constant shaking for 20 min, and centrifuged at 1000 × G, for 10 min. The upper phase containing the lipids was collected into new vials and evaporated to dryness under N_2 stream. Dried extracts were then stored at -20 °C for subsequent cholesterol and triglyceride quantification.

Dry extracts were re-suspended in 100 µL of isopropanol and sonicated in ultra-sound bath (Bandelin Sonorex RK100H) for 15 min at room temperature. Quantification of cholesterol and triglycerides was performed through enzymatical colorimetric assays using Infinity Cholesterol Liquid Stable Reagent and Infinity Triglycerides Liquid Stable Reagent, respectively, both purchased from Thermo Sientific, Biognóstica and following the manufacturer's protocols. Samples were measured in duplicate and absorbance determined at λ 490 nm using a microplate reader (Biotech Synergy HT) coupled with software Gen5 (version 2.0). In every run, a standard curve was performed for the optical quantification of cholesterol and triglycerides. Cholesterol and Triolin standards were prepared and subjected to 6 serial dilutions (from 0.156251 to 5 mg/mL for cholesterol and 0.0625 to 2 mg/mL for triolin).

2.8. Gene expression

2.8.1. RNA isolation and cDNA synthesis

Eight male and eight female Livers from the different treatments (approximately 3 mg) were individually used to isolate total RNA via the Illustra RNAspin Min RNA Isolation Kit (GE Healthcare), according to the manufacturer's protocol. RNA quantification was performed on a

Take3 Micro-Volume Plate Reader (Biotech Synergy HT) coupled with the software Gen5 (version 2.0). RNA quality was verified by electrophoresis in 1.5 % agarose gel and through the measurement of the ratio of absorbance at λ 260 / λ 280 nm. Total cDNA was generated from 1 µg of total RNA extracted using the iScriptTM cDNA Synthesis Kit (Bio-Rad).

2.8.2. qRT-PCR

Fluorescence-based quantitative real time PCR (qRT-PCR) was used to evaluate the transcription profiles of several genes involved in the mevalonate pathway with important roles in cholesterol biosynthesis. The gene encoding 3-hydroxy-3-methylglutaryl-CoA reductase (*hmgcr*) was selected because its product is the primary target of SIM and the limiting step in the mevalonate pathway. According to previous studies on the HMGCR tissue distribution, we chose to assess *hmgcra* instead of *hmgcrb*, due to its predominance in the liver, while *hmgcrb* prevails in the brain (Al-Habsi et al., 2016). Other two genes, those encoding lanosterol 14α-demethylase (*cyp51*) and 7-dehydrocholesterol reductase (*dhcr7*) were chosen as intermediate genes in the mevalonate pathway. The gene encoding ribosomal protein L8 (*rpl8*) was used as the reference gene, as its expression levels remained constant across treatments. All the primers were already described by other authors (Table 1). For each treatment (n = 8), the expression of individual target genes was determined with the Mastercycle ep realplex system (Eppendorf). cDNA of liver samples was amplified in duplicated in 96-well optical plates, containing 10 µL of NZY qPCR Green Master Mix (2x) (NZYTech), 0.8 µL of each primer (forward and reverse), 2 µL of cDNA at 100 nmol and 6.4 µL of water in order to reach a final reaction volume of 20 µL. On each plate, a non-template control was included. In order to determine the efficiency of the reaction, a two-step qRT-PCR was performed: initial denaturation at 95 °C (3 min), followed by 40 cycles of amplification with a denaturation at 95 °C for (15 s) and combined annealing and extension at 58 - 62°C, depending on primer set (25 s) (Table 1). A melting curve (from 55 °C to 95 °C) was generated in each run to confirm the specificity of the reactions. The PCR products were analysed with 2 % agarose gel electrophoresis to check the presence of single bands with the expected size between 136 and 199 bp, depending on the primer sets (Table 1). The PCR efficiency for the genes of interest and for the reference gene was determined by a standard curve, using six serial dilutions of cDNA pools of all samples (from 0.064 to 200 ng of cDNA). The minimum efficiency obtained was 94% (Table 1). Relative change in the transcription abundance of target genes was normalized to *rpl8* and calculated using the Livak method (Livak & Schmittgen, 2001). Control expression levels (ACET treatment) were normalized to 1 and data were then expressed as fold changes relative to solvent control group.

Table 1. Primers, forward (F) and reversed (R), and parameters used in the qRT-PCR for gene expression quantification in the liver of *D. rerio*.

Gene	Sequence (5' – 3')	Expected band size (bp)	Combined annealing and extension temperature (°C)	Average efficiency (%)	Reference
Hmgcra	F: TCGTGGAGTGCCTGGTGATTGGT R: TGGGTCTGCCTTCTCTGCTCTCTC	177	62	98	1
cyp51	F: GCTCGGAGACACTCAGACACATCTT R: AGCAGAACTGAAGTCAGGCTCATCT	138	60	96	1
dhcr7	F: GAGGAGTTCAGGATGGTGCCCGTA R: GTGGACACAGCATAGCCGAGGATG	199	60	94	1
rpl8	F: TTGTTGGTGTTGTTGCTGGT R: GGATGCTCAACAGGGTTCAT	136	58	94	2

Notes: **1** - (Mu et al., 2015); **2 -** (Lyssimachou et al., 2015)

2.9. Analytical quantification of simvastatin by liquid chromatography – tandem mass spectrometry

The actual concentrations of SIM were determined, in each treatment, twice during the bioassay: 30 min after the first SIM spike of the day (T_{0h}) ,and 8 hours after the first SIM spike and immediately before the second spike (T_{8h}). Two samples from each treatment (bulk samples from each replicate) were collected and stored at -20 °C for posterior quantification by solid-phase extraction (SPE) and Liquid Chromatography – Tandem Mass Spectrometry (LC-MS/MS). One hundred millilitres of each water sample were filtered through 0.45µm PVDF filters (Millipore) and spiked with 50 ng/L of Mevastatin (MEV, Sigma-Aldrich) as internal standard. Subsequently, the sample was loaded into a 60 mg Oasis HLB (Waters) cartridge previously conditioned with 5 mL of methanol (MeOH) and 5 mL of Milli-Q water. The cartridges were washed with 5 mL of Milli-Q water, dried under a N_2 stream and eluted with 5 mL of MeOH. The final extracts were evaporated to dryness under N_2 stream, reconstituted using 100 µL of MeOH and injected in the LC-MS/MS system. The quantification was performed by the matrix-matched method, by spiking with SIM real water in the 5 to 1000 ng/L range and submitting these samples to the entire protocol. Due to the fast conversion of SIM and MEV to their corresponding hydroxyacid forms (SIMHA and MEVHA) in aqueous media (Bews at al., 2014), these latter forms were also quantified and the final concentration was calculated as sum of the lactone and hydroxyacid forms, since it is analytically not feasible to report individual concentrations (Bews at al., 2014).

The LC–MS/MS system (Varian) was equipped with two ProStar 212 high-pressure mixing pumps, a vacuum membrane degasser, an autosampler and a thermostated column compartment ProStar 410 module (Varian). A volume of 10 µL of extract was directly injected into a Phenomenex Luna C18 (50 mm × 2.1 mm, 3 µm particle diameter) maintained at a constant temperature of 30 °C. The target compounds were separated at a flow rate of 0.2 mL min^{-1} using 0.1% of formic acid in both, Milli-Q water (A) and MeOH (B) as eluents. The applied gradient was as follows: 0–1 min, 30% B; 1–15 min, linear gradient to 100% B; 15–18 min 100% B; 18–18.5 min, linear gradient to 30%B and 18.5–23 min, 30% B. The system was interfaced to a Varian 320-MS triple quadrupole mass spectrometer equipped with an electrospray interface. Nitrogen was used as nebulizing and drying gas and Argon was used as collision gas. The analytes were determined in the electrospray positive (SIM and MEV) and negative (SIMHA and MEVHA) and multiple-reaction monitoring (MRM) mode of acquisition. Two MRM transitions were used for each compound quantifier and qualifier respectively (precursor > product ion, m/z values): 441 > 325 and 419 > 199 for SIM, 413 > 311 and 391 > 185 for MEV, 435 > 319 and 435 > 341 for SIMHA and finally, 407 > 305 and 407 > 327 for MEVHA.

The method limits of quantification (LOQs) were 1.5 and 1.2 ng/L for SIM and SIMHA, respectively. The linearity, evaluated in terms of correlation coefficient for the sum of SIM and SIMHA, was R^2 0.9964 and the recovery was 91%.

2.10. Statistical analysis

The obtained data were checked for homogeneity of variances (Leven's test) and normality (Kolmogorov-Smirnov test) and subsequently analysed by one-way ANOVA. Post-hoc comparisons were carried out using Fisher's least significant difference (LSD) test. Each treatment was compared with the solvent control. Repeated Measures ANOVA was used to analyse fecundity. Significant differences were set as $p < 0.05$. A Power Analysis and Sample Size Calculation were performed to verify the reliability of data. All the statistics were computed with Statistica 13 (Statsoft, USA).

