Universidade de Aveiro Departamento de Biologia
Ano 2019

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Effects of the acute and chronic exposures to simvastatin and clofibric acid in *Danio rerio*

Efeitos das exposições aguda e crónica a sinvastatina e ácido clofíbrico em *Danio rerio*

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Marinha, realizada sob a orientação científica do Doutor Bruno André Fernandes de Jesus da Silva Nunes, Equiparado a Investigador Auxiliar do Centro de Estudos do Ambiente e do Mar (CESAM) da Universidade de Aveiro, e coorientação do Prof. Doutor Alberto Teodorico Rodrigues Moura Correia, Professor Auxiliar da Faculdade de Ciências da Saúde da Universidade Fernando Pessoa e Investigador do Centro Interdisciplinar de Investigação Marinha e Ambiental (CIIMAR).

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presidente	Doutora Ana Isabel Lillebo Batista Diretora do Mestrado em Biologia Marinha Aplicada Investigadora Principal, Departamento de Biologia, Centro de Estudos do Ambiente e do Mar (CESAM), Universidade de Aveiro
arguente	Doutora Sara Raquel Boaventura Rodrigues Investigadora, Centro Interdisciplinar de Investigação Marinha e Ambiental (CIIMAR)
orientador	Doutor Bruno André Fernandes de Jesus da Silva Nunes Equiparado a Investigador Auxiliar, Departamento de Biologia, Centro de Estudos do Ambiente e do Mar (CESAM), Universidade de Aveiro

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palavras-chave

Peixes; antihiperlipidémicos; disruptores endócrinos; biomarcadores; histologia; peixe-zebra

resumo

Os antihiperlipidémicos são dos fármacos mais prescritos no mundo para o controlo dos níveis de colesterol, a mais de 20 milhões de pacientes. Devido ao seu amplo uso, os fármacos podem ser descartados, metabolizados e excretados no ambiente, potencialmente afetando organismos aquáticos. Apesar desta ampla utilização de antihiperlipidémicos, particularmente a sinvastatina e o ácido clofíbrico, os seus potenciais efeitos toxicológicos ao nível ambiental não estão totalmente caracterizados e compreendidos, sendo assim necessário investigá-los. Portanto, surge uma nova preocupação quanto ao seu potencial impacto ambiental, particularmente no ambiente aquático. Este trabalho visa caracterizar a toxicidade decorrente da exposição aguda (120 horas pós-fertilização) e crónica (60 dias) a drogas antihiperlipidémicas, nomeadamente sinvastatina (92.45, 184.9, 369.8, 739.6 e 1479.2 ng L^{-1}) e ácido clofíbrico (10.35, 20.7, 41.4, 82.8 e 165.6 µg L^{-1}), na espécie de peixe de água doce peixe-zebra (*Danio rerio*). As concentrações selecionadas foram implementadas em ambas as exposições. A análise dos efeitos incidiu sobre a observação histológica de tecidos dos indivíduos, no que diz respeito à determinação do sexo e dos estádios de maturação das gónadas, comportamento (movimentos curtos e longos, tempo de natação e distância total de natação), e biomarcadores de stress oxidativo (superóxido dismutase, catalase e glutationa peroxidase), biotransformação (glutationa S-transferases) e peroxidação lipídica (substâncias reativas ao ácido tiobarbitúrico). No caso da exposição aguda, foram observadas alterações de comportamento em animais expostos a ambos os compostos, sendo que a sinvastatina originou hiperatividade e o ácido clofíbrico provocou hipoatividade em todos os parâmetros comportamentais. Para além disso, foi observada a inibição significativa em todos os biomarcadores em indivíduos expostos a sinvastatina em concentrações de 184.9 a 1479.2 ng L^{-1}, exceto na catalase, parâmetro para o qual não foram reportadas diferenças significativas. A atividade da glutationa peroxidase selénio dependente também aumentou significativamente para níveis de 92.45 ng L^{-1}. Por outro lado, em indivíduos expostos a ácido clofíbrico, houve um aumento significativo em todos os biomarcadores, geralmente em peixes expostos a concentrações de 41.4 a 165.6 µg L^{-1}. No entanto, no caso da catalase e das glutationa S-transferases, na concentração mais elevada, a atividade diminuiu significativamente. Este estudo sugere que as exposições crónicas de *Danio rerio* a sinvastatina e ácido clofíbrico não interferem na proporção de sexo e nos estádios de maturação dos indivíduos. Como as defesas antioxidantes são importantes relativamente à capacidade do organismo no combate ao stress oxidativo, juntamente com efeitos a nível da locomoção, podem afetar o metabolismo ou até mesmo a sobrevivência dos organismos. Assim, terão de ser investigados o modo de ação destes dois compostos, incluindo os efeitos ao nível da disrupção reprodutiva em exposições mais prolongadas, de forma a observar e caracterizar os efeitos a longo-prazo da sinvastatina e do ácido clofíbrico no compartimento aquático e nos seus organismos.

keywords Fish; antihyperlipidemic; reproductive disruptors; biomarkers; histology; zebrafish

abstract

Lipid-regulating drugs are one of the most prescribed medications around the world, to control human cholesterol levels, to more than 20 million patients. Due to their wide usage, pharmaceuticals can be discarded, metabolized and excreted into the environment, potentially affecting aquatic organisms. Despite this increasing use of lipid-regulating drugs, particularly simvastatin and clofibric acid, are not fully characterized and understood in terms of their potential toxicological effects at the environmental level, therefore being necessary to investigate them. Therefore, it emerges a new concern on their effects related to the potential environmental impact, particularly in the aquatic environment. This work intended to characterize the toxicity due to an acute (120 hours post-fertilization) and chronical (60 days) exposure to antihyperlipidemic drugs, namely simvastatin (92.45, 184.9, 369.8, 739.6 and 1479.2 ng L^{-1}) and clofibric acid (10.35, 20.7, 41.4, 82.8 and 165.6 µg L^{-1}), in the freshwater species of zebrafish (*Danio rerio*). The concentrations hereby selected were implemented in both exposures. The analysis of effects focused on the histological observation of tissues in individuals, concerning sex determination and maturation stages of gonads, behavior (small and large distance, total distance and swimming time), biomarkers of oxidative stress (superoxide dismutase, catalase and glutathione peroxidase), biotransformation (glutathione S-transferases) and lipid peroxidation (thiobarbituric acid reactive substances). In terms of acute exposure, it was observed behavioral alterations in both compounds, simvastatin caused hyperactivity and clofibric acid provoked hypoactivity in all behavioral parameters. Moreover, it was observed a significant decrease in all biomarkers in individuals exposed to simvastatin from 184.9 to 1479.2 ng L^{-1}, except for catalase, for which no significant differences were found. Glutathione peroxidase selenium-dependent activity also showed a significant increase at 92.45 ng L^{-1}. On the other hand, in individuals exposed to clofibric acid, there was a significant increase in all biomarkers, typically from 41.4 to 165.6 µg L^{-1}. However, in catalase and glutathione S-transferases, in the highest concentration, the activity was significantly decreased. This study suggests that the chronic exposure of *Danio rerio* to simvastatin and clofibric acid does not interfere with the sex ratio and maturation stages of individuals. As antioxidant defenses are important in terms of the capacity of the organism to overcome oxidative stress, along with effects in locomotion, it can affect the metabolism or even the survival of organisms. Therefore, further studies in terms of mode of action of these two compounds, including reproductive disruption effects in longer exposures, are required to observe and characterize the long-term effects of simvastatin and clofibric acid in the aquatic compartment and its organisms.

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List of Acronyms

CA – Clofibric acid; 2-(p-Chlorophenyl)-2-methylpropanoic acid

CAT – Catalase

CDNB - 1-chloro-2,4-dinitrobenzene

CNS – Central nervous system

Cu-ZnSOD – Copper-zinc superoxide dismutase

CYP450 – Cytochrome P450

dpf – Days post-fertilization

EDCs – Endocrine disrupting chemicals

FET – Fish embryo toxicity test

GPx – Glutathione peroxidase

GR – Glutathione reductase

GSH – Reduced glutathione

GSSG – Oxidized glutathione

GSTs – Glutathione S-transferases

HDL – High-density lipoproteins

HMG-CoA - 3-hydroxy-3-methylglutaryl-coenzyme A

hpf – Hours post-fertilization

LDL – Low-density lipoproteins

MDA – Malondialdehyde

MnSOD – Manganese superoxide dismutase

MS-222 - Ethyl 3-aminobenzoate methanesulfonate

PCBs – Polychlorinated biphenyls

PNS – Peripheral nervous system

POPs – Persistent organic pollutants

PPARs - Peroxisome proliferator-activated receptors

ROS – Reactive oxygen species

SIM – Simvastatin; [(1*S*,3*R*,7*S*,8*S*,8*aR*)-8-[2-[(2*R*,4*R*)-4-hydroxy-6-oxooxan-2-yl]ethyl]-3,7-dimethyl-1,2,3,7,8,8*a*-hexahydronaphthalen-1-yl] 2,2-dimethylbutanoate

SOD – Superoxide dismutase

SSRIs – Selective serotonin reuptake inhibitors

TBA – 2-thiobarbituric acid

TBARS – Thiobarbituric acid reactive substances

TCA – Trichloroacetic acid

UGTs – Uridine 5'-diphospho-glucuronosyltransferases

VLDL – Very -low-density lipoproteins

VTG – Vitellogenin

WWTPs – Wastewater treatment plants

1. INTRODUCTION

1.1. Pharmaceuticals in the environment

The worldwide dispersion of xenobiotics, i.e., foreign substances or chemicals that are not normally produced or present in living organisms, leads to environmental polution. This phenomenon is generally a result of human activities, such as industrial and agropecuary undertakings (Embrandiri et al., 2016). Some examples of xenobiotics are heavy metals, fertilizers, insecticides, herbicides, dyes, solvents, agrochemicals, industrial chemicals, such as polychlorinated biphenyls (PCBs), hydraulics, halogenated substances, fire retardants, pigments, persistent organic pollutants (POPs), and pharmaceuticals (Reineke & Knackmuss, 1988; Dinka, 2018). The use of pharmaceuticals, namely human treatment drugs, and those used in veterinary medicine, to prevent and treat several diseases, has led to a worldwide increase in their consumption and environmental presence (Jelic et al., 2011). Some pharmaceuticals are discarded directly into the wild, or metabolized and excreted into the environment, potentially affecting aquatic organisms and causing short and long-term effects, analogous to those observed in humans, including toxicological interactions with varied pathways and receptors (Henschel et al., 1997). Ecotoxicology describes the relation between chemical pollutants, the environment where they are released and the organisms present (Segner, 2011). The human excretion of pharmaceutical compounds, in some cases, reaches 40% to 90% in its original form or/and as their active metabolites, through urine and feces, providing an entrance source of these compounds into the sewage, such as in the case of direct discard into the environment, when pharmaceuticals are not properly eliminated (Jones et al., 2005; Kumar & Xagoraraki, 2010). The scientific knowledge concerning the environmental fate and effects of pharmaceuticals has increased significantly in the past several years, demonstrating the toxicity of these compounds in the aquatic ecosystem (Richardson et al., 2005; Kumar & Xagoraraki, 2010; Ojemaye & Petrik, 2019).

The presence of pharmaceuticals, namely the parental substances, metabolites, and transformation products, in the aquatic compartment is, at present, a concerning issue, due to the increase and extensive use of this type of contaminants in medicine.

Environmental exposure to drugs that are continuously released into the environment can have various deleterious effects on living individuals. The occurrence of pharmaceuticals has been detected worldwide, in sewage treatment plants, seawater, surface water and groundwater (Nikolaou et al., 2007). The most common pharmaceuticals found in the environment are included in specific pharmaco-therapeutic classes, such as analgesics, anti-inflammatories, antidepressants, antiepileptics, antibiotics, beta-blockers, lipid regulators (Jelic et al., 2011). Among the pharmaceuticals most commonly detected in water treatment plant effluents, one can find simvastatin (Portugal, <369.8 ng $L^{-}1$) (Salgado et al., 2010), naproxen (Germany and Taiwan, 0.017 – 0.52 μg L^{-1}) (Ternes, 1998; Lin et al., 2005), diclofenac (Greece and Germany, 0.01 – 0.56 μg L^{-1}) (Heberer et al., 2001), metoprolol (Germany, 0.025 – 2.2 μg L^{-1}) (Hirsch et al., 1996; Ternes, 1998), triclosan (USA and Canada, 0.01 – 2.7 μg L^{-1}) (McAvoy et al., 2002; Boyd et al., 2003), ibuprofen (Switzerland and UK, 0.002 – 2.972 μg L^{-1}) (Buser et al., 1998; Roberts & Thomas, 2006), clofibric acid (Germany and UK, 0.044 – 4.55 μg L^{-1}) (Stan & Heberer, 1997; Roberts & Thomas, 2006), benzafibrate (Germany and Canada, 0.02 – 4.6 μg L^{-1}) (Ternes, 1998; Miao et al., 2002) and carbamazepine (Germany and Taiwan, 0.042 – 6.3 μg L^{-1}) (Ternes, 1998; Lin et al., 2005). This scenario is a direct consequence of the general inefficacy of wastewater treatment plants (WWTPs) in removing these substances. The results of previous investigations show that WWTPs are mostly ineffective in the removal of pharmaceuticals (Hignite & Azarnoff, 1977). Therefore, WWTPs constitute the main source of entry of these compounds into the environment (Lin et al., 2010). Physicochemical properties of drugs, such as biodegradability, lipophilicity, solubility, photosensitivity, and volatility, as well as operation and climate conditions during the process of treatment, can affect the removal rate of pharmaceuticals from sewage water (Boxall, 2004; Gracia-Lor et al., 2012). However, pharmaceuticals are resistant to metabolic degradation and to most commonly used wastewater treatment processes, being persistent in the environment (Hignite & Azarnoff, 1977).

Pharmaceutical acute and chronic exposure can have various effects on organisms, especially in the gills, one of the primary organs in direct contact with contaminants, and

liver, which is responsible for the detoxification and metabolism of the organisms (El-Saad & Elgerbed, 2010; Omar & Mahmoud, 2017). Drugs also act in the central nervous system (CNS), damaging its proper functioning, affecting behavior (Monat-Descamps & Deschamps, 2012). Many pharmaceuticals, such as antidepressants, selective serotonin reuptake inhibitors (SSRIs), hormones, antihistamines, and various psychiatric drugs, can alter the behavior of individuals. Changes in behavior can be related to alterations in feeding, movement, reproduction, activity and can have various sources of action (McFarland et al., 2014). Therefore, behavior can affect the survival of organisms, having consequences even at the population level, being a relevant issue to investigate (Brewer et al., 2001). Pharmaceuticals may also alter the intracellular homeostasis, i.e., self-regulating processes by which living organisms try to maintain stability, assuring their survival (McEwen, 2016), disrupting mitochondrial proper function and forming free radicals (Nihat Ozaydin, 2017). Some drugs are also genotoxic, acting directly or indirectly in the DNA (Prieto Garcia et al., 2012), or nephrotoxic, causing deleterious modifications in excretion, metabolism, or favoring endocrine disruption (Voss et al., 2005). Endocrine disruption is commonly characterized by alterations in hormonal activity, through changes in the general development and reproduction of individuals. Generally, endocrine disruptors (EDCs) are highly lipophilic, with considerable bioaccumulation potential, i.e., significant tendency of this pharmaceuticals to accumulate gradually in living organisms (Wang, 2016). EDCs have also been associated with the disruption of mental and immune systems and impaired behavior. Therefore, it is important to understand the effects that endocrine disruptors have in individuals, which are not yet fully understood (Embrandiri et al., 2016; Lauretta et al., 2019).

As previously exposed, the exposure to contaminants may result in a huge variety of effects, at the cellular and biochemical levels, that can be measured by biochemical biomarker assessment (Committee on Biological Markers of the National Research Council, 1987). A biomarker is any measurable response that reflects cellular or biochemical alterations according to the exposure to xenobiotics or of host response (Committee on Biological Markers of the National Research Council, 1987). Biomarkers can include various types of analysis, including behavioral, biochemical and histological

assessment (Committee on Biological Markers of the National Research Council, 1987). Biochemical and physiological processes can result from alterations from endogenous or exogenous sources, e.g., after the exposure to contaminants, enabling the evaluation of toxicological effects and being possible to imply the impact in different trophic levels, from the individual to the environment level. The presence of xenobiotics in the wild suggests a pollution frame, which leads to changes in organisms resulting in toxicity (Ayas et al., 2007). Biomarkers can detect the primary effects in the individual exposed to contaminants, e.g. at the behavioral and biochemical level, even at low concentrations, which makes them important tools to assess environmental pollution and consequent effects (Lionetto et al., 2003).

1.2. Endocrine disruptors

The reproductive physiology in fish consists of sexual and gonadal development of both males and females. Various organs are involved in the sex development and maturation of individuals. The primary organ that regulates these processes is the hypothalamus, which produces gonadotropin-releasing hormones, which act in the pituitary gland, which releases gonadotropins. These hormones stimulate gametogenesis, i.e., the development of oocytes or spermatozoids in the gonads in fish (Bogers, 2008). Some xenobiotics, known as Endocrine-Disrupting Chemicals (EDCs), can disrupt the regulation of the hypothalamus-pituitary-gonadal axis, by mimicking endogenous hormones. Generally, EDCs bind and/or activate receptors for hormones, mimicking the original hormone (agonist) or blocking the receptors, leading to its inhibition (antagonist) (Leino et al., 2005). Some of the effects observed after exposure to EDCs are the development of intersex, inhibition of gametogenesis, decrease in fertility and modification of the gonadosomatic index (Carnevali et al., 2018). EDCs can also alter the normal functioning of the endocrine system, interfering with hormone activity and, as mentioned before, altering the development, reproduction, and behavior of individuals (Embrandiri et al., 2016; Lauretta et al., 2019). EDCs can affect the early life stages of individuals, through gametogenesis disruption, and may also disrupt the ontogenic development (Mnif et al., 2011).

EDCs that disrupt the action of sex hormones may result from natural sources, such as natural estrogens or phytoestrogens present in leguminosae and soy (isoflavones), or be man-made. From hundreds of thousands of anthropogenic chemicals, one thousand may have endocrine-acting properties, by the disruption of hormone activity in living organisms (Bogers, 2008; Crusselle-Davis & Archer, 2010). Some examples of this type of EDCs of anthropogenic origin are pharmaceuticals, including estrogens, antibiotics, beta-blockers, antiepileptics, pesticides, plastics, metals, industrial chemicals and lipid regulating drugs, such as statins and fibrates (Louis & Stoker, 2015). Some of the biological endpoints often investigated after exposure to EDCs are gross morphology, including secondary sexual features, as well as secondary sexual characteristics, hepato or gonadosomatic index, levels of liver vitellogenin (VTG), plasma concentrations of androgens and estrogens, and histology of gonads or liver (Bogers, 2008). One of the most sensitive and important endpoints of exposure to EDCs is the histology of gonads, for example, through the determination of sex ratio and maturation stages of individuals (Bogers, 2008). According to Leino et al. (2005), and considering the ovaries, exposure to estrogen receptor agonists, androgen receptor agonists, androgen receptor antagonists, and steroid metabolism can affect the development and maturation of oocytes, which can reduce or cease ovulation. EDCs may also affect the testes, and in this case, their effects are more variable, possibly resulting in the hyper-production of sperm, caused by androgen receptor agonists and aromatase inhibitors, and degenerative alterations, caused by estrogen receptor agonists (Leino et al., 2005). Several guidelines conducted in fish use sex ratio and gonadal maturation stages as an endpoint, indicating estrogen, androgen and steroidogenic activity, such as the Medaka Extended One-Generation Reproduction Test (OECD, 2015) and the Fish Sexual Development Test (OECD, 1984). *Danio rerio* was suggested as a model organism for the assessment of EDCs effects in terms of gonad development (Eimon & Ashkenazi, 2010).

1.3. Behavior

Behavior is an orderly sequence of actions controlled by the peripheral (PNS) and central nervous system (CNS), as well as a display of processes that assure the survival of the individual, at the biochemical, genetic and physiological levels (Benson et al., 2010). Internal and external stimuli can alter the behavior of the organisms, thus having an impact on their survival (Sloman & Mcneil, 2012). Behavioral traits may result from altered physiological processes due to exposure to external contaminants, and consequently, behavioral changes have been generally used to assess the effects of xenobiotics in the aquatic compartment (Scott & Sloman, 2004). Aquatic organisms, namely fish, represent ideal models for behavioral evaluation after the exposure to contaminants. They have complex behaviors, are in direct and constant contact with the source of contamination present in their environment, are ecological relevant in terms of natural systems, are easy to maintain in the laboratory, and are able to come into reproductive readiness in short periods of time, i.e., they have several reproductive periods during the year in several species of fish (Kane et al., 2005). Consequently, the behavioral assessment in fish is considered as an integral part of modern toxicological assays, because this can be a specific, reliable and sensitive method to evaluate chemical effects (Moser, 2010).

