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Chronic life-cycle studies with the priority pharmaceutical metformin: molecular and biochemical assessment with *Danio rerio*

Marta Duarte Ribeiro

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Marta Duarte Caldas Ribeiro

INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR

FACULDADE DE CIÊNCIAS

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Orientador – Doutora Maria Teresa Garrett Silveirinha Sottomayor Neuparth

Categoria – Investigadora Auxiliar

Afiliação – Centro Interdisciplinar de Investigação Marinha e Ambiental (CIIMAR)

Co-orientador – Professor Doutor Miguel Alberto Fernandes Machado Santos

Categoria – Professor Auxiliar com Agregação

Afiliação – Centro Interdisciplinar de Investigação Marinha e Ambiental (CIIMAR) / Departamento de Biologia da Faculdade de Ciências da Universidade do Porto

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Abstract

More than 451 million people worldwide have diabetes mellitus, and the vast majority is affected by type 2 diabetes (T2DM). Metformin (MET) is the first-line pharmaceutical to treat patients with T2DM. Therefore, it has become one of the most prescribed pharmaceuticals worldwide with a consequent emerging concern in rivers and coastal areas given that aquatic ecosystems are the final destiny of both treated and untreated waste waters.

Despite the ubiquitous nature of MET in the aquatic ecosystems, significant uncertainties exist about chronic effects of environmental concentrations of the drug in non-target aquatic organisms. Therefore, the main objective of this research is to integrate up-to-date ecological, biochemical and molecular endpoints to address the mode(s) of action of MET on the freshwater fish *Danio rerio* (zebrafish) after a full life-cycle exposure to three environmentally relevant concentrations of MET (361 ng/L, 2166 ng/L and 13000 ng/L). In this study, the underlying toxicity mechanisms of MET were investigated by the characterizing key morphometric endpoints (length, weight, condition factor, liver weight and hepatosomatic index of adult zebrafish), biochemical markers (cholesterol and triglycerides of adult zebrafish) and the transcription level of key genes, involved in the energetic metabolism of zebrafish larvae and adults.

The exposure to low concentrations of MET produced stimulating effects on growth parameters (length, weight and condition factor) of adult zebrafish, which suggest the occurrence of Hormesis. Despite the possible hormetic effects observed in growth and weight, liver weight and/or hepatosomatic index were significantly increased in adults, which seems to indicate that MET in these concentrations, is just near the toxicity threshold and hormesis. Biochemically, MET proved to be effective in reducing cholesterol levels in 9 mpf females and males, as well as in triglycerides levels in males. Most of gene expression results exhibited a non-monotonic dose-response curve with significant change in *acaca*, *idh3a*, *cox5aa* and *hmgcra* mRNA transcription levels in adults. In 20 dpf larvae, the *igf1* also showed significant changes. Our results also showed that the MET concentration that caused the most effects was the intermediate (2166 ng/L) and that females and males responded differently to MET exposure.

Taken together, these findings expand our understanding of MET effects in teleost fishes, demonstrating significant impacts at environmentally relevant concentrations and highlight the importance of addressing the effects of chemicals under chronic low-level concentrations. Additionally, this study provide important data for environmental risk

assessment, given that aquatic organisms are chronically exposed to MET during multiple generations.

Resumo

Mais de 451 milhões de pessoas em todo o mundo têm diabetes mellitus, em que a grande maioria é afetada pela diabetes tipo 2 (DM2). A metformina (MET) é o fármaco de primeira linha para o tratamento de pacientes com DM2 e, portanto, tornou-se um dos fármacos mais prescritos em todo o mundo, apresentando preocupação emergente em rios e áreas costeiras, uma vez que os ecossistemas aquáticos são o destino final para ambas as águas residuais tratadas e não tratadas.

Apesar da omnipresença da MET nos ecossistemas aquáticos, existem incertezas significativas sobre os efeitos crónicos das concentrações ambientais deste fármaco em organismos aquáticos não-alvo. Portanto, o principal objetivo deste trabalho é integrar metodologias ecológicas, bioquímicas e moleculares atualizadas, para abordar o(s) modo(s) de ação da MET no peixe de água doce *Danio rerio* (peixe-zebra), após uma exposição abrangendo o ciclo de vida completo a três concentrações ambientalmente relevantes de MET (361 ng/L, 2166 ng/L e 13000 ng/L). Neste estudo, os mecanismos de toxicidade subjacentes da MET foram investigados pela caracterização dos principais *endpoints* ecológicos (comprimento, peso, fator de condição, peso do fígado e índice hepatossomático do peixe-zebra adulto), marcadores bioquímicos (colesterol e triglicerídeos do peixe-zebra adulto) e o nível de transcrição de genes-chave, envolvidos no metabolismo energético de larvas e adultos do peixe-zebra.

A exposição a baixas concentrações de MET produziu efeitos estimuladores de parâmetros de crescimento (comprimento, peso e fator de condição) do peixe-zebra adulto, o que sugere a ocorrência de Hormesis. Apesar dos possíveis efeitos horméticos observados no crescimento e no peso, o peso do fígado e/ou o índice hepatossomático aumentaram significativamente em adultos, o que parece indicar que a MET, nessas concentrações, está próxima do limiar de toxicidade e de hormesis. Bioquimicamente, a MET provou ser eficaz na redução dos níveis de colesterol nas fêmeas e machos de 9 meses, bem como nos níveis de triglicerídeos nos machos. A maioria dos resultados da expressão génica exibiu uma curva dose-resposta não monotónica, com mudança significativa nos níveis de transcrição de mRNA dos genes *acaca*, *idh3a*, *cox5aa* e *hmgcra* em adultos. Nas larvas de 20 dpf, o gene *igf1* também mostrou mudanças significativas. Os resultados também mostraram que a concentração de MET que causou a maioria dos efeitos foi a intermediária (2166 ng / L) e que as fêmeas e os machos responderam de forma diferente à exposição à MET.

Todos estes resultados em conjunto, expandem a nossa compreensão dos efeitos da MET em peixes teleósteos, demonstrando impactos significativos em concentrações ambientalmente relevantes e destacam também para a importância de abordar os efeitos dos produtos químicos em baixas concentrações de forma crónica. Além disso, este estudo fornece dados importantes para avaliação de risco ambiental, uma vez que os organismos aquáticos são cronicamente expostos à MET durante várias gerações.

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List of Abbreviations and Acronyms

11KT – 11-ketotestosterone

acaca – Acetyl-CoA carboxylase alfa

acadm - Acyl-CoA dehydrogenase medium chain

ACC – Acetyl-CoA carboxylase

AMP – Adenosine 5′-monophosphate

AMPK – AMP-activated protein kinase

ATP – Adenosine triphosphate

BLAST – Basic Local Alignment Search Tool

BOGA – Biotério de organismos aquáticos

bql – below quantification limit

cAMP – Cyclic adenosine monophosphate

cDNA – Complementary DNA

cG3PD – Cytosolic glycerol-3- phosphate dehydrogenase

Chol – Cholesterol

CIIMAR – Centro Interdisciplinar de Investigação Marinha e Ambiental

cox5aa – Cytochrome c oxidase subunit 5Aa

DHAP – Dihydroxyacetone phosphate

dpf – Day(s) post fertilization

dph – Day(s) post hatch

DM – Diabetes mellitus

EC – Effective Concentration

ECs – Emergent contaminants

EDTA – Ethylenediaminetetraacetic acid

EE2 – Ethinylestradiol

F – Foward

FBPase – Fructose 1,6-bisphosphatase

hmgcra – 3-hydroxy-3-methylglutaryl-CoA reductase a

HMGCR – 3-hydroxy-3-methylglutaryl-CoA reductase

hpf – Hour(s) post fertilization

HSI – Hepatosomatic index

IDF – International Diabetes Federation

idh3a – Isocitrate dehydrogenase (NAD(+)) 3 catalytic subunit alpha

igf1 – Insulin-like growth factor 1

IGF1R – Insulin-like growth factor 1 receptor

ins – Preproinsulin

INSR – Insulin receptor

IRS – Insulin receptor substrate

K – Fulton's condition factor

LC – Lethal concentration

MET – Metformin

mG3PD – Mitochondrial glycerol-3-phosphate dehydrogenase

MoA – Mode of action

mRNA – Messenger ribonucleic acid

MS-222 – Tricaine methanesulfonate

n.a – not available

NAD^+ – Nicotinamide adenine dinucleotide

NADH - nicotinamide adenine dinucleotide + hydrogen

NCBI – National center for biotechnology information

NMDRC(s) – Non-monotonic dose-response curve(s)

NOEC - No observed effect concentration

OCT1 – Organic cation transporter 1

OECD – Organisation for Economic Co-operation and Development

PBS – Phosphate-buffered saline

PCR – Polymerase chain reaction

PhACs – Pharmacologically active compounds

PKA – Protein kinase A

PMFs – Pharmaceutical manufacturing facilities

prkaa1 – Protein kinase, AMP-activated, alpha 1 catalytic subunit

qRT-PCR – Quantitative real time PCR

R – Reverse

RNA – Ribonucleic acid

rpl8 – Ribosomal protein L8

rpl13 – Ribosomal protein L13

SREBP1c – Sterol regulatory element binding protein-1c

STP – Sewage treatment plant

TCA – Tricarboxylic acid

TGL – Triglycerides

T1DM – Type 1 diabetes mellitus

T2DM – Type 2 diabetes mellitus

USA – United States of America

WHO – World Health Organization

WWTPs – Wastewater treatment plants

CHAPTER I. Introduction

1.1 General Introduction

1.1.1 Pharmaceuticals in the environment

The improvements in life expectancy and life quality over the last century are achievements of the constant upgrading in socioeconomic conditions and scientific and technological advances. Despite this global development, the population ageing and the lifestyle choices have been accompanied by increases in the incidence of a number of chronic diseases (Coimbra et al., 2015; Lee & Choi, 2019; Marengoni et al., 2011; Van Oostrom et al., 2014). Chronic diseases, such as diabetes mellitus, obesity, cancer, cardiovascular and pulmonary diseases, depression and anxiety disorders, have been growing exponentially over the last years in human population and are considered one of the main challenges for healthcare systems worldwide (Busse et al., 2010; Marengoni et al., 2011). Along with the increasing healthcare demand due to such diseases, a significant growth in the number of human pharmaceuticals prescribed and consumed worldwide have also been observed (Arnold et al., 2014). In 2019, the USA accounted for 48.7% of world pharmaceutical sales, followed by Europe with 22.9% (EFPIA, 2020).

During the second half of the 20th century, legacy pollutants, such as pesticides and industrial chemicals, were the central focus of chemical pollution due to their toxic/carcinogenic effects and persistence in the environment (Daughton & Ternes, 1999). Pharmaceuticals, however, were disregarded as potential pollutants, since they were detected at trace levels in the environment, and therefore it was assumed that these chemicals did not significantly affect non-target organisms (Arnold et al., 2014; Barros et al., 2018; Daughton, 2016). Currently, there is a growing a concern that pharmaceuticals, for which little knowledge is available, can pose a significant ecological risk for non-target organisms (Elizalde-Velázquez & Gómez-Oliván, 2020). Pharmaceuticals are among some of the emergent contaminants (ECs) of concern, since they target specific pathways at very low concentrations, and therefore behave quite differently from conventional pollutants. Taking in consideration that many signaling pathways are conserved across different taxa, these compounds may cause biologically active effects in a wide range of non-target organisms. Therefore, with the increase of pharmaceutical consumption and the consequent discard into aquatic ecosystems, comes the need to investigate about the fate and potential effects of these chemicals in the environment (Arnold et al., 2014).

1.1.2 Environmental contamination process

Pharmaceuticals represent a complex and important part of the ECs group and are considered as persistent or “pseudo-persistent” pollutants, as a result of their continuous

release into the environment through various pathways (Desbiolles et al., 2018; Fent et al., 2006).

