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Maria De Jesus Rios Sousa

Avaliação de efeitos ecotoxicológicos de salinidade e metformina em *Gambusia holbrooki*

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Marinha Aplicada, realizada sob a orientação científica da Doutora Sara Cristina Ferreira Marques Antunes, Professora auxiliar convidada do Departamento de Biologia da Faculdade de Ciências da Universidade do Porto e da Doutora Rosa de Fátima Lopes de Freitas, Professora auxiliar com Agregação em regime laboral do Departamento de Biologia da Universidade de Aveiro.

o júri

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palavras-chave

Alterações climáticas, Salinidade, Metformina, *Gambusia holbrooki*, stress oxidativo, reservas energéticas, neurotoxicidade.

resumo

Os ecossistemas aquáticos são ameaçados por vários fatores relacionados com as alterações climáticas, levando a modificações nas propriedades físicas e químicas da água. Entre essas alterações, e como consequência do aquecimento global e de eventos climáticos extemos, os ecossistemas costeiros estão a enfrentar flutuações repentinas na salinidade. Os ecossistemas aquáticos são também ameaçados por compostos orgânicos e inorgânicos que aumentam a poluição da água. A metformina é um medicamento antidiabético usualmente utilizado por pacientes com diabetes tipo 2. É também utilizada no tratamento da síndrome do ovário poliquístico e como um medicamento anticancerígeno. A metformina não é metabolizada pelo corpo humano e tem uma alta mobilidade aquosa. Devido à baixa eficiência na remoção destes compostos pelas estações de tratamentos de águas residuais, a metformina pode chegar aos ambientes aquáticos. O objetivo deste estudo é avaliar a influência dos diferentes níveis de salinidade (17, 24- controlo, 31) nos efeitos da metformina (0- Controlo, 1.5, 15, 150 µ/L) em *Gambusia holbrooki* após uma exposição aguda (96h). Uma exposição crónica também foi realizada para aferir os efeitos da metformina (0- Controlo, 0.5, 1, 5, 10 µ/L) em *G. holbrooki* na salinidade 17 durante 28 dias. Para ambos os ensaios, vários biomarcadores foram quantificados nos organismos expostos para aferir: a capacidade antioxidante (superóxido dismutase- SOD, catalase- CAT, glutationa peroxidase- GPx, glutationa redutase- GRed) e capacidade de biotransformação (glutationa S-transferases- GSTs), dano celular (níveis de peroxidação lipídica- TBARs), conteúdo de reservas energéticas (níveis de proteína do corpo e da cabeça- PROT; conteúdo de glicogénio- GLY) e efeitos neurotóxicos (acetilcolinesterase- AChE).

Os resultados obtidos da exposição aguda mostram interações entre a salinidade e a metformina na atividade da SOD, níveis de PROT do corpo e conteúdo de GLY em *G.holbrooki*. Os dados extraídos mostram que um aumento da salinidade pode alterar a resposta de *G.holbrooki* à metformina. Os resultados obtidos da exposição crónica, mostraram que a exposição de *G.holbrooki* à metformina levou a uma diminuição significativa da atividade da SOD em quase todas as concentrações testadas. Além disso, a atividade da GPX e das GSTs aumentou, significativamente, nas concentrações de metformina de 10 e de 5 µ/L, respetivamente. Assim sendo, a metformina pode levar ao stress oxidativo em *G.holbrooki*. No geral, o conteúdo de GLY em *G.holbrooki* aumentou após a exposição ás concentrações de metformina. Não foram observados efeitos significativos nos restantes biomarcadores medidos.

Apesar deste trabalho não mostrar um cenário completo de stress oxidativo em *G.holbrooki*, após a exposição à metformina em diferentes salinidades, outros estudos já revelaram que a metformina pode levar a danos oxidativos em espécies aquáticas. Assim, estudos ecotoxicológicos adicionais devem ser realizados para descobrir se diferentes concentrações de metformina

combinadas com um aumento da salinidade podem ter impactos em espécies não alvo.

keywords

Climate change, Salinity, Metformin, *Gambusia holbrooki*, Oxidative stress, Energy reserves, Neurotoxicity

abstract

Aquatic ecosystems are threatened by factors related to climate change, leading to changes in the physical and chemical water properties. Between these changes, and as a consequence of global warming and extreme weather events, coastal systems are facing sudden fluctuations in salinity. Aquatic ecosystems are also threatened by organic and inorganic compounds that increase water pollution. Metformin is an antidiabetic drug commonly used by patients of diabetes type 2. It is also used as a treatment for polycystic ovary syndrome and as an anti-cancer drug. Metformin is not metabolized by the human body and has high watery mobility. Because wastewater treatment plants have low efficacy in removing these compounds, they may reach in the aquatic environment. The aim of this study was to evaluate the influence of different salinity levels (17, 24 means control, 31) on the effects of metformin (0-Control, 1.5, 15, 150 µ/L) in *Gambusia holbrooki* after acute exposure (96h). A chronic exposure was also performed to assess the effects of metformin (0-Control, 0.5, 1, 5, 10 µ/L) in *G. holbrooki* at salinity 17 for 28 days. For both assays, several biomarkers were quantified in the exposure organisms to assess: antioxidant capacity (superoxide dismutase-SOD, catalase-CAT, glutathione peroxidase-GPx, glutathione reductase-GRed) and biotransformation capacity (glutathione S-transferases-GSTs), cellular damage (levels of lipid peroxidation-TBARS), content of energy reserves (head and body protein levels- PROT, glycogen content- GLY) and neurotoxic effects (acetylcholinesterase-AChE). The results obtain from acute exposure showed interactions between salinity and metformin in SOD activity, body PROT levels and GLY content in *G. holbrooki*. The data obtain showed that an increase in salinity can modulate the response of the *G. holbrooki* to metformin. The results obtain from the chronic assay, showed that exposure of *G. holbrooki* to metformin led to a significant decrease in SOD activity at almost all concentrations tested. In addition, GPx and GSTs activity increased significantly at the concentration of metformin 10 and 5 µ/L, respectively. Therefore, metformin can lead to oxidative stress in *G. holbrooki*. Overall, the GLY content in *G. holbrooki* increased after exposure to metformin concentrations. No significant effects were observed in the remaining biomarkers measured. Although this work does not show a full oxidative stress scenario in *G. holbrooki* after exposure to metformin under different salinities, other studies have already shown that metformin alone can lead to oxidative damage in aquatic species. Therefore, additional ecotoxicological studies should be performed to find out if different metformin concentrations combined with salinity increase might impact non-target species.

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List of abbreviations

GHG – greenhouse gases

WWTPs – wastewater treatment plants

WFD – water framework directive

WL – watch list

ROS – reactive oxygen species

SOD – superoxide dismutase

CAT – catalase

GPx – glutathione peroxidase

GRed – glutathione reductase

$O_2^{\bullet-}$ – superoxide anion radical

H_2O_2 – hydrogen peroxide

GSH – reduced glutathione

GSSG – glutathione disulfide

GSTs – glutathione-S-transferases

LPO – lipid peroxidation

TBARs – thiobarbituric acid reactive substances

GLY– glycogen

PROT– protein

ChE – cholinesterase

AChE – acetylcholinesterase

SDS – sodium dodecyl sulphate salt

SPE – solid phase extraction

AVMA – American veterinary medical association

BDL– below detection limit

MDS – multidimensional scaling

NBT – nitroblue tetrazolium

CDNB – 1-chloro-2,4-dinitrobenzene

DTNB – 5-50-dithio-bis-2-nitrobenzoate

MDS – multidimensional scaling

EDCs – endocrine disrupting chemicals

OPs – organophosphate compounds

1. Introduction

Aquatic ecosystems provide humans with several services, namely provisioning, regulating and cultural services, including different resources for food (such as fisheries and aquaculture), climate regulation, water purification, recreation, and livelihoods, contributing to human well-being (FAO, 2021). Nevertheless, the aquatic environment is at the same time subjected to various pressures such as disturbance of the hydrographic basin, invasive species introduction, aquaculture, organic and inorganic pollution, human-induced climate change, among others (Pistocchi et al., 2017; Vörösmarty et al., 2010). In regards to Climate Change, it is of great concern as it has worsened in recent years and its effects are felt across the planet (Bindoff et al., 2019). Human activities such as industry, burning fossil fuels, agriculture, among others, induce climate changes through anthropogenic emissions of greenhouse gases (GHG). Greenhouse gases emissions increase the concentrations of gases like methane and carbon dioxide in the atmosphere, which prevent the heat from solar radiation from leaving the earth, leading to a greenhouse effect that, consequently, leads to global warming on earth (Kweku et al., 2018). Human activities induced global warming increases at a rate of 0.2 °C per decade, and it is predicted that between 2030 and 2052 it will likely increase up to 1.5 °C due to the continuous rate of GHG emissions (IPCC, 2018). As a consequence of global warming, earth temperature has been increasing leading to an increase in evaporation rate in surface waters and, consequently, the water becomes more saline (Mhawej et al., 2020). Global warming also causes instability in the sea ice sheets (for example, in Antarctica and Greenland), leading to an increase in the average sea level (IPCC, 2018). Moreover, the increase in the average sea level leads to seawater intrusion into estuarine and freshwater environments (Haddout et al., 2019; Nicholls, 2010). On the other hand, the coastal and inland environments are also subject to other factors that induce changes in salinity, a consequence of drought and rainy periods (Monteiro et al., 2021; Nyitrai et al., 2013). Extreme weather events related to drought and rainy periods are increasing in frequency and intensity, resulting in rapid salinity shifts in transitional systems, especially in the ones where the connection to the sea is limited (Akter et

al., 2019; Bindoff et al., 2019; Cardoso et al., 2008; Haddout et al., 2019). Salinity is an environmental stressor that can influence the growth, development, reproduction, metabolic pathways and physiological functions of aquatic species (Bal et al., 2021; Evans & Kültz, 2020). Although studies suggest that aquatic organisms may have an adaptative potential to the increase of salinity (Chervinski, 1983; Venâncio et al., 2019), variations on water salinity may be much faster than biota can adapt (Nielsen et al., 2003). A study performed by Tian et al. (2020) showed that high salinity may have negative effects on the growth and survival of the yellow drum (*Nibea albiflora*), a euryhaline species. Sablefish (*Anoplopoma fimbria*) juveniles also showed changes in antioxidant and immune responses when the organisms were exposed to high water temperature and salinity (Kim et al., 2017), indicating a potential physiological and metabolic stress.