3. Results

3.1. Analytical quantification of simvastatin

Table 2 summarizes the actual SIM concentrations measured during the bioassay at times T_{0h} and T_{8h}. Due to the high conversion of SIM to SIMHA in aqueous media (Bews et al., 2014), the final concentration was calculated as the sum of both active forms and given as total SIM, although the major detected fraction was SIMHA (>99.5%). No SIM, nor SIMHA were detected in solvent control

1 group. Results show that SIM concentrations at the initial time (T_{0h}), were marginally lower than
2 nominal ones. At T_{8h}, total SIM concentration decays as expected, but was still present in all
3 treatments (except control) with a minimum and maximum decay of 27.9% and 57.3% in the 8 and
4 1000 ng/L SIM treatments, respectively.
5

6 **Table 2.** Nominal and measured concentrations of SIM (expressed as sum of SIM and SIMHA) in water
7 samples collected in duplicate from each treatment after the first contamination of the day (T_{0h}) and
8 immediately before the second one (T_{8h}), Data are expressed as mean ± standard error.

Time	Solvent control	SIM 8 ng/L[a]	SIM 40 ng/L[a]	SIM 200 ng/L[a]	SIM 1000 ng/L[a]
T 0 h	n.d.[b]	6.8 ± 0.45	37.9 ± 3.21	108.6 ± 3.70	751.7 ± 40.08
T 8 h	n.d.[b]	4.9 ± 0.08	23.0 ± 0.08	74.0 ± 1.47	343.4 ± 23.14

9 [a] Nominal SIM concentrations
10 [b] Not detected
11

12 **3.2. Survival, growth, body weight and length**

13 Table 3 displays the survival percentages, weight and length of zebrafish males and females
14 following the 90-days chronic exposure to environmentally relevant SIM concentrations. No
15 significant differences among treatments were observed in the survival percentages; almost 100 %
16 of the fish were alive at the end of the bioassay. In SIM-exposed fish, a significant decrease of 8.6
17 and 12.5 % in the body weight was observed in males and females exposed to the highest SIM
18 concentration (1000 ng/L), compared to the solvent control (ACT). A decrease of 9.9% (p=0.09) and
19 11.4 % (p=0.08) in body weight was also observed in females exposed to 40 and 200 ng/L of SIM,
20 respectively, and a decrease of 6.7 % (p=0.06) in males exposed to 40 ng/L of SIM without reaching
21 statistical significance. A significant decrease in female body length was observed in the highest SIM
22 concentration (1000 ng/L).
23

24 **3.3. Reproductive capability**

25 Females from all tested SIM concentrations exhibited a trend towards an increase in fecundity
26 when compared with females from solvent control (Table 3), however statistically significance was
27 not reached. This increase was followed by an increment in the percentage of fertilization, for which
28 significant differences were found in the 200 ng/L SIM treatment.

29

30

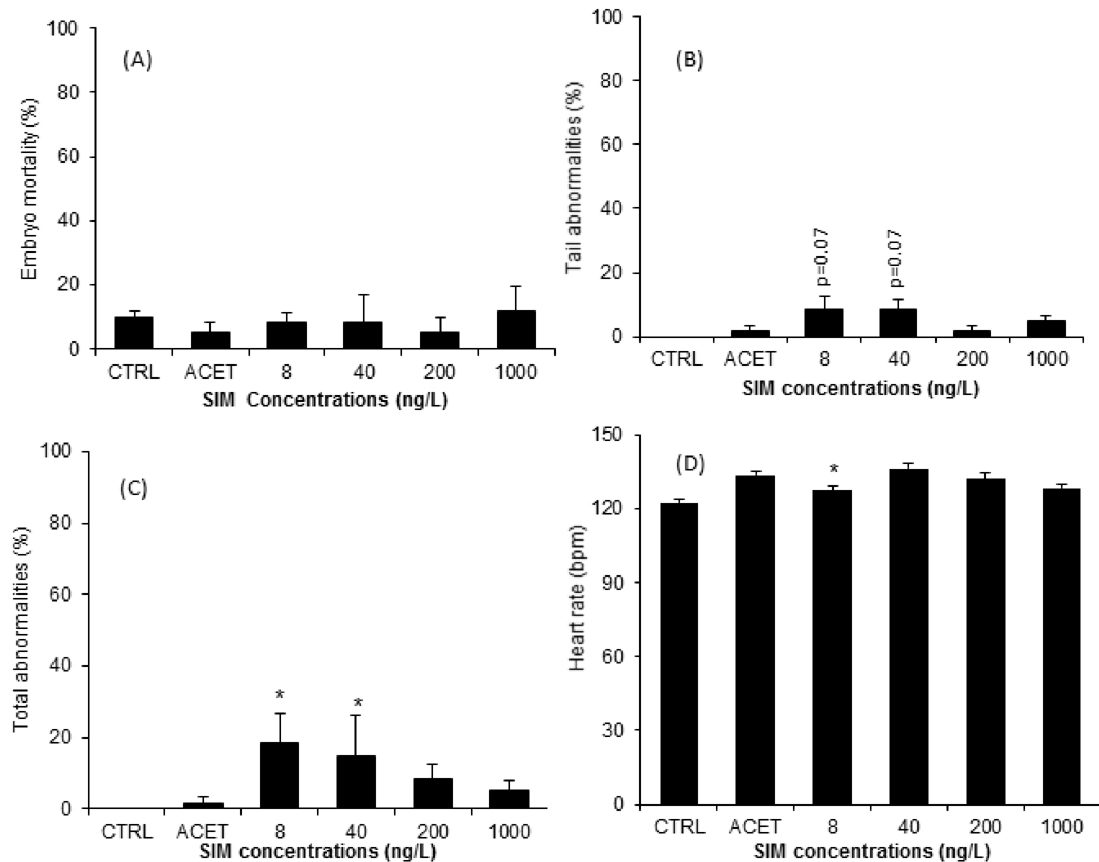
Table 3. Chronic effects of SIM on survival, weight, length, Fulton's condition factor (K), fecundity (number of embryos/ female/ day), and % of fertilization (% of fertilized eggs/female/day) of *D. rerio* after 90 days of exposure (M, males; F, females).

Endpoint		Control	Acet	8 ng/L	40 ng/L	200 ng/L	1000 ng/L
Survival (%)	—	100%	100%	100%	100%	100%	98%
Weight (mg)	M	**611.5** ± 21.9	**642.1** ± 12.7	**615.3** ± 25.3	**598.5**$^{\#}$ ± 13.6	**630.6** ± 9.6	**588.0*** ± 12.9
	F	**769.0** ± 35.7	**839.2** ± 38.6	**810.6** ± 38.6	**756.2**$^{\#}$ ± 30.8	**742.9**$^{\#}$ ± 35.6	**733.8*** ± 40.0
Length (mm)	M	**38.3** ± 0.4	**38.7** ± 0.3	**38.2** ± 0.5	**38.1** ± 0.3	**38.8** ± 0.2	**38.2** ± 0.4
	F	**39.0** ± 0.4	**39.9** ± 0.4	**40.0** ± 0.5	**38.9** ± 0.4	**38.9**± 0.4	**38.7*** ± 0.6
K	M	**1.09** ± 0.03	**1.11** ± 0.01	**1.10** ± 0.03	**1.08** ± 0.02	**1.08** ± 0.01	**1.06** ± 0.01
	F	**1.29** ± 0.04	**1.30** ± 0.04	**1.25** ± 0.03	**1.27** ± 0.03	**1.24** ± 0.03	**1.24** ± 0.03
Fecundity	—	**51.9** ± 9.7	**76.3** ± 20.5	**94.7** ± 18.5	**93.3** ± 17.1	**67.6** ± 24.0	**109.1** ± 15.5
Embryo fertilization (%)	—	**94%** ± 2.9	**90%** ± 4.4	**94%** ± 2.6	**97%** ± 0.8	**98%*** ± 0.6	**95%** ± 1.8

* indicates significant difference from the solvent control group (ACET). Values presented as mean ± standard error; $p < 0.05$; # - values near significance – female weight at 40ng/L (p=0.09) and 200 ng/L (p=0.08), male weight at 40 ng/L (p=0.06). (n=50; ~25 for males and 25 females per treatment) (Power=1.000 for all endpoints; mean sample size calculation = 2.4, for expected power = 0.8).