Behavioral alterations can be a toxic response to exposure to certain compounds (Moser, 2010). There are a large number and types of environmental chemicals that can alter behavior. According to Grue et al. (2002), they can be classified into eight categories. Narcotic chemicals, i.e., low molecular weight solvents, can cause narcosis, and consequently, they result in hypoactivity and reduced responsiveness of the individual, while excitatory agents can cause oxidative phosphorylation, resulting in hyperactivity and hyperreactivity (Grue et al., 2002). Metals originate tissue damage or biochemical impairment, disrupting feeding activity and reproduction, whereas organometals can cause nerve tissue damage, affecting reproduction (Grue et al., 2002). Cholinesterase inhibitors, i.e., organophosphates, carbamates, and many others, can cause hypoactivity and behavioral depression (Grue et al., 2002). On the other hand, reactive chemicals, such as acrolein and benzaldehyde, can cause electrophilic reactions, affecting coordination

and causing hyporeactivity, while central nervous system (CNS) seizure agents, i.e., pyrethroids and organochloride pesticides, act directly on the CNS, causing seizures, hyperactivity, ataxia, convulsions and affect coordination (Grue et al., 2002). Finally, endocrine disruptors, affect mainly reproductive behavior (Grue et al., 2002).

One example of behavioral testing developed with fish is avoidance and attraction, as the individual normally avoids the contaminated area if the chemical induces avoidance, as other contaminants can have attractive responses (Brewer et al., 2001). Respiratory patterns are also behavioral biomarkers, as the contaminant can have several effects on the frequency and amplitude of opercular movements (Diamond et al., 1990). Intra and interspecific interactions are other examples of the behavioral monitoring. The hypoactivity and hyperactivity, prey capture, predator avoidance, scototaxis (preference for light or dark), and reproduction can have a causal linkage with the fate of the population (Laurence, 1972; Steele, 1983). Another type of testing involves the analysis of swimming patterns, as the individuals can develop altered locomotory responses, and the frequency of swimming movements and duration of activity can change due to the exposure to xenobiotics (Little & Finger, 1990; Brewer et al., 2001). Movement analysis is often conducted by automated biomonitoring systems because of their sensitivity, involving the quantification of swimming patterns and videography, such as Zebrabox from Viewpoint (Smith & Bailey, 1988; Miller et al., 2009). Using this automated system, several parameters can be measured, including velocity, acceleration, total distance traveled, angles, swimming time, and horizontal and vertical distribution of individuals.

1.4. Biochemical parameters

One of the most recurrent responses to chemical compounds involves the activation of metabolic defenses, generally by the activation of phase I of enzymatic complexes, modulating phases II and III of metabolism (Holth et al., 2008). Metabolic defense mechanisms usually increase the hydrophilicity of xenobiotics through hydrolysis, reduction, oxidation and/or especially conjugation, which ultimately, but not always, can lead to the transport and excretion of the toxicant (McFarland et al., 2014). Cytochrome monooxygenases P450 enzymes (CYP450) are the major enzymes in phase I of

metabolism, oxidizing lipophilic into hydrophilic compounds (Sharma et al., 2012). Commonly, the poor coupling of the CYP450 catalytic cycle results in the continuous production of reactive oxygen species (ROS), that are relevant in signaling pathways and can be responsible for oxidative stress (Banerjee & Ghosh, 2016).

Most xenobiotics are known to cause hyperproduction of reactive oxygen species (ROS) and oxidative stress after being metabolized. Oxidative stress is defined as an imbalance between the production of ROS and antioxidant defenses, through the activity of several enzymes in the cytoplasm, mitochondria and peroxisomes (Betteridge, 2000). ROS are chemically reactive molecules that are produced during the oxidative metabolism in mitochondria and as a cellular response to xenobiotics (Ray et al., 2012). Although ROS are often associated with oxidative stress, they also are signaling molecules that regulate biological and physiological processes (Schieber & Chandel, 2014). Redox signaling is relevant in terms of cellular differentiation, prevention of aging and tissue regeneration (Schieber & Chandel, 2014). Thus, oxidative stress is present when there is a hyperproduction of ROS that exceeds the capacity of the cell to deploy an antioxidant response (Ray et al., 2012). Large varieties of substances that were already found in the water, including pharmaceuticals, can cause oxidative stress to aquatic species (Valavanidis et al., 2006). Some examples of enzymes that are involved in the antioxidant defense mechanisms, and that are used as biomarkers of oxidative stress, are superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx).

Superoxide dismutase is found in three different forms: extracellular SOD, SOD containing manganese in the mithocondria (MnSOD) and SOD containing copper and zinc, found in the cytoplasm (Cu-ZnSOD) (Lumb, 2017). SOD constitutes protection against the radical superoxide (O_2^-) toxic effects, as it catalyzes its dismutation into hydrogen peroxide (H_2O_2) and molecular oxygen (O_2). Although hydrogen peroxide is not a reactive oxygen species, it is still an oxidizing agent that causes oxygen toxicity. In that way, SOD consists of cellular defense against ROS (Hayyan et al., 2016).

As the hydrogen peroxide is continuously produced in the organism, two enzymes ensure its removal, i.e. catalase (CAT) and glutathione peroxidase (GPx) (Lumb, 2017). CAT acts only against hydrogen, methyl and ethyl peroxides (Lumb, 2017). It is part of the

antioxidant defense system that exists in peroxisomes (Modesto & Martinez, 2010), where many enzymes that are responsible for the elimination of hydrogen peroxide are located (Djordjević, 2004). Its primary function is the reduction of hydrogen peroxide, into water and molecular oxygen (Aebi, 1984), which prevents the conversion to hydroxyl radical and other even more toxic ROS (Djordjević, 2004).

GPx is found in multiple forms, all containing selenium as the catalytic metal (Blondet et al., 2018). It is found in the cytoplasm and mitochondria and acts in a wider range of peroxides, in the way that it can reduce organic peroxides unlike CAT and reactive species originated by lipid peroxidation (Cohen & Hochstein, 1963; Tappel, 1984; Flohé, 1985). GPx also acts against hydrogen peroxide, by the conversion of reduced glutathione (GSH) and hydrogen peroxide into water and oxidized glutathione, a dimer of reduced glutathione (GSSG) (Blondet et al., 2018). The tripeptide glutathione in its reduced form (GSH) has many biological roles including protection against ROS and electrophiles, or as a cofactor for many other enzymes (Ray et al., 2012). It acts directly as antioxidant defense, and it is present in various metabolic processes. Glutathione stores cysteine and nitric oxide, transporting also the latter, and it takes part in the functioning of transcription factors involved in redox signaling, being also responsible for the detoxification of various xenobiotics and endogenous substances (Lushchak, 2012). Glutathione reductase is responsible for the maintenance of the supply of reduced glutathione, at a cellular level, recycling the oxidized into the reduced form, and in turn maintaining the antioxidant capacity of glutathione (Couto et al., 2016). Considering their intimate interconnection, GPx, glutathione (both forms) and glutathione reductase are altogether present in hepatocytes, allowing glutathione-mediated detoxification (Blondet et al., 2018).

As mentioned before, drug metabolism is characterized by three phases (Banerjee et al., 2016). The activity of metabolic components of phase I and II have regularly been used as biomarkers, i.e., by the increase or decrease in enzymes` activity, as a result of the presence of xenobiotics, such markers can be used to estimate the toxic effects in aquatic organisms (Holth et al., 2008). One example of this type of enzymes is phase II metabolic isoenzymes glutathione S-transferases (GSTs). Glutathione S-transferases are a

set of enzymes that lead to the conjugation of reduced glutathione with electrophilic centers, resulting in the metabolism and detoxification of xenobiotics (Ray et al., 2012). Consequently, it is considered one of the best examples of enzymes involved in phase II of biotransformation (Bradford, 1976; Modesto & Martinez, 2010). In that way, GSTs prevent the interaction of xenobiotic substrates with nucleic acids and cellular proteins (Dzoyem et al., 2014). GSTs can also transport proteins and some of their isoenzymes can reduce organic hydroperoxides and are responsible for the isomerization of unsaturated substances, protecting the organism against oxidative stress, and therefore, oxidative damage (Rahman, 2007; G. S. Smith, Walter, & Walker, 2013; Dzoyem et al., 2014).

Lipid peroxidation can result because of the depletion or inefficacy of the previous oxidative damage preventive measures (Castell et al., 1997). Oxidative damage can occur due to the hyperproduction of ROS, by an imbalance between ROS production and their scavenging (Sharma et al., 2012). It affects mainly proteins (denaturation), nucleic acids, carbohydrates and lipids (peroxidation) (Sharma et al., 2012). Therefore, oxidative damage can affect lipoproteins, cellular membranes and molecules containing lipids, and can lead to structural tissue damage and eventually to cell death by apoptosis or necrosis (Meena & Naik, 2019). Free radicals interact with lipids present in membranes, particularly unsaturated fatty acids (Halliwell, 2009; Sies, 1985). This process involves hydrogen removal from a carbon atom, with the insertion of oxygen, leading to the formation of lipid peroxyl radicals and hydroperoxides (Ayala et al., 2014). As secondary products, various aldehydes are formed, such as malondialdehyde-like substances (Ayala et al., 2014). The production of aldehydes (malondialdehyde-like substances) and hydrocarbons lead eventually to the loss of membrane integrity (Dzoyem & Eloff, 2014). Malondialdehyde is the main chemical produced by the oxidation of polyunsaturated fatty acids (Lykkesfeldt, 2007). It can be enzymatically metabolized or it may react with DNA, cells or tissue proteins, forming adducts, which results in lipid peroxidation (Ayala et al., 2014). In that way, malondialdehyde is mainly produced by the oxidation of polyunsaturated fatty acids, being a more reliable and comprehensive indicator of the total amount of final products of lipid peroxidation (Lykkesfeldt, 2007). Lipid peroxidation may be expressed as the content of thiobarbituric acid reactive substances (TBARS), i.e.,

malondialdehydes and other thiobarbituric acid reactive substances. It is the oldest but still the most used methodology for the measurement of lipid peroxidation, by the quantification of malondialdehyde-like compounds (Dasgupta & Klein, 2014).

1.5. Simvastatin

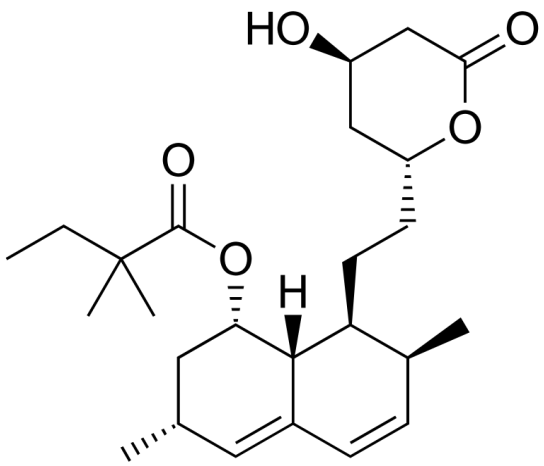

Figure 1 – Chemical structure of SIM (Adapted from Alarfaj et al., 2012)

Lipid-regulating drugs are one of the most prescribed medications around the world, to control human cholesterol levels, to more than 20 million patients (Davidson, 2009), with an expected global market size of 22.6 billion USD by 2022 (Research and Markets, 2018). According to the Infarmed – Portuguese National Authority for Medicines and Health Products, I.P., the consumption of lipid-regulating drugs has increased for decades. Over the years, the cost of antihyperlipidemic drugs has decreased drastically, favoring the increase in its consumption. If the price of such drugs remained constant between 2010 and 2013, the Portuguese population would have spent 129 million € in that period (Infarmed I.P., 2013).

There are several lipid-regulating drugs, such as inhibitors of absorption of cholesterol, nicotinic acid, fish oil derivatives and fibrates, but statins are the predominant group (Rang et al., 2007). In 2013, 90% of lipid-regulating drugs sold in Portugal, corresponded to statins, and the other 10% were generally fibrates (Infarmed

I.P., 2013). Some examples of statins are lovastatin, pravastatin, atorvastatin, rosuvastatin, fluvastatin, pitavastatin, and simvastatin (Rang et al., 2007). Statins exert their therapeutic activity by inhibiting 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase (Rang et al., 2007; Davidson, 2009). HMG-CoA reductase catalyzes the conversion of HMG-CoA to mevalonic acid. Thus, the most evident effect of statins is the reduction of plasma low-density lipoproteins (LDL), some reduction in plasma triglycerides and an increase in high-density lipoproteins (HDL) (Rang et al., 2007). They constitute the first therapeutic option to lower LDL (Stroes, 2005).

According to Infarmed I.P. (2013), the most sold statin in Portugal is simvastatin (SIM). SIM (Figure 1) acts as an inhibitor of the production of cholesterol in the liver (Germershausen et al., 1989). After ingestion, SIM is hydrolyzed, producing a metabolite, in a β-hydroxy acid form, that is structurally similar to HMG-CoA (Vickers et al., 1990). By competition, it inhibits HMG-CoA reductase, reducing the quantity of mevalonic acid, a cholesterol precursor (Germershausen et al., 1989).

Simvastatin can also increase mitochondrial and peroxisomal β-oxidation of fatty acids (Park et al., 2016). β-oxidation and the Krebs cycle are closely related to the electron transport chain. In turn, the final electron acceptor of the electron transport chain consists in O_2, which leads to the production of H_2O, and ultimately to the formation of ROS, such as O_2^-, HO^- and H_2O_2 (Speijer et al., 2014). The production of reactive oxygen species can, therefore, lead to oxidative stress, and ultimately to oxidative damage. The degradation of fatty acids has the primary goal of the formation of acetyl-CoA, to serve as a carbon and energy source, essential for the metabolism of the individual. This process can lead to behavioural changes in organisms due to changes in the input of energy (Orton & Parker, 1982).

SIM is biotransformed by the cytochrome P450 in the liver o fish, particularly by the CYP3A4 isoform. Subsequently, it undergoes glucuronidation, a reaction catalyzed by UDP-glucuronosyltransferases (UGTs) (Prueksaritanont et al., 2002). The metabolism of SIM produces at least four primary metabolites (K. H. Kim et al., 2011).

Considering its use, SIM is released into the sewage system, and ultimately may be discharged into the aquatic compartment, where its photodegradation rate is minimal

(<0.45% during three days) and no degradation product is detected (Sawant & Barge, 2014). Water hydrolysis is dependent on pH and temperature. The higher these values, i.e., higher than pH=7 and 60°C, the faster is the hydrolysis process (Álvarez-Lueje et al., 2005).

The occurrence of SIM in wastewater has been reported in various studies, in multiple countries, such as Greece, with concentrations reaching 718 ng L^{-1}, in wastewater influents (Papageorgiou et al., 2016); in the UK, reaching 115 and 5 ng L^{-1}, in wastewater influents and effluents, respectively (Kasprzyk-Hordern et al., 2009); and in Spain, 7.5 ng L^{-1}, in a drinking water treatment plant (Boleda et al., 2011). Samples from the river Danube also reported a concentration of 0.04 to 0.7 ng L^{-1} (Martín et al., 2011).

There are only a few studies regarding the effects of SIM in aquatic organisms. Some of them showed that this compound can lead to a disruption in the reproduction and development (endocrine disruption), compromising the populational growth of the amphipod *Gammarus locusta* (Neuparth et al., 2014); it can also affect the development time and growth rate of harpacticoid copepods (Dahl et al., 2006). Ellesat et al. (2010) reported a dose-response toxic effect in primary hepatocytes of *Oncorhynchus mykiss* exposed to simvastatin (1 to 200 mg L^{-1}), by the reduction of metabolic activity and stability of membranes. A significant decrease in the development rate was identified in *Nitocra spinipes* exposed to SIM (810 mg L^{-1} during 96h) (Dahl et al., 2006). A decrease in larval length and an increase in morphological abnormalities were also identified in *Danio rerio* (Ribeiro et al., 2015). Strzyzewski et al. (2013) have identified the reduction of oxidative stress and lipid peroxidation by simvastatin. On the other hand, there was an increase in GSTs activity and a decrease in SOD and CAT activity in *Danio rerio* larvae, during 80h of exposure (Cunha et al., 2016). Thus, SIM seems to have antioxidant properties.

1.6. Clofibric acid

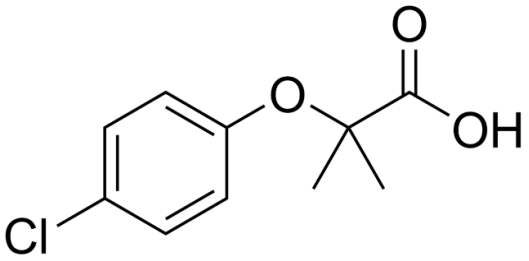

Figure 2 - Chemical structure of CA (Adapted from Kim et al., 2014)

Some of the fibric acid derivatives, commonly known as fibrates, are bezafibrate, ciprofibrate, gemfibrozil, fenofibrate, and clofibrate. Fibrates reduce the levels of very-low-density lipoproteins (VLDL, precursors of LDL) and triglycerides, reducing LDL and increasing HDL, as well. Fibrates can interact with hepatic peroxisome proliferator-activated receptors (PPARs), that regulate the transcription of genes encoding enzymes involved in the synthesis and secretion of lipids, and of the β-oxidation of fatty acids (Michalik et al., 2006). The main principle of action of fibrates is the activation of PPARα, that activates lipoprotein lipase, which results in the decreasing formation of VLDL (Rang et al., 2007). The reduction of plasmatic cholesterol is probably the outcome of two major processes: the increase in excretion of cholesterol derived from body tissues and the inhibition of the hepatic synthesis of cholesterol (Rang et al., 2007).

Clofibrate, etofibrate, and etofyllinclofibrate can be hydrolyzed into clofibric acid (2-(4-chlorophenoxy)-2-methylpropanoic acid) (Figure 2), which is the active metabolite that circulates in plasma and it is believed to be responsible for the hypolipidemic properties of these fibrates (Daughton & Ternes, 1999). Clofibric acid induces the enzymatic activity of hepatic peroxisomes, through binding to the nuclear receptor PPAR, increasing the number and size of the peroxisomes, and consequently their enzyme load and activity. This leads to an increase in β-oxidation of fatty acids (Orton & Parker, 1982). This process can take place in the mitochondria and the peroxisomes. The degradation of fatty acids has the primary goal of the formation of acetyl-CoA, to serve as a carbon and energy source, essential for the metabolism of the individual. Subsequently, CA

undergoes biotransformation, by the cytochrome P450, particularly the CYP4A isoform and glucuronidation (Orton & Parker, 1982). The excretion of CA occurs mostly through urine. While 3 to 20% of this compound can be excreted in its original form, it is generally conjugated and/or excreted as a glucuronic acid conjugate, after the glucuronidation process (Walmsley, 1985). Three metabolites have been identified for clofibric acid: hydroxyisobutyric acid, lactic acid and 4-chlorophenol (Salgado et al., 2012).

The occurrence of CA in wastewater has been reported in several studies, in many countries, such as the UK, with concentrations ranging from 1 to 57 ng L^{-1}, in wastewater influents (Kasprzyk-Hordern et al., 2009); in Canada, ranging from 101 to 175 ng L^{-1}, in surface water of the Detroit River and Hamilton Harbour (Metcalfe et al., 2003); in China, with maximum values of 56.2 and 44.9 ng L^{-1}, in wastewater influents and effluents, respectively (Sui et al., 2011); and in Korea, with concentration reaching 65 and 6 ng L^{-1}, in wastewater influents and effluents, respectively. According to the results obtained by Buser et al. (1998), there is evidence of resistance to sewage treatment and degradation in water, where CA is predicted to persist for approximately 21 years. However, a more recent study reported a high photodegradation rate of this compound, with a half-life period of 50 h when exposed to natural light. Its persistence in the aquatic compartment seems to be related to the quantity of organic matter and nitrates, or by the presence of hydroxyl and other free radicals, that can lead to the photochemical loss (Packer, Werner, Latch, McNeill, & Arnold, 2003).

There are only a few studies regarding the effects of CA in aquatic organisms. Some of them showed that this compound can decrease the growth rate of rainbow trout by 50% (from 5.27 to 2.67% per day), in organisms exposed to clofibric acid ranging from 0.1 to 10 µg L^{-1} during 28 days (Owen et al., 2010); it can also increase the production of superoxide radicals and MnSOD and SOD total activity in humans (Strzyżewski et al., 2013); increase in the CAT activity in *Gambusia holbrooki* (Nunes et al., 2004) and it can lead to the hyperproduction of ROS in rodents (Qu et al., 2001). There is evidence that CA can disrupt the reproductive system, affecting the development and reproduction of individuals, through alterations in endocrine control, i.e., by the reduction of cholesterol, that is used to synthesize hormones (Dahl et al., 2006; Owen et al., 2010).