The aquatic ecosystems are also threatened by several organic and inorganic compounds conducted to increase aquatic pollution. The use of pharmaceutical products to treat diseases has increased in tandem with population growth (Bartolo et al., 2021). Among human diseases of most concern is Diabetes mellitus; an epidemic disease that affects more than 463 million people worldwide, and the number of patients has been rising, with an estimation to increase up to 578 million by 2030 (IDF, 2019). Diabetes is a chronic degenerative metabolic disease characterized and identified by hyperglycemia (high glucose levels in the blood) in the absence of treatment (WHO, 2020). Diabetes occurs when the insulin production by the pancreas is insufficient or does not exist (Diabetes type 1) or when the human body cannot effectively use the insulin that the pancreas produces (Diabetes type 2) (IDF, 2019). Long-term complications in the kidneys, heart, eyes, and autonomic nervous systems are consequences of this disease when patients cannot control blood sugar levels (Nathan, 1993; WHO, 2020). About 90% and 95% of diabetics are type 2 (Chatterjee et al., 2017), and this diabetes is related to environmental factors like sedentary behaviour, obesity, hypertension, heart disease, lack of physical exercise, consumption of food with sugar and fat, among others (WHO, 2019). Along with urbanization growth and unhealthy lifestyle habits, type 2 diabetes has been increasing at a societal level (IDF, 2019), while can be prevented through healthy lifestyle routines, such as a balanced diet and physical exercise (Nathan,

2015). The treatment of type 2 diabetes results from healthy lifestyle routines, oral drugs, or insulin administration (in some cases) to control blood glucose levels. Metformin is an example of an oral antidiabetic compound used worldwide to treat type 2 diabetes (Nathan, 2015) when without increasing insulin secretion, reduces glucose levels (Hundal & Inzucchi, 2003). Studies suggested that this drug can reduce the risk of cancer in patients with type 2 diabetes (Kasznicki et al., 2014; Mallik & Chowdhury, 2018; Morales & Morris, 2015) and can be used as a treatment for regularization of the menstrual cycle in women with polycystic ovary syndrome (Yang et al., 2018). However, metformin has been used for other diseases, leading to an increase in this drug prescription worldwide. As a result of its wide use in the treatment of human diseases, metformin has been identified as the highest pharmaceutical released into the environment (Kolpin et al., 2002; Niemuth & Klaper, 2018; Oosterhuis et al., 2013). This compound is not metabolized by the human body and is excreted unchanged through feces and urine (Oosterhuis et al., 2013; Scheurer et al., 2012). Although wastewater treatment plants (WWTPs) have a rate of removal between 41% to over 98% (Blair et al., 2013; Scheurer et al., 2009; Trautwein & Kümmerer, 2011), metformin reaches the aquatic environment through the discharge of effluents from WWTPs and, due to its properties, high effluent loads and incomplete biodegradation (Briones et al., 2016). The decomposition of metformin in water is approximately 10% at the end of 9 days at 30 ºC (Lee et al., 2019; Sharma et al., 2010). Metformin has a high-water solubility (300 g/L at 25 ºC) (Desai et al., 2014) and a low octanol-water partition coefficient (log K_{ow} -4.9) (Eggen & Lillo, 2012), which suggests that metformin have a high distribution in the aqueous phase (Briones et al., 2016; Elizalde-Velázquez & Gómez-Oliván, 2020).

The European Commission and the Water Framework Directive (WFD) made the surface water watch list (WL) to monitoring data on substances that can have significant impacts on the aquatic environment (Cortes et al., 2020). Every 2 years, the WL is updated, and new substances can be added, regarding some criteria such as intensive use, insufficient monitoring data, low levels of biodegradation, and ecosystem bioaccumulation. Metformin is a candidate for the next WL for 2022 (Cortes et al., 2020); therefore, member states must carefully monitor this water pollutant to determine the risk they pose to the aquatic environment. Although

metformin has been identified as one of the most detected contaminants in the environment (Blair et al., 2013; Bradley et al., 2019; Huber et al., 2016), most of the developing countries did not detect concentrations levels of metformin, unlike the developed countries with high human, economic and social development (Ambrosio-Albuquerque et al., 2021). Metformin concentrations levels have been reported in WWTP influent and effluents, groundwater, sludge, drinking water, and surface water, in different countries such as Greenland, Canada, the USA, Portugal, Poland, among others (Huber et al., 2016; Kot-Wasik et al., 2016; Tong et al., 2015). In Portugal, metformin concentration levels were detected in the range between 1.568 and 325 µg/L in WWTPS influents (de Jesus Gaffney et al., 2017; Santos et al., 2013), and between 0.7 and 6.5 µg/L in WWTPS effluents (de Jesus Gaffney et al., 2017). In surface water, metformin concentrations ranged from 0 µg/L, reported in German by Scheurer et al. (2012), to 9.2 µg/L in the USA (Ambrosio-Albuquerque et al., 2021; Blair et al., 2013) and the highest metformin concentration found in surface water was 33.6 µg/L in the U.S. Great Lakes (Elliott et al., 2017). Metformin was also found in red sea surface waters in concentrations of 4.801 µg/L (Ali et al., 2017).

Regarding the toxicity effects of metformin in non-target organisms, some studies already showed toxic effects (endocrine disruption, reduced fecundity, and oxidative stress) in aquatic species (Elizalde-Velázquez et al., 2021; Lee et al., 2019; Niemuth et al., 2015; Niemuth & Klaper, 2018; Ussery et al., 2018). Niemuth et al. (2015) exposed fathed minnow (*Pimephales promelas*) for 4 weeks to a metformin concentration of 40 µg/L, the results showed that metformin may act as an endocrine disruptor. Niemuth & Klaper (2018) conducted an exposure of *P. promelas* to the same concentration of metformin (40 µg/L) for one year. The results obtained showed further evidence of endocrine disruption (through the analysis of gene expression) in this species. A study conducted by Ussery et al. (2018) demonstrated that metformin can also have negative impacts on fish development (weight loss) when the larvae and embryonic stages of Japanese rice fish (*Oryzias latipes*) were exposed to 3.2 µg/L concentration of this pharmaceutical. Other studies revealed that metformin can also induce oxidative stress in aquatic organisms through the production of reactive oxygen species (ROS) (e.g., Elizalde-

Velázquez et al., 2021; Lee et al., 2019). Oxidative stress can occur when there is an imbalance between oxidants (ROS) and antioxidants (Yoshikawa et al., 2002). To prevent or neutralize oxidative damage, the organisms have a network of enzymes with antioxidant capacity; superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GRed), all important indicators of oxidative stress (Regoli et al., 2011; Regoli & Giuliani, 2014) (Figure 1). Superoxide dismutase provides the first line of antioxidant defense, and this enzyme catalyzes the reduction of superoxide anion radical ($O_2^{\bullet-}$) to hydrogen peroxide (H_2O_2) (Ighodaro & Akinloye, 2018). Catalase is an enzyme that catalyzes the reduction of H_2O_2, which results from the degradation of $O_2^{\bullet-}$ by SOD to H_20 (Ramos et al., 2014). Hydrogen peroxide is eliminated by GPx through reduced glutathione (GSH) which supplies electrons for the degradation of H_2O_2 in H_2O, while GSH is oxidized to glutathione disulfide (GSSG) (Nunes et al., 2006). Glutathione reductase is responsible for the reduction of GSSG to GSH, leading to the formation of two GSH molecules (Nunes et al., 2006). Glutathione reductase is also responsible for maintaining the GSH/GSSG ratio. By catalyzing the conjugation reaction of GSH and electrophilic compounds, Glutathione-S-transferases (GSTs) are a group phase II enzymes involved in detoxification processes (Gröner et al., 2015). Furthermore, this group uses GSH for the degradation of organic hydroperoxides, revealing the dual role in organism protection: antioxidant and detoxification.