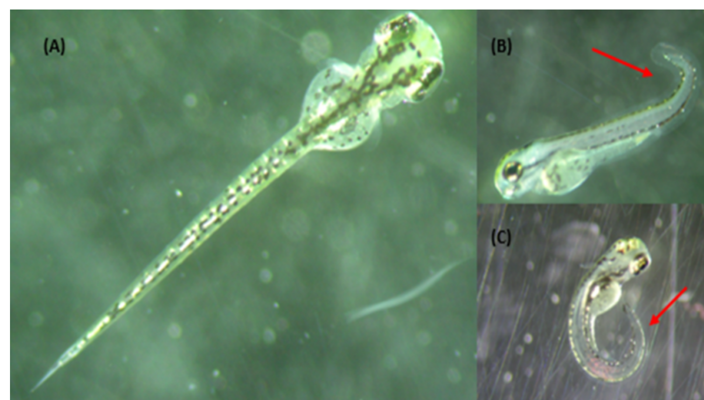
3.4. Embryogenesis

The cumulative percentage of embryo mortality at 80 hpf was similar among treatments and remained at low levels, ranging from 5 % (solvent control and parental exposure to 200 ng/L SIM) to 11.7 % (parental exposure to 1000 ng/L SIM) (Figure 2. A). From all the analysed abnormalities, tails anomalies (shortened and/or curled tails - Figure 2. B) were the most frequently recorded in SIM-exposed groups. Ant increase in tail anomalies was observed at the lowest SIM concentrations, i.e. 5-fold increase in both 8 and 40 ng/L, which was in the rage of significance (p=0.07), compared with the solvent control group. A significant increase of total abnormalities (sum of head, tail, eyes and yolk-sac anomalies plus pericardial oedema) was also observed in embryos from the lowest SIM concentration (8 and 40 ng/L), with 18.3 % and 15% of abnormal embryos in comparison with 1.67 % of the solvent control group (Fgure 2. C). Furthermore, parental exposure to SIM significantly decreases heart beat by 6.5 bpm at 8 ng/L exposure treatment relative to solvent control (Figure 2. D).



1

2 **Figure 2.** Cumulative mortality – n=60 per treatment (A); Anomalies in the tail – n=60 per treatment (B);
3 Total abnormalities – n=60 per treatment (C); and Heart rate – n=16 per treatment (D) observed in zebrafish
4 embryos at 80 hpf, following parental chronic exposure to SIM. Error bars indicate standard errors; *
5 indicates significant difference from the solvent control group (ACET) ($p < 0.05$). (Power=1.000 for all
6 endpoints; mean sample size calculation = 3, for expected power = 0.8).



7

8 **Figure 3.** Tail abnormalities, at 80 hpf, after *D. rerio* parental exposure to SIM for 70 days.
9 Comparison between Solvent Control (A), 40 ng/L SIM (B), 8ng/L SIM (C).

10

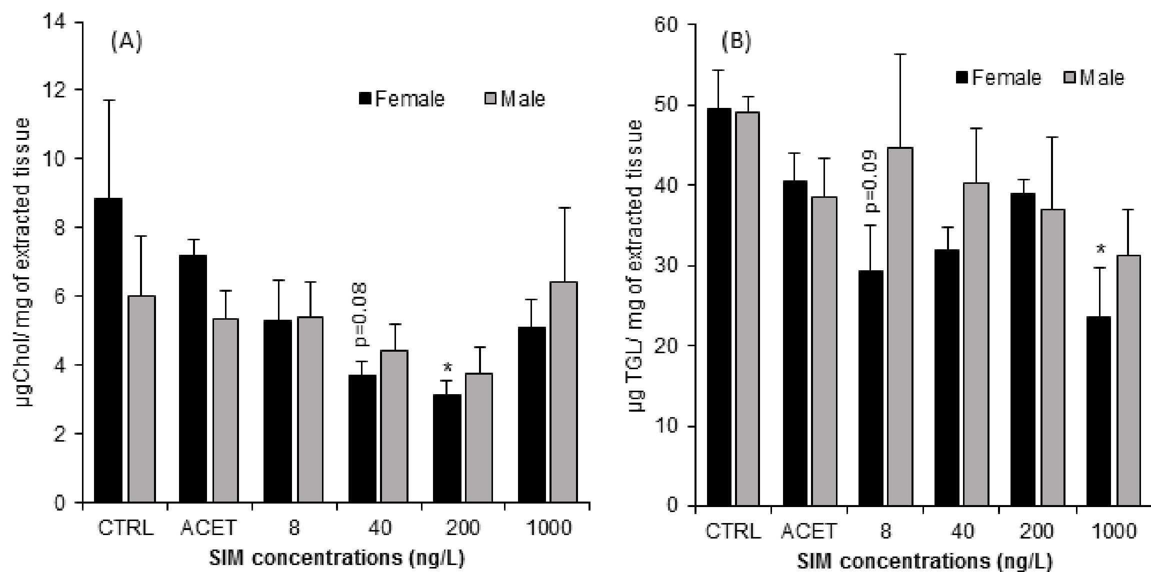
11

12

13 **3.5. Hepatic cholesterol and triglyceride content**

1 SIM significantly decreased liver cholesterol (Chol) levels in female zebrafish at 200 ng/L, 2 with a 56.4 % decrease in comparison with the solvent control group (Figure 4. A). A 48.3% decrease 3 was also observed in females exposed to 40 ng/L SIM, although significance was not reached 4 (p=0.08). Males exhibited the same Chol patterns observed in females at 40 and 200n ng/L yet 5 without significance differences from solvent control group. Both males and females exhibited a 6 slight Chol level increased in the highest SIM concentration (1000 ng/L) when compared with the 7 intermediate SIM treatments, nevertheless Chol levels remained lower or similar to the ones 8 observed the solvent control. Chol levels did not follow a concentration-response curve with a 9 decrease at intermediate SIM concentrations.

10 Triglyceride (TGL) levels in female liver were significantly lower (42.2 %) in the highest SIM 11 concentration (1000 ng/L), when compared with solvent control group, (Figure 4. B). Exposure to 8 12 ng/L of SIM was able to decrease TGL levels by 28 %, however, significance was not reached 13 (p=0.09). No significant differences in TGL levels were observed in males.



14

15 **Figure 4**. Chronic effects of SIM on cholesterol (A) and triglyceride (B) content of *D. rerio* liver after 16 90 days of exposure. Error bars indicate standard errors; * indicates significant difference from the 17 solvent control group (ACET) (p < 0.05) (n=8 per sex/treatment). (Power=0.999; mean sample size 18 calculation = 5).

19

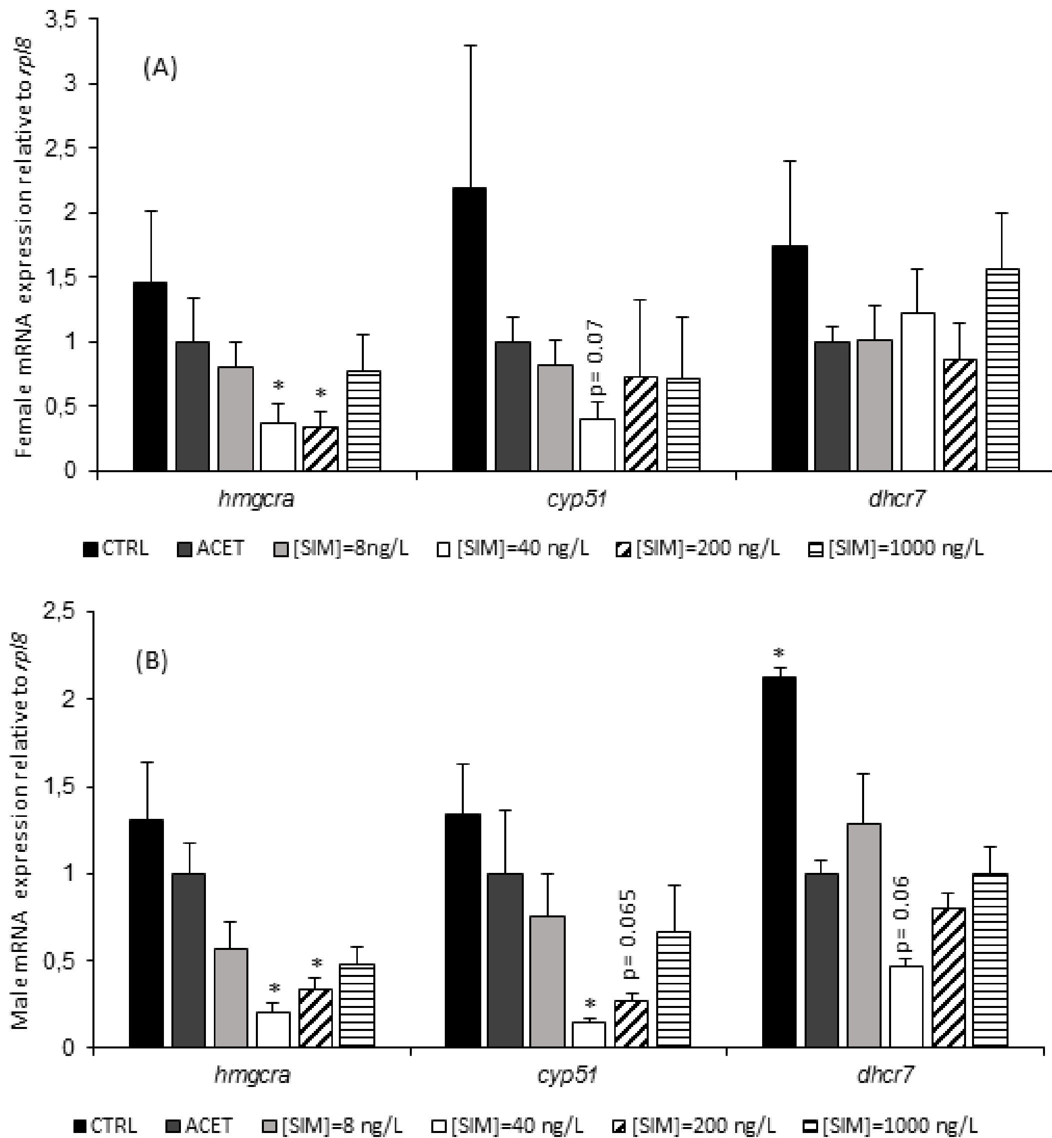
20

21 **3.6. Gene expression**

22 Both *D. rerio* females and males showed significant differences in the transcription levels 23 of several genes of the mevalonate pathway after 90 days of exposure to SIM (Figure 5). Similar to