1.7. Zebrafish

Zebrafish, *Danio rerio,* is a freshwater fish species, originally from South Asia belonging to the family Cyprinidae. This species constitutes an alternative animal model in several areas of biological sciences, including Toxicology and Ecotoxicology. Zebrafish is a good bioindicator in terms of ecotoxicological effects of contaminants in living organisms, because of its sequenced genome, also being similar to humans in terms of biological systems (Dooley & Zon, 2000). Breeding and maintenance of individuals are easy to perform in the laboratory, allowing it to be considered as a model organism for vertebrate biology studies (Dooley & Zon, 2000). Its rearing is made under controlled conditions, regarding the temperature of the water, pH, conductivity, dissolved oxygen and other factors (OECD, 2013). Its use in ecotoxicology testing is due mainly to its easily understandable and observable behavioral responses, as well as the morphological characteristics of its embryos, that are relatively large and transparent, which enables the analyses of alterations or malformations (Dahm, 2006). Also, under controlled conditions, it allows the production of a large number of eggs, having a short reproductive cycle, in which the full maturity is reached in 3 months (Hill et al., 2005). *D. rerio* is a standard species in ecotoxicology assays, assessing the relationship between xenobiotics and biological systems (OECD, 2013). The use of zebrafish embryos is yet to be regulated by current European Union legislation in terms of animal welfare, being zebrafish-based bioassays an alternative to animal experiments and an opportunity to assess the environmental risk of xenobiotics in a small-scale (Scholz et al., 2008). The fish embryo toxicity test (FET) conducted with this species is one of the most used tests performed with zebrafish embryos, and some of its advantages are related to the required small amounts of test compounds, short periods of exposure and single breeding stock (OECD, 2013).

1.8. Objectives

The present study aimed to evaluate the effects of the two drugs, SIM and CA, in zebrafish larvae and juveniles. To achieve this purpose, an acute (120 hpf) and chronic (60 dpf) exposures were conducted to a range of concentrations of each pharmaceutical

compound, at ecologically relevant concentrations, i.e., concentrations already found in the environment. In order to evaluate their toxic effects, behavioral analysis (zebrabox testing for the small and large distance traveled, as well as the swimming time and total distance) and biochemical markers of oxidative stress (determination of the activity of the enzymes SOD, CAT and GPx), biotransformation (GSTs), and lipid peroxidation (TBARS) and histological assessment (sex determination and gonadal developmental stages) were evaluated.

2. MATERIAL AND METHODS

2.1. Chemicals

The chemicals used in this study, namely SIM ([(1*S*,3*R*,7*S*,8*S*,8*aR*)-8-[2-[(2*R*,4*R*)-4-hydroxy-6-oxooxan-2-yl]ethyl]-3,7-dimethyl-1,2,3,7,8,8*a*-hexahydronaphthalen-1-yl] 2,2-dimethylbutanoate; CAS: 79902-63-9; purity ≥ 97 %) and CA (2-(p-Chlorophenyl)-2-methylpropanoic acid; CAS: 882-09-7; purity ≥ 99 %) were acquired from Sigma-Aldrich (Schnellforf, Germany). Exposure media were prepared by successive dilution of the stock solutions, with water provided from Tecniplast ZebTEC Zebrafish Facility recirculating system. Two stock solutions of SIM were prepared in ultrapure water (Milli-Q-Water), one with a concentration of 4 mg L^{-1}, which was used to prepare a second stock solution, with a concentration of 4 µg L^{-1}. However, only one stock solution of CA was prepared, with a concentration of 1,2 mg L^{-1}. Ethyl 3-aminobenzoate methanesulfonate (MS-222; CAS: 886-86-2), reduced glutathione (GSH; CAS: 70-18-8), 1-chloro-2,4-dinitrobenzene (CDNB; CAS: 97-00-7), hydrogen peroxide (H_2O_2; CAS: 7722-84-1), trichloroacetic acid (TCA; CAS: 76-03-9), 2-thiobarbituric acid (TBA; CAS: 504-17-6), glutathione reductase (GR; CAS: 9001-48-3), cumene hydroperoxide (CAS: 80-15-9), cytochrome c (CAS: 9007-43-6), potassium cyanide (CAS: 151-50-8) and bovine γ-globulin (CAS: 9007-83-4) were purchased from Sigma-Aldrich. Bradford reagent was purchased from Bio-Rad UK. The chemicals used for histological analyses were ethanol absolute (CAS: 64-17-5), acquired from AGA – Álcool e Géneros Alimentares S.A.; Appliclear (Xylene substitute) (CAS: 64742-49-0), Bouin liquor for clinical diagnosis, and Histofix decalcifier 1 for clinical diagnosis, all were purchased from PanReac AppliChem ITW Reagents; Hematoxylin solution modified acc. to Gill III, Certistain (Eosin y) (CAS: 15086-94-9) and DPX new (non-aqueous mounting medium for microscopy) were acquired from Merck-Millipore; Hydrochloric acid standard solution 1M was purchased from Fluka Analytical (Sigma-Aldrich). All other chemicals for buffers preparation were obtained either from Sigma-Aldrich or Merck-Millipore.

2.2. Test organisms

Individuals of the test species (*Danio rerio*) used in this study were kept in a Tecniplast ZebTEC Zebrafish Facility recirculating system under controlled conditions (OECD, 2013). Water temperature was 26 ± 1°C, pH was 7.5 ± 0.5, conductivity was 750 ± 50 µS cm^{-1}, and dissolved oxygen was equal or above 95% of saturation. The culture water was tap water filtered and purified by activated carbon and reverse osmosis, to which "Instant Ocean Synthetic Sea Salt" (Spectrum Brands, USA) was added to regulate the conductivity to 800 µS cm^{-1}. The photoperiod cycle was upheld at 16h:8h (light: dark). The adult individuals were fed twice daily during the week, once on the weekend, with an artificial diet [GEMMA Micro 500, acquired from Skretting Zebrafish (Maine, United States)]. To protect eggs from predation by adult individuals, marbles were placed in the bottom of the aquarium for refuge. After the removal of the marbles, eggs were collected, rinsed with water and malformations or absence of cleavage were identified under a stereomicroscope (Nikon, SMZ 1500) for eggs to be discarded.

2.3. Concentrations of chemicals

The concentrations to which test organisms were exposed, in both chronic and acute exposures, were 92.45, 184.9, 369.8, 739.6 and 1479.2 ng L^{-1} for SIM, and 10.35, 20.7, 41.4, 82.8 and 165.6 μg^{-1} L for CA. The most concentrated solution of each contaminant was directly prepared from the stock solution, and submitted to successive dilution, as mentioned before. The intermediate concentration values for each of the contaminants (SIM – 369.8 ng L^{-1}; CA – 41.4 µg L^{-1}) were based on ecologically relevant concentrations, i.e., the maximum level found in effluents from wastewater treatment plants in Portugal (Salgado et al., 2010) and in the predicted environmental concentrations for Portuguese surface waters (Pereira et al., 2015), as well as the levels found in WWTP influents (11.7±3.2 µg L^{-1}), effluents (2.65±0.8 µg L^{-1}) and Apies River (1.585±0.3 µg L^{-1}), all located in Pretoria, South Africa (Tete et al., 2019), respectively. Lower concentrations were thus considered realistic and ecologically relevant; higher concentrations allowed testing for the organisms´ responsiveness towards both chemicals.

2.4. Acute exposure

The method used in acute exposure was a modified fish embryotoxicity test (FET; OECD, 2013). In the present study, eggs were transferred to 96-well plates, each well containing 300 µL of each test solution and one egg, five replicates (20 pools, a total of 100 eggs) for each concentration and control group, for each contaminant. For the measurement of the behavioral and biomarker activities, the larvae were exposed during 120 h, for behavioral assessment and subsequent biochemical analysis.

CA has a half-life of 50 h in natural water irradiated by sunlight (Packer et al., 2003). Therefore, every two days (approximately 48h), exposure media for both contaminants were newly made, with water retrieved from the Tecniplast ZebTEC Zebrafish Facility recirculating system, including control group, and partially renewed by approximately 80%, according to the OECD guidelines (OECD, 1984; OECD, 1992; OECD, 2011; OECD, 2013).

2.4.1. Behavioral test

The period of exposure to both drugs was extended to 120 hpf, as mentioned before, to allow the complete development of the swim bladder. Only then it was possible to evaluate locomotor activity using the Zebrabox (Viewpoint, Lyon, France) tracking system under stabilized temperature (26±1 °C). The larvae were observed for the identification of morphological alterations or deformations that could compromise locomotory capacity, being removed from the test if such conditions were observed (Şişman et al., 2008). The test was conducted in a cycle of four alternating periods during twenty minutes, i.e., 1200 s [0 to 300 s (light), 300 to 600 s (dark), 600 to 900 s (light), 900 to 1200 s (dark)]. The alternate light and dark cycles are justified by the natural behavior of *D. rerio*; normally developing larvae show little to no movement in the light periods and increase their swimming activity in dark periods (Collier et al., 2017). Data outputs were obtained at the end of each cycle and the following parameters were calculated: relatively small (less than 0.5 mm s^{-1}) and large distance, total distance traveled (sum of small and large distance traveled) and relative swimming time.

After the motor activity test, pools of 20 larvae (as mentioned before) were allocated into Eppendorf microtubes and kept at -80 °C. For the biomarker analysis, the larvae were homogenized in 1 mL of phosphate buffer (50 mM, pH = 7.0, with Triton X-100 0.1 %) by sonication (Branson S-250A), on ice, during 30 seconds, and centrifuged (refrigerated centrifuge Thermo Fisher Scientific - Heraeus mod. Megafuge 8R) at 15.000 g during 10 min at 4°C. After centrifugation, the remaining pellets were discarded, and the supernatants were collected and stored at -80 °C.

2.4.2. Biochemical analyses

2.4.2.1. Superoxide dismutase activity quantification

The SOD activity quantification (total, Cu-ZnSOD, and MnSOD) was based on the method described by Flohé & Ötting (1984). A xanthine-xanthine oxidase system produced superoxide radicals. The result of the reaction of these radicals with cytochrome c represented SOD's total activity. Potassium cyanide inhibited the activity of Cu-ZnSOD, allowing the quantification of the isolated activity of MnSOD. Thus, results for Cu-ZnSOD were obtained as the difference between total SOD activity and MnSOD activity, being compared with MnSOD activity. The enzymatic activity in all of these cases was spectrophotometrically followed at λ = 550 nm and was then expressed in mmol min^{-1} mg^{-1} of protein.

2.4.2.2. Catalase activity quantification

CAT activity quantification was based on the protocol by Aebi (1984). The decomposition of H_2O_2 was catalyzed by CAT. This decomposition was spectrophotometrically followed at λ = 240 nm, by the decrease of absorbance. The enzymatic activity was then expressed in µmol min^{-1} mg^{-1} of protein.

2.4.2.3. Glutathione peroxidase activity quantification

The GPx activity quantification was based on the method proposed by Flohé & Günzler (1984). The reduction of oxidized L-glutathione (GSSG) to its reduced form, GSH, by glutathione reductase, through the oxidation of NADPH (ε = 6.2 mM cm^{-1}), was

spectrophotometrically followed at λ = 340 nm. GPx selenium-dependent activity was measured using H_2O_2 as a substrate, whereas GPx total activity (selenium-dependent + non-dependent) was measured using cumene hydroperoxide as a substrate. The enzymatic activity was then expressed in mmol min^{-1} mg^{-1} of protein.

2.4.2.4. Glutathione S-transferases activity quantification

GSTs activity quantification was based on the method described by Habig et al. (1974). The conjugation of CDNB with GSH was catalyzed by GSTs, which led to the formation of a thioether (ε = 9.6 mM cm^{-1}). This formation was spectrophotometrically followed at λ = 340 nm, by the increase of the absorbance. The enzymatic activity was then expressed in µmol min^{-1} mg^{-1} of protein.

2.4.2.5. Thiobarbituric acid reactive substances quantification

The TBARS quantification was based on the protocol described by Buege & Aust (1978). In the presence of thiobarbituric acid (TBA) and heat (water bath at approximately 100 °C), malondialdehyde (MDA)-like compounds reacted with TBA, which led to the formation of a colored end product. This formation was spectrophotometrically quantified at λ = 535 nm. TBARS concentrations, expressed as MDA equivalents, were then calculated in µmol min^{-1} mg^{-1} of protein.

2.4.2.6. Protein determination

The quantification of protein of all the samples was based on the protocol by Bradford (1976). The conjugation of the Bradford reagent with the total protein led to the formation of a colored and stable complex, which was spectrophotometrically measured at λ = 595 nm. Protein standards were prepared with bovine γ-globulin in a concentration of 1 mg mL^{-1}.

2.5. Chronic exposure

Chronic exposure was based on OECD guidelines (OECD, 2011; OECD, 2013), with some modifications. In the present study, seven hundred and twenty eggs were transferred into twenty-four Petri dishes (with 15 mL of exposure medium each), twenty-

five eggs per plate, at the beginning of the experiment. At 96 hpf, larvae were transferred into the exposure aquariums (1 L each). Twenty-five larvae were assigned per aquarium, two replicates for each concentration, including a control group (unexposed individuals), for each contaminant. The aquaria were randomly distributed in the exposure room, under wide spectrum fluorescent bulbs.

The exposure period ended at 60 dpf (chronic exposure) according to the OECD guideline (OECD, 2011). Fish were fed twice daily during the week, and once during the weekend, with synthetic rations, particularly GEMMA Micro 150 (9 to 30 dpf), GEMMA Micro 300 (30 to 60 dpf), acquired from Skretting Zebrafish (Maine, United States), according to Martins et al. (2016).

The exposure media for both contaminants was partially renewed by approximately 80%, according to the OECD guidelines and as mentioned before for the acute exposure, i.e., each 48 hours (OECD, 2011; OECD, 2013).

The water used to prepare the exposure media was under controlled conditions (water temperature = 26 ± 1°C, pH = 7.5 ± 0.5, conductivity = 750 ± 50 µS cm^{-1} and dissolved oxygen saturation ≥ 95%). During the exposure, mortality was below 70% in all treatment groups, with no mortality in the control group, in agreement with the requirements of the adopted OECD guideline (OECD, 2011).

2.5.1. Fish sacrifice

After the chronic exposure, five specimens from each aquarium were euthanized by immersion into a solution of MS-222 (250 mg L^{-1}), according to the OECD guideline (OECD, 2011) until there was no observable opercular movement and the specimens could not swim. Experiments took into consideration the AVMA Guidelines for the euthanasia of animals and the Portuguese animal welfare law (Decreto-Lei 113/2013). This facility is certified by the Portuguese veterinarian authority and the experiments were conducted according to guidelines set up by the animal welfare commission of the University of Aveiro.

2.5.2. Sample processing

Fish were dissected, the head and tail were removed and discarded, for the incorporation of Bouin solution into the entire organism, and the specimens (five per replicate) were held in plastic tissue cassettes, followed by immersion into Bouin solution for 24h. After chemically fixed in Bouin, the specimens in tissue cassettes were decalcified (24h), dehydrated through an increasing series of alcohols (70 %, 80 %, 90 % and 100 %, one hour each), cleared with xylene (2 h), impregnated in paraffin wax (56 to 58 °C), and sectioned (5 to 7 µm) using a microtome (Leica, Reichert-Jung 2030). These sections were stained with hematoxylin-eosin, mounted with DPX in coverslips, and analyzed by light microscopy (Olympus CX41) for the sex determination and identification of the gonadal developmental stages, according to Brown-Peterson et al. (2011). Female individuals were classified, such as immature (only oogonia and primary growth oocytes present), early developing subphase (only primary growth and cortical alveolar oocytes present), developing (primary growth, cortical alveolar, primary vitellogenic and secondary vitellogenic oocytes present), spawning capable (tertiary vitellogenic oocytes or postovulatory follicle complex present), regressing and regenerating. Male individuals were classified as immature (only primary spermatogonia present), early developing subphase (only primary spermatogonia, secondary spermatogonia, and primary spermatocyte present), developing (secondary spermatogonia, primary spermatocyte, secondary spermatocyte, spermatids and spermatozoa present), spawning capable (spermatozoa in lumen; the various stages of spermatogenesis can be present), regressing and regenerating (Brown-Peterson et al., 2011).

2.6. Statistical analyses

Data from behavioral and biochemical testing was evaluated for equal variance (Levene test) and normality (Shapiro-Wil test) prior to the statistical analyses, with attempted transformations to the data, when needed. Behavioral results were compared by a One-way ANOVA on Ranks, and in some cases by a One-Way ANOVA, when data transformations made it possible. On the other hand, biochemical results were only compared by or One-way ANOVA (Murphy et al., 2003). These statistical analyses were

followed by a Dunnett or Dunn multi-comparison test, after One-way ANOVA or One-way ANOVA on Ranks, respectively, to identify significant differences between the treatments and the control group. Data from sex determination was evaluated statistically for homogeneity with the heterogeneity chi-square analysis, whereas gonadal developmental stages were also statistically evaluated for homogeneity and independence of occurrence through a G test, according to Zar (2010). The adopted level of significance for all statistical methods was 0.05. Data are presented as mean and standard error. The analyses were performed with the software SPSS 24.0.

3. RESULTS

3.1. Simvastatin

3.1.1. Acute exposure

3.1.1.1. Behavioral results

According to the results obtained in terms of small distance traveled by fish exposed to SIM (Figure 3), there were significant differences between treated groups and the control animals, in all light and dark periods. During the first light cycle (0 to 300s), there was a significant decrease in the small distance travelled, except for the organisms exposed to the second highest concentration (739.6 ng L^{-1}), compared with control treatment (One-Way ANOVA on Ranks followed by a Dunn's test: H_5=91.651, p<0.001). During the first dark period (300 to 600s), there was a significant increase, particularly in animals exposed to the intermediate (369.8 ng L^{-1}) and highest concentrations (1479.2 ng L^{-1}), compared with control (One-Way ANOVA on Ranks followed by a Dunn's test: H_5=67.317, p<0.001). During the second light cycle (600 to 900s), there was a significant decrease in fish exposed to the lowest concentration (92.45 ng L^{-1}), followed by a significant increase in those exposed to the highest concentration (1479.2 ng L^{-1}), comparing to the control (One-Way ANOVA on Ranks followed by a Dunn's test: H_5=35.1, p<0.001). In the final dark period (900 to 1200s), there was a significant decrease, namely in animals exposed to the lowest (92.45 ng L^{-1}) and second highest concentrations (739.6 ng L^{-1}), followed by a significant increase in individuals subjected to the highest concentration (1479.2 ng L^{-1}), compared with control (One-Way ANOVA on Ranks followed by a Dunn's test: H_5=101.299, p<0.001).

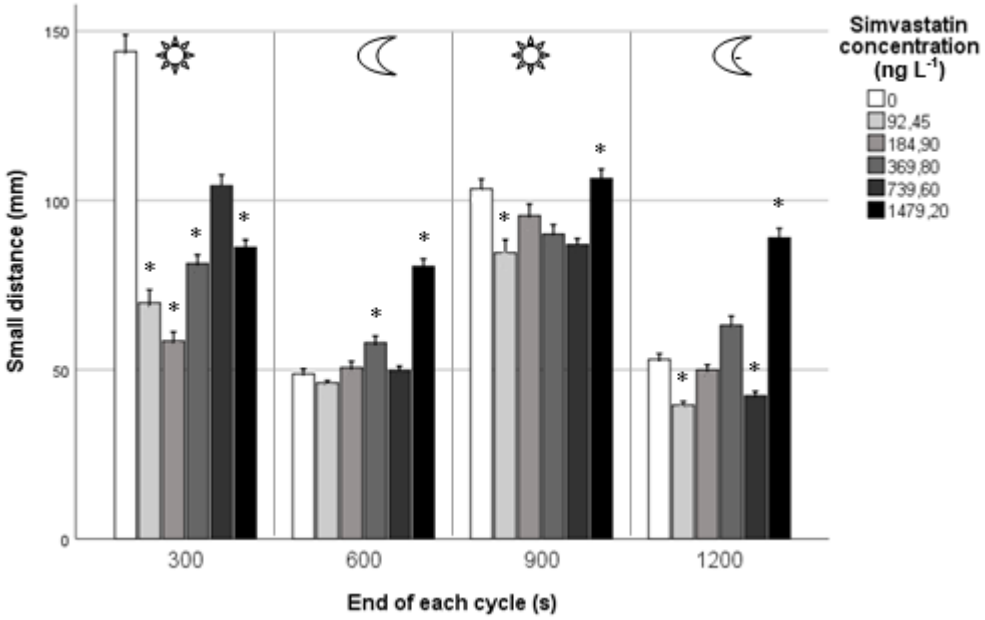

Figure 3 - Small distance traveled for individuals exposed to SIM, in cycles of light and dark periods. Data are Mean±SE (n=20 fish/treatment). ☼ *Stands for light cycles while* ☾ *stands for dark periods. * Stands for significant differences among treatments, compared with the control, during the respective light or dark period (Dunn's test, p<0.05).*

Considering the large distance traveled by fish exposed to SIM, there were significant differences between the treatment groups and the control fish, for all the light and dark periods (Figure 4). During the first light cycle (0 to 300s), there was a significant increase, except for the organisms exposed to the lowest (92.45 ng L^{-1}) and highest (1479.2 ng L^{-1}) concentrations, compared with control (One-way ANOVA followed by a Dunnett's test: H_5=111.148, p<0.001). During the first dark period (300 to 600s), there was a significant decrease in the individuals subjected to the lowest concentration (92.45 ng L^{-1}), followed by a significant increase in those exposed to the second highest concentration (739.6 ng L^{-1}), compared with control (One-Way ANOVA on Ranks followed by a Dunn's test: H_5= 59.682, p<0.001). During the second light cycle (600 to 900s), there was a significant decrease in organisms exposed to the lowest concentration (92.45 ng L^{-1}), followed by a significant increase in those subjected to the two highest concentrations (739.6 and 1479.2 ng L^{-1}), compared with control (One-Way ANOVA on Ranks followed by a Dunn's test: H_5=86.617, p<0.001). In the final dark period (900 to 1200s), there was a

significant increase in individuals exposed to the three highest concentrations (369.8, 739.6 and 1479.2 ng L^{-1}), compared with control (One-Way ANOVA on Ranks followed by a Dunn's test: H_5=61.514, p<0.001).