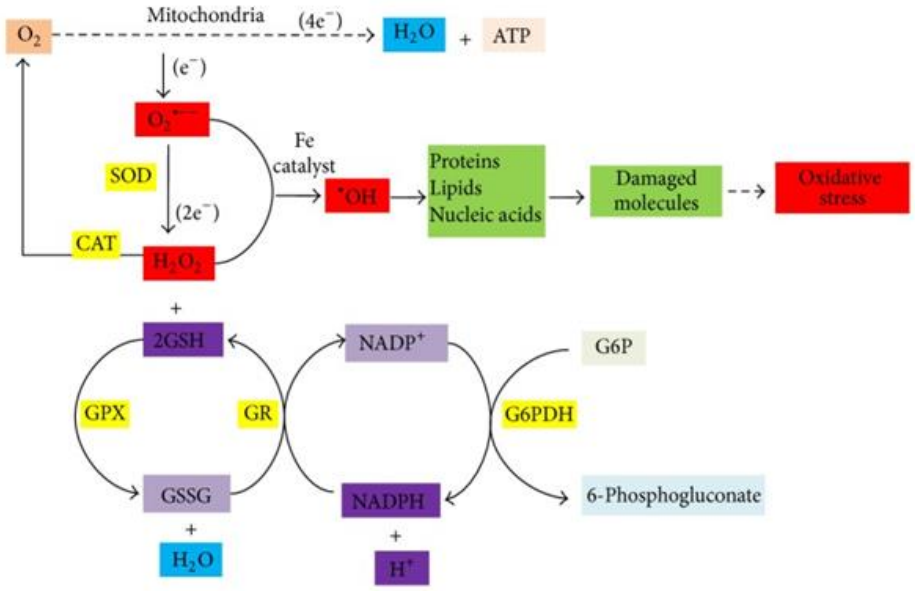

Figure 1: Schematic representation of Superoxide anion radical ($O_2^{\bullet-}$) removal by the antioxidant defense systems and possible damage from this radical. SOD - superoxide dismutase; CAT - Catalase; GPx - glutathione peroxidase; GR - glutathione reductase; GSH - reduced glutathione; GSSG - oxidized glutathione. *Adapted from* Chainy et al. (2016)

Oxidative stress biomarkers, like the ones mentioned previously, are used to assess effects on fish subjected to variations of salinity and temperature (effects of e.g., climate changes) (Kim et al., 2017) and contaminants (Pandey et al., 2003), and therefore they are widely used in ecotoxicological studies. As an example, Lee et al. (2019), exposed two generations of Japanese rice fish (F0 and F1) to 3 metformin concentrations (40, 120, and 360 µg/L) for 133 days. The results showed that in the male of the F0 generation, ROS content increased, and the glutathione content (GSH) decreased, while F0 female fish the CAT activity increased when exposed to metformin treatment. In another study by Elizalde-Velázquez et al. (2021) zebrafish (*Danio rerio*) embryos were exposed to different concentrations of metformin (1, 10, 20, 30, 40, 50, 75, 100 µg/L) until 96 h post-fertilization (hpf). The results showed that SOD, CAT and GPx increased the activity when the embryos were exposed to the tested metformin concentrations (excepted in the concentration of 100 µg/L). When antioxidant defense mechanisms fail to prevent oxidative stress, oxidative damage, including lipid peroxidation (LPO) of biological membranes, can occur in macromolecules such as DNA, cell proteins, and lipids (Pereira et al., 2010), which can lead to cell death. Lipid peroxidation leads to an increase in thiobarbituric acid reactive substances (TBARs), which are formed due to the

presence of malondialdehyde. When organisms are subjected to oxidative stress, energy reserve levels (such as glycogen and protein) are rapidly utilized to activate mechanisms of antioxidant defenses and detoxification (Smolders et al., 2004; Smolders et al., 2003). In this way, glycogen (GLY) and protein (PROT) content reflect the energetic status of the organism and are also used as biomarkers in ecotoxicological studies (Cruz et al., 2016; Emre et al., 2013). Another level of biomarker effects is assessed by the evaluation of neurotoxicity with the analysis of cholinesterase (ChE) activity, usually employed as a biomarker. Acetylcholinesterase (AChE) is an enzyme present in nervous tissues that regulate neurotransmission and plays an important role in neuronal differentiation (Nunes et al., 2011). Changes in the activity of this enzyme can lead to behavioral changes, like delay in growth and changes in metabolism, which can lead to death (Rhee et al., 2013). In aquatic organisms, the activity of AChE can be inhibited by some pharmaceuticals (Li et al., 2012; Solé et al., 2010), suggesting its sensitivity and usefulness for providing an integrative measurement of the overall neurotoxic damage.

Although there are studies that show the interactions between climate change and chemical pollution (Maulvault et al., 2018; Serra-Compte et al., 2018; Wiles et al., 2020), there are no published studies that address the influence of salinity on the impacts caused by metformin. Therefore, this study aimed to evaluate the ecotoxicological metformin effects in *Gambusia holbrooki* under different salinity conditions, to understand the ecotoxicological effect that metformin can have on non-target organisms and the influence of salinity on the sensitivity of the organisms to this drug. To assess the ecotoxicological effects, acute and chronic assays were performed, and a multi-biomarker approach was adopted.

2. Material and methods

2.1. Chemicals

Metformin (1,1-Dimethylbiguanide hydrochloride) used in this study was purchased from Alfa Aesar (Kandel, Germany) (LOT: N27F021; CAS 115-70-4). The stock solutions of metformin (1.5, 15, and 150 µg/L) were prepared with artificial seawater (Red Sea salt and tap water) adjusted to a salinity of 24 (salinity of the sampling site – Ria de Aveiro) for acute exposure. For chronic exposure, the metformin concentrations (0.5, 1, 5 and 10 µg/L) were prepared at salinity 17.

2.2. Study organism

The Eastern mosquitofish, *Gambusia holbrooki* (Girard), is a freshwater fish native from United States of America, already disseminated over the world and categorized as an invasive species (Alcaraz et al., 2008; Kats & Ferrer, 2003). This species has a widespread and cosmopolitan distribution, introduced in several countries for control of populations of malaria mosquitos (Krumholz et al., 2015; Pyke, 2005). The use of the mosquitofish as a model species showed many advantages including its high abundance in wildlife, its small size, and easy maintenance under laboratory conditions (Nunes et al., 2008).

To conduct this study, individuals of *G. holbrooki* were captured, with a hand net, at Esteiro, Ria de Aveiro lagoon (northeast of Portugal, 40.7181679, -8.5934359). Mosquitofish specimens were transported to the laboratory in refrigerated plastic boxes with local water and aerification. The abiotic parameters (pH, conductivity, temperature, salinity, and dissolved oxygen) of the water from the sampling site were quantified for the characterization of water conditions and quality. The acclimation period occurred for two weeks during which the organisms were maintained under laboratory-controlled conditions in a refrigerated box with artificial seawater (Red Sea salt) at salinity 24 (for acute exposure, resembling the sampling site) and at 17 (for chronic exposure), continuous aeration, photoperiod 16h light: 8h dark and constant temperature (22 ± 2 °C). During this period, once a day *G. holbrooki* was feed with fish food commercially available (Tetra Goldfish), and ≈80% of the water was changed. The mortality was monitored during the acclimatization period, and in the absence of mortality for more than a week, organisms were

selected for the assay. For acute exposure, feeding was interrupted before 24 h of the beginning of the bioassays.

2.3. Acute assay

The acute assay was performed under laboratory-controlled conditions during a period of 96 h (in agreement with the OECD test guideline n° 203; OECD, 1992). The metformin concentrations tested were: 0 (CTL), 1.5, 15 and 150 µg/L, and each concentration was tested with different water salinities (17, 24, 31). A blank treatment (without organisms) was performed to evaluate the degradation of metformin during the exposure period (96 h). Metformin concentrations were selected according to information on the literature considering the range of values detected (0.01 – 33.6 µg/L) in the environment (e.g., surface waters) (Briones et al., 2016; Elizalde-Velázquez & Gómez-Oliván, 2020; Santos et al., 2013). The salinities were chosen by increasing and decreasing 7 units in the water salinity at the capture site to recreate the predicted climate change scenarios and consequent salinity variations. Individuals of *G. holbrooki* (length = 1.9 ± 0.2 cm, and weight = 0.067 ± 0.004 g) were exposed in 700 mL of the exposure medium (plastic bottles), and with three replicates per treatment with four organisms in each replicate (twelve per treatment). During the exposure period, abiotic conditions were controlled (continuous aeration, photoperiod 16 h light: 8 h dark, and temperature 20 ± 2°C) and the organisms were not fed. After 48 h from the beginning of the test, the water was completely renewed.

For test validation purposes (OECD, 1992 guidelines) the water parameters: pH, conductivity (µS/cm), temperature (°C), salinity and dissolved oxygen (mg/L) were measure daily (Table 1), using a multiparametric probe. Additionally, water samples were collected from each replicate at 24 and 72 h after spiking, for quantification of nitrites and ammonium, according to the standard protocols of Spectroquant ® Multy (test 114776 and test 114752 respectively). For evaluation of metformin degradation along the exposure period, water samples (30 mL) were collected immediately after spiking, 24 h after spiking, and before the water change on plastic vials for chemical analysis, and immediately stored at -20°C.

2.4. Chronic assay

The chronic assay was performed following OECD guideline No. 215 (OECD, 2000). *G. holbrooki* (length = 4.2 ± 0.22 cm and weight = 0.95 ± 0.22 g) was exposed, for 28 days, to different concentrations of metformin: 0 (CTL), 0.5, 1, 5 and 10 µg/L at salinity 17. The selection of salinity for the chronic assay was based on the results obtained from the acute exposure. A blank treatment was also done to assess metformin degradation during the exposure period. The metformin concentrations selected for the chronic test were below those found in surface waters, for ecological relevance (Ali et al., 2017; Ambrosio-Albuquerque et al., 2021; Elizalde-Velázquez & Gómez-Oliván, 2020; Tong et al., 2015).The organisms were exposed in plastic bottles with 700 mL of the exposure medium, in a total of three replicates per treatment, each with one organism. Abiotic conditions were similar to described for acute exposure, and every 48 h the organisms were fed to satiation, and the water completely renewed.

The parameters for test validation were the same used in the acute test, and the measurements occurred once a week during the exposure period (Table 1). Water samples (30 mL) were collected two times during the exposure period for chemical analysis. The samples were collected immediately after metformin addition and before the water change (only in the blank treatments).

2.5. Chemical analysis

Metformin hydrochloride powder, potassium dihydrogen phosphate and sodium dodecyl sulphate salt (SDS) were purchased from Sigma Aldrich. The standard stock solution was prepared by dissolving 10 mg of metformin hydrochloride in 10 mL of mobile phase and stored in the dark at 4 °C. The working solutions were prepared daily by dilution of the stock solution with mobile phase. For chromatographic analysis (Tahara et al., 2006) distilled water and acetonitrile were of HPLC grade and purchased from VWR International Srl (Milano, Italia). Solid-phase extraction cartridges, packed with 200 mg of Oasis HLB, were purchased from Waters (Milford, MA, USA).