1 Chol levels, *hmgcra* and *cyp51* genes presented, for both sexes, a response that did not follow a
2 concentration-response curve with a similar decrease at both 40 and 200 ng/L SIM. In females,
3 *hmgcra* was significantly downregulated (2.64 (p= 0.01) and 2.89-fold (p= 0.01)) after exposure to
4 40 and 200 ng/L of SIM, respectively, when compared to the solvent control treatment (ACET).
5 Similarly, in males, significant decrease (4.86 (p= 0.02) and 2.94-fold) in *hmgcra* expression levels
6 was observed at the same concentrations. In females, none of the remaining genes showed altered
7 transcript levels. Nevertheless, a trend towards a decrease in *cyp51* gene expression was observed
8 at 40 ng/L SIM, although significance was not reached (p = 0.07). Males, on the other hand,
9 exhibited a 6.80-fold significant decrease of *cyp51* expression when exposed to 40 ng/L SIM. A
10 similar downregulation pattern was observed at 200 ng/L SIM, just in the range of significance
11 (p=0.06). Neither males nor females presented significant alterations in *dhcr7* transcription levels.
12 No significant differences were observed between control and solvent control for the analysed
13 genes, with the exception of the *dhcr7* in males.

1
2



3

4 **Figure 5** Relative mRNA expression of *hmgcra*, *cyp51*, and *dhcr7* in adult *D. rerio* livers after 90 days SIM
5 exposure. Females (A) and Males (B). Error bars indicate standard errors; asterisks (*) indicate significant
6 differences from the solvent control treatment (ACET = 1-fold) ($p < 0.05$) (n=8 per sex/treatment)
7 (Power=0.999; mean sample size calculation = 6)

8

9 **3. Discussion**

10 Statins, such as SIM, are designed to produce specific biological effects in humans at low
11 concentrations and, therefore, once in the aquatic environment may affect aquatic *taxa*, which
12 consequently might be at risk. Despite the ubiquitous nature of statins in the aquatic ecosystems
13 and the liability of the scientific community to improve their risk assessment, the chronic effects of
14 these pharmaceuticals in aquatic organisms has not yet been adequately studied. In order to

1 address this knowledge gap, the present study aimed at investigating the chronic partial life-cycle
2 and early offspring embryonic developmental effects of SIM exposure, using zebrafish as a model
3 organism. Thus, several fundamental questions were addressed: Does chronic SIM exposure affect
4 ecologically relevant endpoints in zebrafish? Are these effects related with alteration of cholesterol
5 and triglyceride levels? Is the cholesterol homeostasis disturbed and correlated with changes in the
6 transcription of key genes from the mevalonate pathway?

7 In mammals, SIM and other statins operate by competitively inhibiting the rate-limiting
8 enzyme of the mevalonate pathway (HMGCR), thereby reducing Chol biosynthesis (Blumenthal,
9 2000; Endo et al., 1976a; Endo et al., 1976b; Sehayek et al., 1994). Given the conservation status of
10 the mevalonate pathway among mammals and all vertebrates including fish (Pertusa et al., 2007;
11 Santos et al., 2016), the high degree of conservation of HMGCR with respect to its catalytic activity
12 and statin interactions across all metazoans (Santos et al., 2016), it is expected that fish display
13 similar responses to those described in mammals, i.e. an impact on Chol synthesis and alterations
14 in the transcription of genes from the mevalonate pathway. The results of this study show that
15 chronic exposure to low levels of SIM impacts key ecologically-relevant endpoints of zebrafish,
16 particularly embryonic development, concomitantly with alterations in the Chol and TGL levels in
17 the liver. Furthermore, an impact in the transcription of key genes from the mevalonate pathway
18 was also observed. Importantly, several of the analysed endpoints did not follow a concentration-
19 response curve, with significant effects observed only in the low-intermediate SIM concentrations.
20 The results also indicate for some endpoints, such as Cho and TGL, sex-dependent differences,
21 suggesting that females and males might not respond similarly to SIM exposure.

22 The sub-lethal exposure to the highest SIM concentration (1000 ng/L) significantly
23 decreased the body weight of zebrafish for both sexes and the length of females. To our knowledge,
24 there is no scientific study reporting effects of SIM, or other statins, on the weight and growth
25 control of fish or mammals; and this alteration was not reflected in the levels of Chol nor in the
26 transcription level of the screened genes. However, the significant decrease in weight and growth,
27 in females, integrates well with the significant reduction of TGL observed in 1000 ng/L SIM. In fact,
28 TGL constitutes the main source of energy for physiological needs of zebrafish, therefore, with low
29 TGL level available, less energy reserves were allocated for the normal growth and weight of
30 females.

31 Our results on the embryonic development showed that chronic parental exposure to 8 and
32 40 ng/L SIM resulted in a significant increase of morphologic abnormalities, with tail anomalies as
33 the most frequent abnormalities observed. Also, a decrease in the heart rate at parental exposure
34 to 8 ng/L SIM was observed. Some previous studies showed that the embryonic development of
35 zebrafish is a target of SIM in the tested higher SIM concentrations with described embryo

1 malformations similar to those reported here in addition to abnormalities in eyes and yolk-sac,
2 pericardial oedema and impairment of primordial germ cell migration (Campos et al., 2016; Cunha
3 et al., 2016; Ribeiro et al., 2015; Thorpe et al. , 2004). It is plausible to hypothesize that the parental
4 exposure might be responsible for the significant embryonic abnormalities observed. Thus, the
5 observed disruption in the zebrafish embryonic development induced by SIM could have important
6 ecological impacts at the population level in fish, affecting the fitness and survival of fish, since the
7 impact of SIM can be passed to the second generation as the embryos were raised in SIM-free
8 water.

9 The findings recorded on the embryonic development agree well with the observed
10 decrease of Chol and TGL levels in the liver of females exposed to the intermediate SIM
11 concentrations. In fact, the most abundant lipids in vertebrate embryos (including zebrafish) are
12 Chol and TGL, which are transferred from the mother to the embryo yolk, having an important role
13 in providing energy and structural cellular components during embryogenesis (Fraher et al., 2016).
14 On the other hand, the study of Cunha et al. (2017) reported that zebrafish embryos exposed to 5
15 and 50 µg/l of SIM presented a transcriptional downregulation of the genes encoding retinoic acid
16 receptor (RAR) and retinoid X receptor (RXR), which have been implicated in lipid homeostasis (RXR)
17 and development (RXR and RAR) and thus could give a possible explanation for the present
18 observations (Castro & Santos, 2014). Overall, our findings and the ones from Cunha et al. (2017)
19 point to an impact of SIM in the lipid homeostasis of zebrafish embryos that might be implicated in
20 the observed abnormalities observed during in the embryogenesis.

21 Chol and TGL are also essential in fish adulthood for the maintenance of several biological
22 processes like growth, reproduction and cell integrity (van der Wulp et al., 2013; Vergauwen et al.,
23 2010). The administration of statin leads to a reduction of those lipids, at least in mammals.
24 Although the most prominent effects attributable to statins are their potent Chol lowering
25 properties by the inhibition of HMGCR enzyme, it is also well established that statins significantly
26 reduce TGL levels (Funatsu et al., 2001). The reduction of TGL levels by statins seems to be related
27 with the scarce secretion of very low-density lipoprotein (VLDL) from the liver and the increase in
28 clearance of TGL-rich lipoprotein via induced LDL receptors from plasma (Funatsu et al., 2001). As
29 expected from the studies with mammals, our results show that chronic low level exposure to SIM
30 reduce the Chol and TGL content in zebrafish. Interestingly, SIM treatment was more effective in
31 females than males, with differences registered for both lipids only in females at the lower SIM
32 concentrations. Although not exhibiting significant decreases in Chol and TGL levels at the SIM
33 treatments tested, males present similar response patterns as females, at least for Chol levels.
34 Similar sex-dependent effects on Chol and TGL levels have been reported by Al-Habsi et al. (2016)
35 after feeding zebrafish with 0.53 µg/g of atorvastatin for 30 days; registering a significant reduction

of both Chol and TGL only for females. Altogether, these results suggest that female zebrafish are more prone to the effects of SIM on Chol and TGL content than males. In fact, female zebrafish need higher lipid content for reproduction than males due to the importance of these components in egg production (Landgraf et al., 2017).