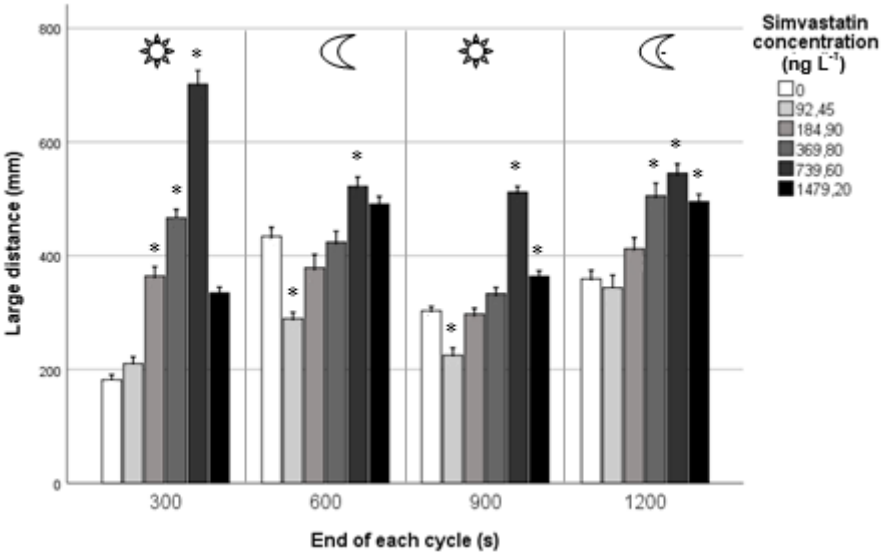

Figure 4 - Large distance traveled for individuals exposed to SIM, in cycles of light and dark periods. Data are Mean±SE (n=20 fish/treatment). ☼ *Stands for light cycles while* ☾ *stands for dark periods. * Stands for significant differences among treatments, compared with control, during the respective light or dark period (Dunnett's test in the first light cycle and Dunn's test in the other periods, p<0.05).*

According to the results of swimming time measured in fish exposed to SIM, there were significant differences between treatment groups and the control, in the light and dark cycles (Figure 5). During the first light cycle (0 to 300s), there was a significant decrease in organisms exposed to the intermediate (369.8 ng L^{-1}) and second highest concentration (739.6 ng L^{-1}), compared with control (One-Way ANOVA on Ranks followed by a Dunn's test: H_5=18.426, p=0.002). During the first dark period (300 to 600s), there was a significant decrease in the organisms exposed to the two lowest concentrations (92.45 and 184.9 ng L^{-1}), followed by a significant increase in the two highest concentrations (739.6 and 1479.2 ng L^{-1}), compared with control (One-Way ANOVA on Ranks followed by a Dunn's test: H_5=99.196, p<0.001). During the second light cycle (600 to 900s), no significant differences were found between animals from the treatment groups and from the control. In the final dark cycle (900 to 1200s), there was a significant

decrease in individuals exposed to the lowest concentration (92.45 ng L^{-1}), followed by a significant increase in those subjected to the two highest concentrations (739.6 and 1479.2 ng L^{-1}), compared with control (One-Way ANOVA on Ranks followed by a Dunn's test: H_5=92.713, p<0.001).

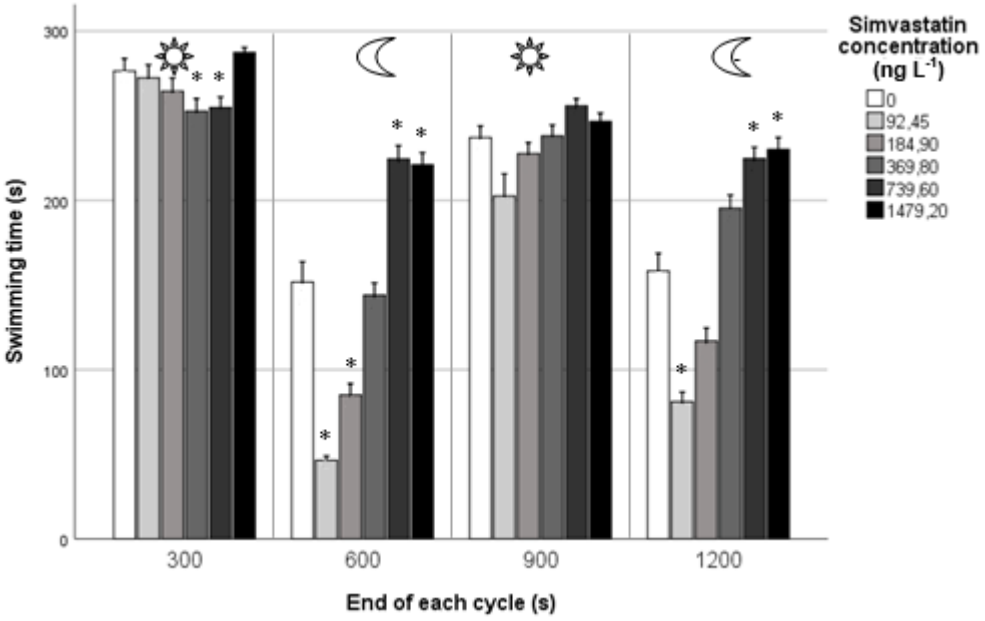

Figure 5 - Swimming time for individuals exposed to SIM, in cycles of light and dark periods. Data are Mean±SE (n=20 fish/treatment). ☼ *Stands for light cycles while* ☾ *stands for dark periods. * Stands for significant differences among treatments, compared with control, during the respective light or dark period (Dunn's test, p<0.05).*

Considering the total distance traveled by fish exposed to SIM, there were significant differences found between the treatments and the control group in all the light and dark periods (Figure 6). During the first light cycle (0 to 300s), there was a significant increase, particularly in the individuals exposed to the lowest (92.45 ng L^{-1}), intermediate (369.8 ng L^{-1}) and second highest concentration (739.6 ng L^{-1}), compared with control (One-Way ANOVA on Ranks followed by a Dunn's test: H_5=57.018, p<0.001). During the first dark period (300 to 600s), there was a significant decrease in the organisms exposed to the lowest concentration (92.45 ng L^{-1}), followed by a significant increase in those exposed to the two highest concentrations (739.6 and 1479.2 ng L^{-1}), compared with control (One-Way ANOVA on Ranks followed by a Dunn's test: H_5=82.244, p<0.001). During the second light cycle (600 to 900s), there was a significant decrease in the

individuals exposed to the lowest concentration (92.45 ng L^{-1}), followed by a significant increase in those exposed to the second highest concentration (739.6 ng L^{-1}), compared with control (One-Way ANOVA on Ranks followed by a Dunn's test: H_5=75.903, p<0.001). During the second and final dark period (900 to 1200s), there was a significant increase in the organisms exposed to the two highest concentrations (739.6 and 1479.2 ng L^{-1}), compared with control (One-Way ANOVA on Ranks followed by a Dunn's test: H_5=60.931, p<0.001).

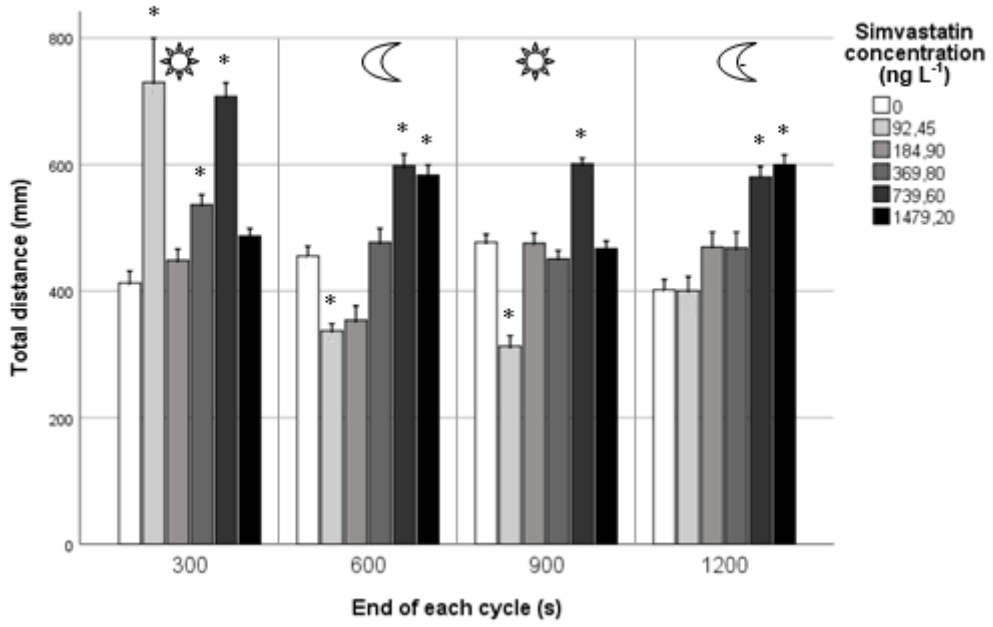

Figure 6 - Total distance traveled for individuals exposed to SIM, in cycles of light and dark periods. Data are Mean±SE (n=20 fish/treatment). ☼ *Stands for light cycles while* ☾ *stands for dark periods. * Stands for significant differences among treatments, compared with the control, during the respective light or dark period (Dunn's test, p<0.05).*

3.1.1.2. Biochemical results

3.1.1.2.1. Superoxide dismutase

According to the results obtained in terms of copper-zinc SOD (Cu-ZnSOD) activity in fish exposed to SIM, there was a significant decrease, except for the individuals exposed to the lowest concentration (92.45 ng L^{-1}), compared with control (One-way ANOVA followed by a Dunnett's test: $F_{5.15}$=37.728, p<0.001). Manganese SOD (MnSOD)

activity in the same individuals revealed no significant differences between treatment groups and the control (Figure 7).

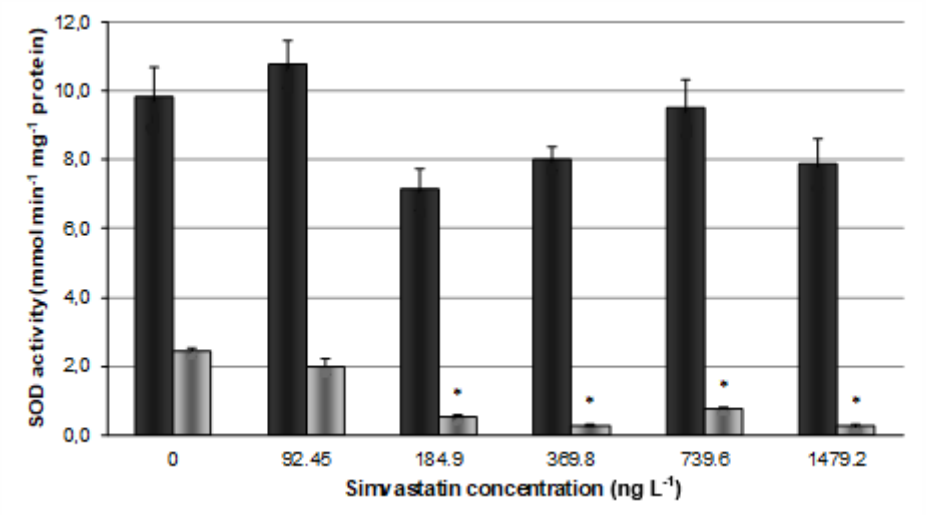

*Figure 7 – Cu-ZnSOD activity (grey) and MnSOD activity (black) in individuals exposed to SIM. Data are Mean±SE (n=20 fish/treatment). * Stands for significant differences among treatments, compared with control, in each SOD activity (Cu-Zn or Mn) (Dunnett's test, p<0.05).*

3.1.1.2.2. Catalase

Considering the results obtained in terms of the CAT activity in fish exposed to SIM, there were no significant differences found between the treatment groups and the control animals (One-way ANOVA: $F_{5.12}$=3.784, p=0.027) (Figure 8).

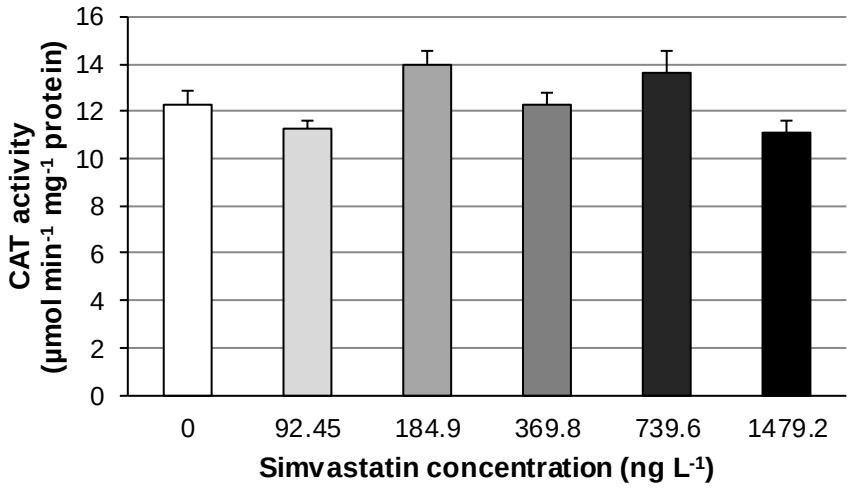

Figure 8 - CAT activity in individuals exposed to SIM. Data are Mean±SE (n=20 fish/treatment).

3.1.1.2.3. Glutathione peroxidase

According to the results of GPx total activity in fish exposed to SIM, there was a significant decrease, except for animals subjected to the lowest concentration (92.45 ng L^{-1}), compared with control (One-way ANOVA followed by a Dunnett's test: $F_{5.15}$=8.567, p<0.001). GPx selenium-dependent activity reported a significant increase in organisms exposed to the lowest concentration (92.45 µg L^{-1}), compared with control (One-way ANOVA followed by a Dunnett's test: $F_{5.13}$=16.685, p<0.001) (Figure 9).

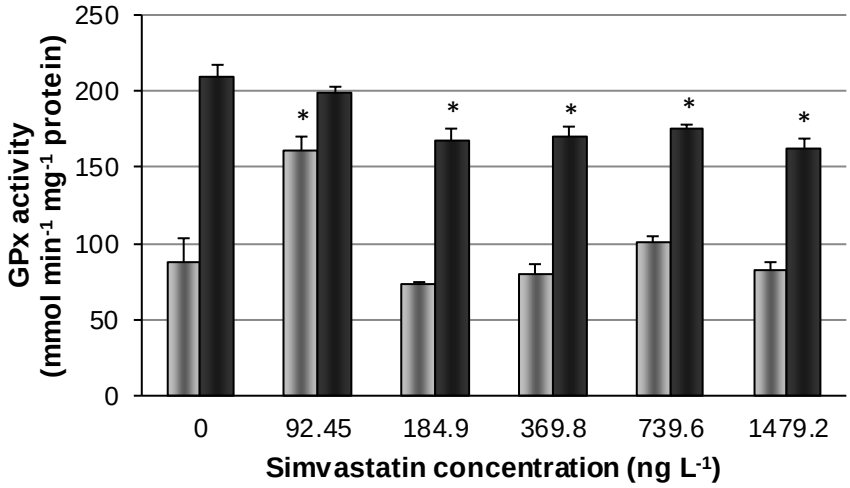

*Figure 9 - GPx total activity (black) and selenium-dependent activity (grey) in individuals exposed to SIM. Data are Mean±SE (n=20 fish/treatment). * Stands for significant differences among treatments, compared with control, in each GPx activity (total or selenium-dependent) (Dunnett's test, p<0.05).*

3.1.1.2.4. Glutathione S-transferases

Considering the GSTs activity of fish exposed to SIM, there was a significant decrease in animals of all the treatment groups, compared with control (One-way ANOVA followed by a Dunnett's test: $F_{5.18}$=17.955, p<0.001) (Figure 10).

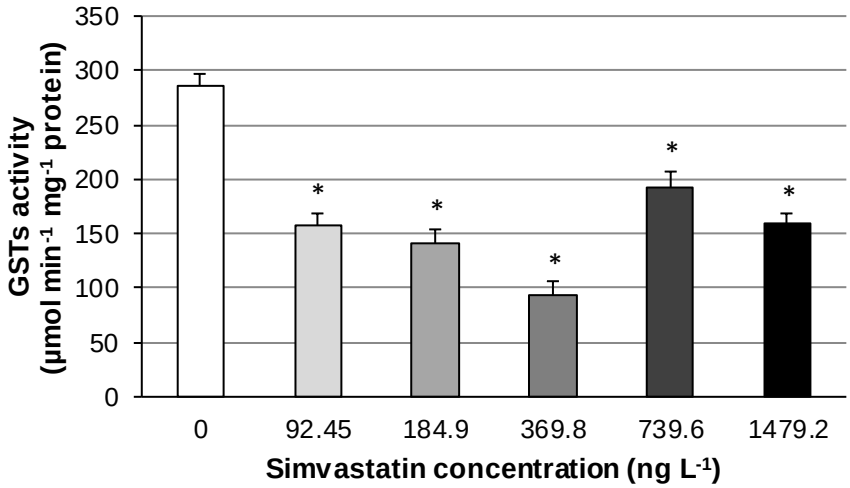

*Figure 10 – GSTs activity in individuals exposed to SIM. Data are Mean±SE (n=20 fish/treatment). * Stands for significant differences among treatments, compared with control (Dunnett's test, p<0.05).*

3.1.1.2.5. Thiobarbituric acid reactive substances

The results obtained in terms of TBARS in fish exposed to SIM, evidenced a significant decrease, except for the individuals subjected to the lowest concentration (92.45 ng L^{-1}), compared with control (One-way ANOVA followed by a Dunnett's test: $F_{5.13}$=12.107, p<0.001) (Figure 11).

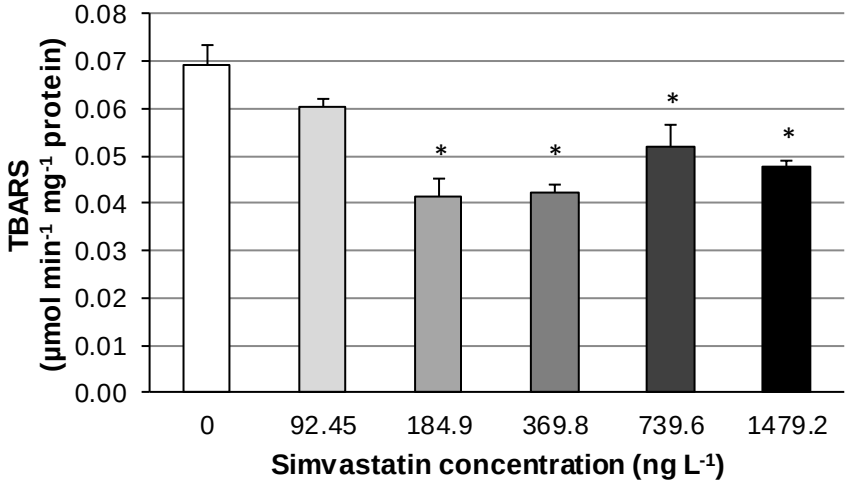

*Figure 11 - TBARS in individuals exposed to SIM. Data are Mean±SE (n=20 fish/treatment). * Stands for significant differences among treatments, compared with control (Dunnett's test, p<0.05).*

3.1.2. Chronic exposure

3.1.2.1. Sex determination

Taking into consideration the visual inspection of the HE glass slides of gonads, and after appropriate statistical treatment, no significant differences were found, in terms of sex determination (i.e. the percentage of males vs females) of the individuals exposed to SIM (χ^2=7.433, df=5, p=0.190) (Figure 12).