2.5.1 Chromatographic analysis

The chromatographic analyses were performed using an HPLC system with a PerkinElmer Series 200 variable flow pump, coupled to a PerkinElmer UV-VIS detector (PerkinElmer), which was set at 236 nm. The system was controlled by a PerkinElmer interface module (NCI 900 Network Chromatography Interface) and chromatograms were processed by a PerkinElmer TotalChrom Navigator software. Separation was carried out on a 250x4.6 mm chromatographic column X-Bridge C18 5µm (Waters) at room temperature. The mobile phase consisted of 0.01M phosphate buffer (pH 5.0) and acetonitrile (90:10 v/v) at a flow rate of 0.8 ml/min and an injection volume of 100 µL was used.

2.5.2 Water Sample analysis

The solid phase extraction (SPE) procedure was performed by using Oasis HLB Cartridges 6cc extraction columns as described by use manual with some modification. Before extraction, each HLB cartridge was pre-conditioned with 3 mL of methanol followed by rinsing with 3 mL deionized water. Then 3 mL aqueous solution of an ion-pair reagent (SDS 10mM) was added and drawn through the cartridge. After treatment of cartridge with an ion-pair reagent, the water sample, 50 ml, was loaded through the HLB-cartridge at a flow rate of about 1 mL/min. When the extraction was completed, the cartridge was washed with 3 ml of water and subsequently air dried under a vacuum. The metformin was then eluted from the cartridge with 3 mL of methanol. The extract was completely evaporated to dryness with a stream of nitrogen at a temperature of 40 ° C. The residue was redissolved with 1 mL of mobile phase and injected into HPLC (Tahara et al., 2006).

The recovery was >80% and the detection limit, calculated as a signal-to-noise ratio of 3:1, was 1.0 µg/L.

2.6. Sacrifice and sample collection

At the end of the exposure periods, the organisms were euthanized by rapid cooling, placed in a bucket with approximately equal amounts of ice and water (≤4 °C). When organisms lose their opercular movements and the ability to swim, they were sacrificed by decapitation. The procedure is effective and is not stressful for the organisms (Wilson et al., 2009). The use of decapitation as a physical method to euthanize animals is following the American Veterinary Medical Association (AVMA).

Head samples were used for acetylcholinesterase (AChE) activity determinations (neurotoxicity biomarker). Body samples were used for the determination of oxidative stress related biomarkers and energy content.

2.7. Biomarkers

2.7.1 Sample preparation

Head samples were kept in a falcon tube with ice-cold phosphate buffer (0.1 M, pH 7.2) and then homogenized with a mechanical homogenizer (Yellowline DI 18 basic). After homogenization, samples were centrifuged at 3300 *g* for 5 min at 4 °C, and neurotoxicity effects were determined by the Acetylcholinesterase (AChE) quantification in the supernatant.

Body samples were kept in falcon tubes with a phosphate buffer (50 mM, pH 7.0, with 0.1% TRITON X-100) and after homogenization were centrifuged at 15000 *g* for 10 min at 4 °C. After homogenization, aliquots of the supernatant were transferred to microtubes and kept at -80 °C for further measurement of antioxidant and biotransformation (superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GRed), glutathione S-transferases (GSTs) activities), cellular damage (lipid peroxidation levels), and energetic reserves (glycogen (GLY) and protein (PROT) contents) biomarkers.

2.7.2. Biochemical determinations

The activity of SOD was determined according to the protocol described by Beauchamp and Fridovich (1971). The activity was measured spectrophotometrically at a wavelength of 560 nm through the conversion of nitroblue tetrazolium (NBT) to NBT diformazan (formazan dye) and expressed at U per mg of body PROT.

The activity of CAT was determined following the protocol described by Aebi (1984), by monitoring the decomposition of H_2O_2 at 240nm. Results were expressed as µmol of H_2O_2 consumed per min per mg of body PROT.

According to the protocol described by Flohé and Günzler (1984), the total activity of GPx was measured spectrophotometrically through the oxidation of NADPH at a wavelength of 340 nm, when glutathione reductase, reduces glutathione disulfide (GSSG) to glutathione (GSH). The GPx activity is expressed in nmol of NADPH per min per mg of body PROT.

The activity of GRed was determined according to the protocol described by Carlberg and Mannervik (1985) by monitoring 340 nm of NADPH oxidation and expressed in µmol per min and per mg of body PROT.

The activity of GSTs was determined according to Habig et al. (1974). These enzymes catalyze the conjugation of the substrate 1-chloro-2,4-dinitrobenzene (CDNB) with glutathione, and the thioether formed by this reaction is measured in the absorbance of 340 nm and is expressed per mmol of thioether produced per min per mg of body PROT.

The quantification of TBARS content was performed to measure the extent of LPO according to the protocol described by Buege & Aust (1978). Absorbance readings of each sample were measured at a wavelength of 535nm and the content of TBARS was expressed in mmol per mg of body PROT.

The quantification of GLY content was performed according to the protocol described by Lo et al. (1970). Quantification was performed spectrophotometrically at a wavelength of 490 nm. A standard curve was created with GLY standards, and the GLY values were determined by interpolation. The results were expressed in mg of GLY per mg of body PROT.

Protein content (head and body) was quantified with Bradford reagent according to described by Bradford (1976), by spectrophotometric measurement at a wavelength of 595 nm.

According to the protocol described by Ellman et al. (1961), AChE activity was determined using acetylthiocholine as substrate, by spectrophotometric measurement at a wavelength of 412 nm, where the colored compound results from the conjunction of thiocholine with 5-50-dithio-bis-2-nitrobenzoate (DTNB). The quantification was performed because is the predominant form present in the head of *G. holbrooki* (Nunes et al., 2005). The results were expressed in µmol per min per mg of head PROT.

2.8. Statistical analysis

The biomarker results of acute exposure data were submitted to multidimensional scaling (MDS) analysis performed with the software PRIMER v6 to allow for a clearer distinction among treatments.

After performing the normality and homogeneity tests, the acute effects of metformin concentrations and salinities were analyzed for all biochemical parameters (SOD, CAT, GPx, GRed, GSTs, TBARS, GLY, head PROT, body PROT, and AChE) employing a two-way analysis of variance (ANOVA) in the software JAMOVI, followed by a simple main effects analysis whenever metformin concentration and salinities interaction occurred ($p \leq 0.05$).

The chronic effects of metformin were analyzed for all biochemical parameters using a one-way ANOVA in the SPSS software, followed by a Dunnett test to discriminate differences between metformin and control treatment ($p \leq 0.05$).

3. Results and Discussion

3.1. Water quality

All the physical and chemical parameters recorded along the acute and chronic exposure (Table 1) were within the standard quality parameters to assay validation (OECD, 2000). Moreover, no mortality of *G. holbrooki* occurred during the acute and chronic exposure.

Table 1: Average (n=3) and standard error of the physical and chemical results measured during the acute and chronic exposure. (OECD, 2000)

Exposure	Salinity	Metformin concentrations (µg/L)	pH	Temperature (°C)	Salinity	Conductivity (µS/cm)	Dissolved oxygen (mg/L)	Ammonium (mg/L)	Nitrites (µg/L)
Acute exposure	17	0	8.39 ± 0.01	19.23 ± 0.08	17.08 ± 0.03	27.97 ± 0.03	8.72 ± 0.05	0.46 ± 0.26	136.00 ± 71.68
		1.5	8.26 ± 0.03	19.33 ± 0.03	17.00 ± 0.00	27.85 ± 0.02	8.19 ± 0.22	0.68 ± 0.30	80.00 ± 17.05
		15	8.35 ± 0.03	19.42 ± 0.05	17.25 ± 0.07	28.20 ± 0.09	8.57 ± 0.12	0.70 ± 0.23	82.50 ± 14.66
		150	8.38 ± 0.01	19.35 ± 0.08	17.12 ± 0.04	28.03 ± 0.05	8.78 ± 0.06	1.78 ± 0.56	89.50 ± 42.04
	24	0	8.42 ± 0.01	19.16 ± 0.07	24.26 ± 0.02	38.43 ± 0.05	8.89 ± 0.03	0.58 ± 0.24	78.50 ± 22.18
		1.5	8.34 ± 0.02	19.15 ± 0.07	24.20 ± 0.04	38.42 ± 0.04	8.63 ± 0.07	0.70 ± 0.25	66.75 ± 25.78
		15	8.41 ± 0.01	19.22 ± 0.05	24.15 ± 0.02	38.38 ± 0.02	8.70 ± 0.06	0.75 ± 0.20	104.25 ± 62.66
		150	8.43 ± 0.03	19.22 ± 0.06	24.23 ± 0.02	38.42 ± 0.04	8.77 ± 0.08	0.51 ± 0.19	89.50 ± 18.39
	31	0	8.39 ± 0.02	19.18 ± 0.04	31.27 ± 0.08	48.33 ± 0.12	8.65 ± 0.05	0.44 ± 0.16	81.00 ± 03.56
		1,5	8.43 ± 0.02	19.15 ± 0.06	31.33 ± 0.08	48.43 ± 0.11	8.91 ± 0.03	0.45 ± 0.17	176.35 ± 53.35
		15	8.44 ± 0.01	19.32 ± 0.05	29.45 ± 1.64	48.10 ± 0.25	8.75 ± 0.07	0.41 ± 0.10	158.75 ± 24.67
		150	8.43 ± 0.03	19.27 ± 0.05	31.22 ± 0.09	48.23 ± 0.09	8.95 ± 0.13	0.46 ± 0.09	132.25 ± 29.81
Chronic exposure	17	0	8.10 ± 0.06	19.07 ± 0.05	17.00 ± 0.02	26.24 ± 0.34	8.85 ± 0.06	0.55 ± 0.09	75.60 ± 10.00
		0.5	8.03 ± 0.03	19.14 ± 0.05	17.01 ± 0.02	25.94 ± 0.31	8.82 ± 0.11	0.35 ± 0.04	98.67 ± 13.85
		1	8.02 ± 0.05	19.23 ± 0.03	16.99 ± 0.03	26.07 ± 0.36	8.77 ± 0.07	0.50 ± 0.04	81.47 ± 11.54
		5	7.98 ± 0.02	18.96 ± 0.05	17.00 ± 0.03	26.43 ± 0.41	8.80 ± 0.05	0.67 ± 0.19	127.43 ± 30.83
		10	7.99 ± 0.02	18.88 ± 0.03	17.02 ± 0.02	26.21 ± 0.37	8.70 ± 0.06	0.44 ± 0.05	89.47 11.55

3.2. Chemical analysis

Metformin concentrations in water samples were collected during acute exposure (Table 2). At salinity 17, metformin concentrations were similar to nominal concentrations. At salinity 24, metformin concentrations were similar to nominal concentrations and similar to salinity 31. At salinity 31, metformin concentrations showed to be lower than the nominal concentrations and lower then in salinity 17 or 24. At low concentrations of metformin (1.5 µg/L), metformin values were below detection limit.