This study shows that the observed SIM-induced biological and biochemical effects occurred in parallel, with significant alterations in gene transcription in male and female liver of two key mevalonate pathway genes, *hmgcra* (the rate limiting step in the mevalonate pathway) and *cyp51* (an intermediate gene in the mevalonate pathway). A downregulation of these two genes was observed in males and females at the intermediate concentrations of SIM (40 and 200 ng/L for *hmgcra* and 40ng/L for *cyp51*). Interestingly, these are the SIM concentrations that significantly reduced the levels of liver Chol and therefore, the observed changes in transcript levels of key genes of the mevalonate pathway appear to be linked with the alterations in Chol levels. The mechanism of SIM here observed is similar to those reported in mammals, i.e. decrease of Chol production concomitantly with changes in the transcription levels of gene encoding the enzyme responsible for the limiting step of mevalonate pathway - HMGCR. However, the majority of the available studies in the literature, for humans and mammalian animal models, report a transcriptional up-regulation of *hmgcr* after SIM administration (Conde et al., 1999; Gouni-Berthold et al., 2008; Rudling et al., 2002). Also, one study with zebrafish fed with the statin, atrovastin, for 30 days presented the same *hmgcr* pattern to that reported in mammals (Al-Habsi et al., 2016), in contrast with the downregulation observed in the present study. Therefore, the potential link between the transcriptional down-regulation of the *hmgcr* and *cyp51* and the decrease of Chol production observed here with zebrafish should be confirmed in further studies involving longer exposure periods and additional SIM concentrations.

The contrasting expression profile of *hmgcr* observed in the present study suggests that possibly, the low SIM concentrations used in the present study affects zebrafish transcription levels of *hmgcr* in a time-specific manner i.e. fluctuations in gene expression over time. To the best of our knowledge, there are not studies available that investigate the time effects of SIM on *hmgcr* gene expression. However, a clear time-dependent gene expression response is described in the literature for distinct genes in several aquatic model organisms exposed to different compounds, including SIM (Cunha et al., 2017; Hamadeh et al., 2002; Kim et al., 2015). Thus, the absence of data on the temporal *hmgcr* gene expression highlights the importance of using multiple time-points in gene expression studies with statins. This issue should be explored in future studies. It is worth noting that SIM induced significant toxic responses at the lowest concentrations (8, 40 and/or 200 ng/L) in most of the analyzed endpoints, while at the highest concentration (1000 ng/L) the responses were similar to the control conditions. Therefore, the intermediate SIM exposures

1 appeared more effective in the disruption of lipid homeostasis than the highest exposure. These
2 responses appeared to be non-monotonic, showing an inverted U-shape for the embryonic
3 development abnormalities and a U-shape for the cholesterol levels and for the expression of
4 *hmgcra* and *cyp51* genes. This is a common pattern observed in low concentration exposures during
5 chronic experiments for some endocrine disrupting chemicals; therefore the non-monotonicity
6 responses should not be ignored in environmental studies and hazard assessment (Vandenberg et
7 al., 2012). The mechanism(s) that cause this apparent non-monotonicity of SIM in the present study
8 are currently unknown and should be further investigated. However, we hypothesize that the
9 interactions of the endogenous feedback mechanism to maintain the homeostatic control of Chol
10 and the mechanisms of SIM to reduce the Chol levels might have a role in the responses reported
11 here. The lowest SIM exposure may result in the activation of SIM associated-pathways to reduce
12 the Chol levels, however, at the higher concentrations, the endogenous mechanisms to control the
13 levels of Chol must have compensated the effects of SIM in order to achieve homeostasis and, thus,
14 the effect of SIM was less evident.

15 In summary, taken together the findings of the present study indicate that low
16 environmentally realistic concentrations of SIM have a significant impact in Chol metabolism of
17 zebrafish with some similarities to the effects reported in mammals. Chronic exposure to SIM
18 reduced the levels of Chol and TGL, mainly in females, concomitantly with changes in the
19 transcription levels of key genes involved in the Chol biosynthesis. These alterations could be
20 associated with the disruption of embryonic development, which might have implications at the
21 ecological level, given that the effects were observed in the low ng/L range. Given that SIM is
22 expected to impact the mevalonate pathway in a wide range of animal taxa (Santos et al., 2016),
23 the results of the present study highlight the need to expand chronic low-level exposure studies to
24 other groups of animals.
25

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32

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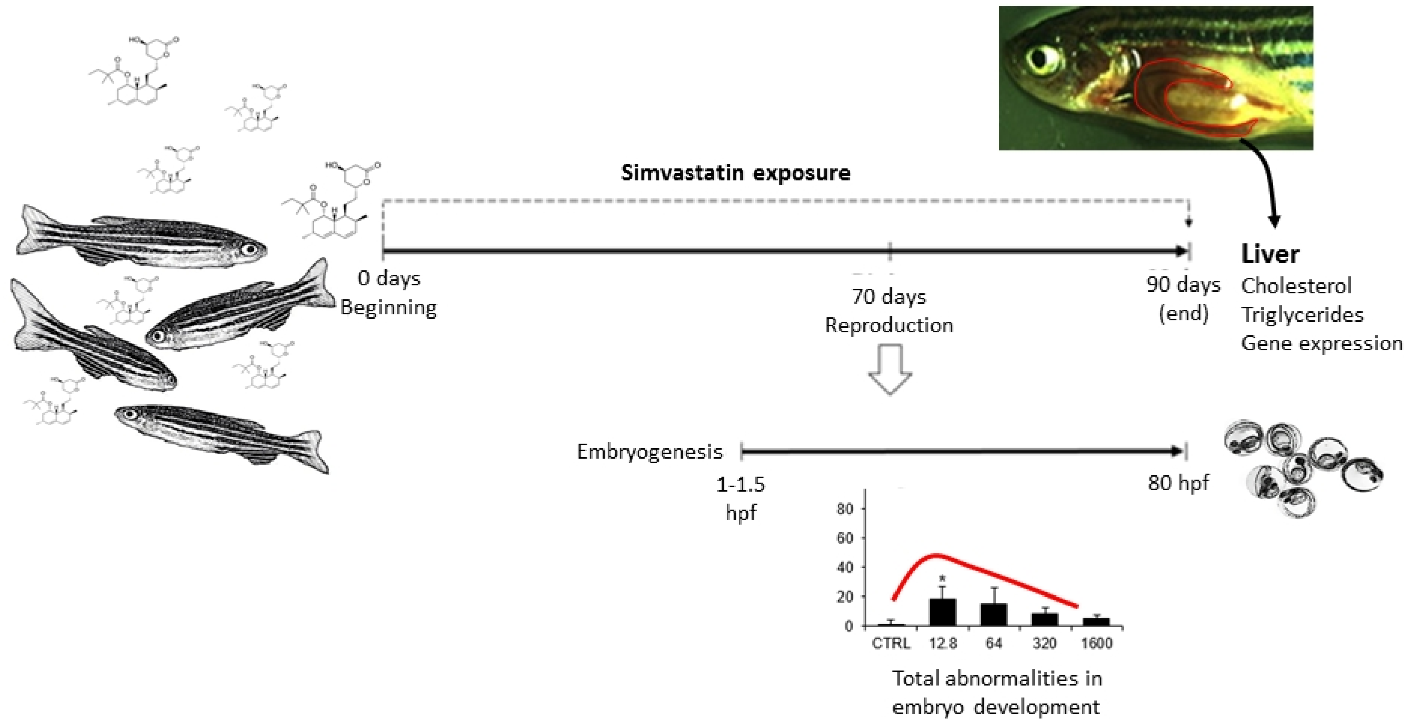
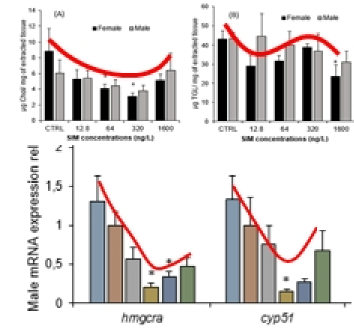

Simvastatin exposure
0 days
Beginning
70 days
Reproduction
90 days
(end)
Liver
Cholesterol
Triglycerides
Gene expression
Embryogenesis
1-1.5
hpf
80 hpf
80
60
40
20
0
CTRL
12.8
64
320
1600
Total abnormalities in
embryo development