Potential sources from which pharmaceuticals can enter the environment are scattered along the trajectory of a drug's life cycle (Figure 1). Nevertheless, it is possible to name three main sources: pharmaceutical manufacturing, consumption/administration (human and veterinary) and waste management (Archer et al., 2017; Arnold et al., 2014; Fent et al., 2006). For many years, contamination via pharmaceutical manufacturing facilities (PMFs) was thought to be minimal due to good practice regulations and therefore, was neglected (Larsson, 2014; Scott et al., 2018). However, it has been recently demonstrated that PMFs can indeed release significant amounts of pharmacologically active compounds (PhACs) into the environment, through direct or indirect discharges, not only in countries with developing economies, but also in European countries and in the United States of America (Larsson, 2014; Scott et al., 2018; Trautwein et al., 2014). Data from 2014 showed that more than 4000 pharmaceuticals were being used worldwide, in human and veterinary healthcare, and that number is expected to increase in the future (Arnold et al., 2014). Once administrated and due to the incomplete assimilation of the drugs, it is expected for organisms to excrete the unused amount in its original forms and/or in active metabolites, after suffering biotransformation (Rivera-Utrilla et al., 2013; Scott et al., 2018). In cases of livestock, aquaculture or pets, PhACs enter directly into the environment after excretion. As for human patients, these compounds are then released into municipal sewage systems and reach wastewater treatment plants (WWTPs) as domestic and hospital influents (Arnold et al., 2014; Rivera-Utrilla et al., 2013; Xie et al., 2019). However, a large amount of the bioactive compounds that get to WWTPs are not completely removed, due to the absence of adequate methodologies to eliminate this class of chemicals (Ortiz de García et al., 2014). As a consequence, these chemicals remain present in treated wastewaters which will be distributed in surface waters, groundwaters and sediments. Thereby, WWTPs effluents are the major source of aquatic contamination (Archer et al., 2017; Fekadu et al., 2019; Lee & Choi, 2019). Even though pharmaceutical take-back programs exist for over a decade, in order to collect unused and out-of-date medications in an environmentally safe way, reports around the world suggest that community participation is often below 50%. As a result, mismanagement of pharmacological waste by the improper disposal through household waste, sinks and toilets, is still a big source of pollution, mainly due to the lack of education and public awareness for this issue (Bound & Voulvoulis, 2005; Trautwein et al., 2014; Vatovec et al., 2021).

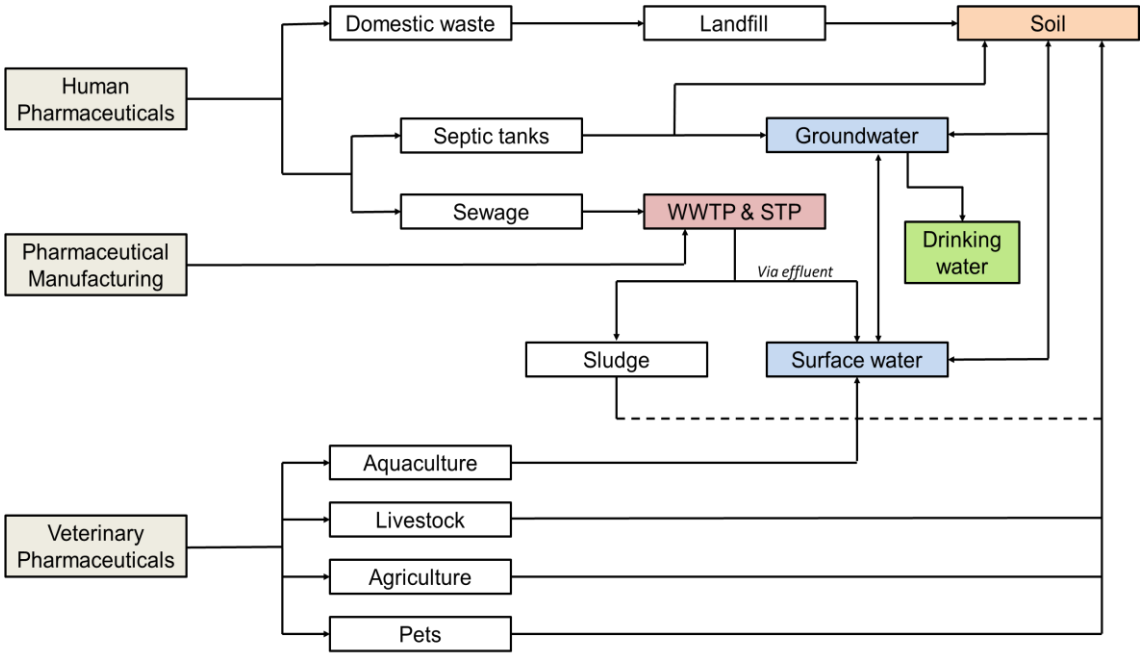

Figure 1. Representative scheme of the sources, pathways and fate of pharmaceuticals in the environment. Adapted from Boxall et al., 2012; Kümmerer, 2010; Santos et al., 2010.

1.1.3 Effects of pharmaceuticals in non-target organisms

The occurrence of PhACs in the aquatic ecosystems, as a consequence of the increasing pharmaceutical consumption rate over the past decades, has become one of the main emerging concerns in ecotoxicological and environmental risk assessment studies (Ortiz de García et al., 2014). In general, pharmaceuticals are designed with a specific mechanism of action and to interact with specific receptors, in human and animals, to trigger the desired effect. However, after entering into the environment, they can also induce unintended and unpredictable effects on non-target organisms after exposure (Boxall et al., 2012; Quesada et al., 2019). Although risk assessment studies regarding the potential side effects of pharmaceuticals residues in the environment are still limited, this issue has become a focus of concern, largely due to the best-documented cases of the danger of environmental concentrations to which non-target organisms are subjected. These two studies are the ones involving ethinylestradiol [17α-ethynylestradiol (EE2)] and diclofenac (EEA, 2010). EE2, a synthetic estrogen present in contraceptive pills found in WWTPs effluents, demonstrated to be a potent endocrine disruptor after inducing feminizing effects, especially in male fish, after exposure to low ng/L concentrations (Gross-Sorokin et al., 2006; Kidd et al., 2007; Purdom et al., 1994; Soares et al., 2009; Versonnen & Janssen, 2004). Soares et al. (2009) exposed zebrafish during their entire life cycle (from egg to 8 mpf) to environmentally relevant concentrations of EE2 (0.5, 1 and 2 ng/L) and reported a significant increase in egg mortality (from 8 to 24 hpf) for all EE2 concentrations, and a

significant increase in vitellogenin (*vgt1*) transcription levels of males, after exposure to 2 ng/L of EE2. Moreover, starting in the 1990s, the population of vultures in the Indian subcontinent experienced a decline of >95% due to renal failure, consequence of their feeding on cattle carcasses treated with diclofenac, an anti-inflammatory drug strongly used in veterinary (Oaks et al., 2004; Pain et al., 2003).

Given these two well investigated and documented events, awareness of the ubiquity and danger of pharmaceuticals in ecosystems began to grow. On the other hand, most ecotoxicological studies only focus on the acute or sub-lethal toxicity effects. Most PhACs are detected in surface waters between ng/L and low µg/L levels and the concentrations considered necessary to produce acute effects are far superior to those actually environmentally relevant (Barros et al., 2018; Fent et al., 2006; Neuparth et al., 2014). Additionally, aquatic organisms can be chronically exposed to pharmaceuticals as they spend their entire life-cycle in contaminated water. Therefore, such standard acute ecotoxicity data are not adequate to represent the true environmental effects and subsequently, risk assessment of PhACs (Barros et al., 2018; Fent et al., 2006).

As mentioned above, pharmaceuticals are bioactive compounds designed to generate biological reactions in known signaling pathways. Thus, when assessing the aquatic toxicity of a PhAC in non-target aquatic organisms, its mode of action (MoA) needs to be taken in consideration (Barros et al., 2020). Hence, chronic life-cycle studies with environmentally relevant concentrations, where the PhAC's mechanism of action is also identified, are a requirement to have an ecologically relevant view of the compounds' impact (Neuparth et al., 2019).

1.2 Anti-diabetic agents and Metformin

Along with the increase of chronic diseases' cases, an increase of the various categories of pharmaceuticals is also inevitable (Arnold et al., 2014). Anti-diabetic drugs is the pharmaceutical group has been exponentially growing in its consumption in OECD countries (Figure 2A and 2B). Between the years 2010 and 2020, Portugal and Chile more than doubled its percentage of antidiabetic drugs in total sales and in several other OECD countries, such as Costa Rica and Estonia, consumption of this pharmaceutical group doubled in the last 12 years. Such growth can be explained in part by the rising prevalence of Diabetes mellitus (DM) which can also be linked with other diseases, for example obesity. According with the World Health Organization (WHO) and the International Diabetes Federation (IDF) the number of people over 18 years old living with DM increased substantially from 108 million in 1980, to 463 million in 2019 and it is projected that by 2045

the number of diabetes' cases will rise to 700 million (Cho et al., 2018; IDF, 2021; OECD, 2019, 2021).

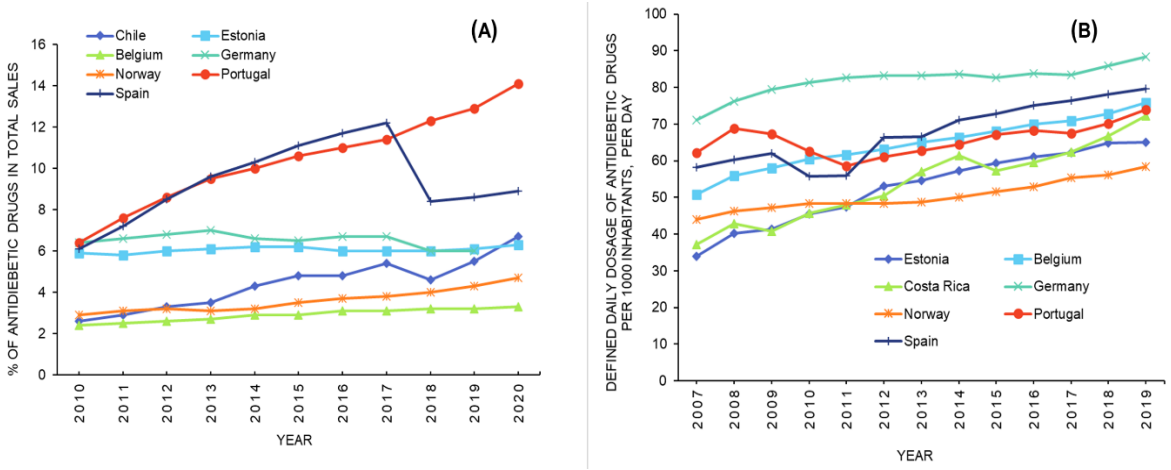

Figure 2. Percentage (%) of total sales, from 2010 to 2020 (A) and defined daily dosage per 1000 inhabitants per day, from 2007 and 2019 (B), of antidiabetic drugs used in OECD countries.

DM is a complex chronic metabolic disease characterized by hyperglycemia (high blood glucose levels), that results from lifestyle and dietary habits, genetic predisposition, as well as environmental factors. Over time DM can increase the risk of having heart and blood vessels complications, neuropathy, nephropathy and retinopathy. Furthermore, these health problems can lead to cardiovascular diseases, lower limb amputation, blindness or kidney failure (Chellappan et al., 2018; Cho et al., 2018; Correia et al., 2008). DM can be divided in two major categories: type 1 diabetes mellitus (T1DM), caused by a decrease in mass and function of ß-cells of the pancreatic islets, leading to insufficient or no insulin production; and type 2 diabetes mellitus (T2DM), which is caused by insulin resistance, along with a pancreatic ß-cells dysfunction. In diagnosed adults, the predominant form is T2DM (Chellappan et al., 2018; Cho et al., 2018; IDF, 2020). In order to achieve adequate glycemic levels and prevent, or at least, delay the development of diabetes complications, most T2DM patients require pharmacological therapy through oral anti-diabetic agents (Chaudhury et al., 2017). These can be used alone or in combination with insulin or other anti-diabetic agents. At the moment, there are several classes of oral anti-diabetic agents available in the pharmaceutical market, including the biguanides (Chaudhury et al., 2017; Cheng & Fantus, 2005).