In general, the concentrations found after 24h were lower compared to those found after spiking (loss percentage >20%). Moreover, lower concentrations were detected in water samples with organisms compared to blank samples after spiking.

Table 2: Metformin concentrations (µg/L) in water samples collected immediately after spiking and after 24h in each tested condition. BDL stands for below detection limit (<1.0 µg/L)

Sampling	Salinity	Treatment	Metformin concentration (µg/L)	Value
After spiking	17	Blank	1.5	<BDL
			15	14.1
			150	147.7
		With organisms	1.5	<BDL
			15	10.1
			150	102.9
	24	Blank	1.5	<BDL
			15	13.9
			150	148.6
		With organisms	1.5	<BDL
			15	9.3
			150	91.3
	31	Blank	1.5	<BDL
			15	11.1
			150	140.7
		With organisms	1.5	<BDL
			15	7.9
			150	76.2
24h after spiking	17	Blank	1.5	<BDL
			15	11.1
			150	101.2
	24		1.5	<BDL
			15	9.6
			150	87.6
	24		1.5	<BDL
			15	6.5
			150	67

3.3. Multidimensional scaling analysis

The biochemical data distribution on the MDS graph obtain from acute exposure (Figure 2) shows a clear separation between metformin concentrations at different salinities, resulting in 3 distinct groups, highlighting the influence of salinity over metformin concentrations. One of the groups includes the CTL treatments (without metformin), exposed to different salinities, and different concentrations at salinity 17; another group was composed by different exposure metformin concentrations at salinity 24; and the third group comprised all tested metformin concentrations at salinity 31. From this analysis it is possible to observe that the organisms showed a similar response when exposed to different salinities (17, 24, 31) in the absence of metformin, which indicates a great adaptation to salinity variations of this species if not under contaminated medium. The results obtained through the MDS further suggest that, among the tested salinities, the lowest salinity allows the organism to adapt better to the presence of metformin in the water since contaminated organisms (regardless the concentration) maintained at salinity 17 presented a similar biochemical performance in comparison to non-contaminated organisms exposed to the different salinities. *G. holbrooki* used for the acute assay were captured in waters with an average salinity between 4 and 16 (Dias et al., 2021). However, at the time of the organism capture, the water salinity was at 24. This increase in salinity occurs due to the seasonal period when the organisms were captured, the late summer when the temperature was high (33 °C). High environmental temperatures lead to a higher rate of evaporation and, consequently, to an increase in local salinity, especially in the shallow waters (Mhawej et al., 2020). Through the MDS results, salinity 17 was chosen to conduct the chronic exposure. This selection was made according to the results obtained in the acute exposure where at salinity 17 the effects at different concentrations of metformin were similar and where smaller differences were observed between exposure only to salinity and metformin concentrations.

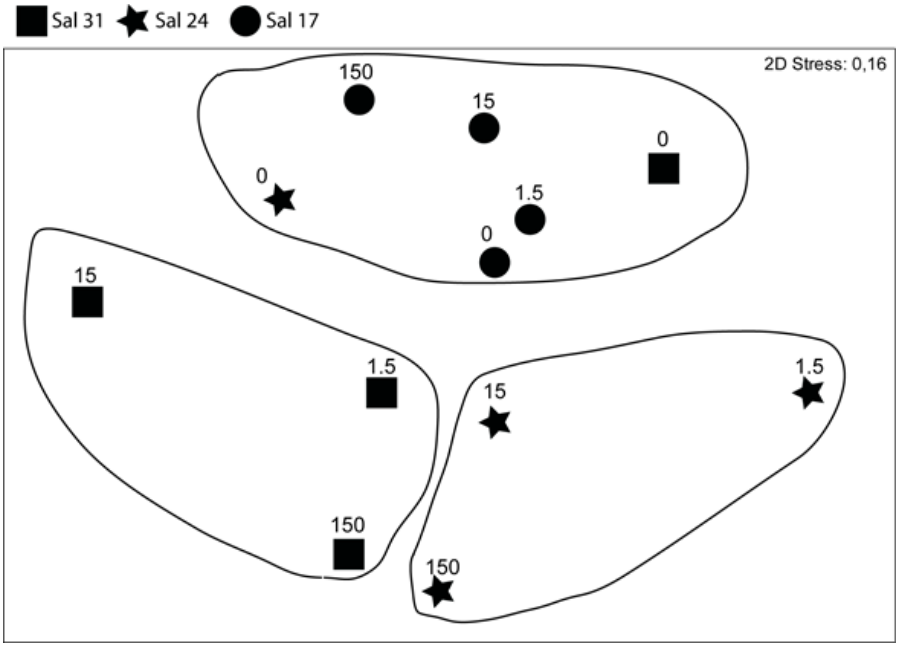

Figure 2: Nonmetric multidimensional scaling (MDS) of the biomarkers data analyzed in the acute exposure. The salinities are represented with figures: ● - salinity 17; ★ - salinity 24; and ■ - salinity 31. The metformin concentrations are represented with 0 (0 µg/L), 1.5 (1.5 µg/L), 15 (15 µg/L), 150 (150 µg/L).

3.4. Stress oxidative parameters

Figure 3 shows the results from oxidative stress related parameters (antioxidant and biotransformation enzymes activities, and cellular damage) quantified after acute exposure of metformin concentrations at different salinities and after chronic exposure of metformin concentrations at salinity 17 in *G. holbrooki*.

Regarding the SOD activity, significant interactions between salinity and metformin concentrations were observed, after acute exposure (Table 3; Figure 3). However, only for the individuals exposed to salinity 24 in the presence of metformin a significant increase in SOD activity was observed. Relatively to the chronic exposure, a significant decrease in SOD activity was recorded in the metformin concentration tested except for 5 µg/L (Figure 3). No significant effects were recorded for CAT activity and TBARS levels of *G. holbrooki* after acute and chronic exposure (Table 3; Figure 3) regardless the conditions tested. Also, no significant acute effects on the activity of GPx were recorded in the different conditions tested (Table 3; Figure 3). However, after chronic exposure, a significant decreased of GPx activity was observed at the highest metformin concentration tested (10 µg/L of metformin, Figure 3). In what regards to GRed enzyme activity, although no significant interaction between the salinity and metformin concentrations were

observed, significant differences were obtained between salinities after acute exposure. In fact, a significant decrease of GRed activity was observed in *G. holbrooki* exposed to salinity 31 (Table 3; Figure 3). In the chronic exposure, no significant differences were observed in GRed activity (Figure 3). Relatively to the GSTs activities, no significant alterations were observed after acute exposure, however a significant increase was observed in *G. holbrooki* after chronic exposure, only for 5 µg/L of metformin concentration (Figure 3).

With climate change, aquatic environments are increasingly subject to sudden changes in salinity, especially in estuarine environments (Gillanders et al., 2011; Vargas et al., 2017). Although organisms that inhabit estuarine environments are likely able to adapt to differences in salinity (Chervinski, 1983; Venâncio et al., 2019), the combination of salinity with other stress factors (such as temperature, toxicants, among others) can negatively affect them. Acute results of GRed activity showed significant differences in salinities tested. *G. holbrooki* is a freshwater species and when exposed to high salinities (such as 31) it resort to antioxidant defense mechanisms to avoid this pressure. Paital & Chainy (2010) showed that crabs exposed to different salinities (17-CTL, 10 and 35) presented an increase in GRed activity in the abdominal muscle and gills at higher salinities. Salinity stress can lead to greater production of ROS (Liu et al., 2007). Thereby, alteration in GRed response may indicate that this enzyme is involved in the process of restoring the GSH needed for the glutathione cycle or involved in other antioxidant defense functions (Nie et al., 2013). In particular, salinity is a stress factor that can change the sensitivity of organisms to contaminants present in aquatic ecosystems, such as metals, endocrine disrupting chemicals (EDCs) and polycyclic aromatic hydrocarbons (Bosker et al., 2017; Hooper et al., 2013; Ramachandran et al., 2006; Wood et al., 2004). Correia et al. (2016) exposed clams (*Ruditapes philippinarum*) to paracetamol concentrations (0.00-CTL, 0.05, 0.5 and 5 mg/L) under different salinities (28-CTL, 14 and 35) for 96 h. The results showed that variations in salinity increased the antioxidant response of clams after exposure to paracetamol. A study conducted by Freitas et al. (2020) showed that in chronic exposure to salicylic acid (4 mg/L) at different salinities (30- CTL, 25 and 35), the SOD activity was higher at salinities (25 and 35). This fact was recorded in both mussels contaminated and

non-contaminated with salicylic acid. In agreement with these studies, after acute exposure, SOD activity in *G. holbrooki* increased at salinity 24 when exposed to metformin concentrations, suggesting that different salinities may modulate the response of these organisms to metformin contamination.