Male mRNA expression rel
2
1,5
1
0,5
0
hmgcra
cyp51

List of Captions

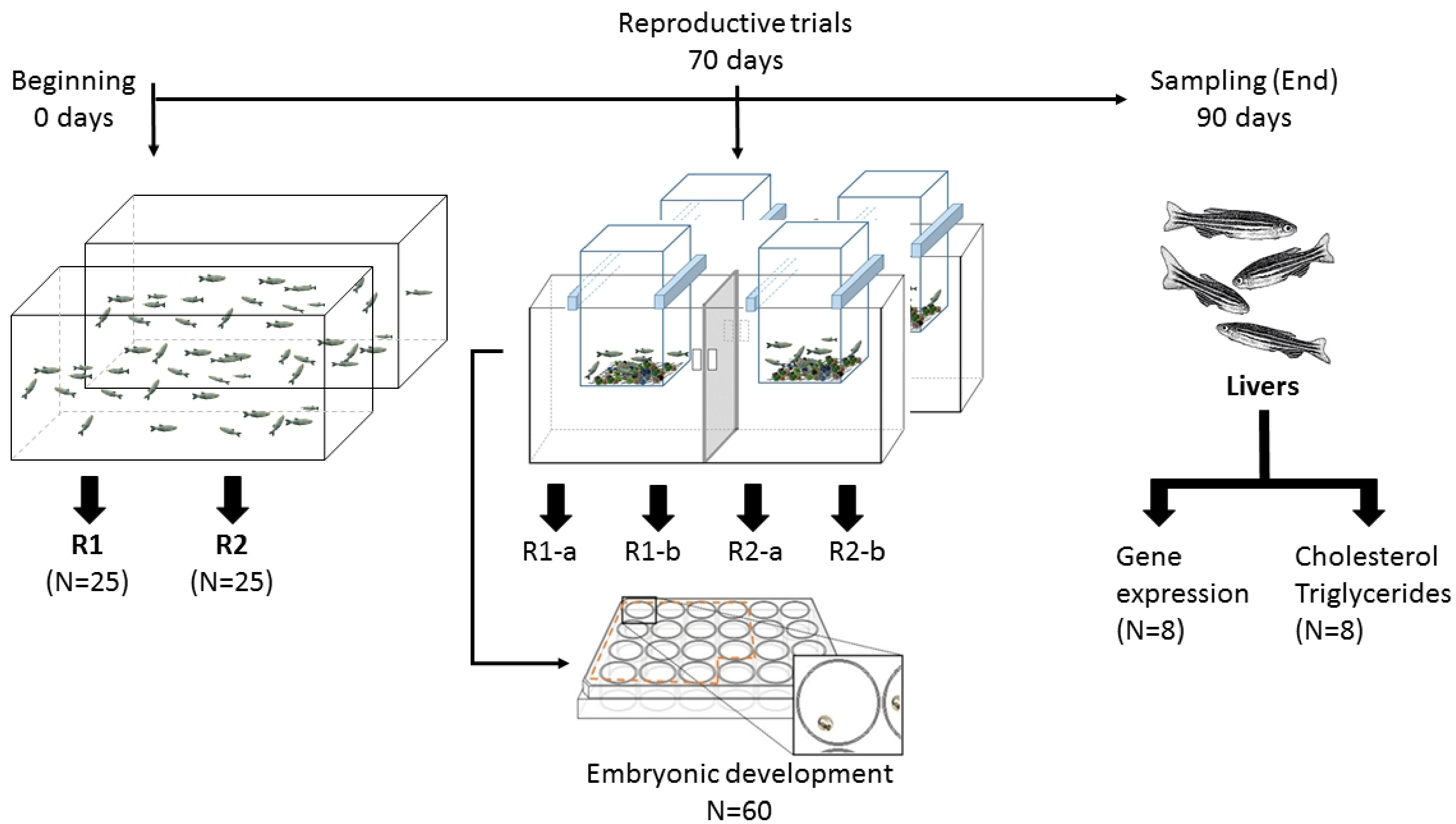
Figure 1. Schematic representation of the experimental setup. Each treatment had two aquaria (R1 and R2), with 25 animals each. Aquaria were then subdivided in two (a and b) for the reproductive trials and embryonic studies at day 70. Embryonic development was analyzed using 60 embryos (15 each in four replicates).The experiment finished at day 90 and all animals were sacrificed, measured, weighted, and livers from both males and females were collected for gene expression analysis (N=8) and for cholesterol/triglyceride quantification (N=8 for males and N=8 for females).

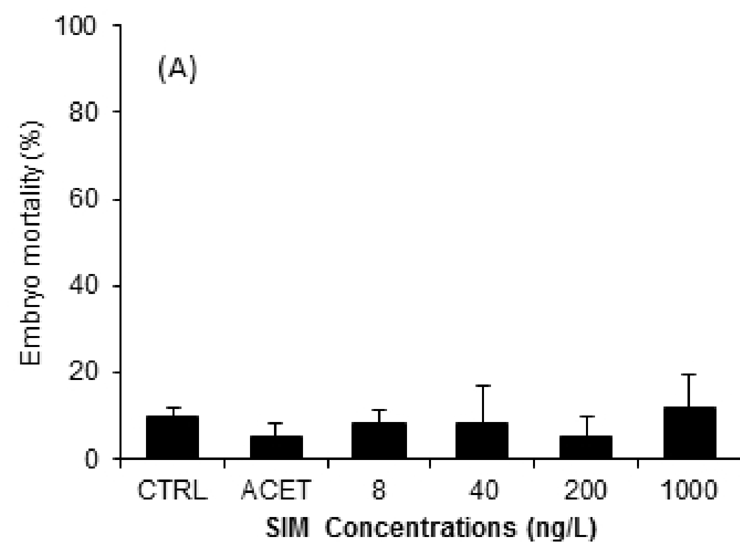
Figure 2. Cumulative mortality – n=60 per treatment (A); Anomalies in the tail – n=60 per treatment (B); Total abnormalities – n=60 per treatment (C); and Heart rate – n=16 per treatment (D) observed in zebrafish embryos at 80 hpf, following parental chronic exposure to SIM. Error bars indicate standard errors; * indicates significant difference from the solvent control group (ACET) ($p < 0.05$). (Power=1.000 for all endpoints; mean sample size calculation = 3, for expected power = 0.8).

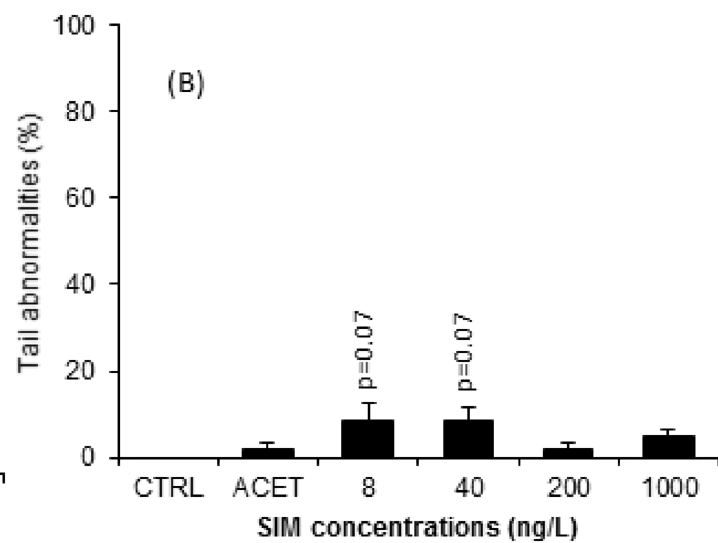
Figure 3. Tail abnormalities, at 80 hpf, after *D. rerio* parental exposure to SIM for 70 days. Comparison between Solvent Control (A), 40 ng/L SIM (B), 8ng/L SIM (C).

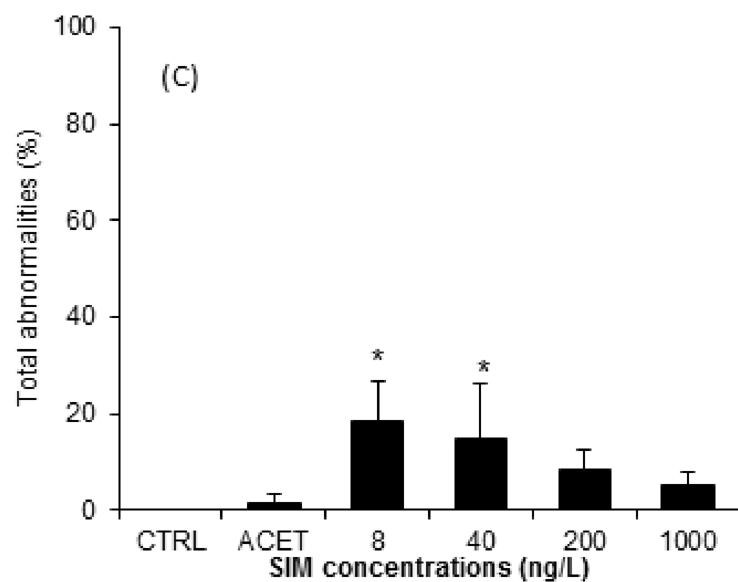
Figure 4. Chronic effects of SIM on cholesterol (A) and triglyceride (B) content of *D. rerio* liver after 90 days of exposure. Error bars indicate standard errors; * indicates significant difference from the solvent control group (ACET) ($p < 0.05$) (n=8 per sex/treatment). (Power=0.999; mean sample size calculation = 5).