Metformin (MET) belongs to the biguanides' class, and it is considered the first-line oral therapy to control glucose levels in T2DM patients, as recommended by international guidelines (Davies et al., 2018; Foretz et al., 2019). Currently, Metformin is one of the most effective and safe anti-diabetic agents as it rarely causes hypoglycemia, reduces the risk of

cardiovascular mortality and has a very low risk of causing lactic acidosis when compared to other biguanides (phenformin and buformin) (Foretz et al., 2019, 2010; Gong et al., 2012). Furthermore, it is expected that the demand for MET will grow even more in the near future, since according to a number of studies, this drug can be used in the treatment of other chronic diseases such as polycystic ovary syndrome and cancer (Elizalde-Velázquez & Gómez-Oliván, 2020; Yang et al., 2018b; Zi et al., 2018). Therefore, MET has become one of the most prescribed and consumed pharmaceuticals worldwide, being also part of the WHO's essential medicine list (Bailey, 2017; Foretz et al., 2019; WHO, 2019).

Metformin (1,1-dimethylbiguanide hydrochloride) is a derivative drug developed from galegine, a toxic chemical compound found in an herbaceous plant – *Galega officinalis* – used in herbal medicine since Middle Ages (Bailey, 2017; Foretz et al., 2014). While most current drugs are obtained in the laboratory, MET is, therefore, derived from a natural product, without a previously defined pathway, and which began by being designated as a safe and effective therapy. Mechanistic studies came later, when science and technology allowed it, and although its clinical use has been around for 60 years, its MoA is still much debated (Rena et al., 2017). According to the human pharmacokinetic data, the target organs of metformin are the liver, kidney and intestines (Rena et al., 2017). MET it is not metabolized by the human body, being excreted completely unchanged, mainly in the urine (Bailey, 2017; Foretz et al., 2014).

1.2.1. Metformin's mechanism of action in humans

In patients with T2DM, the liver plays a crucial role in lowering the blood glucose levels, under the effect of MET. Physiologically, MET has then shown to reduce hyperglycemia by suppressing gluconeogenesis, as well as decrease fatty acid and triglyceride synthesis and improving glucose uptake, fatty acid ß-oxidation and insulin sensitivity (Foretz et al., 2019; Gong et al., 2012). However, the cellular and molecular mechanisms of action of metformin (Figure 3), are still not fully understood (Rena et al., 2017). Given metformin's chemical properties, its uptake through biological membranes requires specific transporters as it does not pass through cell membranes by means of simple passive diffusion (Vial et al., 2019). The organic cation transporter 1 (OCT1) is the main hepatic MET transporter (Figure 3A). Over the six decades after MET's first commercialization, several underlying mechanisms have been suggested for this pharmaceutical, but it is only in recent years that an agreement has begun to emerge, placing the mitochondria at the center of metformin's cellular actions (Vial et al., 2019). After entering the cell, metformin is believed to target and accumulate in the mitochondria (Foretz

et al., 2019). In the year 2000, two independent research groups reported for the first time that metformin inhibits the complex I of the mitochondrial respiratory chain (El-Mir et al., 2000; Owen et al., 2000). This hypothesis, over the past 20 years, has been confirmed in several other studies with different biological models and species (Carvalho et al., 2008; Detaille et al., 2002; Ling et al., 2017; Stephenne et al., 2011). According to El-Mir et al. (2000) and several posterior studies, the inhibition of the complex I does not interfere with the downstream oxidative phosphorylation machinery, although still capable of diminish ATP production, accompanied by a decrease in mitochondrial NADH oxidation, a decrease in the proton gradient around the internal mitochondrial membrane and a reduction in oxygen consumption in the cell. Such mechanism is yet poorly understood (Foretz et al., 2014, 2019). Inhibition of complex I can lead to a decrease in ATP levels, which can induce a reduction of gluconeogenesis in order to create a balance, and can also lead to an accumulation of adenosine 5′-monophosphate (AMP), which can reduce the activity of FBPase (a key gluconeogenic enzyme) (Figure 3B) and at the same time can inhibit adenylate cyclase and cAMP-PKA signaling (Foretz et al., 2014, 2019; Rena et al., 2017; Zhou et al., 2001). In 2001, it was reported that MET increased the activity of AMP-activated protein kinase (AMPK) in hepatocytes (Zhou et al., 2001). AMPK has an important role in the regulation of energy homeostasis and is a phylogenetically conserved serine/threonine protein kinase composed by three subunits (α, ß and γ) where each subunit has several isoforms (α1, α2, β1, β2, γ1, γ2, γ3) (Foretz et al., 2010). AMPK is a protein that senses alterations in ADP:ATP and AMP:ATP ratios in the cell, and can be activated when ATP levels decrease and AMP and ADP levels increase, as consequences of the inhibition of the complex I (Foretz et al., 2019; Rena et al., 2017; Zhou et al., 2001). Such activation can cause the phosphorylation and inactivation of key enzymes such as acetyl-CoA carboxylase (ACC) and 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) (Gong et al., 2012). ACC is a limiting enzyme, responsible for *de novo* lipogenesis and if its activity is suppressed, it will result in lower intracellular levels of fatty acids available for triglycerides synthesis (Yoon, 2009). Moreover, inhibition of ACC also stimulates fatty acids ß-oxidation (Foretz et al., 2010; Zhou et al., 2001). And, therefore, it is expected that at the end of the drug's molecular action, we will have the physiological outcomes mentioned above.

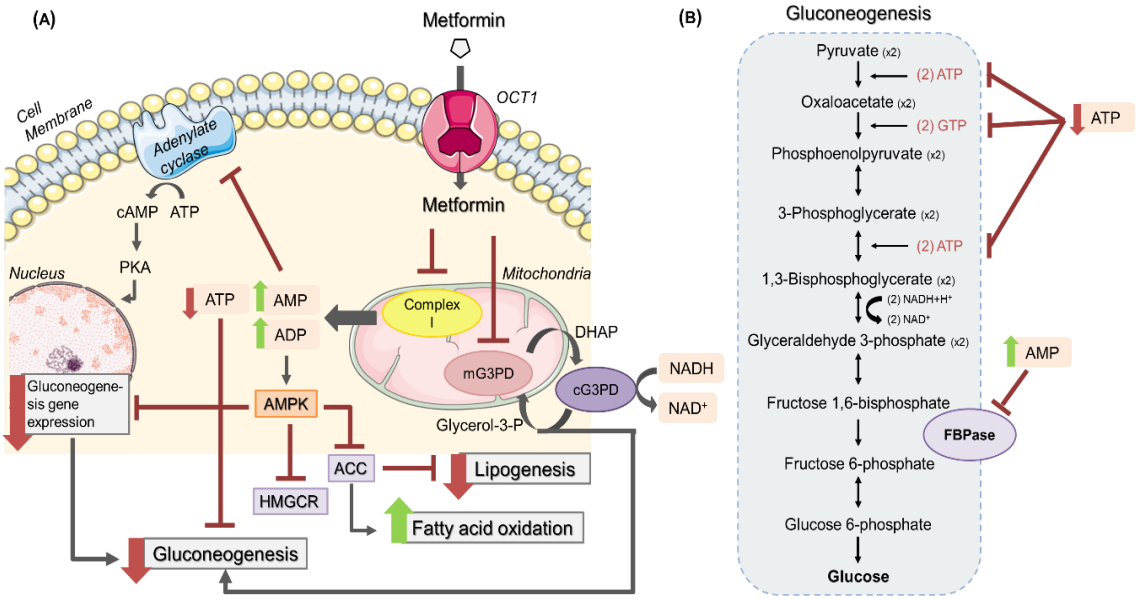

Figure 3. (A) Metformin's mechanisms of action in humans and its consequences; (B) how gluconeogenesis can be affected. Adapted from Foretz et al. (2014, 2019); Rena et al. (2017).

1.2.2 Effects of Metformin in the aquatic environment

As previously mentioned, MET is the primary treatment for type 2 diabetes by reducing glucose levels and it is amongst the most sold PhAC on the Portuguese market according to the annual reports of last years from INFARMED, (Figure 4) (Foretz et al., 2019; INFARMED, 2020).

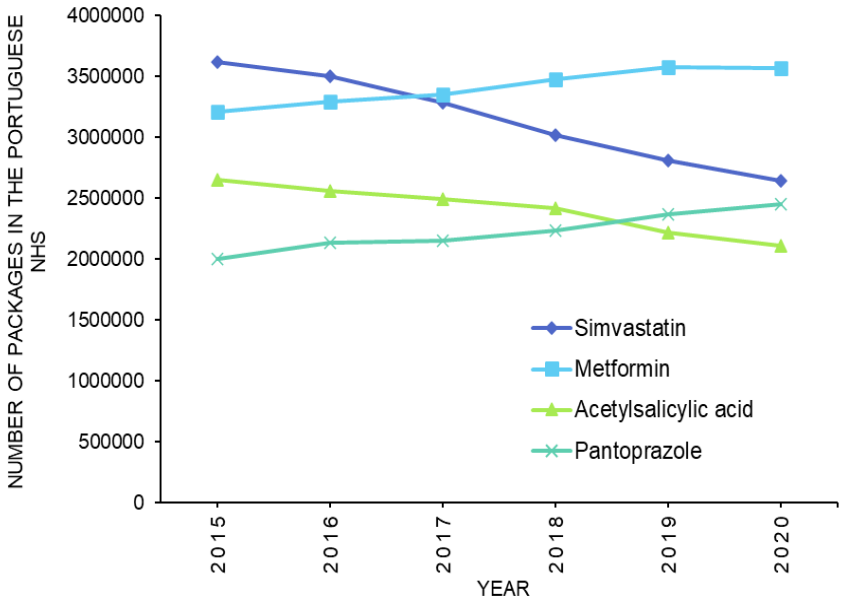

Figure 4. Comparison of the 5 years evolution of number of packages sold, of 4 active substances of the top 10 most used pharmaceuticals in the Portuguese National Health Service (NHS): Simvastatin, Metformin, Acetylsalicylic acid and Pantoprazole.

Considering that: (1) the consumption of MET is exponentially growing, (2) it is not metabolized by the human body, and (3) it has a Log K_{ow} of -2.64, which makes this drug a strong hydrophilic compound with high mobility in the aqueous phase; this pharmaceutical has potential to be found at increasing concentrations in WWTPs effluents and surface waters (Briones et al., 2016; Markiewicz et al., 2017). In fact, the study performed by De Jesus Gaffney et al. (2017) to determine the efficiency of the Beirolas WWTP (Portugal), on the removal of several PhACs, verified that during the 7-month monitoring program, MET was found in all WWTP's influents and effluents analyzed, and was one of the few PhACs detected at very high levels, with concentrations between 70 µg/L and 325 µg/L in influents and 50 – 58 000 ng/L in effluents. Another Portuguese study monitored the occurrence of several pharmaceuticals of great consumption, in four hospitals influents and effluents of a WWTP in Coimbra, Portugal, that receives and co-treats its wastewaters. The maximum MET concentration detected in Coimbra WWTP influents was 1568 ng/L, whereas in the WWTP effluents was 299 ng/L (Santos et al., 2013).