Metformin is an antidiabetic drug that by suppressing the respiratory chain of mitochondrial complex I can suppress gluconeogenesis in the liver leading to a decrease in glucose levels (Giannarelli et al., 2003; Viollet et al., 2012). Mitochondrial complex I is involved in the formation of $O_2^{\bullet-}$ by forward and reverse electron flux in electron transfer chain, so the inhibition of this complex can lead to a decrease in mitochondrial reactive oxygen species (ROS) production (Batandier et al., 2006; Ouslimani et al., 2005; Vial et al., 2019; Wheaton et al., 2014). These findings corroborate our results after chronic exposure of *G. holbrooki* to metformin at salinity 17, where SOD activity was not activated at most metformin concentrations tested. Superoxide dismutase inhibition can be associated with metformin that by acting on the mitochondrial complex and preventing the formation of $O_2^{\bullet-}$, which is degraded by SOD to H_2O_2 (Ighodaro & Akinloye, 2018). The hydrogen peroxide resulting from the enzymatic action of SOD can be degraded by enzymes such as CAT and GPx. A decrease $O_2^{\bullet-}$ production, can lead to a decrease in SOD activity and consequently to a decrease in H_2O_2 production. The here-obtained results showed that CAT and GPx activities were not affected in acute exposure, however, after chronic exposure to metformin, the GPx activity significantly increased at the highest concentration tested (10 µg/L). Contrary to our findings, in a multi-generational assay with *O. latipes*, metformin exposure (0-CTL, 40, 120 and 360 µg/L) increased the ROS content, decreased GSH levels and CAT activity also increased, which demonstrates that this compound can induce oxidative stress (Lee et al., 2019). Also, a recent study conducted by Elizalde-Velázquez et al. (2021) evaluated the effects of metformin in the embryonic development of zebrafish (*Danio rerio*), also showed that after acute exposure of embryos to metformin concentrations (1, 10, 20, 30, 40, 70 and 100 µg/L) increased the SOD, CAT and GPx activities. Furthermore, Queiroz et al. (2014) reported that a 72 h metformin treatment (10 mM) increased the ROS content if used as a treatment for breast cancer cell lines, and SOD and CAT can improve cell viability in the presence

of metformin. These differences between results obtained in the literature and in the chronic exposure of *G. holbrooki* may be related to the chosen metformin concentrations. The highest metformin concentration chosen for chronic exposure was 10 µg/L, in contrast to Lee et al. (2019) who chose 40 µg/L as the lowest concentration. Higher concentrations are more likely to cause oxidative effects in organisms. Furthermore, the species chosen to conduct the assays and their developmental status may also be a source of variation due to species specificity in oxidative damage. *G. holbrooki* is an organism that adapts to various environmental conditions and was in its full developmental stage during chronic exposure, unlike what happened in Elizalde-Velázquez et al. (2021) that evaluated the effects of metformin on zebrafish embryos.

Glutathione-S-transferases conjugate GSH with electrophilic compounds during phase II detoxification processes (Gröner et al., 2015). In Lee et al. (2019), although metformin exposure increase ROS content and decrease GSH content, GSTs activity were not changed. During chronic exposure to metformin, only *G. holbrooki* exposed to 5 µg/L showed an increase in GSTs activity, meaning that this detoxification pathway may be used by the organisms to eliminate the compound.

Table 3: Summary table of the two-way ANOVA applied to salinity variation and metformin concentrations during acute exposure. Bold values stands for significant differences between tested factors ($p \leq 0.05$)

Endpoint	Source variation	F	d.f.	*p*
SOD	Sal	13.285	2	**<0.001**
	Met	9.817	3	**<0.001**
	Sal x Met	3.505	6	**0.021**
	Residual		16	
CAT	Sal	0.119	2	0.889
	Met	1.453	3	0.252
	Sal x Met	2.265	6	0.071
	Residual		24	
GPx	Sal	1.165	2	0.329
	Met	1.377	3	0.274
	Sal x Met	1.034	6	0.428
	Residual		24	
GRed	Sal	7.507	2	**0.003**
	Met	0.771	3	0.522
	Sal x Met	2.343	6	0.064
	Residual		24	
GSTs	Sal	1.723	2	0.200
	Met	0.372	3	0.774
	Sal x Met	1.346	6	0.276
	Residual		24	
TBARs	Sal	1.293	2	0.293
	Met	0.0562	3	0.982
	Sal x Met	0.710	6	0.645
	Residual		24	
Head PROT	Sal	0.351	2	0.707
	Met	0.834	3	0.488
	Sal x Met	0.311	6	0.925
	Residual		24	
Body PROT	Sal	3.63	2	0.043
	Met	2.27	3	0.107
	Sal x Met	5.09	6	**0.002**
	Residual		23	
GLY	Sal	13.57	2	**<0.001**
	Met	2.86	3	0.059
	Sal x Met	4.91	6	**0.002**
	Residual		23	

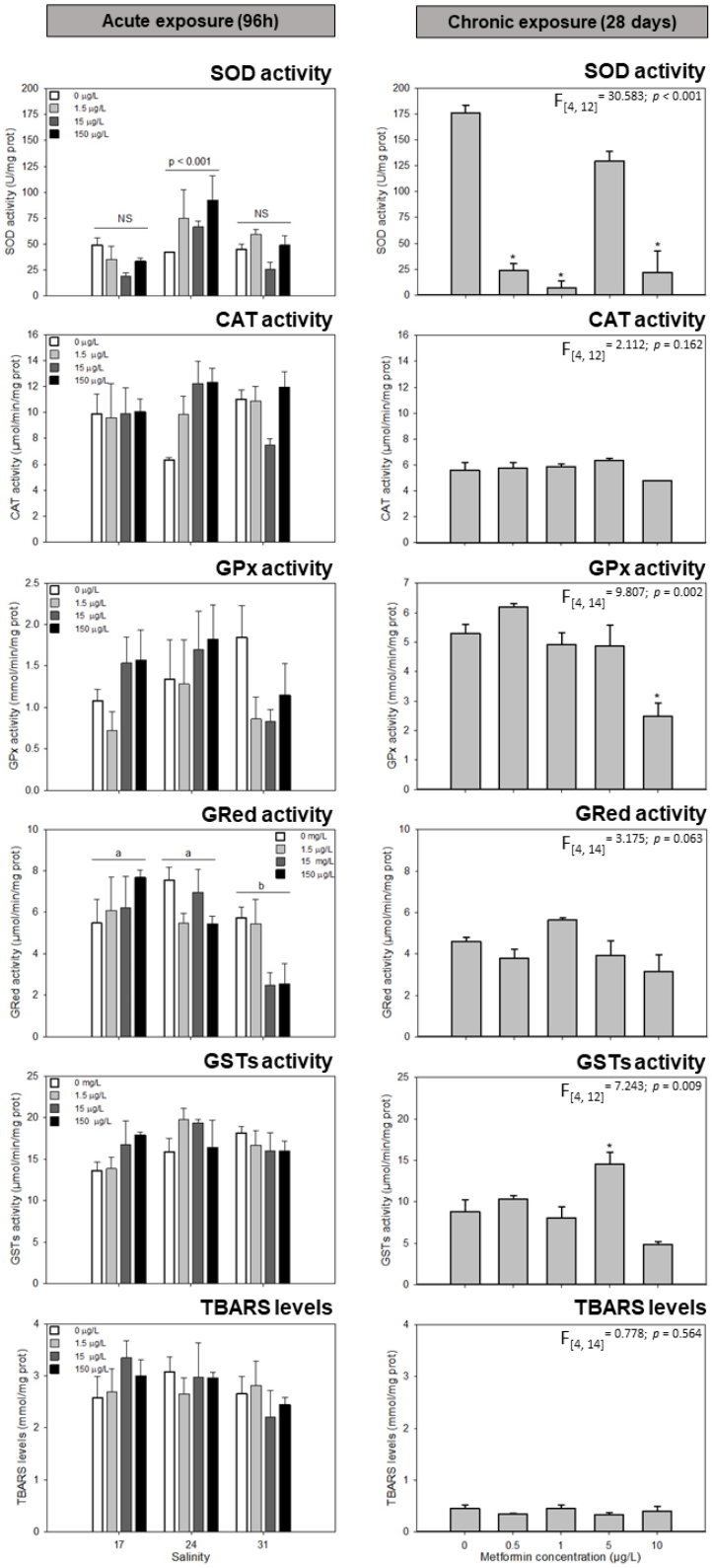

Figure 3: Results of acute (left) and chronic (right) exposure to metformin and salinities in superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GRed), glutathione S-transferases (GSTs) activities, and lipid peroxidation (measured as TBARs levels) in *Gambusia holbrooki*. *stands for discriminate significant differences to the control group (0 µg/L of metformin), Dunnett p ≤ 0.05.

3.5. Energy content

No significant differences in head PROT levels were observed in *G. holbrooki* after acute and chronic exposures (Table 2; Figure 4). However, during acute exposure, a significant interaction between salinity and metformin was observed in the body PROT levels of *G. holbrooki* (Table 2; Figure 4). Regarding salinity 24, significant differences were recorded at body PROT levels along the metformin concentrations tested. No significant differences were observed in head and body PROT levels after chronic metformin exposure (Figure 4).

A significant interaction was recorded in GLY content after acute exposure to metformin at different salinities (Table 2; Figure 4). Indeed, only at salinity 31, a significant increase in GLY contents was observed after acute exposure to metformin concentrations. Overall, in the chronic exposure, a significant increase in GLY content was observed, namely at the lowest metformin concentrations tested (Figure 4).

Organisms may need additional energy to cope with environmental stressors. Lipids, proteins and carbohydrates, when metabolized, can provide energy to the organisms (Tseng & Hwang, 2008). *G. holbrooki*, exposed to salinity 24, showed a decrease in PROT levels when exposed to tested metformin concentrations in the acute assay. Gracia-López et al. (2006) showed that, after exposure of common snook (*Centropomus undecimalis*) to different salinities (35-CTL, 0, 12 and 25), the organisms metabolized proteins, lipids, and carbohydrates to deal with differences in environmental conditions. Furthermore, at low salinities, organisms preferred to use the protein as a substrate for additional energy. *G. holbrooki*, may have used proteins as an energy substrate to deal with salinity conjugation and metformin exposure.