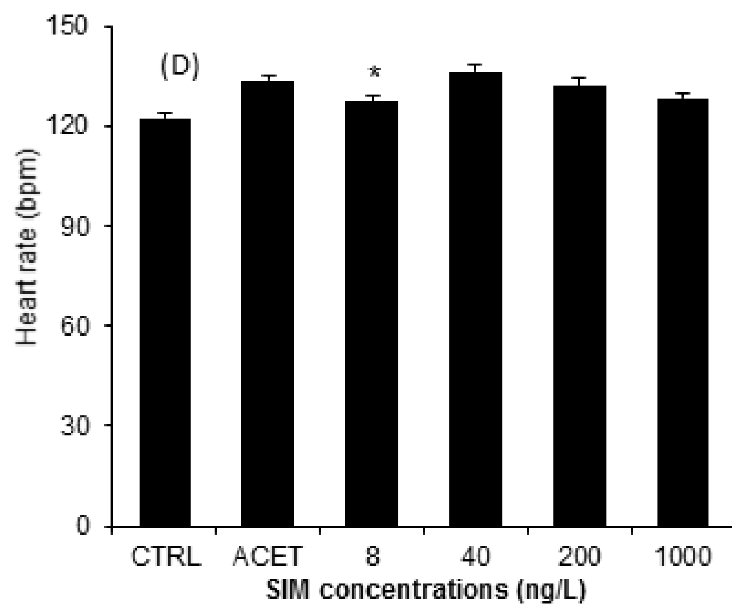
Figure 5 Relative mRNA expression of *hmgcra*, *cyp51*, and *dhcr7* in adult *D. rerio* livers after 90 days SIM exposure. Females (A) and Males (B). Error bars indicate standard errors; asterisks (*) indicate significant differences from the solvent control treatment (ACET = 1-fold) ($p< 0.05$) (n=8 per sex/treatment) (Power=0.999; mean sample size calculation = 6)


Reproductive trials
70 days
Beginning
0 days
Sampling (End)
90 days
Livers
R1
(N=25)
R2
(N=25)
R1-a
R1-b
R2-a
R2-b
Gene
expression
(N=8)
Cholesterol
Triglycerides
(N=8)
Embryonic development
N=60

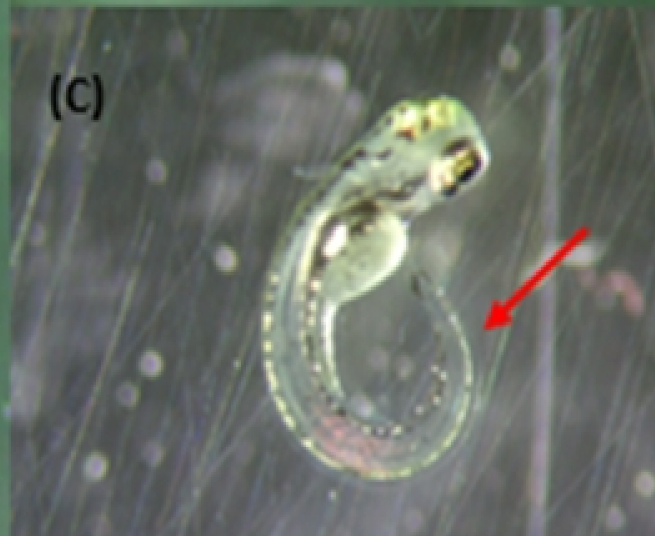

(A)
Embryo mortality (%)
100
80
60
40
20
0
CTRL
ACET
8
40
200
1000
SIM Concentrations (ng/L)

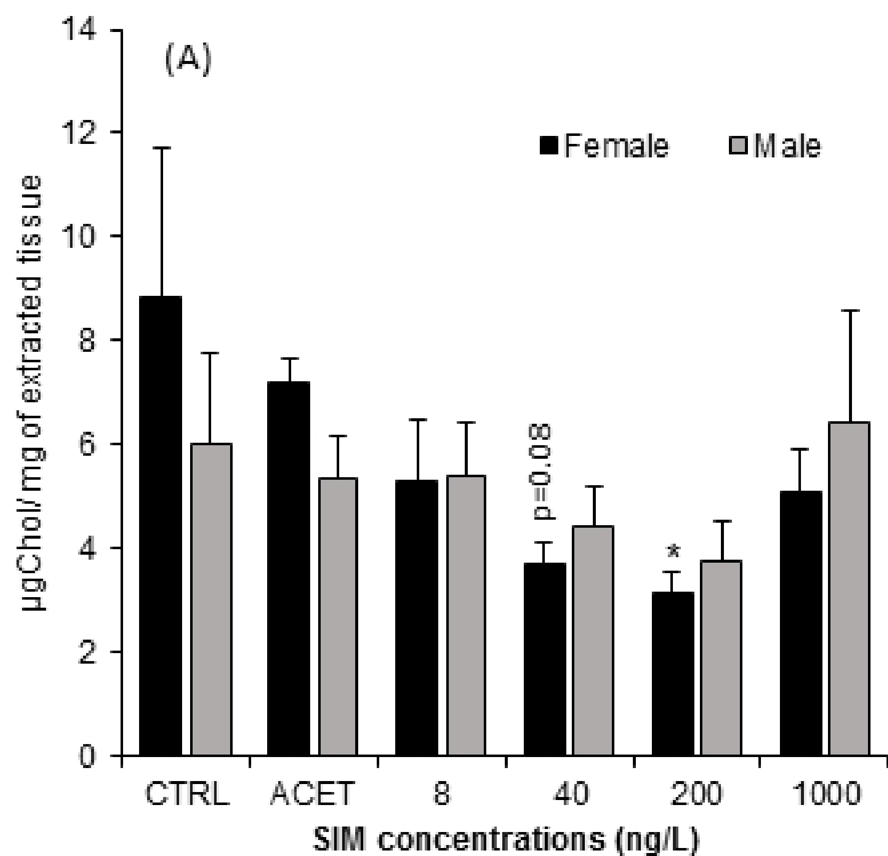

(B)
Tail abnormalities (%)
100
80
60
40
20
0
p=0.07
p=0.07
CTRL
ACET
8
40
200
1000
SIM concentrations (ng/L)


(C)
Total abnormalities (%)
100
80
60
40
20
0
*
*
CTRL
ACET
8
40
200
1000
SIM concentrations (ng/L)

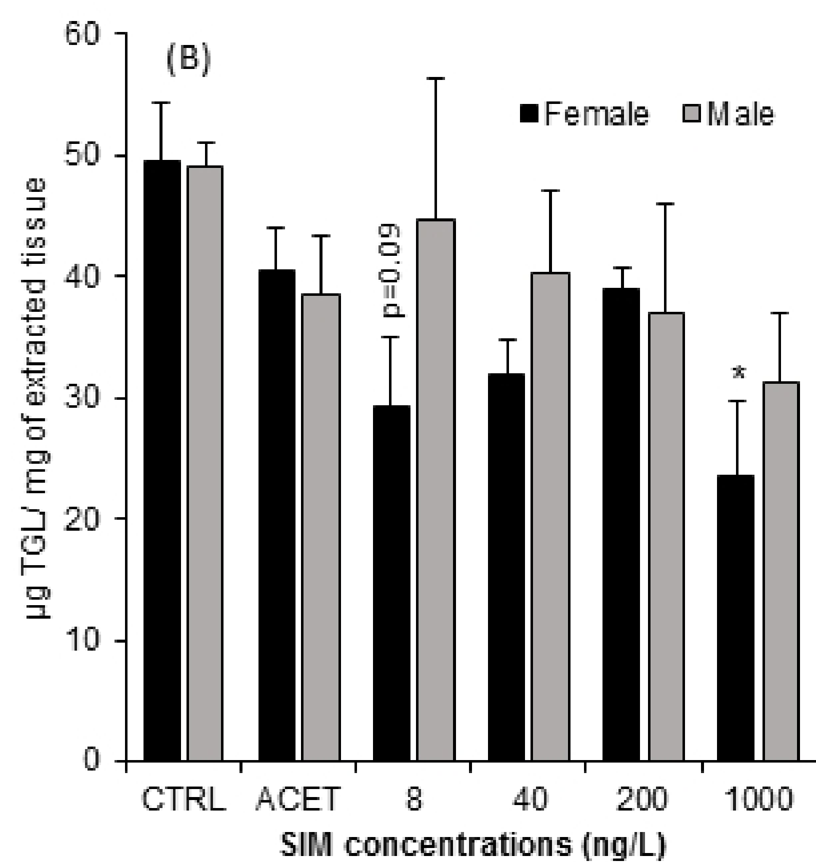

(D)
Heart rate (bpm)
150
120
90
60
30
0
*
CTRL
ACET
8
40
200
1000
SIM concentrations (ng/L)

(A)