Numerous other authors have also reported the presence of MET in influents and effluents of WWTPs across the globe, where the concentrations vary in the mg/L – µ/L range (Table 1). Also, several studies documented the occurrence of considerable high concentrations of MET in surface waters worldwide, with values in the rage of 35 – 33 600 ng/L (Table 1). Even though MET suffers a reduction due to the WWTPs' incomplete treatment and a dilution effect upon arrival to the receiving waters bodies, MET is still one of the most commonly identified pharmaceutical reported in international surface water monitoring studies, possible to observe in Table 1 (Briones et al., 2016; Houtman et al., 2013). In a study conducted by Ghoshdastidar et al. (2015) in Canada's surface waters, reported that MET was found in a range from 12 to 1487 ng/L, in 67% of the water samples. A year later, another Canadian study reported a maximum detection of 10100 ng/L for MET in surface waters (Table 1). In the early 2000s, it was provided by the U.S Geological Survey for the first time, a national recognition of the occurrence of pharmaceuticals, hormones and other contaminants in USA's surface waters. Within the 139 stream samples, 4.8% corresponds to the frequency of MET detections, with a reported mean of 110 ng/L and a maximum concentration of 150 ng/L (Kolpin et al., 2002). However, a more recent North American monitoring study reported the highest detection of MET until now, in surface waters: 33600 ng/L. As for the European countries, the concentrations detected in surface waters are lower and closer to those used in this study (Table 1).

Considering that MET discharge into aquatic environment is a reality and thus, many aquatic taxa might be at risk, considerable concern and interest has grown for studying the impact of this drug on non-target aquatic organisms (Elizalde-Velázquez & Gómez-Oliván,

2020). Previous studies have reported multiple detrimental effects of MET in different taxonomic groups of aquatic organisms at several levels of biologic organization (Table 2). The main relevant effects of MET previously reported were related with alterations in growth parameters (*Chlorella vulgaris, Lemna minor Brachionus calyciflorus, Plationus patulus, Pimephales promelas* and *Salmo trutta*), reproduction inhibition (*Daphnia similis*), endocrine disruption (*P. promelas*, *O. latipes*), embryonic malformations (*Danio rerio*), alteration of biochemical and/or gene expression parameters (*Nothobranchius guentheri*, *Pimephales promelas* and *Oryzias latipes*). With the exception of the aforementioned studies in fish and the ones summarily described in Table 2, which are studies that reported chronic effects of MET in the low/average µg/L range, the majority of the available studies in the literature, including the ones on Table 2 for all the taxa there represented, have been based on acute toxicity tests, with MET concentrations fare above environmental relevance and even above human daily consumption, that varies from 500 to 1000 mg (Bailey, 2017).

Table 1. Compilation of MET concentrations (ng/L) detected in various WWTPs influents and effluents as well as surface waters, around the world. n.a: not available.

Country	Concentrations detected (ng/L)			References
	WWTP Influent (max.)	**WWTP Effluent** (max.)	**Surface water** (max.)	
Spain	5900[(1)]	1252[(1)]	50[(2)]	[(1)]Carmona et al. (2017); [(2)]Sadutto et al. (2020)
Germany	142 300	6400	643	Trautwein et al. (2014)
	95 000	6500	470	Tisler & Zwiener (2018)
Greece	573	23	n.a	Kosma et al. (2015)
Poland	16 800	62.9	n.a	Kot-Wasik et al. (2016)
Iceland	5900	5590	n.a	Huber et al. (2016)
Faroe Islands	9660	7560	77.9	Huber et al. (2016)
USA	100 000[(1)]	47 000[(1)]	33 600[(2)]	[(1)]Blair et al. (2013); [(2)]Elliott et al. (2017)
Canada	n.a	10 608[(1)]	10 100[(2)]	[(1)]Ghoshdastidar et al. (2015); [(2)] de Solla et al. (2016)
China	21 000[(1)]	640[(1)]	20 015[(2)]	[(1)]Yao et al. (2018); [(2)]Kong et al. (2015)

Table 2. Metformin toxicity data for several groups of organisms. Concentration expressed as mg/L.

Organisms	MET concentration (µg/L)	Duration	Observed effects	Reference
Clorophyta				
Chlorella vulgaris	76800 – 767800	96 hours	Growth inhibition Reduction in the electron transport rate	(Cummings et al., 2018)
Macrophyte				
Lemna minor	53700 ([a]EC_{50})	7 days	Growth inhibition	(Godoy et al., 2018)
Rotifera				
Brachionus calyciflorus	25 – 200	16 days	Peak population density and rate of population increase per day, both decreased	(García-García et al., 2017)
Plationus patulus	25 – 200	16 days		
Crustacean				
Daphnia similis	14300 (EC_{50}) 4400 ([b]EC_{10})	48 hours 14 days	Immobilization Inhibition of reprodution	(Godoy et al., 2018)
Daphnia magna	64000 (EC_{50})	48 hours	Immobilization	(Cleuvers, 2003)
Fish				
Danio rerio	600000 ([c]NOEC)	96 hours	Embryonic malformations	(Godoy et al., 2018)
Nothobranchius guentheri	2000	10 months	Decrease in growth parameters. Decrease in Chol and TGL content. Swimming alterations. Alteration in mRNA levels of inflammatory cytokines.	(Wei et al., 2020)
***Salmo truta* (at 11 ºC)**	1 – 100	57 and 95 days	Decrease in growth parameters. Higher liver glycogen content.	(Jacob et al., 2018)
***Salmo truta* (at 7 ºC)**	10 – 100	95 and 108 days	Decrease in growth parameters. Higher liver glycogen content. Swimming alterations.	(Jacob et al., 2018)
Pimephales promelas	40	28 days	Increase in mRNA transcription levels of *vtg* in males	(Niemuth et al., 2015)
	40	365 days	Decrease in growth parameters.	(Niemuth & Klaper, 2015)

			Reduction in fecundity. Intersex in male's gonads.	
	40	365 days	Increase in mRNA transcription levels of *ar*, *3β-hsd*, *17β-hsd*, and *cyp19a1* and *sult2a1* in males	(Niemuth & Klaper, 2018)
	31 – 322	170 days	Increase in growth parameters in juveniles. Increase in gonado-somatic index and ovipositor area in females. Delay in reproduction.	(Parrott et al., 2021)
Oryzias latipes	3.2 – 100	165 days	Decrease in growth parameters. Increase in 11-KT in females.	(Ussery et al., 2018)
	40 – 360	133 days	Increase in mRNA transcription levels of *cyp19a* and *erα,* in males. Decrease in mRNA transcription levels of *erβ1* and *vtg2* in females.	(Lee & Choi, 2019)

Notes: [a]EC_{50} – median effective concentration; [b]EC_{10} – effect concentration at which 10% effect ; [c]NOEC – No Observed Effect Concentration.

1.3 Model Species: *Danio rerio* (zebrafish)

When conducting chronic ecotoxicological bioassays, one of the most important aspects relies on choosing an adequate model organism. For the present work, *Danio rerio* - commonly known as zebrafish - was selected in order to obtain ecologically relevant data to better understand the potential risk of MET in aquatic ecosystems. Taxonomically zebrafish belong to the largest family of freshwater fishes, which is also the second largest vertebrate family, the Cyprinidae, making this species a good representative for a large group of organisms (Padilla & Glaberman, 2020; Segner, 2009).

During the last three decades, zebrafish has become a very popular model species across several disciplines, including development biology, genetics, drug discovery, toxicology and ecotoxicology (Garcia et al., 2016; Lele & Krone, 1996; Williams et al., 2014). There are numerous practical and technical features that make zebrafish an attractive laboratory model species to study biological processes, effects and mechanisms (Segner, 2009). Zebrafish have already a well described life-cycle and individual development, and extensive behavioral, functional, biochemical and molecular available data, including

fundamental resources, such as having the genome fully sequenced (Ruzicka et al. 2019) (Garcia et al., 2016; Kimmel et al., 1995; Padilla & Glaberman, 2020). More advantages offered by this species include easy and economical maintenance, as well as easy manipulation and observation. The short life-cycle facilitates the performance of chronic bioassays, and its small size and high fecundity rates mean having larger sample sizes in toxicity tests and achieve better statistical outcomes, compared, for example, to mammalian studies (Garcia et al., 2016; Lele & Krone, 1996; Segner, 2009). Another aspect that made zebrafish's popularity increase, was the general desire to reduce the number of experiments on mammals. Teleost fishes, such as zebrafish, have a high level of conservation of genomic sequences and in several functional pathways (lipid metabolism, oxidation and inflammation) making them phylogenetically close to mammals. In addition, previous studies showed about 70% of gene homology between the human and zebrafish genomes, and also the presence of approximately 84% of gene associated with human diseases, enabling to extrapolate data to mammals and other vertebrates (Fang & Miller, 2012; Howe et al., 2013; Williams et al., 2014). Given all these characteristics, the model organism zebrafish appeared to us as the appropriate choice to carry out this experimental work.

1.4 Objectives

The main objective of the present work was to investigate several biological responses in zebrafish, after chronic exposure to environmental relevant concentrations of MET (ng/L) during their entire life-cycle in order to simulate a realistic scenario of the effects of MET in non-target aquatic organisms and relate the findings with apical endpoints. For a better characterization of the effects after exposure to MET, two different development stages of the model organism were used for analysis: 20 dpf larvae and 9 mpf males and females. In this work, we integrate multiple key endpoints at ecological level (weight, length, and hepatosomatic index and condition factor – K), biochemical markers of lipid homeostasis (cholesterol and triglycerides) and molecular analysis of genes coding for important enzymes involved in the lipid and energy metabolism (transcription levels of *acaca*, *acadm*, *cox5aa*, *hmgcra*, *idh3a*, *igf1*, *ins* and *prkaa1* genes). These endpoints should give more information regarding the MoA of MET at environmentally relevant concentrations in non-target aquatic organisms, as well as insights over possible long-term effects at individual and population level. Another aim was to increase MET database, in order to better understand and predict its potential adverse effects in wild fish populations under chronic exposure.

CHAPTER II. Materials and Methods

2. Material and Methods

2.1. Chemicals

1,1-Dimethylbiguanide hydrochloride (CAS: 1115-70-4), commonly known as metformin (MET), was purchased from Sigma-Aldrich (Germany). MET stock and working solutions were prepared in distilled water and stored at - 20°C.

2.2 Zebrafish maintenance and eggs production

Wild type zebrafish breeding stock were kept at *Biotério de Organismos Aquáticos* (BOGA) located at CIIMAR under dechlorinated filtered and aerated water at 28 ± 1 ºC and 14:10h (light:dark) photoperiod. Fish were fed, *ad libitum*, three times per day with commercial fish diet Tetramin (Tetra, Melle, Germany), supplemented with live brine shrimp (*Artemia* spp). The amount of food distributed was adjusted according to fish development stage and size, in the same proportion to all aquaria. In the afternoon before breeding, animals were housed in cages with a net bottom covered with marbles, in a proportion of 1:2 (female:male). At the following morning, approximately 1 – 1.5 h after the beginning of the light period, breeding fish were carefully removed from the cages, and resulting eggs collected and cleaned. Only fertilized eggs were used for the experimental procedure.

2.3. Chronic toxicity bioassay

The chronic ecotoxicological bioassay was performed at BOGA. The experiment was subjected to an ethical review process carried out by CIIMAR's animal welfare body (ORBEA) prior to the beginning of the experimental work. The bioassay was performed in compliance with the European Directive 2010/63/EU on the protection of animals used for scientific purposes, and the Portuguese 'Decreto Lei' 113/2013.