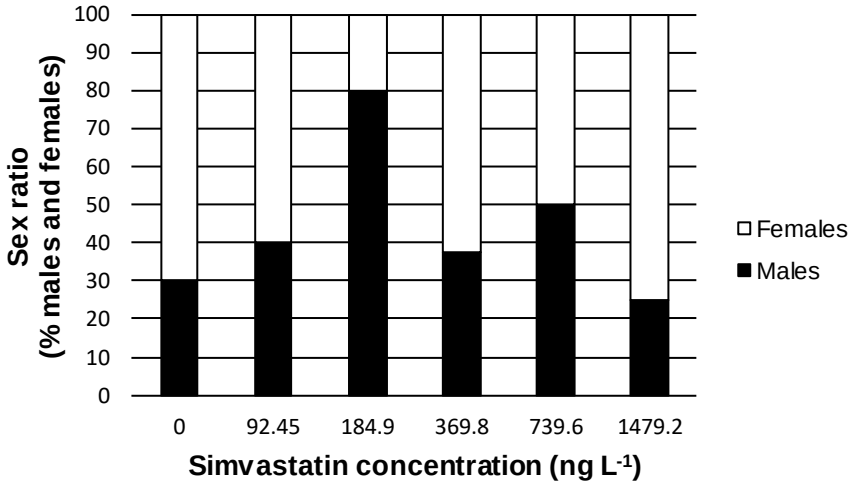

Figure 12 – Sex ratio (percentage of males and females) in individuals exposed to SIM (n=10 fish/treatment).

3.1.2.2. Gonadal developmental stages

Only four of the six stages of maturation were identified in females exposed to SIM, namely immature phase, early developing subphase, developing phase and spawning capable phase (Figure 13).

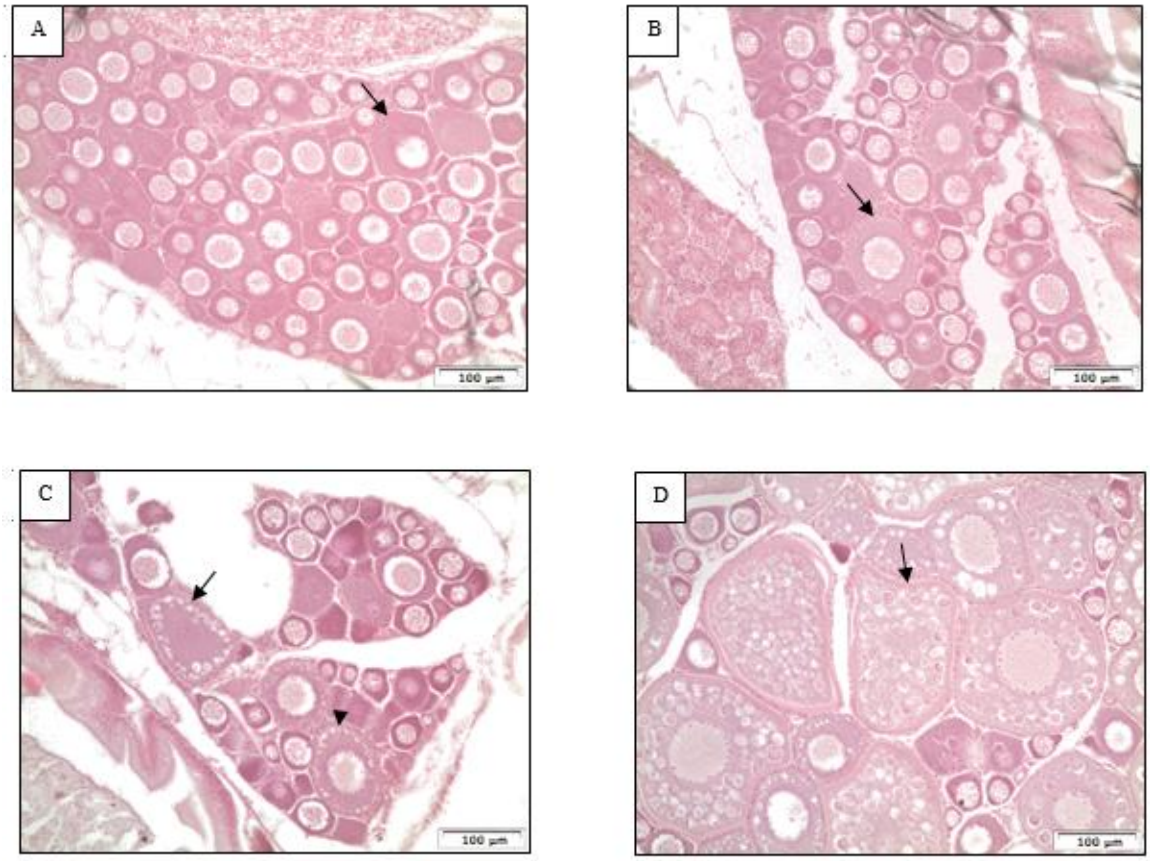

Figure 13 – Histological architecture in terms of maturation stages of females in different treatment groups exposed to SIM (n=10 fish/treatment). ***(A) Immature phase*** *at 1479.2 ng L^{-1}, with primary growth oocytes (arrow) (200x);* ***(B) Early developing subphase*** *at 184.9 ng L^{-1}, with cortical alveolar oocyte (arrow) (200x);* ***(C) Developing phase*** *at 92.45 ng L^{-1}, with primary vitellogenic oocyte (arrowhead) and secondary vitellogenic oocyte (arrow) (200x); and* ***(D) Spawning capable phase*** *at 92.45 ng L^{-1}, with tertiary vitellogenic oocyte (arrow) (200x).*

Regarding the recorded developmental stages, no significant differences were found among groups (G=1.913, df=15, p=0.999) (Figure 14).

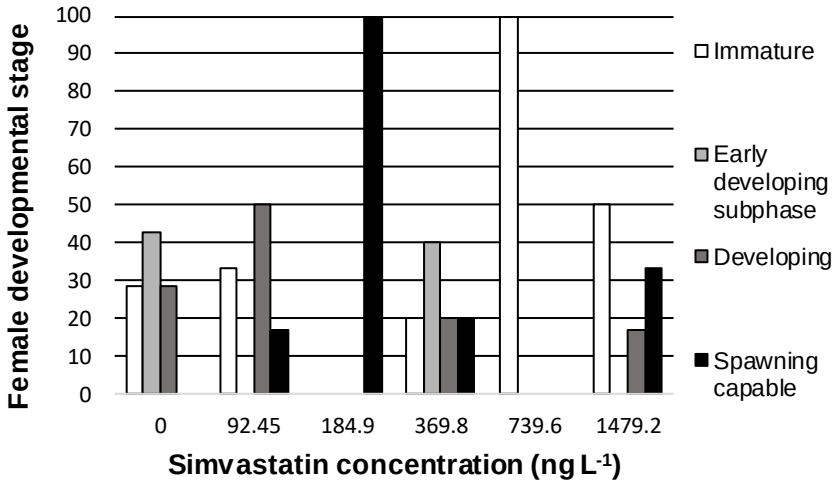

Figure 14 – Female developmental stage in individuals exposed to SIM (n=10 fish/treatment).

Only four of the six stages of maturation of spermatocytes were identified in males exposed to SIM, namely immature phase, early developing subphase, developing phase and spawning capable phase (Figure 15).

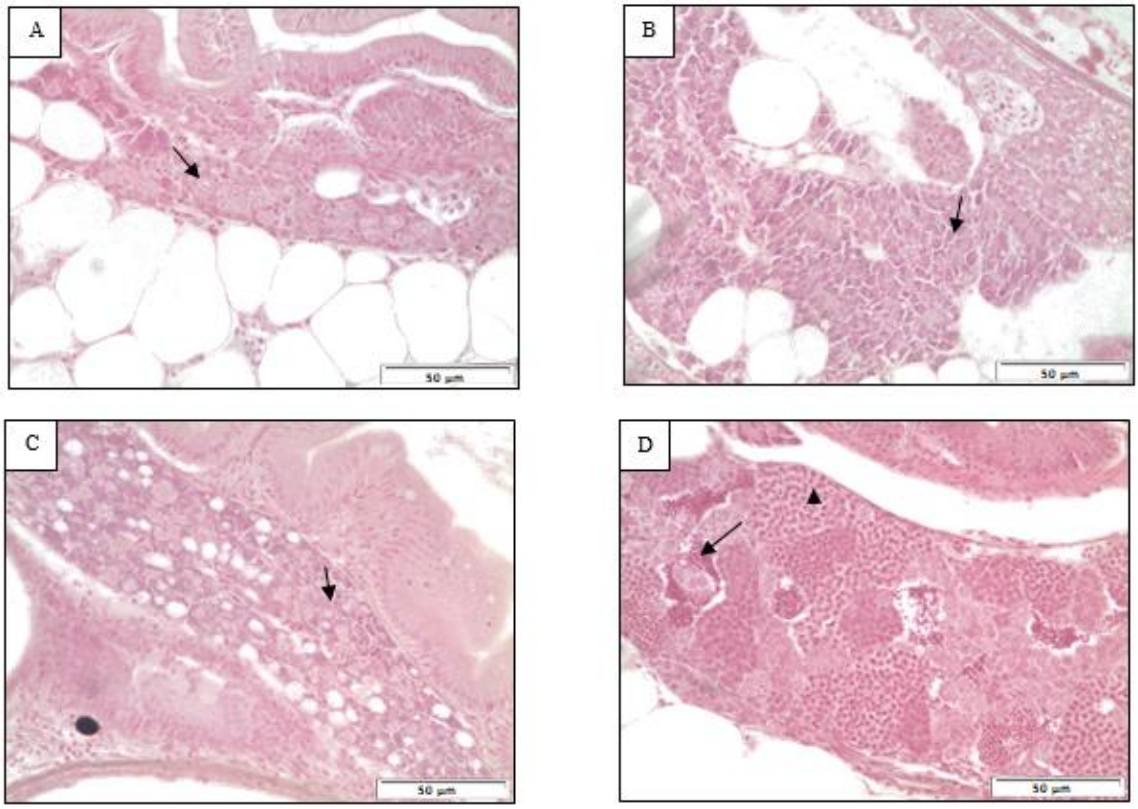

Figure 15 – Histological architecture in terms of maturation stages of spermatocytes identified in different treatment groups exposed to SIM (n=10 fish/treatment). ***(A) Immature phase*** *at 369.8 ng L^{-1}, with primary spermatogonia (arrow) (400x);* ***(B) Early developing subphase*** *at 184.9 ng L^{-1}, with primary spermatocyte (arrow) (400x);* ***(C) Developing phase*** *at 369.8 ng L^{-1}, with secondary spermatocyte (arrow) (400x); and* ***(D) Spawning capable phase*** *in the control group, with spermatids (arrowhead) and spermatozoa (arrow) (400x).*

Taking into consideration the observation of male developmental stages in individuals exposed to SIM, no significant differences were found (G=1.401, df=15, p=0.999) (Figure 16).

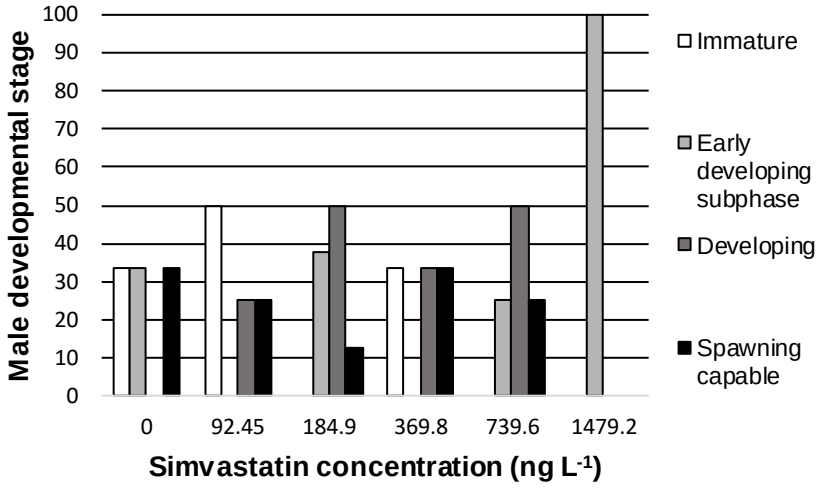

Figure 16 – Male developmental stage in individuals exposed to SIM (n=10 fish/treatment).

3.2. Clofibric acid

3.2.1. Acute exposure

3.2.1.1. Behavioral results

According to the results for small distance traveled by fish exposed to CA, there were significant differences between treatment groups and the control in all of the light and dark periods (Figure 17). During the first light cycle (0 to 300s), there was a significant decrease in organisms exposed to the three lowest concentrations (10.35, 20.7 and 41.4 µg L^{-1}), followed by a significant increase in fish subjected to the highest concentration,

compared with control (One-Way ANOVA on Ranks followed by a Dunn's test: H_5=159.151, p<0.001). During the first dark period (300 to 600s), there was a significant decrease in organisms exposed to second lowest (20.7 µg L^{-1}) and the intermediate concentration (41.4 µg L^{-1}), also followed by a significant increase, namely in those subjected to the two highest concentrations (82.8 and 165.6 µg L^{-1}), compared with control (One-Way ANOVA on Ranks followed by a Dunn's test: H_5=249.941, p<0.001). The second light cycle (600 to 900s) followed the pattern of the first light cycle, with a significant decrease in organisms exposed to the three lowest concentrations (10.35, 20.7 and 41.4 µg L^{-1}), followed by a significant increase in fish subjected to the highest concentration, compared with control (One-Way ANOVA on Ranks followed by a Dunn's test: H_5=95.409, p<0.001). Finally, in the last dark cycle (900 to 1200s), there was a significant decrease in the individuals exposed to the first three treatment groups, compared with the control group (One-Way ANOVA on Ranks followed by a Dunn's test: H_5=234.994, p<0.001).

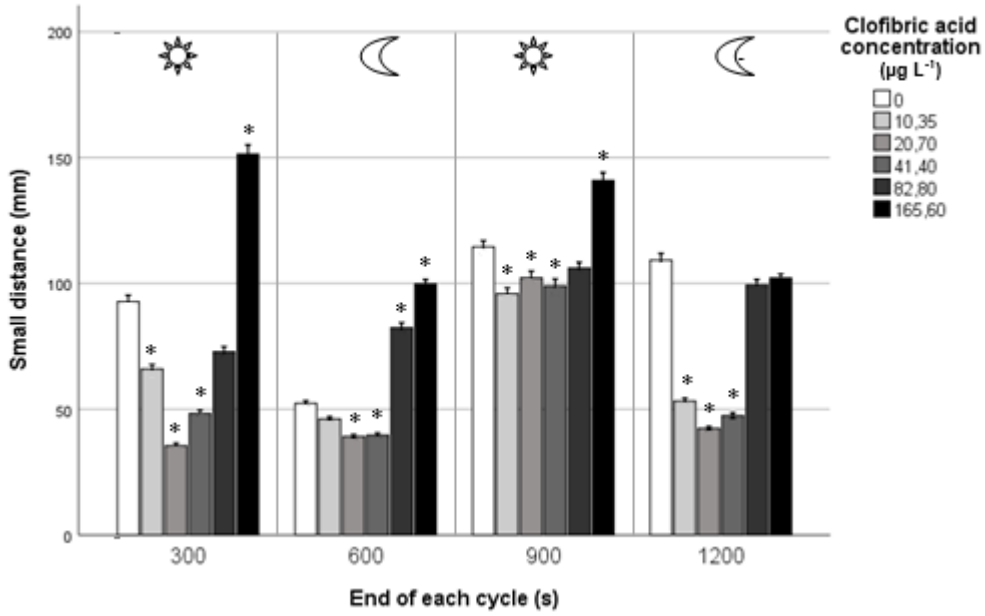

Figure 17 - Small distance traveled for individuals exposed to clofibric acid, in cycles of light and dark periods. Data are Mean±SE (n=20 fish/treatment). ☼ *Stands for light cycles while* ☾ *stands for dark periods. * Stands for significant differences among treatments, compared with the control, during the respective light or dark period (Dunn's test, p<0.05).*

Considering the results for large distance traveled by fish exposed to CA, there were significant differences between treatment groups and the control for all the light and dark periods (Figure 18). During the first light cycle (0 to 300s), there was a significant decrease in the large distance travelled in all treatment groups, except for organisms exposed to the second lowest concentration (20.7 µg L^{-1}), compared with control (One-Way ANOVA on Ranks followed by a Dunn's test: H_5=120.544, p<0.001). During the first dark period (300 to 600s), there was also a significant decrease in all treatment groups, except for individuals subjected to the highest concentration (165.6 µg L^{-1}), compared with control (One-Way ANOVA on Ranks followed by a Dunn's test: H_5=152.207, p<0.001). During the second light cycle (600 to 900s), there was also a significant decrease in all treatment groups, except for organisms exposed to the lowest concentration (10.35 µg L^{-1}), compared with control (One-Way ANOVA on Ranks followed by a Dunn's test: H_5=104.041, p<0.001). Finally, during the second dark period (900 to 1200s), there was a significant decrease in all treatment groups, compared with control, through a directly proportional increase between treatments, according to the concentration (One-way ANOVA followed by a Dunnett's test: $F_{5.251}$=46.171, p<0.001).

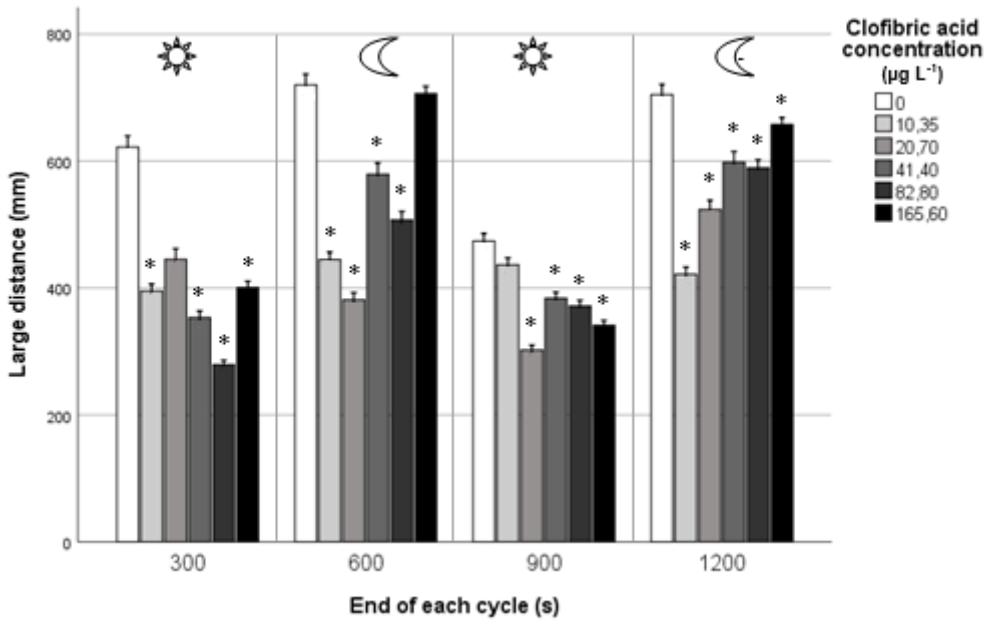

Figure 18 - Large distance traveled for individuals exposed to clofibric acid, in cycles of light and dark periods. Data are Mean±SE (n=20 fish/treatment). ☼ *Stands for light cycles while* ☾ *stands for dark periods. * Stands for significant differences among treatments, compared with the*

control, during the respective light or dark period (Dunn's test in the three first periods, and Dunnett's test in the last dark cycle, p<0.05).

According to the swimming time of fish exposed to CA, there were significant differences between treatment groups and the control, in the dark and light periods (Figure 19). During the first light cycle (0 to 300s), no significant differences were found between treatment groups and the control. During the first dark period (300 to 600s), there was a significant decrease between some of the treatment groups and the control, except for organisms exposed to the intermediate (41.4 µg L^{-1}) and the highest concentration (165.6 µg L^{-1}) (One-Way ANOVA on Ranks followed by a Dunn's test: H_5=118.803, p<0.001). For the second light cycle (600 to 900s), there was a significant decrease in organisms exposed to the lowest concentration (10.35 µg L^{-1}) and a significant increase in those subjected to the highest concentration (165.6 µg L^{-1}), following a directly proportional pattern of swimming time and concentration, compared with control (One-Way ANOVA on Ranks followed by a Dunn's test: H_5=57.447, p<0.001). For the final dark period (900 to 1200s), there was only a significant decrease in individuals exposed to the lowest concentration (10.35 µg L^{-1}), compared with control (One-Way ANOVA on Ranks followed by a Dunn's test: H_5=91.117, p<0.001).