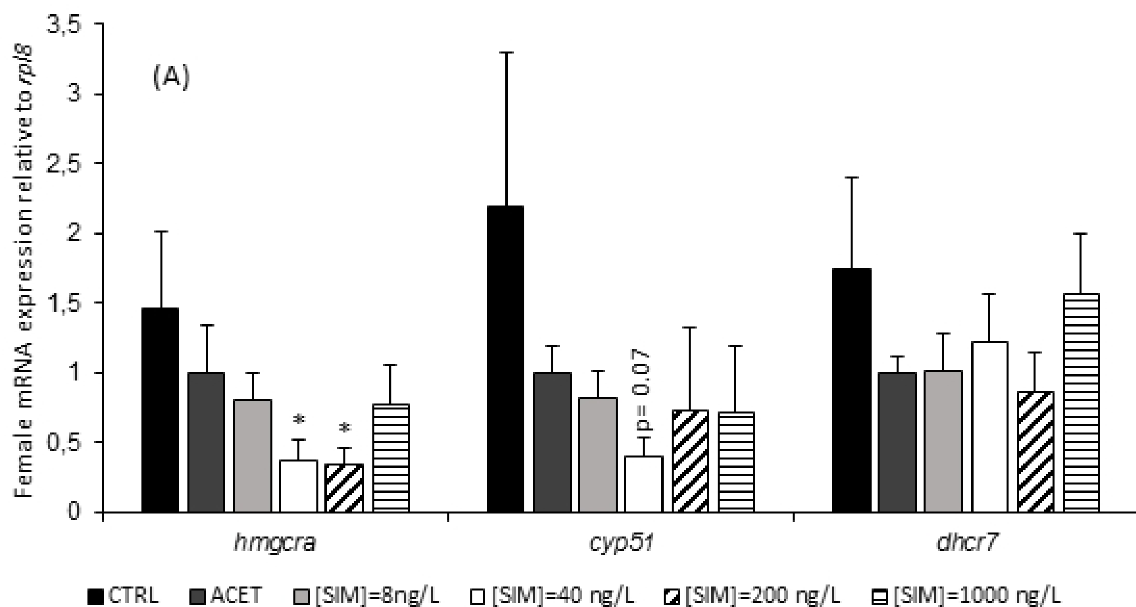




(A)
Female
Male
μgChol/ mg of extracted tissue
14
12
10
8
6
4
2
0
p=0.08
*
CTRL
ACET
8
40
200
1000
SIM concentrations (ng/L)



(B)
Female
Male
μg TGL/ mg of extracted tissue
60
50
40
30
20
10
0
p=0.09
*
CTRL
ACET
8
40
200
1000
SIM concentrations (ng/L)


(A)
Female mRNA expression relative to rpl8
3,5
3
2,5
2
1,5
1
0,5
0
*
*
p= 0.07
hmgcra
cyp51
dhcr7
CTRL
ACET
[SIM]=8ng/L
[SIM]=40 ng/L
[SIM]=200 ng/L
[SIM]=1000 ng/L

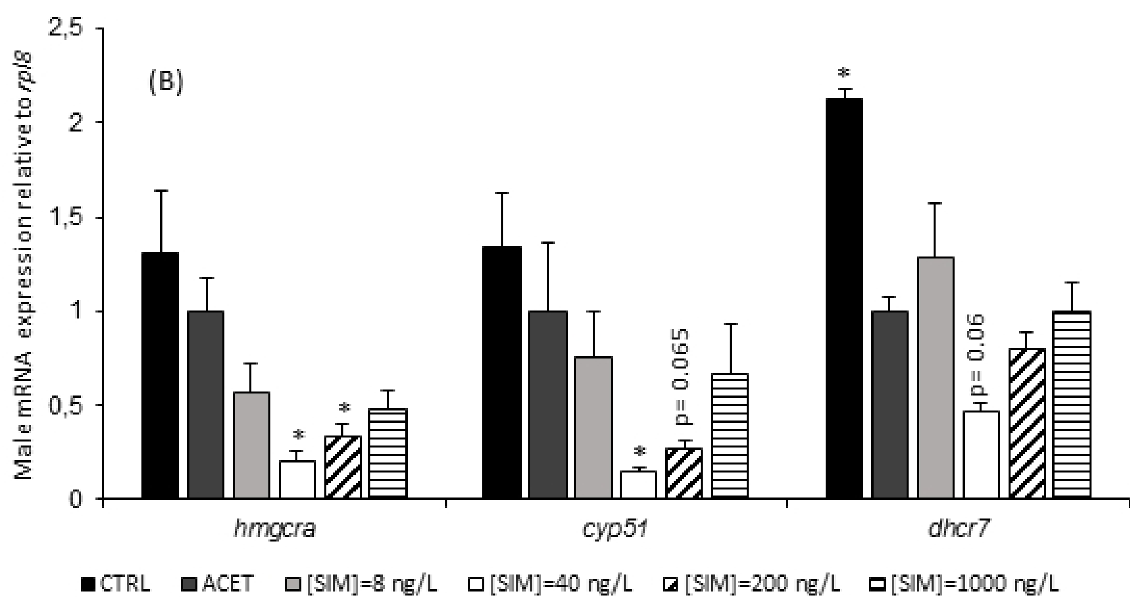

(B)
Male mRNA expression relative to rpl8
2,5
2
1,5
1
0,5
0
*
*
*
*
p= 0.065
p= 0.06
hmgcra
cyp51
dhcr7
CTRL
ACET
[SIM]=8 ng/L
[SIM]=40 ng/L
[SIM]=200 ng/L
[SIM]=1000 ng/L

Table 1. Primers, forward (F) and reversed (R), and parameters used in the qRT-PCR for gene expression quantification in the liver of *D. rerio*.

Gene	Sequence (5' – 3')	Expected band size (bp)	Combined annealing and extension temperature (°C)	Average efficiency (%)	Reference
hmgcra	F: TCGTGGAGTGCCTGGTGATTGGT R: TGGGTCTGCCTTCTCTGCTCTCTC	177	62	98	1
cyp51	F: GCTCGGAGACACTCAGACACATCTT R: AGCAGAACTGAAGTCAGGCTCATCT	138	60	96	1
dhcr7	F: GAGGAGTTCAGGATGGTGCCCGTA R: GTGGACACAGCATAGCCGAGGATG	199	60	94	1
rpl8	F: TTGTTGGTGTTGTTGCTGGT R: GGATGCTCAACAGGGTTCAT	136	58	94	2

Notes: **1** - (Mu et al., 2015); **2 -** (Lyssimachou et al., 2015)

Table 2. Nominal and measured concentrations of SIM (expressed as sum of SIM and SIMHA) in water samples collected in duplicate from each treatment after the first contamination of the day (T_{0h}) and immediately before the second one (T_{8h}), Data are expressed as mean ± standard error.

Time	Solvent control	SIM 8 ng/L[a]	SIM 40 ng/L[a]	SIM 200 ng/L[a]	SIM 1000 ng/L[a]
T 0 h	n.d.[b]	6.8 ± 0.45	37.9 ± 3.21	108.6 ± 3.70	751.7 ± 40.08
T 8 h	n.d.[b]	4.9 ± 0.08	23.0 ± 0.08	74.0 ± 1.47	343.4 ± 23.14

[a] Nominal SIM concentrations
[b] Not detected

Table 3. Chronic effects of SIM on survival, weight, length, Fulton's condition factor (K), fecundity (number of embryos/ female/ day), and % of fertilization (% of fertilized eggs/female/day) of *D. rerio* after 90 days of exposure (M, males; F, females).

Endpoint		Control	Acet	8 ng/L	40 ng/L	200 ng/L	1000 ng/L
Survival (%)	—	**100%**	**100%**	**100%**	**100%**	**100%**	**98%**
Weight (mg)	M	**611.5** ± 21.9	**642.1** ± 12.7	**615.3** ± 25.3	**598.5#** ± 13.6	**630.6** ± 9.6	**588.0*** ± 12.9
	F	**769.0** ± 35.7	**839.2** ± 38.6	**810.6** ± 38.6	**756.2#** ± 30.8	**742.9#** ± 35.6	**733.8*** ± 40.0
Length (mm)	M	**38.3** ± 0.4	**38.7** ± 0.3	**38.2** ± 0.5	**38.1** ± 0.3	**38.8** ± 0.2	**38.2** ± 0.4
	F	**39.0** ± 0.4	**39.9** ± 0.4	**40.0** ± 0.5	**38.9** ± 0.4	**38.9**± 0.4	**38.7*** ± 0.6
K	M	**1.09** ± 0.03	**1.11** ± 0.01	**1.10** ± 0.03	**1.08** ± 0.02	**1.08** ± 0.01	**1.06** ± 0.01
	F	**1.29** ± 0.04	**1.30** ± 0.04	**1.25** ± 0.03	**1.27** ± 0.03	**1.24** ± 0.03	**1.24** ± 0.03
Fecundity	—	**51.9** ± 9.7	**76.3** ± 20.5	**94.7** ± 18.5	**93.3** ± 17.1	**67.6** ± 24.0	**109.1** ± 15.5
Embryo fertilization (%)	—	**94%** ± 2.9	**90%** ± 4.4	**94%** ± 2.6	**97%** ± 0.8	**98%*** ± 0.6	**95%** ± 1.8

* indicate significant differences from the solvent control group (ACET). Values presented as mean ± standard error; p < 0.05; # - values near significance – female weight at 40ng/L (p=0.09) and 200 ng/L (p=0.08), male weight at 40 ng/L (p=0.06). (n=50; ~25 for males and 25 females per treatment) (Power=1.000 for all endpoints; mean sample size calculation = 2.4, for expected power = 0.8).