The experiment consisted of four treatments: a control (dechlorinated water) in triplicate and three MET concentrations in duplicate: 361 ng/L, 2166 ng/L and 13000 ng/L (Figure 3). The selection of these MET concentrations was based on environmental concentrations reported in literature (Table 1). Aquaria were maintained with a water temperature of 28 ± 1°C, 14:10 h (light:dark) photoperiod, pH 7.5 ± 0.2 and a mean ammonia and nitrite concentration of 0.04 ± 0.02 mg/L and 0.03 ± 0.03, respectively. The bioassay was conducted for 9 months and started by randomly allocating 400 newly fertilized zebrafish eggs (<4 hpf) in 7 L aquaria, under a flow-through system, where water flow was maintained at 1.01 L per hour by means of two peristaltic pumps, one supplied with dechlorinated, heated and charcoal filtered tap water (ISM 444, ISMATEC), and another with stock metformin solutions (ISMATEC). At 20 dpf, 100 larvae were allocated to

30 L aquaria and the remaining ones were collected for molecular analysis. Density was again reduced to 35 animals per aquaria at 60 dpf.

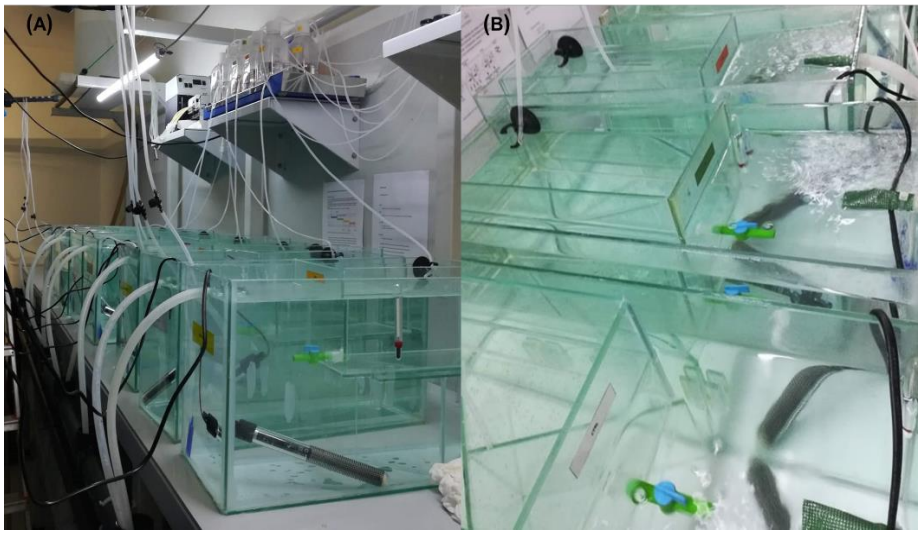

Figure 5. Chronic toxicity bioassay's setup (A and B).

2.4 Sampling

At the end of the chronic bioassay, all animals were sacrificed with an anesthetic overdose of 300 mg/L tricane methanesulfonate (MS-222) buffered with the same amount of sodium hydrogen carbonate. All animals were measured and weighted to calculate the Fulton's condition factor, which represents the relation between weight and length of each individual fish, according to the formula: $K = (\text{weight} / \text{length}^3) \times 100$ (Nash et al., 2006).

Livers of males and females (9 mpf) of each treatment were collected and weighted for the Hepatosomatic index (HSI) determination. The index was calculated according to the following equation: HSI = (Liver weight (g) / Fish weight (g)) x 100. The livers were then frozen in liquid nitrogen and stored at - 80ºC until lipid extraction or preserved in RNALater (Sigma-Aldrich) at - 80ºC for gene expression analysis.

2.5 Lipid extraction and Cholesterol and Triglycerides quantification

Lipids were extracted from the adult liver samples stored at - 80ºC using an extraction protocol, adapted from Schwartz & Wolins (2007), with a low toxicity solvent. The liver tissues were homogenized in separated tubes, using a Precellys 24 homogenizer, with 10 nM PBS buffer pH 7.4, containing 10mM EDTA (10 mg of tissue per 1 mL buffer) and two ceramic beads per tube. The homogenate of each sample was transferred (500 µL), in duplicate, to test glass vials containing 5 mL of isopropanol/hexane solution (4:1 proportion).

All samples were vortexed and incubated at room temperature in the dark, with constant shaking (170/180 rpm) for 1 hour. To prevent lipid peroxidation, samples were always passed through nitrogen current before closing vials. After the 1-hour incubation, samples were washed with 2 mL of petroleum ether/hexane (1:1) solution. Vials were again vortexed and put in the dark for a 10 minutes' incubation, at room temperature. 2.5 mL of Milli-Q H2O was added to each vial, then vortexed and incubated at room temperature in the dark, with constant shaking for 20 min. The phases were then separated by centrifugation at 1000 × G, for 10 min. The upper phase contains the lipids, was collected into new vials and evaporated to dehydration under nitrogen current. At the end, the dried extracts were then stored at - 20°C, until cholesterol and triglycerides quantification.

Before quantification, dry extracts were re-suspended in 100 µL of isopropanol and sonicated in an ultra-sound bath Bandelin Sonorex RK100H, for 15 minutes, at room temperature. Quantification of both parameters was performed through enzymatically colorimetric assays using Infinity Cholesterol Liquid Stable reagent and Infinity Triglycerides Liquid Stable Reagent, (Thermo scientiphic, Biognóstica, Portugal) and following the manufacturer's protocol (Figure 6). Absorbance was determined at 490nm using a microplate reader (Biotech Synergy HT) coupled with Gen5 software (version 2.0). In every run, a standard curve was performed for optimal quantification and all samples were measured in duplicates. Cholesterol and triolein standards were prepared by 6 serial dilutions (from 5 mg/mL to 0.156251 for cholesterol and 2 mg/mL to 0.0625 for triolein).

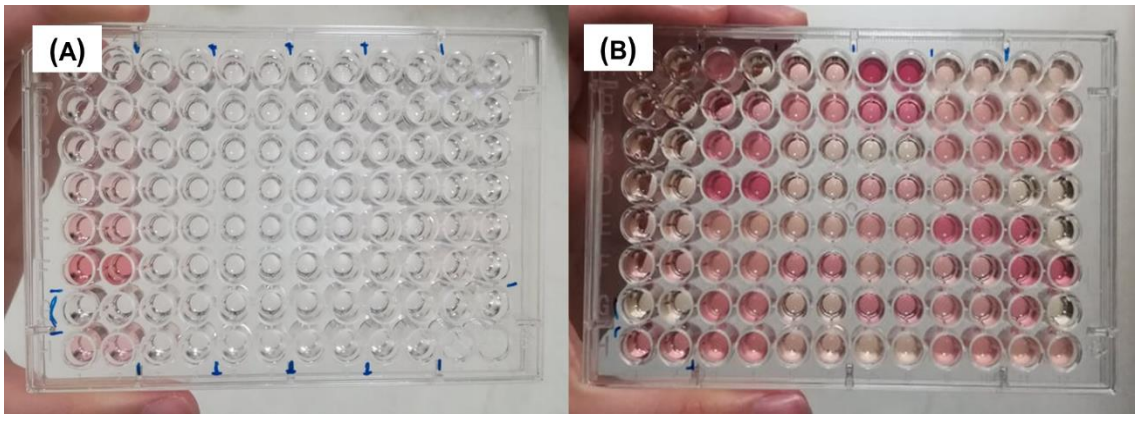

Figure 6. Enzymatic colorimetric assays for cholesterol (A) and triglycerides (B) quantification.

2.6. Gene expression

2.6.1. RNA isolation and cDNA synthesis

20 dpf larvae (pools of 2) and 9 mpf male and female liver samples from each treatment were preserved in RNALater and kept at - 80º C until use. Samples were then

individually homogenized with lysis solution, ß-mercaptoethanol and two ceramic beads on a Precellys 24 homogenizer to lyse the cells. The homogenate was then used to isolate total RNA via the Illustra RNAspin Mini RNA Isolation kit (GE Healthcare), according to the manufacturer's standard protocol (N=6-8). RNA quantification was performed by the measurement of optical density with a Take3^{TM} on a microplate reader (Biotech Synergy HT) coupled with the Gen5 software (version 2.0). RNA quality was verified by electrophoresis in 1.5% agarose gel and through the measurement of the ratio of absorbance at λ260/λ280 nm. All isolated RNA samples were stored at - 80°C until further use. Total cDNA was generated from 600 ng and 1 µg of total RNA extracted from the larvae and adult livers, respectively, using the iScript^{TM} cDNA Synthesis Kit (Bio-Rad).

2.6.2. Primer Design

The qRT-PCR zebrafish primers for the genes *acaca*, *acadm*, *cox5aa*, *idh3a*, *igf1*, *hmgcra* and *rpl8*, were obtained in other published studies (Table 3). For the other genes, where the qRT-PCR primers were not available (the target genes – *ins*, *prkaa1* and reference gene – *rpl13*), the zebrafish mRNA sequences were acquired from NCBI's database (Table 3), and specific primers were designed for qRT-PCR using the Primer designing tool "Primer – BLAST" (NCBI) and Beacon Designer (Premier Biosoft International). To confirm the specificity of each primer, a PCR was performed in a Tgradient thermocycler (Biometra), and the subsequent products were run by electrophoresis in a 1.5% agarose gel. The resulting bands, with the expected size for each gene, were cut out and purified with GelPure (NzyTech), following the manufacturers' protocol and sent for sequencing at GATC (Eurofins Genomics). The sequencing results were then uploaded and analyzed with the BLAST tool from NCBI in order to confirm the amplified sequence (Table 3).

2.6.3. Quantitative Real Time – PCR

Several proteins involved in the MoA of MET, were selected to evaluate their gene expression in each of the 4 treatments performed (control, 361, 2166 and 13000 ng/L MET concentrations (Figure 7). Inside the cell, MET appears to upregulate the cytoplasmatic 5'-AMPK pathway and reducing the cellular energy status (Xu et al., 2015). The protein kinase, AMP-activated, alpha 1 catalytic subunit (*prkaa1*) was one of the first genes to be selected, due to its role in the MoA of metformin. Since MET targets for the mitochondria, two genes that are involved in the cellular respiration were selected. *Idh3a*, a gene encoding isocitrate dehydrogenase (IDH) is a rate-limiting enzyme in the tricarboxylic acid cycle, and *cox5aa* (cytochrome c oxidase subunit 5Aa), which is part of the complex IV of the electron transport

chain and plays a key role in the aerobic mitochondrial energy metabolism (Arnold, 2012; Barros et al., 2020; Kadenbach et al., 2000). Once AMPK is activated, catabolic processes are stimulated (e.g. fatty acid oxidation) and anabolic pathways are suppressed (e.g. gluconeogenesis and fatty acid synthesis). The fatty acids ß-oxidation, that occurs in the mitochondria, can be carried out by the medium-chain acyl-CoA dehydrogenase (*acadm*) (Barros et al., 2020). The gene encoding acetyl-CoA carboxylase alpha (*acaca*) was selected, since this cytosolic enzyme (ACC) catalyzes the carboxylation of acetyl-CoA to form malonyl-CoA, an essential substrate in fatty acids synthesis (Alves-Bezerra & Cohen, 2018; Harwood Jr, 2005). Since cholesterol was one of the biochemical parameters analyzed, the gene that encodes the 3-hydroxy-3-methylglutaryl-CoA reductase protein (HMGCR), a limiting enzyme in the synthesis of cholesterol, was selected to complement the biochemical data – *hmgcra* (Al-Habsi et al., 2016). The remaining genes were only evaluated in the 20 dpf larvae, which are *igf1* and *ins*. The larvae stage is, where major morphological and physiological changes occur. One of the main hormones responsible for regulating growth in zebrafish larvae is the Igf-1 (insulin-like growth factor 1) encoded by *igf1* (Opazo et al., 2017). As for insulin (*ins*), the most powerful anabolic hormone, it also plays a vital role in regulating growth and the overall metabolism of vertebrates. Insulin effects encompass stimulation of glucose uptake and synthesis of lipids, glycogen and other proteins (Yang et al., 2018a; Zhou et al., 2001). Gene expression profiles of *acaca*, *acadm*, *cox5aa*, *hmgcra*, *idh3a*, *igf1*, *ins* and *prkaa1* were assessed by means of quantitative real time PCR (qRT-PCR). Ribosomal protein L8 (*rpl8*) and ribosomal protein L13 (*rpl13*), were used as reference genes since their expression levels did not show significant changes within the different treatments. *rpl8* was used for gene expression analysis of 20 dpf larvae, while rpl13 was used for the analysis of liver samples from adult zebrafish. Primers were already described by other authors or designed and tested for this study (Table 3). The cDNA of 20 dpf and liver samples of each treatment (N=6-8) were amplified in duplicate using the Mastercycler®ep *realplex* system (Eppendorf) in 96-well optical white plates, containing 5 µL of NZY qPCR Green Master Mix (2x) (nzytech), 0.4 µL of each primer (forward and reverse), 2.2 µL of water and 2 µL of cDNA at 100 nmol, in order to reach a final reaction volume of 10 µL. In every plate, a nontemplate control was included. In order to determine the efficiency of the primers, and consequently, of the reaction, a two-step qRT-PCR was performed as follows: 95°C of initial denaturation for 2 minutes, followed by 40 cycles of amplification with a denaturation at 95°C for 5 seconds and combined annealing and extension at 58 – 62°C, depending on the pair of primers previously validated, for 25 seconds. To confirm the specificity of the reactions, a melting curve (from 55°C to 95°C) was generated in each run. PCR products were then analyzed by electrophoresis in 2% agarose gel to check the presence of single bands with expected size