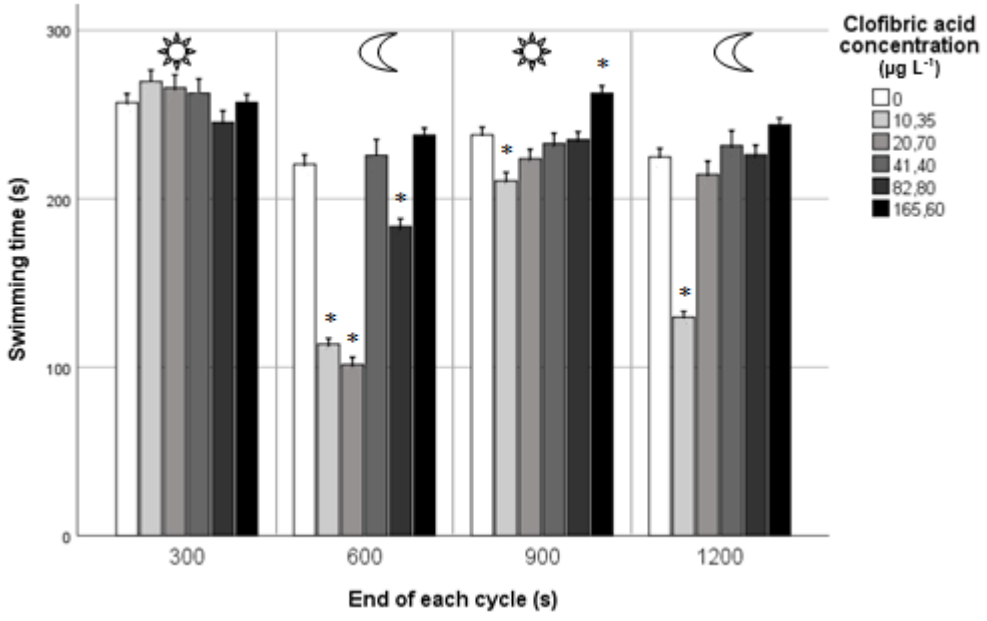

Figure 19 – Swimming time for individuals exposed to CA, in cycles of light and dark periods. Data are Mean±SE (n=20 fish/treatment). ☼ *Stands for light cycles while* ☾ *stands for dark periods. * Stands for significant differences among treatments, compared with control, during the respective light or dark period (Dunn's test, p<0.05).*

Considering the total distance traveled by fish exposed to CA, there were significant differences between fish from all treatment groups and the control treatment, in all the light and dark periods (Figure 20). During the first light cycle (0 to 300s), there was a significant decrease in all treatment groups, compared with control (One-Way ANOVA on Ranks followed by a Dunn's test: H_5=99.626, p<0.001). During the first dark period (300 to 600s), there was also a significant decrease, except for organisms exposed to the highest concentration (165.6 µg L^{-1}), compared with control (One-Way ANOVA on Ranks followed by a Dunn's test: H_5=172.918, p<0.001). During the second light cycle (600 to 900s), there was a significant decrease, except for individuals subjected to the lowest concentration (10.35 µg L^{-1}), compared with control (One-Way ANOVA on Ranks followed by a Dunn's test: H_5=53.170, p<0.001). During the second and final dark period (900 to 1200s), the pattern was similar to the first dark cycle, with a significant decrease, except for organisms exposed to the highest concentration (165.6 µg L^{-1}), compared with control (One-Way ANOVA on Ranks followed by a Dunn's test: H_5=163.561, p<0.001).

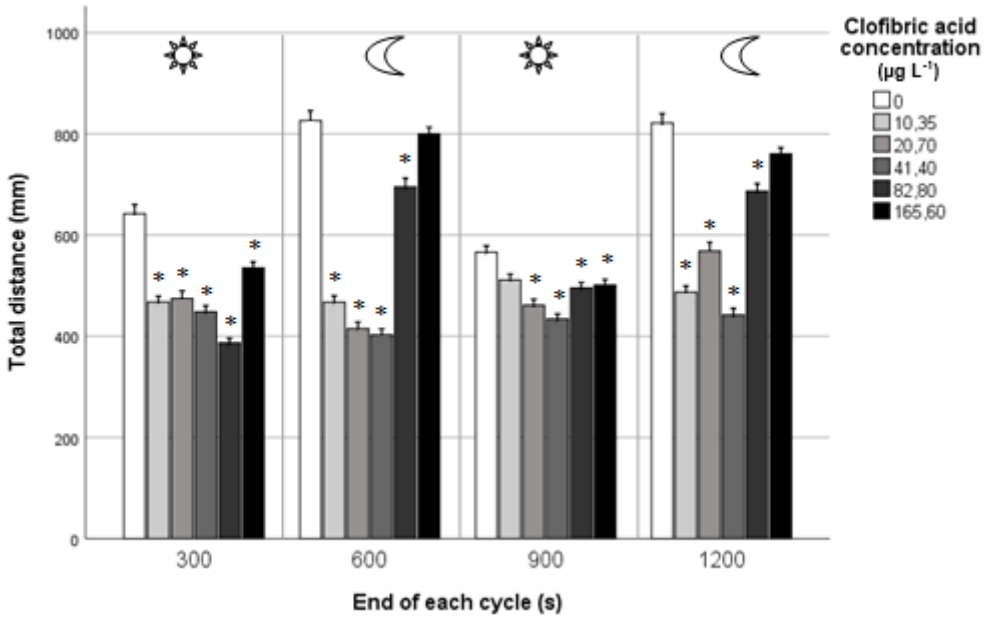

Figure 20 – Total distance traveled for individuals exposed to CA, in cycles of light and dark periods. Data are Mean±SE (n=20 fish/treatment). ☼ *Stands for light cycles while* ☾ *stands for dark periods. * Stands for significant differences among treatments, compared with control, during the respective light or dark period (Dunn's test, p<0.05).*

3.2.1.2. Biochemical results

3.2.1.2.1. Superoxide dismutase

Considering the results obtained for Cu-ZnSOD activity in fish exposed to CA, there was a significant increase, namely in animals subjected to the two highest concentrations (82.8 and 165.6 µg L^{-1}), compared with control (One-way ANOVA followed by a Dunnett's test: $F_{5.12}$=308.926, p<0.001). MnSOD activity in the same individuals showed a significant increase, particularly in fish from the intermediate (41.4 µg L^{-1}) and highest concentrations (165.6 µg L^{-1}), compared with control (One-way ANOVA followed by a Dunnett's test: $F_{5.13}$=12.917, p<0.001) (Figure 21).

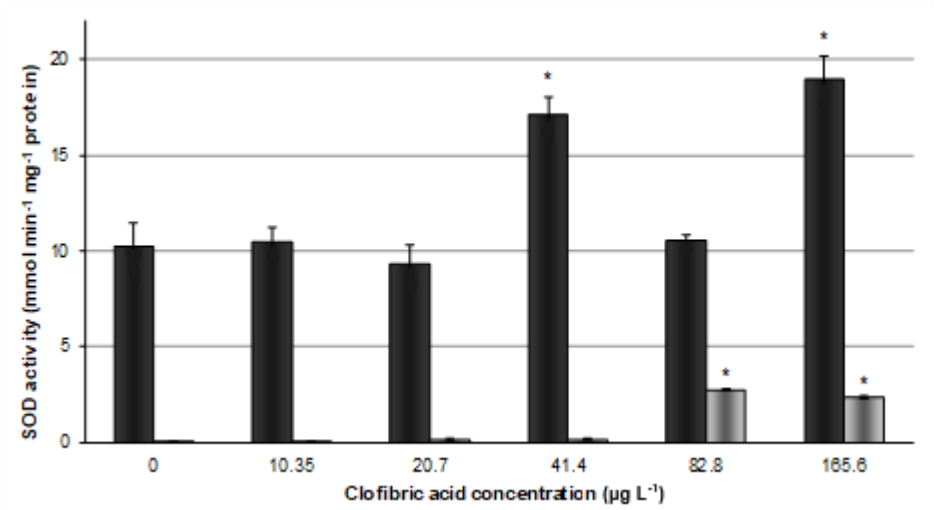

*Figure 21 – Cu-ZnSOD activity (grey) and MnSOD activity (black) in individuals exposed to CA. Data are Mean±SE (n=20 fish/treatment). * Stands for significant differences among treatments, compared with control, in each SOD activity (Cu-Zn or Mn) (Dunnett's test, p<0.05).*

3.2.1.2.2. Catalase

Considering the CAT activity in fish exposed to CA, there was a significant increase for animals exposed to the second lowest concentration (20.7 µg L^{-1}), followed by a significant decrease in fish exposed to the highest concentration (165.6 µg L^{-1}) (One-way ANOVA followed by a Dunnett's test: $F_{5.17}$=11.657, p<0.001) (Figure 22).

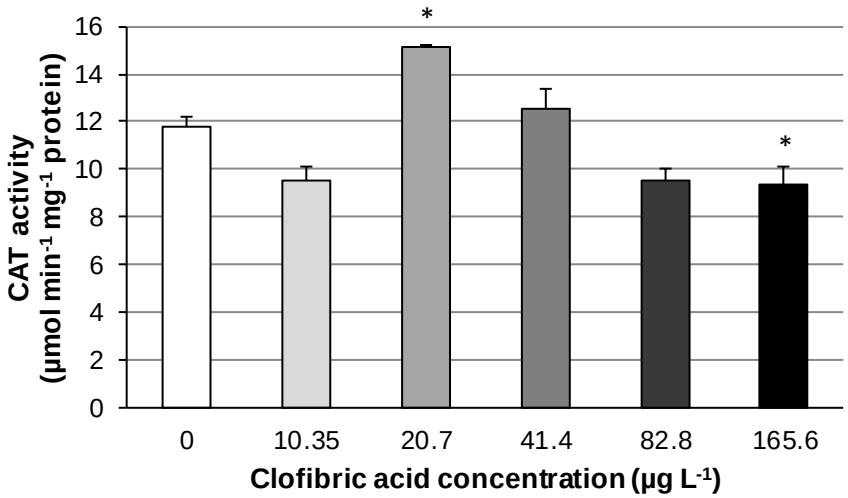

*Figure 22 - CAT activity in individuals exposed to CA. Data are Mean±SE (n=20 fish/treatment). * Stands for significant differences among treatments, compared with control (Dunnett's test, p<0.05).*

3.2.1.2.3. Glutathione peroxidase

Considering the total GPx activity in fish exposed to CA, there was a significant increase for animals exposed to the intermediate concentration (41.4 µg L^{-1}), compared with control (One-way ANOVA followed by a Dunnett's test: $F_{5.13}$=8.123, p=0.001). Considering the selenium-dependent GPx activity, there was a significant increase in organisms subjected to the intermediate (41.4 µg L^{-1}) and highest concentrations (165.6 µg L^{-1}), compared with control (One-way ANOVA followed by a Dunnett's test: $F_{5.14}$=7.507, p=0.001) (Figure 23).

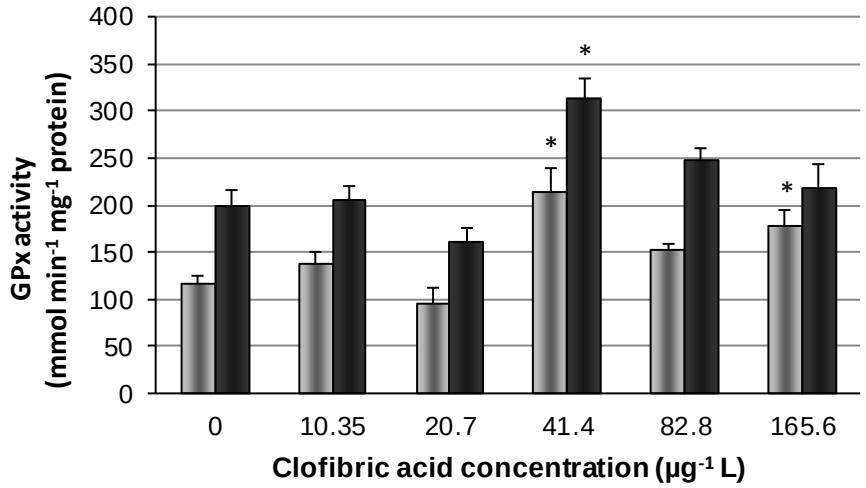

*Figure 23 – Total (black) and selenium-dependent (grey) GPx activity in individuals exposed to CA. Data are Mean±SE (n=20 fish/treatment). * Stands for significant differences among treatments, compared with control, in each GPx activity (total or selenium-dependent) (Dunnett's test, p<0.05).*

3.2.1.2.4. Glutathione S-transferases

In terms of the GSTs activity obtained in fish exposed to CA, there was a significant increase for animals exposed to the second lowest (20.7 µg L^{-1}) and intermediate concentrations (41.4 µg L^{-1}), followed by a significant decrease reported in fish exposed to the highest concentration (165.6 µg L^{-1}), compared with control (One-way ANOVA followed by a Dunnett's test: $F_{5.13}$=27.634, p<0.001) (Figure 24).

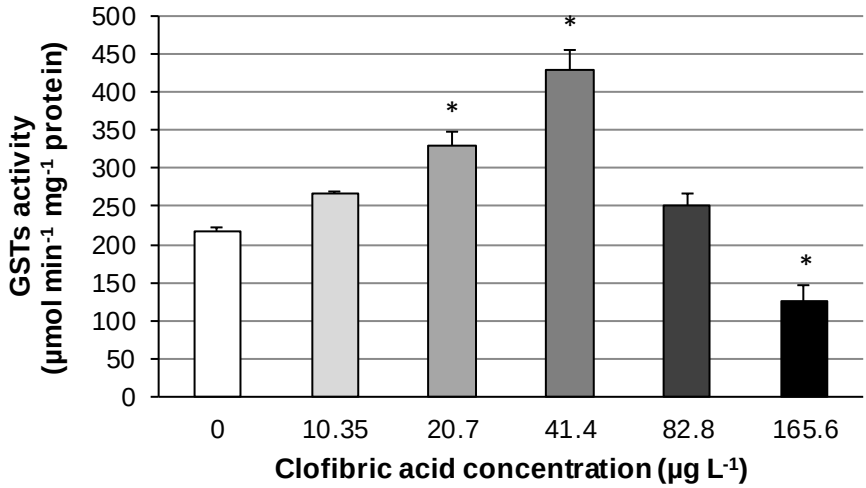

*Figure 24 - GSTs activity in individuals exposed to CA. Data are Mean±SE (n=20 fish/treatment). * Stands for significant differences among treatments, compared with control (Dunnett's test, p<0.05).*

3.2.1.2.5. Thiobarbituric acid reactive substances

Considering the TBARS in fish exposed to CA, there was a significant increase in animals exposed to the intermediate (41.4 µg L^{-1}) and highest concentrations (165.6 µg L^{-1}) compared with control (One-way ANOVA followed by a Dunnett's test: $F_{5.14}$=11.036, p<0.001) (Figure 25).

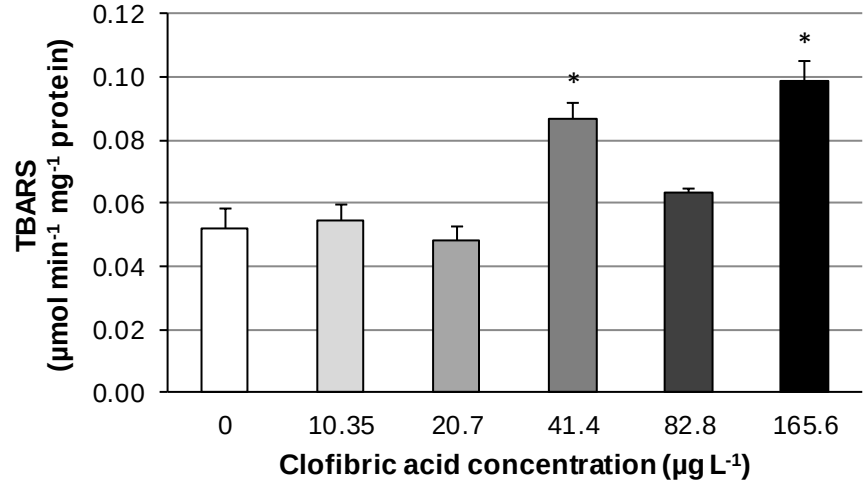

*Figure 25 – TBARS in individuals exposed to CA. Data are Mean±SE (n=20 fish/treatment). * Stands for significant differences among treatments, compared with control (Dunnett's test, p<0.05).*

3.2.2. Chronic exposure

3.2.2.1. Sex determination

Taking into consideration the visual inspection of the HE glass slides of gonadal tissue, and following appropriate statistical treatment, no significant differences were found between individuals exposed to CA, in terms of sex determination (χ^2=7.204, df=5, p=0.206) (Figure 26).

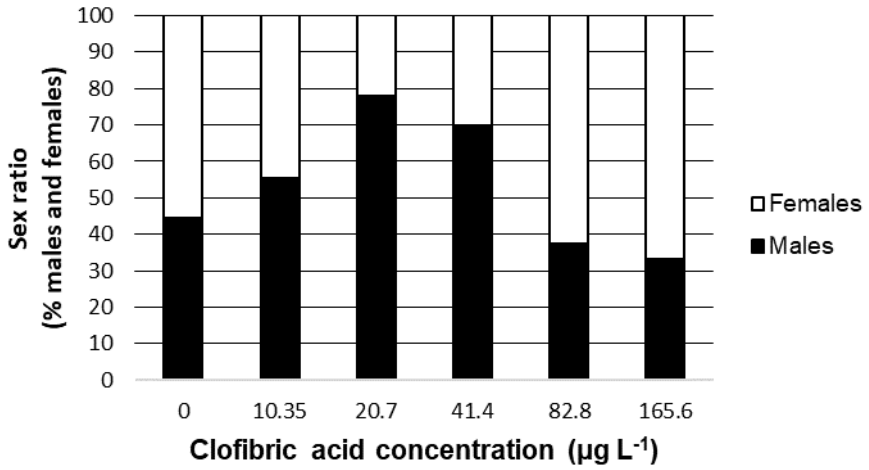

Figure 26 – Sex ratio (percentage of males and females) in individuals exposed to CA (n=20 fish/treatment).

3.2.2.2. Gonadal developmental stages

Only four of the six stages of maturation were identified in females exposed to CA, namely immature phase, early developing subphase, developing phase and spawning capable phase (Figure 27).

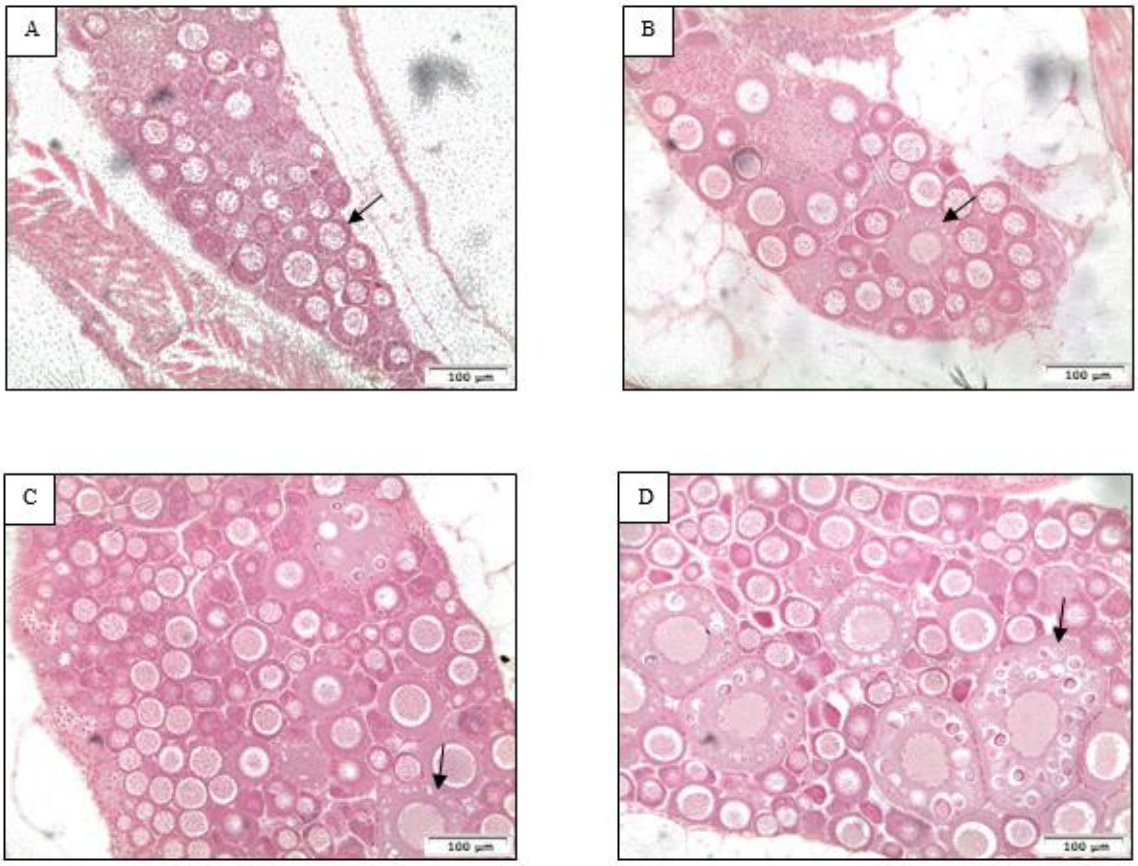

Figure 27 – Histological architecture in terms of maturation stages of females in different treatment groups exposed to CA (n=10 fish/treatment). ***(A) Immature phase*** *at 165.6 µg L^{-1}, with primary growth oocytes (arrow) (200x);* ***(B) Early developing subphase*** *in the control group, with cortical alveolar oocyte (arrow) (200x);* ***(C) Developing phase*** *at 10.35 µg L^{-1}, with primary vitellogenic oocyte (arrow) (200x); and* ***(D) Spawning capable phase*** *at 10.35 µg L^{-1}, with tertiary vitellogenic oocyte (arrow) (200x).*

Moreover, for individuals exposed to CA, no significant differences were found, in terms of maturation stages (G=0.662, df=15, p=1) (Figure 28).