Jacob et al. (2018), exposed brown trout (*Salmo trutta f. fario*) embryos to metformin concentrations (0-CTL, 1, 10, 100 and 1000 µg/L) and showed that the GLY content in the liver was higher in the lowest metformin concentration (1 µg/L), however, at higher doses of metformin, a major inconsistency in GLY content occurred without a concentration-effect relationship. The authors explained these findings by the role of metformin in inhibiting glycogenolysis in humans which can lead to a higher GLY content in the organisms. Also at higher concentrations, the

organisms may need additional energy, leading to a lower GLY content when exposed to higher metformin concentrations (Jacob et al., 2018). This fact was also recorded in the here-presented results where chronic exposure to metformin in a similar range showed an increase in GLY content at the lowest metformin concentrations tested and an opposite response at the highest metformin concentrations tested.

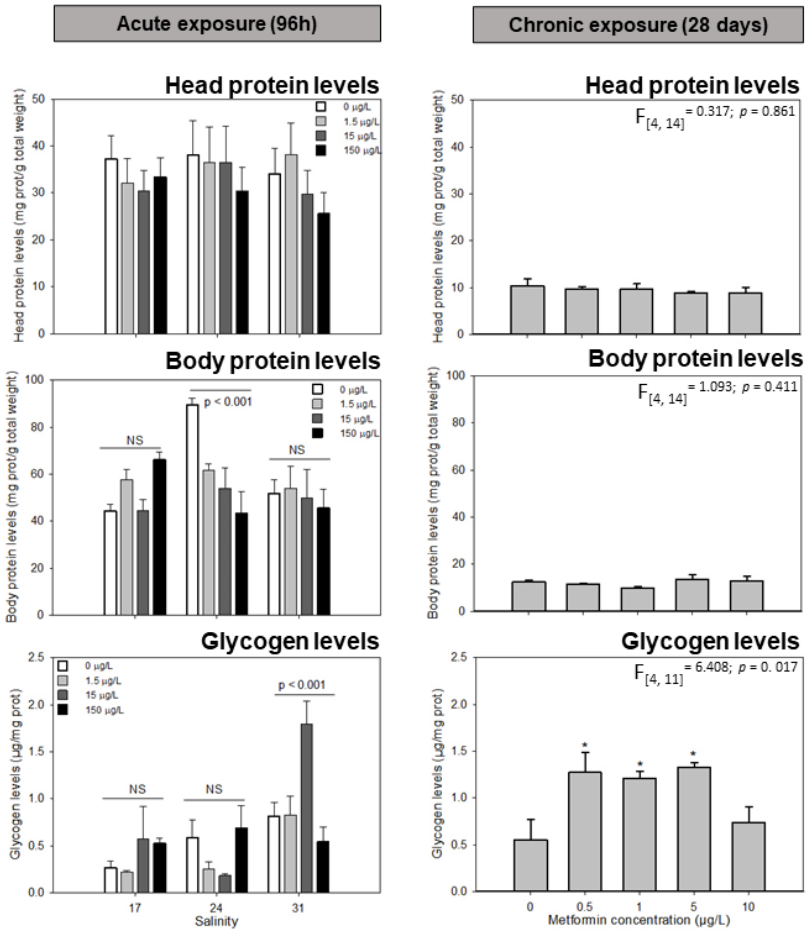

Figure 4: Results of acute (left) and chronic (right) exposure of metformin and salinities in the content of protein head, protein body and glycogen in *Gambusia holbrooki*. *stands for discriminate significant differences to the control group (0 µg/L of metformin), Dunnett p ≤ 0.05.

3.6. Neurotoxicity

No significant effects were recorded in AChE activity after acute and chronic exposure of *G. holbrooki* to metformin at different salinities (Table 2; Figure 5).

Acetylcholinesterase is an essential enzyme in the nervous system and an important indicator of environmental toxicity (Lionetto et al., 2013). Acetylcholinesterase is often targeted by organophosphate compounds (OPs) that are part of the most widely used insecticides (Casida & Durkin, 2013) that inhibited the AChE leading to an excess of acetylcholine. Moreover, there are reports of other contaminants related to AChE inhibition such as some pharmaceuticals (Nunes et al., 2006). However, the here-obtained results (acute and chronic exposure) do not show significant effects after metformin concentrations regardless the salinities tested. Similarly, Doujet (2016) already showed that juveniles of Atlantic salmon (*Salmo salar*) exposed to metformin concentrations (5 and 50 µg/L) did not present significant differences in AChE activity after 10-day of exposure. Acetylcholinesterase activity can also depend on abiotic factors related to climate change like temperature and salinity (Cailleaud et al., 2007; Pfeifer et al., 2005; Sarma et al., 2013). Variations in salinity can be extremely sensitive for osmoregulatory organisms, especially if there is an increase in salinity in freshwater environments (Velasco et al., 2019), which can lead to neurotoxicity damage through inhibition of AChE activity in some species. Walking catfish (*Clarias batrachus*), a freshwater species when subject to salinity (4 and 8) showed inhibition of AChE activity (Sarma et al., 2013). However, in the here-exposure conditions the salinity and metformin concentrations did not inhibit AChE activity in *G. holbrooki*. As previously mentioned, *G. holbrooki* is a species with a high tolerance to salinity variations, so the salinity variations tested in the acute exposure may not be sufficient to cause changes in AChE activity. Also, metformin concentrations tested in both acute and chronic may not be sufficient to impact AChE activity in *G. holbrooki*.

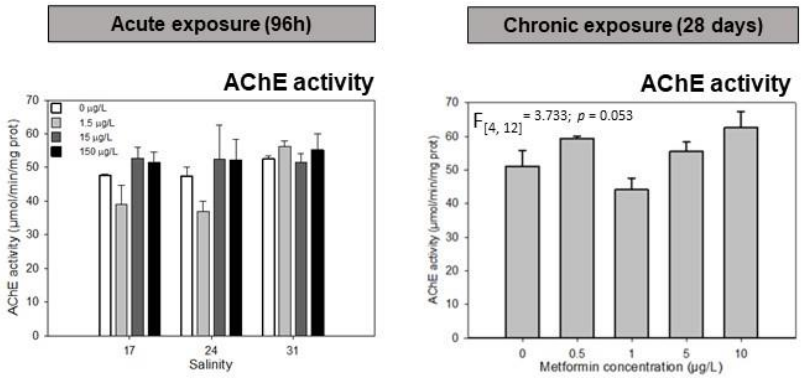

Figure 5: Results of Acetylcholinesterase (AChE) activity after acute (left) and chronic (right) exposure of *Gambusia holbrooki* to metformin and salinities

4. Conclusions

The present study provides information on the combined effects of salinity variations and metformin exposure in *Gambusia holbrooki*. The salinity variations caused by climate change and the worldwide use of pharmaceuticals such as metformin for the treatment of diseases can cause antioxidant and energetic alterations in aquatic organisms such as *G. holbrooki*. The results obtained from the acute exposure showed that the increase in salinity combined with the exposure to metformin concentrations is a stress factor for the organisms, causing effects at the antioxidant and metabolic levels. It is possible to infer that in salinity 17 the organisms managed to adapt to the combined toxicity of metformin and salinity. However, increased salinity and exposure to metformin change the response of organisms leading to a scenario of oxidative stress. During chronic exposure to a salinity of 17, metformin exerted its pharmacological effect, in which it is able to decrease the production of ROS by the mitochondrial complex I in organisms not activating antioxidant defense lines such as SOD and increasing the level of glycogen in the organism. However, metabolic pathways linked to the organism detoxification, such as GSTs, were activated.

Overall, salinity appears to modulate the response of the organism to metformin, leading to an effect more accentuated when salinity is higher. However, further studies need to be carried out to try to understand the effect of metformin concentrations and salinity variation on other non-target aquatic organisms.

5. References

Aebi, H. (1984). Catalase in vitro. Methods in Enzymology, 105, 121-126.

Akter, R., Asik, T. Z., Sakib, M., Akter, M., Sakib, M. N., Al Azad, A. S. M., Maruf, M., Haque, A. & Rahman, M. (2019). The dominant climate change event for salinity intrusion in the GBM Delta. Climate, 7(5), 69.

Alcaraz Cazorla, C., Bisazza, A., & García-Berthou, E. (2008). Salinity mediates the competitive interactions between invasive mosquitofish and an endangered fish. Oecologia, 155 (1), 205-213.

Ali, A. M., Rønning, H. T., Alarif, W., Kallenborn, R., & Al-Lihaibi, S. S. (2017). Occurrence of pharmaceuticals and personal care products in effluent-dominated Saudi Arabian coastal waters of the Red Sea. Chemosphere, 175, 505-513.

Ambrosio-Albuquerque, E. P., Cusioli, L. F., Bergamasco, R., Gigliolli, A. A. S., Lupepsa, L., Paupitz, B. R., Barbieri, P. A., Borin-Carvalho, L. A., & Portela-Castro, A. L. B., (2021). Metformin environmental exposure: A systematic review. Environmental Toxicology and Pharmacology, 83,103588.

Bal, A., Panda, F., Pati, S. G., Das, K., Agrawal, P. K., & Paital, B. (2021). Modulation of physiological oxidative stress and antioxidant status by abiotic factors especially salinity in aquatic organisms. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology, 241, 108971.

Bartolo, N. S., Azzopardi, L. M., & Serracino-Inglott, A. (2021). Pharmaceuticals and the environment. Early Human Development, 155, 105218.