between 94 and 198 bp, depending on the pair of primers (Table 3), in order to confirm the reaction. The PCR efficiency for the target and reference genes was determined by a standard curve, using six serial dilutions of cDNA pools of all samples (from 0.064 to 200 ng of cDNA in the 20 dpf larvae samples and from 500 to 2.058 ng of cDNA in adult liver samples). The average PCR efficiencies obtained for target genes ranged from 87 to 105% (Table 3).

Relative change in transcription abundance of the genes of interest was normalized to *rpl8* or *rpl13* and calculated using the $2^{-\Delta\Delta Ct}$ analysis method (Livak & Schmittgen, 2001). Control expression levels (dechlorinated water treatment) were normalized to 1 and data were then expressed as fold changes of the control group.

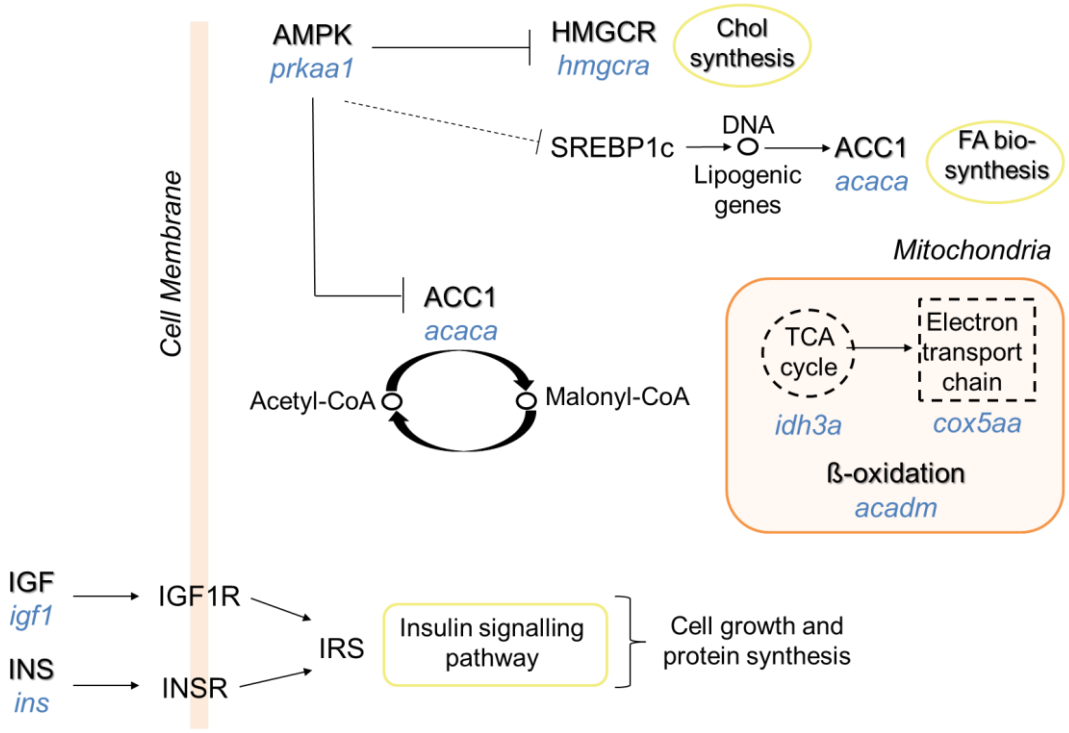

Figure 7. Schematic representation of the sites of action of the selected genes.

Table 3. Primer sequences, forward (F) and reverse (R), and parameters used in the qRT-PCR for gene expression quantification in the 20 dpf zebrafish larvae and in the 9-month-old zebrafish livers.

Gene	Sequence (5' – 3')	Expected band size (bp)	Combined annealing and extension temperature (°C)	Average efficiency (%)	Reference
acaca	F: AGAGAGGGCAGGTTTTACCA R: GCCATCATACGAGAGCAACA	194	60	95	(Lyssimachou et al., 2015)
acadm	F: CCGATCCCAAATGTCCTGCT R: ATGTAATGCCCCTGGTGTCG	129	60	92	(Barros et al., 2020)
cox5aa	F: CGCCCTTCGACTGTCATTCT R: TCATCCGTCTCCTGTTTGCC	111	62	95	(Barros et al., 2020)
idh3a	F: CACCCATCCATGAACCTGCT R: CTCTGAACAACTCCATCCACGA	182	62	96	(Barros et al., 2020)
igf1	F: GGCAAATCTCCACGATCTCTAC R: CGGTTTCTCTTGTCTCTCTCAG	198	62	105	(Lyssimachou et al., 2015)
ins	F: ATCCACCATTCCTCGCCTCT R: TGCTTACACTGGACACGACC	101	60	98	This study
hmgcra	F: TCGTGGAGTGCCTGGTGATTGGT R: TGGGTCTGCCTTCTCTGCTCTCTC	177	62	87	(Barros et al., 2018)
prkaa1	F: GAGTGGGGACGTTCGGAAAA R: CTGCGGATCTTCTGTCGGTT	94	60	96	This study
rpl8	F: TTGTTGGTGTTGTTGCTGGT R: GGATGCTCAACAGGGTTCAT	136	58	99	(Lyssimachou et al., 2015)
rpl13	F: GCTGAAGGAATACCGCACCA R: TCCAGTAAGCTGTGTTGCCAT	109	60	98	This study

2.7. Statistical analysis

Data obtained from this experimental study was checked for normality (Kolmogorov-Smirnov test) and homogeneity of variances (Levene's test) followed by a one-way ANOVA. Post-hoc comparisons were carried out using Fisher's least significant difference (LSD) test. Significant differences were set as $p<0.05$. All the statistical analyses were computed with Statistica 12 (Statsoft, USA).

CHAPTER III. Results

3. Results

3.1. Morphometric condition endpoints

Table 4 shows the length, weight, Fulton's condition factor, liver weight and the hepatosomatic index (HSI) of zebrafish males and females after 9 months of exposure to 361 – 13000 ng/L of MET. The male's average length and weight were significantly increased in the highest MET concentration tested (13000 ng/L) in comparison to control groups, while female's length and weight were not affected by MET exposure. The Fulton's condition factor was significantly increased for both male and female, after exposure to 2166 ng/L and 13000 ng/L in each sex, respectively. Liver weight was significantly higher in males exposed to 13000 ng/L of MET and also in all MET exposed females, while the HSI showed a significant increase for females exposed to 361 and 2166 ng/L MET concentrations. Male's HSI was not affected after MET exposure.

Table 4. Ecological endpoints, for both male and female zebrafish after 9 months of exposure to MET concentrations. Asterisks (*, **, ***) indicate significant differences from the control group (*-p<0.05; **- p<0.01; ***- p<0.001). Values presented as mean ± standard error.

Endpoint	Sex	Control	361 ng/L	2166 ng/L	13000 ng/L
Length (cm)	M	3.76 ± 0.07	3.86 ± 0.03	3.80 ± 0.06	**3.97** ± 0.04**
	F	4.05 ± 0.04	4.12 ± 0.06	4.14 ± 0.04	4.07 ± 0.05
Weight (mg)	M	510 ± 2.0	550 ± 1.0	550 ± 2.0	**610*** ± 2.0**
	F	750 ± 3.0	830 ± 5.0	820 ± 4.0	850 ± 4.0
Fulton's condition factor (K)	M	0.94 ± 0.03	0.94 ± 0.01	**1.02* ± 0.03**	0.97 ± 0.02
	F	1.12 ± 0.03	1.16 ± 0.03	1.15 ± 0.03	**1.25** ± 0.03**
Liver weight (mg)	M	4.39 ± 0.68	5.77 ± 0.58	6.17 ± 0.68	**7.42** ± 0.99**
	F	8.33 ± 0.84	**19.72*** ± 2.81**	**15.58*** ± 0.12**	**12.94*** ± 1.56**
Hepato-Somatic index (HSI)	M	0.85 ± 0.13	1.14 ± 0.13	1.14 ± 0.13	0.97 ± 0.12
	F	1.17 ± 0.12	**2.23** ± 0.29**	**2.20*** ± 0.28**	1.51 ± 0.18

3.2. Cholesterol and Triglycerides quantification

Quantification of cholesterol (Figure 8A) and triglycerides (Figure 8B) in zebrafish males and females revealed significant differences from the control treatment, after 9 months of MET exposure.

Cholesterol's concentration in the liver of the adult males, significantly decreased after exposure to 2166 ng/L and 13000 ng/L of MET, when compared with the control. Adult females also showed a similar pattern, being the cholesterol levels significantly lower for the medium concentration (2166 ng/L).

Triglycerides (TGL) levels in the adult male livers were significantly lower after exposure to the intermediate MET concentration, 2166 ng/L, in comparison to the control, whereas no effects were recorded for the lower (361 ng/L) and highest (13000 ng/L) MET concentrations, with responses were similar to the control conditions. Therefore, the intermediate MET exposure appeared more effective than the highest or lower exposures. This response appeared to be non-monotonic, showing an inverted U-shape. Females' TGL remained unchanged.

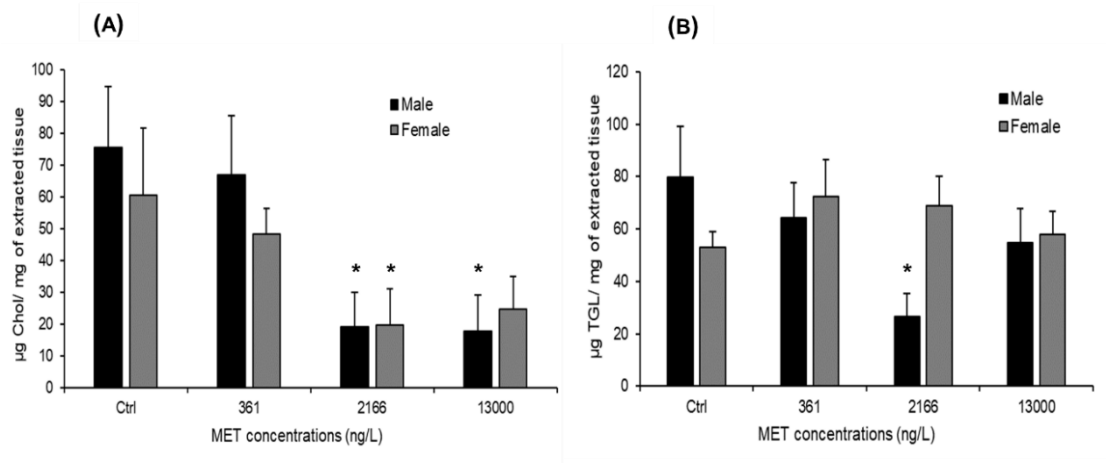

Figure 8. Effects of MET on zebrafish liver cholesterol (A) and triglycerides (B) levels, expressed as µg per mg of extracted tissue, after 9 months of exposure. Error bars indicate standard errors; asterisks (*) indicate significant differences from the control group ($p<0.05$).