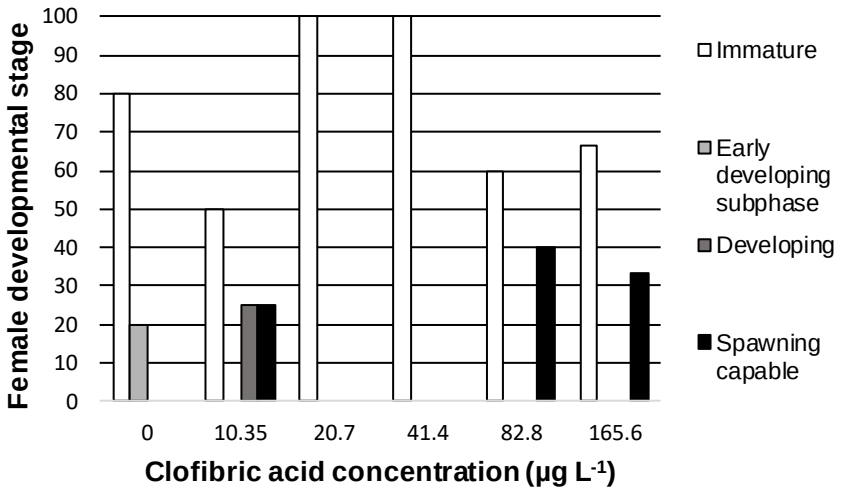

Figure 28 – Female developmental stage in individuals exposed to CA (n=10 fish/treatment).

Only two of the six stages of maturation were identified in males exposed to CA, namely the developing phase and spawning capable phase (Figure 29).

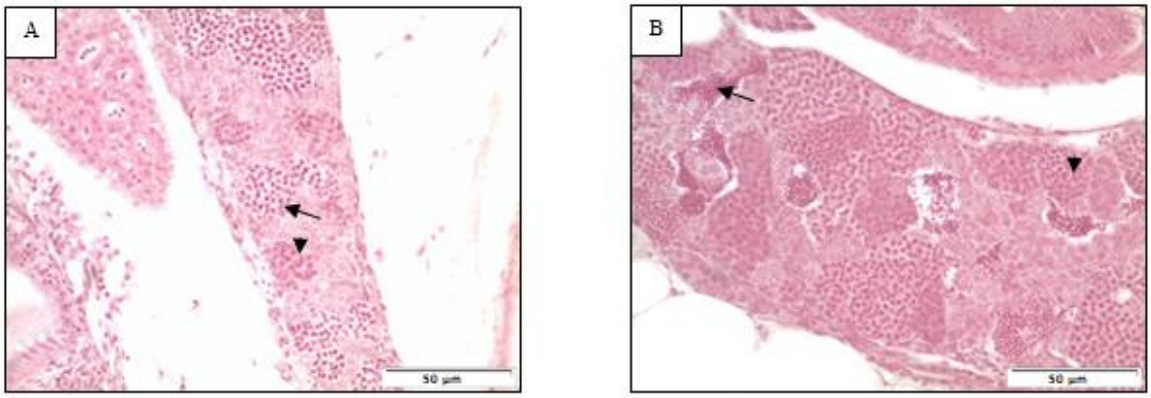

Figure 29 – Histological architecture in terms of maturation stages of spermatocytes identified in different treatment groups exposed to CA (n=10 fish/treatment). ***(A) Developing phase*** *at 20.7 µg* L^{-1}*, with primary spermatocyte (arrowhead) and secondary spermatocyte (arrow) (400x); and* ***(B) Spawning capable phase*** *in the control group, with spermatids (arrowhead) and spermatozoa (arrow) (400x).*

Taking into consideration the observation of male developmental stages in individuals exposed to CA, no significant differences were found, in terms of maturation stages (G=0.753, df=5, p=0.979) (Figure 30).

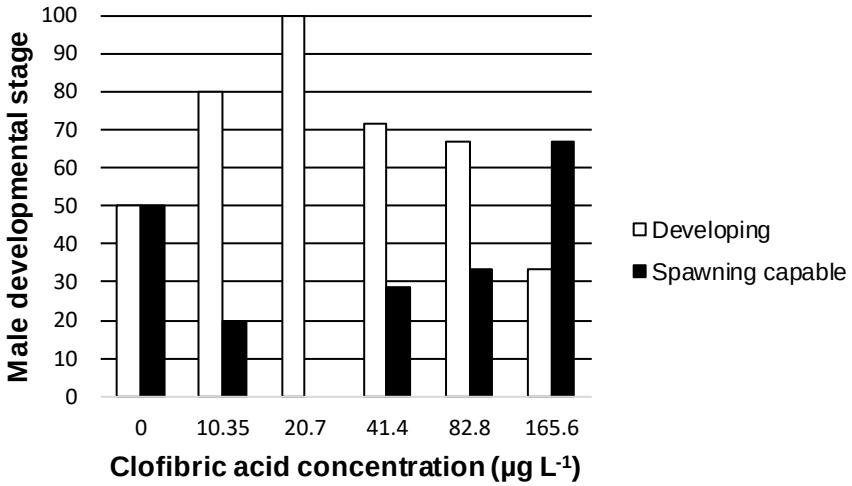

Figure 30 – Male developmental stage in individuals exposed to CA (n=10 fish/treatment).

4. DISCUSSION

4.1. Simvastatin

4.1.1. Behavioral assessment

To our knowledge, there are no previous data from the literature regarding the effects of simvastatin in locomotor ability in early life stages of zebrafish in response to stimulation in light-dark transition.

According to the results obtained for small distance traveled, in individuals exposed to SIM, the general patterns observed were a decrease in the first light period, an increase in the first dark cycle, and a decrease in the second light and dark periods, compared with the control group. Regarding the results obtained in terms of large distance traveled in individuals exposed to SIM, the overall patterns reported were an increase in the first light period, a decrease in the first dark cycle and an increase in the second light and dark periods, compared with control organisms. Overall, small and large distance traveled seem to have a proportionally inverse relation. Generally, small distance traveled (or small movements) decreased in individuals exposed to SIM, compared with control, while large distance traveled increased.

According to the results obtained for swimming time in individuals exposed to SIM, the general patterns observed were a decrease in the first light cycle, an increase in the first dark period, relatively similar values in the second light cycle and an increase in the second dark period, compared with control.

Regarding the results of total distance traveled in individuals exposed to SIM, the overall pattern reported was an increase in the first light and dark cycles, relatively similar values in the second light period, and an increase in the second dark cycle, compared with the control group.

Overall, there was a decrease in erratic movements (small distance) and an increase in the traveled large distance. In terms of swimming time and total distance traveled, even though there were significant differences in the organisms exposed to SIM.

As mentioned before, statins can inhibit HMG-CoA reductase, the rate-limiting enzyme in the mevalonic acid pathway (Santos et al., 2016). This pathway produces five-

carbon building blocks that lead to the formation of isoprenoids, e.g., coenzyme Q10 (CoQ10). In turn, CoQ10 is responsible for the production of ATP in various organs, that require a major demand for energy (Pasha & Moon, 2017). Thus, statins can lead to a decrease in the production of CoQ10, consequently leading to a decrease in the input of energy used for movement, such as evidenced in the parameter small distance traveled. Campos et al. (2016) also reported decreasing movement in zebrafish embryos exposed to SIM in the range of 0.3 nM to 10 µM. It was inferred in this assay, that the decrease in locomotion can also be due to tissue damage, particularly in muscles (Campos et al., 2016). It was evidenced by a recent study that, even in low concentrations, SIM can affect cytoskeleton and adhesion structures involved in myogenesis, by affecting cholesterol withdrawal (Campos et al., 2015). This effect occurs since cholesterol has structural and signaling functions in myogenesis. The capacity for the reduction in cholesterol levels by the exposure to SIM seems to induce a reduction in the amount of extracellular (laminin) and intracellular (vinculin and desmin) components present in the myofibrils, the composing subunits of muscles cells, which leads to shortening and thickening of its structure. SIM can also induce a reduction in the number of differentiated muscle cells (Campos et al., 2015). However, the decrease in small distance and increase in large distance in this assay suggest a reduction of anxiety-like behavior. Some pharmaceuticals with antioxidant properties, such as SIM, are known to decrease erratic movements of individuals, such as in this case, through the reduction of stress and anxiety-like behavior (Bouayed, 2011; Hassan et al., 2014; De Carvalho et al., 2019).

However, individuals showed also an increase in swimming time, as well as increased total distance traveled, which means that they spent more time swimming, while more distance was traveled. This indicates that simvastatin could have caused an increase in the overall activity of the organisms exposed. As mentioned before, simvastatin can increase the β-oxidation of fatty acids (Park et al., 2016). The increasing degradation of fatty acids leads to a higher input of energy in the individuals, leading to more locomotion (Orton & Parker, 1982). The metabolism of fatty acids can influence the behaviour of individuals, including fertilization and growth rate in zebrafish (Spence et al., 2008). Overall, data from behavioural analysis suggests that simvastatin potentiates the

locomotion of individuals, leading also to a decrease in erratic movements (small distance).

After 96h and when exposed to alternating light and dark periods, zebrafish larvae should present increased movement in the dark and decreased in the light (Maximino et al., 2010). However, in this essay, regarding total distance traveled and swimming time, the effect was the opposite, i.e., there was a decrease in movement in dark periods, compared with light periods (Burgess & Granato, 2007). The maturation of the zebrafish larvae blood-brain barrier occurs between 3 dpf and 10 dpf (Fleming et al., 2013). Statins have hydrophilic and hydrophobic areas, being classified as amphiphilic drugs (Tsinman et al., 2011). The hydrophobic region interacts with membrane phospholipids. On the other hand, this type of substance does not require specific transport to cross membranes, being soluble in aqueous biological fluids and membranes, diffusing through the individual (Fong, 2014). SIM is mostly characterized as a lipophilic statin, which has a higher risk of neurological and neurocognitive alterations because of its capacity to cross the blood-brain barrier (Fong, 2014). Lipids are vital for brain function, as the decrease in serum lipid levels can affect the formation of the myelin sheath, neuronal cell membranes, and synapses (McFarland et al., 2014). It has been demonstrated in *Oreochromis niloticus,* that the reduction of cholesterol levels by statins, can affect the structure of neural cells and neurochemical processes, by compromising the effectiveness of the central serotonergic system, leading to behavioral changes (Aguiar & Giaquinto, 2018). A feature already described for fish, altering their capacity to distinguish between light and dark cycles, can be due to neuronal and metabolic disruption, such as mentioned. Overall and considering all the behaviour endpoints determined in this study, simvastatin seemed to increase locomotion in the individuals exposed, especially large distance traveled, and reduce anxiety like-behaviour (small distance).

Moreover, there was a discrepancy between the observed effects when animals were exposed to lower and higher doses of SIM in terms of behavioral assessment, i.e., in fish subjected to lower doses, parameters such as small distance, large distance, and total distance traveled, as well as swimming time, were significantly decreased; while in animals exposed to higher concentrations, there was an increase, compared with control,

of such parameters. Dahl et al. (2006) studied growth-related sublethal endpoints in harpacticoid copepods, which can affect the locomotion of individuals. In this study, it was suggested that differences between effects in low and high concentrations can be due to different ecotoxicological modes of action of SIM, i.e., effects at higher concentrations (5 to 16 μg L^{-1}) seem to be related to energy-mediated processes, while effects in terms of endocrine disruption seem to affect the individuals in lower concentrations (<1.6 μg L^{-1}).

4.1.2. Biochemical assessment

As mentioned before, superoxide dismutase (SOD) can have three different forms: extracellular, MnSOD in the mitochondria, and Cu-Zn SOD in the cytoplasm (Lumb, 2017). It constitutes a primary protection system against ROS, particularly superoxide (O_2^-), and its activity results in the production of H_2O_2 and molecular oxygen (Hayyan et al., 2016).

According to the results obtained, there were no significant differences in MnSOD activity, while Cu-Zn SOD activity was significantly decreased, except for individuals exposed to 92.45 ng L^{-1}, comparing to the control. Cunha et al. (2016) also reported a decrease in SOD activity in zebrafish embryos exposed to 5 to 50 μg L^{-1} SIM. The decreasing activity of Cu-Zn SOD can be due to the antioxidant properties and synergism of SIM with antioxidants. It has been hypothesized that the mechanisms underlying these properties of SIM may be due to the inhibition of oxidant formation (NADPH-oxidase) and the increase in bioavailability of nitric oxide that can neutralize radicals (Stoll et al., 2004). The increase in antioxidant defenses, reduces significantly the quantity of ROS, leading to a decrease in antioxidant enzymes activity (Strzyzewski et al., 2013). Statins can decrease the production of superoxide anion through a direct effect on the enzyme's structure and consequently its function, thus reducing SOD activity (Delbosc et al., 2002; Wassmann et al., 2001). Decreased SOD activity can also be a response to increased production of H_2O_2 and O_2^-, by autooxidation of excess glucose and nonenzymatic glycation of proteins (Aragno et al., 1997). Moreover, hydroxyl radicals and hydrogen peroxide can lead to partial inactivation of SOD (Pigeolet et al., 1990).

As mentioned before, CAT ensures the removal of hydrogen peroxide from the organism (Lumb, 2017). It constitutes an antioxidant defense, located in the peroxisomes, by reducing H_2O_2 and molecular oxygen (Aebi, 1984; Modesto & Martinez, 2010). Regarding the results obtained in terms of CAT activity in individuals exposed to SIM, no significant differences were found, compared with control. The results from this study are not in agreement with previously reported data, where exposure to SIM caused an increase in CAT activity, in humans (Kaminsky et al., 2010; Piechota-Polanczyk et al., 2012). De Sotomayor et al. (2005) suggested that HMG-CoA reductase inhibitors, such as SIM, inhibit the gp91 phox-containing NADPH oxidase, which is involved in the generation of superoxide anions, and in turn increases CAT activity. In this case, and considering the absence of increase of CAT activity, the formation of H_2O_2 seems not to have been favoured by the exposure to SIM, despite previous indications. The antioxidant properties of SIM allied to the absence of production of H_2O_2, related to the inhibition of SOD activity, can explain the obtained results (Strzyzewski et al., 2013). The lack of significant results in our study can also be due to the duration of exposure used in our assay, justifying the contradictions in relation to other studies from the literature, which indicated an increase in CAT activity, conducted in humans (De Sotomayor et al., 2005; Piechota-Polanczyk et al., 2012). It was also suggested by Federici et al. (2007) that the absence of significant differences may be due to the capacity of the here used organism to use other antioxidant enzymes to avoid oxidative stress, namely GPx, to counteract H_2O_2 levels.

As mentioned above, GPx acts in a wider range of substrates when compared to CAT, reducing organic peroxides, unlike CAT, and reactive oxygen species originated by lipid peroxidation (Cohen & Hochstein, 1963; Flohé, 1985; Tappel, 1984). However, selenium-dependent GPx can reduce H_2O_2, while non-selenium dependent GPx does not (Battello, 2016). GPx selenium-dependent converts H_2O_2 and reduced glutathione into water and oxidized glutathione (Blondet et al., 2018).

According to the results obtained in individuals exposed to SIM, in terms of GPx total activity, there was a significant decrease at 184.9, 369.8, 739.6 and 1479.2 ng L^{-1}, while in the GPx selenium-dependent activity there was a significant increase in

individuals at 92.45 ng L^{-1}, compared with control. Some studies demonstrate that the exposure to SIM leads to a decrease in GPx total activity, in very distinct animal models, in organisms such as rodents (mg kg^{-1}) (Srinivasa Rao et al., 2012) and humans (10 mg day^{-1} during 8 months) (Ungureanu et al., 2003). The significant decrease in GPx total activity corroborates the above-mentioned results in terms of the antioxidant properties of SIM (Strzyzewski et al., 2013), which may contribute to a reduction of the activity of antioxidant enzymes, including GPx. Moreover, while CAT is present in the peroxisomes, GPx is located in the cytoplasm. Such as in the case of MnSOD, mostly found in the mitochondria, CAT activity was not significantly different from the individuals of the control group, which suggests that SIM did not act in the mitochondria to generate oxidative stress (Modesto & Martinez, 2010).

As far as we know, there are no studies regarding GPx selenium-dependent in aquatic organisms exposed to statins. The significant increase in selenium-dependent GPx activity in individuals exposed to the lowest concentration of SIM could represent an increase in H_2O_2 levels. However, in higher concentrations, the two mechanisms mentioned before, i.e. the inhibition of NADPH-oxidase and the increase of nitric oxide levels, could have lead to a decrease in ROS, including H_2O_2, which in turn produced non-significant results in GPx selenium-dependent activity above this concentration.

As mentioned before, GSTs are responsible for the conjugation of reduced glutathione with electrophilic centers present in endogenous and exogenous compounds, leading to the formation of hydrophilic compounds, that can be partially metabolized and excreted (Bradford, 1976; Modesto & Martinez, 2010).

According to the obtained results, individuals exposed to SIM experienced a significant decrease in GSTs activity, compared with control organisms. Apart from Cunha et al. (2016), that reported an increase in GSTs activity in zebrafish embryos after exposure to SIM, to the best of our knowledge, there are no other studies that investigate this enzyme's activity in aquatic organisms exposed to SIM. However, other studies have reported similar results to those encountered in this study, where GSTs levels decreased, namely in human patients with pancreatic damage, when treated with SIM (Matalka et al., 2013; Prokop'eva & Gulyaeva, 2000). There is evidence that in phase 2 of metabolism,

SIM undergoes glucuronidation, i.e., glucuronic acid conjugation by UDP-glucuronosyltransferases (UGTs) (Prueksaritanont et al., 2002), which leads to the conclusion that SIM probably is not metabolized via GSTs. So, SIM should not be metabolized in *D. rerio* via GSTs.

Alternatively, the role of GSTs is not entirely related to phase II (conjugation) metabolism, since GSTs can also prevent the interaction of xenobiotics with nucleic acids and proteins, also protecting against oxidative stress and damage (Dzoyem et al., 2014; Rahman, 2007; Smith et al., 2013). GSTs are thus antioxidant enzymatic forms that play a protective role. The lack of modification of GSTs levels reinforces the previously made assumption, concerning the putative absence of clear pro-oxidative effects. In that way, as it was mentioned before for SOD and GPx enzymes activity, the antioxidant properties of SIM could have led to the hereby observed results.

As mentioned before, oxidative damage can lead to lipid peroxidation, where free radicals interact with lipids containing carbon-carbon double bonds, particularly polyunsaturated fatty acids (Halliwell, 2009; Sies, 1985). Considering the results obtained for lipid peroxidation there was a significant decrease in this parameter in individuals exposed to SIM in levels of 184.9, 369.8, 739.6 and 1479.2 $ng^{.1}$ L, compared with control. As far as we know, there are only a few studies that investigated lipid peroxidation, being in the context of treatment of a specific condition with statins and conducted only in humans and rodents. Some of these studies reported a decrease in lipid peroxidation, through oral administration of SIM, at dosages of 10 mg kg^{-1} (Mohamadin et al., 2011) and administration of 20 mg day^{-1} during 4 to 12 weeks (Broncel et al., 2006), both studies were conducted in humans. The decrease in lipid peroxidation can allow inferring the decrease in ROS levels, through the alteration in the process of interaction of free radicals with polyunsaturated fatty acids, leading to a decrease in malondialdehyde-like compounds. Compounds with antioxidant properties, such as SIM, have been shown to lead to less oxidative damage linked to lipid peroxidation (Venturini et al., 2010; Golbidi et al., 2011).

4.1.3. Sex determination and gonadal developmental stages

This study was conducted with the primary goal of understanding the potential impacts of chronic and acute exposure of simvastatin and clofibric acid in *Danio rerio*.
The number of studies about the toxicity of simvastatin in aquatic species in terms of endocrine disruption is scarce (Dahl et al., 2006; Neuparth et al., 2014), and none of them was performed with *Danio rerio*.