3.3 Gene expression

20 dpf zebrafish larvae, showed significant differences compared to the control group in the expression levels of *igf1* at the concentration of 13000 ng/L (Figure 9) with a fold induction of 1.79. *ins*, *hmgcra* and *prkaa1* did not showed significant alterations on their mRNA levels.

Adult zebrafish females (Figure 10) and males (Figure 11) livers also showed significant alterations from the control group on their gene's expression levels after 9 months of exposure to MET, with females exhibiting more changes than males. In females, the *acaca* gene was up regulated by 3.05 fold from the control group after exposure to 2166 ng/L of MET. The transcription levels of *cox5aa* showed an up regulation of 1.45 fold for 364 ng/L of MET exposure. *idh3a* was also up regulated by 1.81 and 2.53 fold from the control treatment after exposure to 364 ng/L and 2166 ng/L of MET, respectively. *hmgcra* expression was found to be highly up regulated in females, with a 4.73 and 13.45 fold increase from the control group after exposure to 364 ng/L and 2166 ng/L of MET. On the other hand, a 5.88 fold downregulation of *hmgcra* was observed in males exposed to 2166 ng/L of MET. In both male and female livers the mRNA levels of all significantly altered genes followed a non-monotonic dose-response curve (NMDRC), with a U-shaped curve in males, and inverted U-shaped curve in females. Neither females nor males exhibited significant differences in *acadm* and *prkaa1* mRNA expression levels.

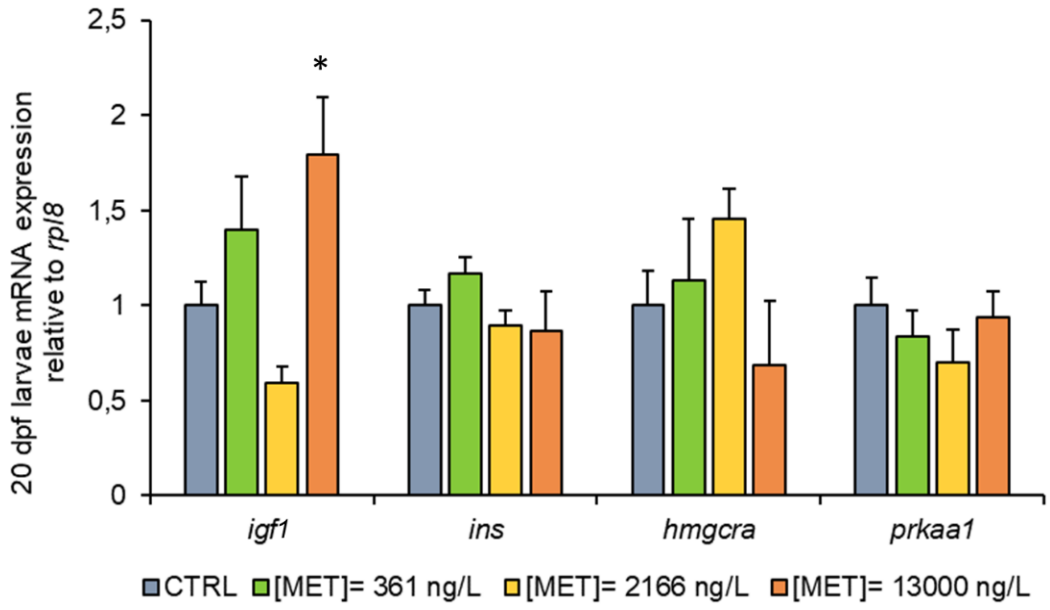

Figure 9. 20 dpf zebrafish larvae's relative gene expression of igf1, ins, hmgcra and prkaa1, after 20 days of MET exposure. Error bars indicate standard errors; asterisks (*) indicate significant differences from the control group ($p<0.05$).

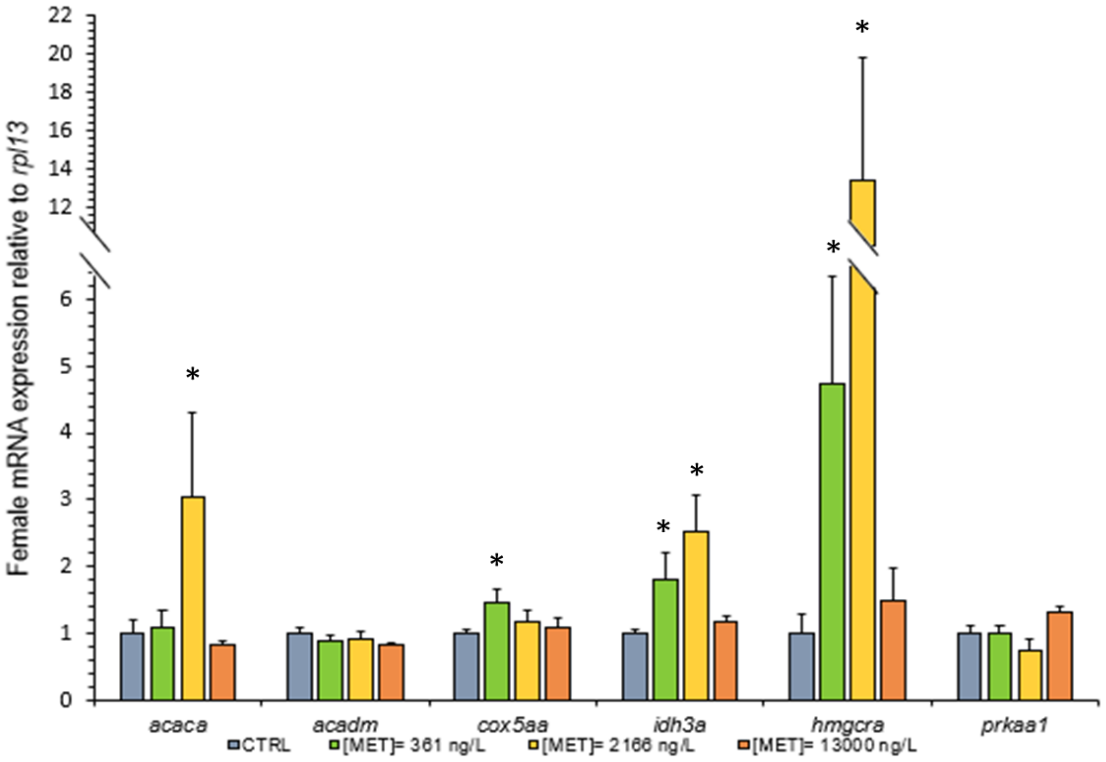

Figure 10. Females' relative gene expression of acaca, acadm, cox5aa, idh3a, hmgcra and prkaa1, in adult zebrafish livers, after 9 months of MET exposure. Error bars indicate standard errors; asterisks (*) indicate significant differences from the control group ($p<0.05$).

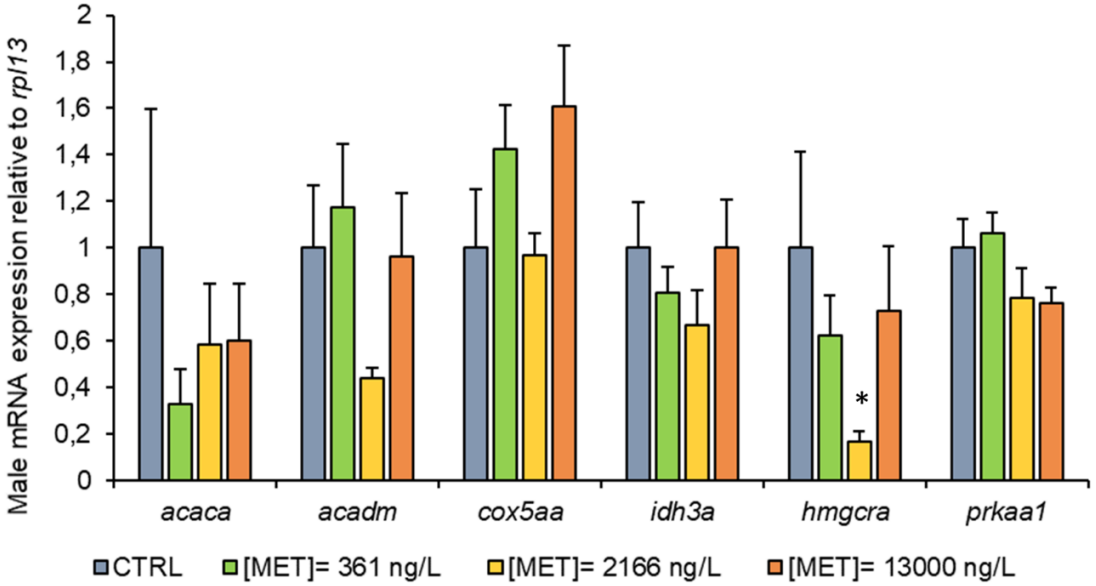

Figure 11. Males' relative gene expression of acaca, acadm, cox5aa, idh3a, hmgcra and prkaa1, in adult zebrafish livers, after 9 months of MET exposure. Error bars indicate standard errors; asterisks (*) indicate significant differences from the control group ($p<0.05$).

CHAPTER IV. Discussion

4. Discussion

For the past decades, metformin has become extremely popular since it is prescribed for the treatment of several chronic diseases, such as T2DM, polycystic ovary syndrome and some types of cancer where insulin resistance is present (Briones et al., 2016; Yang et al., 2018b; Zi et al., 2018). As a result of its high rate of prescription and consumption, MET discharge into aquatic ecosystems has increased exponentially, and therefore, MET is now one of the most abundant pharmaceuticals found in these environments (Table 1). Being MET a PhAC (pharmacologically active compound), it is meant to produce biological responses at low concentrations, potentially leaving non-target organisms that share conserved targeted pathways with mammalian at risk (Arnold et al., 2014). Regardless of the growing number of studies concerning the acute toxicity of MET in aquatic ecosystems, there is a major knowledge gap concerning the chronic effects of this emerging contaminant (Ussery et al., 2018). In order to address the current gap regarding the effects following a chronic exposure of non-target aquatic species to MET, the present study aimed to investigate biological responses at various levels in the model organism zebrafish, after a chronic exposure of 9 months to environmentally relevant concentrations of MET (361, 2166 and 13000 ng/L). This work integrated key ecological endpoints such as length, weight, and liver weight, complemented with the determination of cholesterol and triglycerides levels and the expression of key genes related to the MoA of MET in liver. The results of this study showed that the 9-month chronic exposure to the selected concentrations, MET induced a series of disruptive effects, in several ecological endpoints, as well as at biochemical and molecular level. Results also revealed a few sex-dependent differences, which can suggest that females and males may not respond similarly to MET. The chronic exposure to MET in ng/L to µg/L range lead to an increase of male length and weight at the highest concentration, while these two parameters were not affected in the females. Both male and female had their Fulton's condition factor increased as well. The endpoints related to the liver also showed remarkable differences, with females showing a significant increase, both in terms of liver weight and HSI. So far these parameters, clearly affected in zebrafish as a consequence of exposure to environmentally relevant concentrations of MET, are not concordant with the few MET chronic studies available in the literature that reported decrease of length and weight or no effects after chronic exposures to MET (Niemuth et al., 2015; Parrott et al., 2021; Ussery et al., 2018). Ussery et al. (2018) reported a decrease in growth parameters (length and weight) on juveniles *Oryzias latipes* (Japanese medaka) following a MET exposure to 1.0, 3.2, 10, 32 and 100 µg/L from 6 hpf through 28-days post hatch, where differences started to be observed at concentrations on 3.2 µg/L and above. Similarly, a study with exposed *Pimephales promelas* (fathead minnows) juveniles with 30 dph to 40 µg/L for 365 days, also

resulted in a decrease in weight and K (Niemuth & Klaper, 2015). Nonetheless, a recent study performed by Parrott et al. (2021), revealed that *P. promelas* generational exposure to MET concentrations ranging from 3 to 322 µg/L did not lead to differences in neither growth nor weight of adults.