Zebrafish gonadal differentiation initiates with a juvenile ovary (Liew & Orbán, 2014). In zebrafish, genetic sex-determining systems, as well as environmental factors seem to regulate the gender of the individuals (Devlin & Nagahama, 2002; Liew et al., 2012), such as dissolved oxygen (hypoxia) (Shang et al., 2006) and temperature (Abozaid et al., 2011; Abozaid et al., 2012; Uchida et al., 2004). At 15 dpf, the juvenile ovary develops into ovaries or testes (Pradhan & Olsson, 2016).

According to the results obtained in terms of sex determination in individuals exposed to SIM, no significant differences were found between groups. As far as we know, no studies were yet conducted in terms of sex ratio in organisms exposed to this pharmaceutical. However, a few studies reported development disruption in *Gammarus locusta* (Neuparth et al., 2014) and harpacticoid copepods (Dahl et al., 2006). HMG-CoA reductase inhibitors, such as statins, are thought to affect sex hormone biosynthesis, by inhibition of synthesis of cholesterol, a precursor of estradiol and androstenedione (Ser et al., 2010). LDLs biosynthesis seems also to be affected by statins. As mentioned before, HMG-CoA reductase catalyzes the conversion of HMG-CoA into mevalonic acid. In that way, SIM's inhibition of HMG-CoA reductase leads to a decrease in the production of LDL (Rang et al., 2007). LDL is mainly a precursor for ovarian steroid biosynthesis (Vandeputte et al., 2007). Thus, statins properties of reduction of LDL can result in a decrease in sex hormones, such as androgens and estrogens (Pradhan & Olsson, 2016). In zebrafish, estrogen induces ovarian differentiation and maintains femaleness in adulthood, while androgen induces testicular differentiation (Pradhan & Olsson, 2016). This means that exposure to statins could cause a disruption in the development and gonadal differentiation of individuals. Considering the lack of significant results in terms of sex determination, and the differences from the results of this assay from others in terms of

the development of individuals exposed to SIM, we can assume that major differences in terms of development alterations may be due to the distinct levels tested. Development changes may only be attained when SIM is present in high levels, such as in these investigations, where SIM was used at mg L^{-1} range, well above the ecologically relevant concentrations to which zebrafish were exposed in this assay (ng L^{-1} range). Moreover, taxonomic differences between the tested species and those used in the above-mentioned investigations could also lead to differences in terms of endocrine disruption.

As far as we know, there are no assays that investigate particularly gonadal maturation after exposure to statins. Zebrafish is an asynchronously spawning fish, which means that oocytes and spermatocytes can be observed at various stages of development in each individual, which in turn characterize each maturation stage (Okuthe et al., 2014). As mentioned before, SIM can decrease LDL levels, which can lead to a decrease in sex hormones, such as estrogen and androgen. Estrogen levels can alter gonadotropin secretion, which regulates oocyte maturation (Nagahama et al., 1995), while androgens stimulate spermatogenesis, i.e., spermatocyte maturation (Singh et al., 1995). Previous investigations have shown that statins can interfere with the reproductive development of individuals and are able to decrease testosterone levels (Bustan et al., 2017; Leite et al., 2014). Given the lack of results in terms of gonadal maturation phases in females and males exposed to SIM, we can assume that SIM did not have any effect in terms of reproductive disruption. Although the concentrations used in this assay were based on the maximum level found in effluents from WWTPs in Portugal, the range used was at ng L^{-1}, which compared to previous studies, might have been a low dosage to produce endocrine disruption effects. Moreover, SIM does not seem to have reproductive disruption effects, in terms of sex ratio and maturation stages.

Considering all the above-mentioned results, in terms of behavior, biochemical assessment, and reproductive disruption, in individuals exposed to SIM, we can conclude that this compound, in these particular conditions, caused alterations in the first two parameters determined, namely behavior and biochemical biomarkers. In terms of

behavioural alterations, SIM does not seem to have a well-defined mechanism of action, as behavioural alterations may derive from various sources, as mentioned before. Locomotion changes seem to be potentially due to alterations in energy deficits (biosynthesis of ATP) and neuronal changes, altering movement patterns. On the other hand, the biochemical assessment revealed decreasing activity in several enzyme activities, such as Cu-Zn SOD, GPx total activity, GSTs, and TBARS. The antioxidant properties of SIM might explain this phenomenon, since the presence of an antioxidant drug (such as SIM), may counteract the overproduction of ROS, consequently conducting to a decrease in the activity of antioxidant enzymes. In that way, the mechanisms behind these two biomarkers (behavior and biochemical) do not seem to have a relationship with one another. Reproductive disruption through changes in sex ratio and maturation stages of individuals exposed to SIM did not reveal any significant differences, compared with the control group. The ability of SIM to lower LDL levels should reduce the production of sex hormones, such as estrogen and androgen, which ultimately should lead to a lower number of juvenile females, through the decrease in estrogen, responsible for the maintenance of femaleness in adulthood. However, this outcome was not reported. Moreover, a lower concentration of estrogen and androgen should also lead to a decrease in ovarian and testicular differentiation, which should be translated in terms of maturation stages of individuals. Again, effects of this nature were not observed after exposing *D. rerio* to SIM under the proposed conditions.

4.2. Clofibric acid

4.2.1. Behavioral assessment

As mentioned before, fibrates can interact with hepatic PPARs, that regulate β-oxidation of fatty acids (Michalik et al., 2006). As far as we know, there are no previous investigations regarding the effects of clofibric acid in locomotor ability in early life stages of zebrafish in response to stimulation in light-dark transition.

According to the results obtained for small distance traveled in individuals exposed to CA, the general patterns observed were an increase in the first light period, an

increase in the first dark cycle and a decrease in the second light and dark periods, compared with control.

Regarding the results for large distance traveled in individuals exposed to CA, the overall pattern reported was a decrease in all light and dark cycles, compared with control organisms. Energy deficits can cause a decrease in more "vigorous movements" as shown by Henriques et al. (2016) in zebrafish exposed to gemfibrozil.

According to the results obtained in terms of swimming time in organisms exposed to CA, the general patterns observed were: relatively similar values in the first light cycle, a decrease in the first dark period, and similar values also for the second light and dark cycle, compared with the control group. Considering the results in terms of total distance traveled in individuals exposed to CA, the overall pattern reported was a decrease in all light and dark periods, compared with control. Overall, individuals exposed to CA spent more time swimming, however, comparing to the total distance traveled, we can assume that organisms swam slower, even though they had more activity than the control group. Overall, behavioral effects may result from CA's mode of action which resulted in a delay in the development, directly affecting the physiology and capacity of the individual to mobilize nutrients (energy) needed for movements. However, behavior changes in locomotor activities depend on various physiological systems (Scott & Sloman, 2004; Tierney, 2011). Behavior disruption detected for clofibric acid can, therefore, be derived from various sources. Substances that alter the metabolism of nutrients during development, such as CA, through changes in beta-oxidation pathways, can have relevant consequences on organogenesis (Embrandiri et al., 2016; Henriques et al., 2016). CA interacts with hepatic PPARs, leading to an increase of β-oxidation of fatty acids, and consequently to an increment in energy, that can be translated in higher activity of individuals, which did not happen in this case, maybe because of the short period of exposure to which animals were subjected (Kawashima et al., 1985). As lipid regulator, CA may delay embryo development, being more evident at 120 hpf, which can compromise the swimming ability of larvae (Henriques et al., 2016). The delayed consumption of the yolk sac can also delay the swim bladder inflation and gut morphogenesis, compromising locomotor ability (Raldúa et al., 2008). The inhibition of mobilization of nutrients and

energy from the yolk sac, as well as the decrease in lipoprotein levels by exposure to CA, may limit the capacity for locomotion (Raldúa et al., 2008).

The discrepancy between animals exposed to lower and higher doses, with a decrease followed by an increase in behavioral traits, maybe due to a delay in development in larvae exposed to higher doses of CA, which can lead to repercussions in locomotor behavior, as it has been described for gemfibrozil by Henriques et al. (2016).

4.2.2. Biochemical assessment

According to the results obtained in terms of Cu-Zn SOD activity, there was a significant increase in animals exposed to 82.8 and 165.6 µg L^{-1}, while MnSOD activity was also significantly increased at 41.4 and 165.6 µg L^{-1}, compared with control. Except for some studies with *Cyprinus carpio* (Corcoran et al., 2015) and *Artemia parthenogenetica* (Nunes et al., 2006), in which SOD activity was not altered, as far as we know, there are only a few number of studies that reported similar results in terms of SOD activity, namely in *Gambusia holbrooki exposed* to CA at a mg L^{-1} range during 96h (Nunes et al., 2008). Oxygen is consumed in several cellular locations such as the mitochondria, endoplasmatic reticulum and peroxisomes, where oxygen is reduced into hydrogen peroxide, which is further reduced to H_2O (Schrader & Fahimi, 2006). The high consumption of molecular oxygen, allied with the production of hydrogen peroxide and other free radicals, and the presence of various ROS-metabolizing enzymes, make peroxisomes a very important organelle in the production and scavenging of ROS, in particular, H_2O_2 (Schrader & Fahimi, 2006). β-oxidation of fatty acids also contributes to the generation of H_2O_2, which is mainly reduced by CAT into water and molecular oxygen (Boveris et al., 1972). The metabolic activity of peroxisomal oxidases leads to the production of ROS (Schrader & Fahimi, 2006). As clofibric acid is a peroxisome proliferator, it can cause an increase in peroxisome proliferation and β-oxidation of fatty acids, leading to the hyperproduction of ROS (Goel et al., 1986; Qu et al., 2001). In that way, CA can increase superoxide levels, which in turn increases SOD activity, which reduces superoxide into H_2O_2 and O_2.

Considering the results obtained in terms of CAT activity, there was a significant increase in test organisms exposed to 20.7 μg L^{-1}, followed by a significant decrease at 165.6 μg L^{-1}, compared with the control group. Several studies also reported an increase in CAT activity in individuals exposed to fibrates (Jones & Neill, 1982; Klucis et al., 1984; Yang et al., 1990). CAT has been used as an indicative parameter of peroxisome proliferation, that constitutes an antioxidant defense, located in the peroxisomes (Aebi, 1984; Modesto & Martinez, 2010). As a peroxisome proliferator, CA can induce CAT (Schrader & Fahimi, 2006). It has been suggested that peroxisome proliferators induce the hyperproduction of ROS, in particular, H_2O_2, which justifies the increase in CAT activity (Yeldandi et al., 2000). However, there was a decrease in CAT activity in animals exposed to the highest concentration of CA, which could suggest that other antioxidant defenses (such as GPx) could have been involved in the reduction of H_2O_2 concentrations, leading to lower levels of this peroxide in this case.

According to the results obtained in terms of total GPx activity, there was a significant increase at 41.4 μg L^{-1}, while selenium-dependent GPx activity reported a significant increase at 41.4 and 165.6 μg L^{-1}. To our knowledge, there are only a few studies that report effects in terms of GPx activity in organisms exposed to clofibric acid, and only some reported an increase in GPx total activity after exposure to ciprofibrate in different subcellular compartments (Dhaunsi, 1994), and an increase in GPx selenium-dependent in rodents exposed to gemfibrozil (O'Brien et al., 2001). On the other hand, there was also a study with *Gambusia holbrooki* that reported a significant inhibition of GPx in fish exposed to clofibric acid at the mg L^{-1} range (Nunes et al., 2008). As CA can stimulate beta-oxidation of fatty acids, with the release of hydrogen peroxide and superoxide, as well as other ROS, GPx increase may intend to reduce the levels of hydrogen peroxide (Perevoshchikova et al., 2013).

Considering the results obtained in terms of GSTs activity, there was a significant increase in fish exposed to 20.7 and 41.4 μg L^{-1} of CA, followed by a decrease at 165.6 μg L^{-1}, compared with the control group. There are only a few studies that report similar results as the ones observed, in organisms exposed to bezafibrate and gemfibrozil, such as *Mytilus galloprovincialis* at ng g^{-1} range (Canesi et al., 2007) and in *Cyprinus carpio*

exposed to clofibric acid at µg and $mg^{\cdot 1}$ L, where the expression of genes encoding GSTs increased (Corcoran et al., 2015). While GPx is more efficient in scavenging H_2O_2, GSTs have more affinity with other organic peroxides (Mannervik & Guthenberg, 1981; Levander, 1992). Moreover, H_2O_2 production in peroxisomes can also lead to the formation of other organic peroxides (Mennes et al., 1994). Thus, the increase in CAT activity can be related to an excess of H_2O_2, which in turn produces organic peroxides, which can also increase GSTs activity. GSTs are mainly enzymes of phase II biotransformation, as they can also indirectly infer oxidative stress. However, there is evidence that in phase II of metabolism, CA undergoes glucuronidation in fish, which may indicate that this compound is not metabolized strictly via GSTs (Walmsley, 1985). GSTs can also detoxify some of the secondary ROS formed when ROS reacts with cells. The oxidation of membranes generates toxic reactive compounds that GSTs are able to conjugate with glutathione (Veal et al., 2002). Three metabolites have been identified for clofibric acid: hydroxyisobutyric acid, lactic acid and 4-chlorophenol (Salgado et al., 2012). 4-chlorophenol is known to induce GSTs activity as well as the production of ROS (Tamburro et al., 2004), which may explain the hereby obtained results.

Regarding the results obtained in terms of lipid peroxidation, there was a significant increase in fish exposed to levels of 41.4 and 165.6 µg L^{-1}, when compared to the control. Some studies have reported similar results in terms of lipid peroxidation in rodents exposed to ciprofibrate (Goel et al., 1986) and in *Gambusia holbrooki* exposed to clofibric acid (Nunes et al., 2008). Clofibric acid and other peroxisome proliferators increase beta-oxidation of fatty acids and peroxisome proliferation, which may result in oxidative stress, with the occurrence of lipid peroxidation (Goel et al., 1986). Antioxidant defenses prevent the formation of ROS and prevent lipid peroxidation and DNA damage (Livingstone, 2003). However, oxidative stress can still arise, leading to lipid peroxidation. In this case, the results obtained seem to be due to the hyperproduction of ROS, as a consequence of the lack of capacity to neutralize oxidative stress through the previous oxidative stress preventive measures, resulting in peroxidative damage.

4.2.3. Sex determination and gonadal developmental stages

To our knowledge, there are few studies that report endocrine disruption in aquatic organisms exposed to clofibric acid, and none report the exposure of this pharmaceutical in *D. rerio*. As mentioned before, the fibrate's main function is to reduce VLDL and triglycerides, which reduces LDL and increases HDL. They are PPARα agonists, which activate lipoprotein lipase and decreases VLDL (Rang et al., 2007). Clofibric acid is the hydrolyzed form of clofibrate, being the active metabolite that circulates in plasma and believed to be responsible for the hypolipidemic properties of clofibrate (Daughton & Ternes, 1999).

According to the results obtained in terms of sex determination in individuals exposed to CA, no significant differences were found, compared with control. However, there are contradictory results, compared with other investigations, that report endocrine disruption (Runnalls et al., 2007; Coimbra et al., 2015). Apparently, fibrates can have estrogenic activity (Isidori et al., 2009), as there was a dose-dependent decrease of plasma testosterone in *Carassius auratus* exposed to 1.5 and 1500 g L^{-1} (Mimeault et al., 2006). This would mean a decrease in the number of males and an increase in the number of females. Fibrates, being PPARα ligands, can affect reproduction indirectly. They can interfere with biosynthesis and breakdown of cholesterol and fatty acids, which alters the availability of cholesterol for the production of steroids (Cameron et al., 2011; Velasco-Santamaría et al., 2011; Manibusan & Touart, 2017). Fibrates seem to impact in cholesterol movement, by interfering in the release of cAMP and the mitochondrial transport of cholesterol, limiting steps of steroidogenesis (Gazouli et al., 2002; Cameron et al., 2011). There was also a study that reported a male-biased population in individuals exposed to CA at 1 and 10 mg g^{-1} (through food) (Coimbra et al., 2015). This evidence could suggest that the exposure to CA would cause a disruption in development and, therefore, in gonadal differentiation of individuals. Considering the lack of significant results in terms of sex determination, and the differences from the results of this assay from others in terms of reproductive disruption in individuals exposed to CA, we can assume that major differences in terms of development alterations may be due to the variations in concentrations, exposure and model organisms tested.

Considering the results obtained in terms of female and male developmental stages, there were also no significant differences, compared with control. However, other studies reported different results in terms of female maturation stages, where there was a suppression of the expression of genes involved in steroidogenesis by exposure to fibrates (Toda et al., 2003). Ovarian follicle development is composed of two phases: follicular growth, where the immature oocyte grows in size, and a maturation stage, where immature oocytes transition into a mature egg (Selman et al., 1993). According to (Ibabe et al., 2005), there are three PPARs (α, β, and γ) in the zebrafish ovary. Their expression decreases follicular development (Ibabe et al., 2005). PPARα seems to be involved in regulating follicular growth and maturation (Komar et al., 2001; Komar, 2005). As PPARα agonists, fibrates can alter these processes (Rang et al., 2007). PPARs in Sertoli and Leydig cells seem to have a role in spermatogenesis and to be directly involved in maturation (Ibabe et al., 2005). Thus, PPAR ligands can affect spermatogenesis and sperm activities (Huang, 2008).

Differences in terms of tested organisms, duration of exposure and concentrations may explain the lack of results in terms of female and male maturation stages, after exposure to CA.

Considering all the above-mentioned results, in terms of behavior, biochemical assessment, and reproductive disruption, for individuals exposed to CA, we can conclude that this compound, in these particular conditions, caused alterations in the first two biomarkers (behavior and biochemical) determined.

In terms of behavioral alterations and according with the information mentioned before, the decrease in activity seems to be related to the lack of nutrient mobilization and energy, with lower lipoprotein levels, that were reflected in delayed development of the individual, and consequently of its swimming ability. (Handy et al., 1999), also reported that metabolic costs due to detoxification processes can also compromise locomotory responses of individuals, which could also explain the behavioral changes observed. On the other hand, the biochemical assessment revealed increased activity in several enzyme activities, such as SOD, CAT, GPx, which constitutes evidence for oxidative

stress. Moreover, GSTs increased activity also suggests the occurrence of conjugation and biotransformation (phase II) and the increase in lipid peroxidation levels (TBARS) indicate peroxidative damage in individuals. As mentioned before, the metabolic activity of peroxisomal oxidases leads to the hyperproduction of ROS. Moreover, CA can produce three metabolites, being one of them 4-chlorophenol, which is also known to induce the production of ROS. In that way, the increase in ROS levels leads to an increase in antioxidant enzyme activity. Reproductive disruption through changes in sex ratio and maturation stages of individuals exposed to CA did not reveal any significant differences, compared with the control group. As a PPAR ligand, CA should be able to affect spermatogenesis, as well as follicular growth and maturation, which does not seem to have happened in this case.

5. CONCLUSION

Ecotoxicological data for lipid-regulating drugs, such as simvastatin, are still lacking, and these drugs have only been confusingly characterized in terms of modes of action and consequences in aquatic organisms. This assay provided information about the ecotoxicity of two lipid-regulating drugs, simvastatin, and clofibric acid, in both embryonic and juvenile stages of *D. rerio*, which are of ecological relevance. Results from this study prove that zebrafish early-life stages and juvenile individuals can serve as model organisms in ecotoxicological assays (Scholz et al., 2008; Lammer et al., 2009). This species is easy to maintain, and behavioral responses can be monitored with high precision. Ecotoxicity assessment can be easily determined in zebrafish larvae, which makes it a relevant tool for this type of investigation. Lipid-regulating drugs, such as those used in this assay, are still lacking and confusingly characterized in terms of modes of action and consequences in aquatic organisms.

Overall, zebrafish larvae were more sensitive to the exposure to clofibric acid, which showed hypoactivity and prooxidant effects, while simvastatin caused hyperactivity, and antioxidant effects. Thus, oxidative stress responses, as well as lipid peroxidation effects, seem to be related to behavioral changes in individuals. In terms of reproductive disruption, no evident alterations were reported after the here-defined exposure conditions to both compounds.

Statins and fibrates have different properties, including their mechanism of action and especially metabolism, which could explain the distinct results obtained for both compounds. The results here obtained, with concentrations of ecological relevance of these two pharmaceuticals, can suggest deleterious effects in non-target individuals. Further investigations with other methods for endocrine disruption assessment in adult individuals exposed to simvastatin and clofibric acid, e.g. the quantification of vitellogenin content, should be relevant to determine the role of these lipid-lowering drugs in the reproductive disruption, if there is one. Moreover, cholinesterase activity could be a good endpoint to determine if there is a relationship between these enzymes activity and behavioral effects in both compounds.

In summary, this assay contributes to a better understanding of the effects of these lipid-regulating drugs, often present in the aquatic compartment, on zebrafish individuals. The tools here developed, overall seem to be highly responsive and may be relevant in the ecotoxicological assessment of xenobiotics in the aquatic environment.

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