Overall, together with the present study, the available literature on the effects of chronic exposure to MET in growth parameters of non-target organisms has revealed to be inconsistent. This may be a consequence of the scarce amount of chronic life-cycle studies with MET that used different species, concentrations and development stages. In fact, results of the existing studies in the literature are not in agreement, may be a consequence from the scarce chronic life-cycle studies with MET, which lead us to speculate that ecological endpoints may well be disrupted by MET, but results will vary depending on the concentrations used, the development stage and the species selected for the assay. It should also be noticed that the MET concentrations used in the current study are environmentally relevant and much lower than the ones used on the studies mentioned above. The hypothesis that we advance to explain the stimulating effects of MET contamination on adult zebrafish growth parameters (length, weight and K) is Hormesis. Hormesis is thought to result from an overcompensation of the homeostatic regulatory control mechanisms induced by low doses of toxicants which in consequence will be responsible for stimulatory effects in a population (i.e enhancement of survival, growth and/or reproduction). The event of hormesis has been documented in vertebrates and invertebrates in response to a variety of stressors (Calabrese, 2004, 2008; Neuparth et al., 2005). Despite the possible hormetic effects observed in adult zebrafish growth and weight, liver weight and/or hepatosomatic index were significantly increased, which seems to indicate that MET in these concentrations, is just near the toxicity threshold and hormesis. The remaining endpoints analyzed (biochemical parameters and mRNA expression levels) did not show a hormetic effect, although NMDRC can be observed in some of the results.

The biochemical results revealed that MET was effective in significantly decreasing cholesterol levels in males exposed to 2166 and 13000 ng/L and in females exposed to 2166 ng/L of MET. A similar behaviour was observed in the TGL content of exposed males, being the levels significantly lower for the intermediate concentration (2166 ng/L). The quantification of TGL of exposed females did not show alterations. The results are in agreement to the MoA reported in mammalians, where MET is described to inhibit the synthesis of both cholesterol and triglyceride content (Fang & Miller, 2012; Gong et al., 2012). Two studies that evaluated the lowering power of MET in cholesterol and TGL levels, verified that MET caused an accentuated reduction on both parameters on the organisms, such as mice and fish (*Nothobranchius guentheri*) (Geerling et al., 2014; Wei et al., 2020).

It should also be noted that in the present study the females' response to MET was quite interesting, since the cholesterol levels were decreased, the TGL content was not altered and yet they showed a significant increase in liver weight, HSI and Fulton's condition factor (K). Since cholesterol and triglycerides are extremely important for the well-being of many aquatic organisms including the zebrafish, due to their role in the lipidic homeostasis and consequently, the maintenance of various biological processes (e.g. growth and reproduction), alterations in these parameters, such as a decrease, can implicate harmful consequences for these organisms (Barros et al., 2018). The liver is one of the target organs of MET and the organ responsible for lipid homeostasis, hence it will be important to further investigate this topic in future studies.

With the aim of providing a more complete analysis of the long-term effects of MET, which so far are rather scarce, several genes directly or indirectly involved in the putative MoA of MET were selected for molecular analysis. At the end of 9 months exposure to MET, zebrafish presented alterations in the mRNA levels of *acaca*, *idh3a*, *cox5aa* and *hmgcra* genes. Interestingly, taking together with the results of biochemical parameters, it is possible to see that the intermediate concentration of MET, 2166 ng/L, was responsible for most of these changes in both analyses. Also, although males seemed to be more susceptible to biochemical changes, at the molecular level females exhibited more alterations in gene expression. At a physiological level, MET has shown to reduce hyperglycemia by suppressing gluconeogenesis and fatty acid synthesis and, on the other hand, stimulate glycolysis and fatty acid ß-oxidation. However, the underlying mechanisms by which MET operates are still elusive (Foretz et al., 2019; Gong et al., 2012). Taking into account the existing literature, since MET is expected to decrease cellular ATP levels by inhibiting the complex I and glycolysis is an important source of energy as the primary step of cellular respiration, it is expected that glycolysis emerges as a compensatory pathway (de Souza Silva et al., 2010; El-Mir et al., 2000; Foretz et al., 2010). In fact, de Souza Silva et al. (2010) demonstrated an increased in glycolysis on MET fed rats' livers as one plausible mechanism to restore oxidative phosphorylation. Additionally, Cai et al. (2020) analyzed protein levels of key enzymes of the glycolysis pathway, in hepatocellular carcinoma cells and revealed that these proteins abundance was ~3 to 4 folds higher after treatment with MET. The results of the present study seem to be in line with the literature, since mRNA transcript levels of *idh3a* and *cox5aa* reveal an upregulation in female livers, which indicate alterations in phases of the cellular respiration such as the tricarboxylic acid cycle and the electron transport chain, respectively, which may be a consequence of a stimulation of glycolysis. It is believed that changes in the ATP:ADP and AMP:ATP ratios lead to the activation of AMPK. mRNA transcriptional results revealed that *prkaa1* (AMPKα1

encoding gene) expression levels were not altered in neither of the two zebrafish developmental states analyzed (20 dpf larvae and 9 mpf females and males). This is an unexpected result, however, it has been previously shown that the several isoforms of AMPK (α1, α2, β1, β2, γ1, γ2, γ3) may play different roles/functions in relation to the link between the protein and the AMP:ATP ratio and that the activation of each one of the isoforms can vary from species to species and according with the conditions to which the tissue and/or organism is subject (Hawley et al., 2010; Stephenne et al., 2011). On the other hand, Activation of AMPK implies that enzymes such as HMGCR and ACC are phosphorylated and inactivated (Gong et al., 2012). Inhibition of HMGCR prevents the conversion of HMG-CoA into mevalonate, reducing cholesterol biosynthesis in the organism (Al-Habsi et al., 2016; Barros et al., 2018). This study shows MET-induced biochemical and molecular effects, with significant alterations on cholesterol levels and on gene expression in female and male livers of the rate limiting gene of the mevalonate pathway – *hmgcra*. We hypothesized that the mRNA expression of hmgcra would be increased, as a negative feedback response to lower levels of cholesterol, given the fact available studies in the literature, using mammalian models as well as zebrafish, report an upregulation of transcriptional levels of *hmgcr* after administration/exposure to HMGCR inhibitors, such as statins (Al-Habsi et al., 2016; Conde et al., 1999; Gouni-Berthold et al., 2008). Females in fact showed an upregulation of *hmgcra*, accompanied by a decrease in liver cholesterol levels. However, although male cholesterol levels decreased as anticipated, a downregulation of *hmgcra* was observed at the intermediate concentration of MET (2166 ng/L). Moreover, with the biochemical and molecular results related to cholesterol combined, we hypothesize that a change in AMPK activity due to MET exposure, could explain the observation, given that it was expected for MET to interfere in cholesterol biosynthesis through activation of AMPK. The expression profiles of *hmgcra* obtained in the present study may suggest that the low MET concentrations used in this experimental work, affects zebrafish transcription levels of *hmgcra* in a time specific manner i.e fluctuations in gene expression over time (Barros et al., 2018). Several studies have already addressed and described this time-dependent gene expression response, in a variety of aquatic organisms after exposure to different compounds (Cunha et al., 2017; Hamadeh et al., 2002; Kim et al., 2015). The expression profile of *hmgcra* in this study is an example of this matter, since 20 dpf larvae did not show differences in transcription levels, but 9-month-old females and males did. Therefore, the absence of studies on the temporal gene expression of *hmgcra* and other genes involved in the MoA of MET (i.e *prkaa1*), emphasizes the necessity of using multiple time-points in gene transcription studies with this pharmaceutical.

No differences were observed in the results of mRNA expression levels of *acaca* (enables ACC activity) and *acadm* (catalyzes fatty acids ß-oxidation) in adult males, contrary to what was expected, as TGL levels are significantly lower. However, *acaca* mRNA were significantly upregulated in females exposed to the intermediate concentration (2166 ng/L), even though a downregulation of this gene was expected (Fullerton et al., 2013; Gong et al., 2012; Zhou et al., 2001). It is possible that such effect could also be an example of fluctuations in gene expression over time.

Lastly, *igf1* levels of mRNA expression were upregulated in 20 dpf larvae. A 2021 study revealed that MET did in fact, induced the transcription levels of *igf1* (Seneviratne et al., 2021). In teleost fishes, Igf-I plays a crucial role in the growth and development of the organism, and it was already stated that environmental contaminants may target the IGF system in fish (Lyssimachou et al., 2015). More interestingly, the concentration where the upregulation of *igf1* was verified in the 20 dpf larvae, is the same where it is possible to observe an increase in length and weight in adult males and in the K of females – 13000 ng/L of MET. These results highlight the hypothesis placed above, where low concentrations are thought to have stimulatory effects in growth parameters.

Most of our results in molecular and biochemical analysis demonstrated a non-monotonic dose-response curve (NMDRC) with an inverted U-shaped curve in females' gene expression of *acaca*, *cox5aa*, *idh3a* and *hmgcra*, and a U-shaped for males' gene expression of *hmgcra* and males' TGL levels. The mathematical definition of an NMDRC states that, within the range of doses assessed, such phenomenon occurs when the curves' slope changes from negative to positive, or vice versa (Kohn & Melnick, 2002). For a long period of time, the premise that "the dose makes the poison" was the starting point for rational thinking and argumentation and therefore, it was assumed that contaminants would always present a linear monotonic dose-response (Vandenberg et al., 2012). However, several long-term studies for the past years, have been documenting more regularly the occurrence of NMDRC as a response to low concentrations of several compounds, mainly endocrine-disrupting chemicals (Andrade et al., 2006; Barros et al., 2020, 2018; Crépeaux et al., 2017; Faigón et al., 2014; J. Kim et al., 2014). The mechanism(s) behind this non-monotonicity are not totally understood, however, the explanatory basis of NMDRCs is based on adaptation mechanisms, generally dependent on the exposure time and the range of concentrations tested. As of today, there is no record of this type of response for MET, and so, we raise the possibility that this is the first study to demonstrate NMDRCs in response to an exposure to MET. The reason behind this may be that, in most cases, MET studies are of acute exposure, as well as with higher concentrations, when compared to the conditions in the present study.

CHAPTER V. Conclusion

5. Conclusion

The present study revealed numerous new and interesting effects of MET on aquatic non-target organisms, still not reported in the literature for this pharmaceutical. The long-term exposure to MET affected multi biological responses of the model organism *Danio rerio* from molecular to ecological level, including disruption of important mechanisms related with energy metabolism, lipid homeostasis and biological processes associated with growth. Moreover, the findings provided in the present study demonstrating significant impacts of MET at environmentally relevant concentrations and highlight the importance of addressing the effects of chemicals under chronic low-level concentrations. Additionally, important data were here generated with relevance for environmental risk assessment, given that aquatic organisms are chronically exposed to MET during multiple generations.

Regardless the value of this study, many questions still remain about MET's MoA and its chronic effects. Taking in consideration the contradictory results available in the literature about the effects of MET in non-target organisms, further long term studies with several environmental concentrations should be undertaken to provide additional insight into the impact of MET across different non-target aquatic organisms and development stages and to improve the knowledge of the MET underlying mechanism(s) of toxicity.

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