Batandier, C., Guigas, B., Detaille, D., El-Mir, M., Fontaine, E., Rigoulet, M., & Leverve, X. M. (2006). The ROS production induced by a reverse-electron flux at respiratory-chain complex 1 is hampered by metformin. Journal of Bioenergetics and Biomembranes, 38(1), 33-42.

Beauchamp, C., & Fridovich, I. (1971). Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. Analytical Biochemistry, 44(1), 276-287.

Bindoff, N. L., Cheung, W. W., Kairo, J. G., Arístegui, J., Guinder, V. A., Hallberg, R., Hilmi, N., Jiao, N., Karim, M. S., Levin, L., O'Donoghue, S., Purca Cuicapusa, S. R., Rinkevich, B., Suga, T., Tagliabue, A., & Williamson, P. (2019). Changing Ocean, Marine Ecosystems, and Dependent Communities.

IPCC Special Report on the Ocean and Cryosphere in a Changing Climate, 447–588.

Blair, B. D., Crago, J. P., Hedman, C. J., Treguer, R. J., Magruder, C., Royer, L. S., & Klaper, R. D. (2013). Evaluation of a model for the removal of pharmaceuticals, personal care products, and hormones from wastewater. Science of the Total Environment, 444, 515-521.

Bosker, T., Santoro, G., & Melvin, S. D. (2017). Salinity and sensitivity to endocrine disrupting chemicals: A comparison of reproductive endpoints in small-bodied fish exposed under different salinities. Chemosphere, 183, 186-196.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry, 72(1-2), 248–254.

Briones, R. M., Sarmah, A. K., & Padhye, L. P. (2016). A global perspective on the use, occurrence, fate and effects of anti-diabetic drug metformin in natural and engineered ecosystems. Environmental Pollution, 219, 1007-1020.

Buege, J. A., Aust, S. D. (1978). Microsomal lipid peroxidation. Methods in Enzymology, 52, 302–310.

Cailleaud, K., Maillet, G., Budzinski, H., Souissi, S., & Forget-Leray, J. (2007). Effects of salinity and temperature on the expression of enzymatic biomarkers in *Eurytemora affinis* (Calanoida, Copepoda). Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 147(4), 841-849.

Cardoso, P. G., Raffaelli, D., & Pardal, M. A. (2008). The impact of extreme weather events on the seagrass *Zostera noltii* and related *Hydrobia ulvae* population. Marine Pollution Bulletin, 56(3), 483-492.

Carlberg, I., Mannervik, B. (1985). Glutathione reductase. Methods in Enzymology,113, 484–490.

Casida, J. E., & Durkin, K. A. (2013). Anticholinesterase insecticide retrospective. Chemico-biological interactions, 203(1), 221-225.

Chainy, G. B. N., Paital, B., & Dandapat, J. (2016). An overview of seasonal changes in oxidative stress and antioxidant defence parameters in some invertebrate and vertebrate species. Scientifica, 2016, 6126570, 1-8.

Chatterjee, S., Khunti, K., & Davies, M. J. (2017). Type 2 diabetes. The lancet, 389(10085), 2239-2251.

Chervinski, J. (1983). Salinity tolerance of the mosquito fish, *Gambusia affinis* (Baird and Girard). Journal of Fish Biology, 22(1), 9-11.

Correia, B., Freitas, R., Figueira, E., Soares, A. M., & Nunes, B. (2016). Oxidative effects of the pharmaceutical drug paracetamol on the edible clam *Ruditapes philippinarum* under different salinities. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology, 179, 116-124.

Cortes, L. G., Marinov, D., Sanseverino, I., Cuenca, A. N., Niegowska, M., Rodriguez, E., & Lettieri, T. (2020). Selection of substances for the 3rd Watch List under the Water Framework Directive. Office of the European Union: Luxembourg.

Cruz, D., Almeida, Â., Calisto, V., Esteves, V. I., Schneider, R. J., Wrona, F. J., Soares, A. M., Figueira, E. & Freitas, R., (2016). Caffeine impacts in the clam *Ruditapes philippinarum*: Alterations on energy reserves, metabolic activity and oxidative stress biomarkers. Chemosphere, 160, 95-103.

de Jesus Gaffney, V., Cardoso, V. V., Cardoso, E., Teixeira, A. P., Martins, J., Benoliel, M. J., & Almeida, C. M. M. (2017). Occurrence and behaviour of pharmaceutical compounds in a Portuguese wastewater treatment plant: Removal efficiency through conventional treatment processes. Environmental Science and Pollution Research, 24(17), 14717-14734.

Desai, D., Wong, B., Huang, Y., Ye, Q., Tang, D., Guo, H., Huang, M. &Timmins, P. (2014). Surfactant-mediated dissolution of metformin hydrochloride tablets: wetting effects versus ion pairs diffusivity. Journal of Pharmaceutical Sciences, 103(3), 920-926.

Dias, J. M., Pereira, F., Picado, A., Lopes, C. L., Pinheiro, J. P., Lopes, S. M., & Pinho, P. G. (2021). A Comprehensive Estuarine Hydrodynamics-Salinity Study: Impact of Morphologic Changes on Ria de Aveiro (Atlantic Coast of Portugal). Journal of Marine Science and Engineering, 9(2), 234.

Doujet, T. (2016). Uptake, organ distribution and physiological effects of an anti-diabetic II drug (metformin) in Atlantic salmon (*Salmo salar*) (Master's thesis, Norwegian University of Science and Technology).

Eggen, T., & Lillo, C. (2012). Antidiabetic II drug metformin in plants: uptake and translocation to edible parts of cereals, oily seeds, beans, tomato, squash, carrots, and potatoes. Journal of Agricultural and Food Chemistry, 60(28), 6929-6935.

Ellman, G. L., Courtney, K. D., Andres Jr, V., & Featherstone, R. M. (1961). A new and rapid colorimetric determination of acetylcholinesterase activity. Biochemical Pharmacology, 7(2), 88-95.

Elizalde-Velázquez, G. A., & Gómez-Oliván, L. M. (2020). Occurrence, toxic effects and removal of metformin in the aquatic environments in the world: Recent trends and perspectives. Science of The Total Environment, 702, 134924.

Elizalde-Velázquez, G. A., Gómez-Oliván, L. M., García-Medina, S., Islas-Flores, H., Hernández-Navarro, M. D., & Galar-Martínez, M. (2021). Antidiabetic drug metformin disrupts the embryogenesis in zebrafish through an oxidative stress mechanism. Chemosphere, 285, 131213.

Elliott, S. M., & VanderMeulen, D. D. (2017). A regional assessment of chemicals of concern in surface waters of four Midwestern United States national parks. Science of the Total Environment, 579, 1726-1735.

Emre, I., Kayis, T., Coskun, M., Dursun, O., & Cogun, H. Y. (2013). Changes in antioxidative enzyme activity, glycogen, lipid, protein, and malondialdehyde content in cadmium-treated *Galleria mellonella* larvae. Annals of the Entomological Society of America, 106(3), 371-377.

Evans, T. G., & Kültz, D. (2020). The cellular stress response in fish exposed to salinity fluctuations. Journal of Experimental Zoology Part A: Ecological and Integrative Physiology, 333(6), 421-435

FAO (2021). Food and Agricultural Organization of the United Nations. Fisheries and Aquaculture Department.

Flohé, L., & Günzler, W. A. (1984). Assays of glutathione peroxidase. Methods in enzymology, 105, 114-120.

Freitas, R., Silvestro, S., Coppola, F., Meucci, V., Battaglia, F., Intorre, L., Soares, A.M., Pretti, C. & Faggio, C. (2020). Combined effects of salinity changes and salicylic acid exposure in *Mytilus galloprovincialis*. Science of the Total Environment, 715, 136804.

Ghoshdastidar, A. J., Fox, S., & Tong, A. Z. (2015). The presence of the top prescribed pharmaceuticals in treated sewage effluents and receiving waters in Southwest Nova Scotia, Canada. Environmental Science and Pollution Research, 22(1), 689-700.

Giannarelli, R., Aragona, M., Coppelli, A., & Del Prato, S. (2003). Reducing insulin resistance with metformin: the evidence today. Diabetes & metabolism, 29(4), 6S28-6S35.

Gillanders, B. M., Elsdon, T. S., Halliday, I. A., Jenkins, G. P., Robins, J. B., & Valesini, F. J. (2011). Potential effects of climate change on Australian estuaries and fish utilising estuaries: a review. Marine and Freshwater Research, 62(9), 1115-1131.

Gracia-Lopez, V., Rosas-Vazquez, C., & Brito-Perez, R. (2006). Effects of salinity on physiological conditions in juvenile common snook *Centropomus undecimalis*. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 145(3), 340-345.

Gröner, F., Ziková, A., & Kloas, W. (2015). Effects of the pharmaceuticals diclofenac and metoprolol on gene expression levels of enzymes of biotransformation, excretion pathways and estrogenicity in primary hepatocytes of Nile tilapia (*Oreochromis niloticus*). Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology, 167, 51-57.

Habig, W. H., Pabst, M. J., & Jakoby, W. B. (1974). Glutathione S-transferases: the first enzymatic step in mercapturic acid formation. Journal of biological Chemistry, 249(22), 7130-7139.

Haddout, S., Baimik, I., Maslouhi, A., Igouzal, M., Magrane, B., & Marah, H. (2019). The influence of spring and neap tide on salt intrusion and stratification in Sebou estuary (Morocco). International journal of river basin management, 17(1), 131-142.

Hooper, M. J., Ankley, G. T., Cristol, D. A., Maryoung, L. A., Noyes, P. D., & Pinkerton, K. E. (2013). Interactions between chemical and climate stressors: A role for mechanistic toxicology in assessing climate change risks. Environmental Toxicology and Chemistry, 32(1), 32-48.

Huber, S., Remberger, M., Kaj, L., Schlabach, M., Jörundsdóttir, H. Ó., Vester, J., Arnórsson, M., Mortensen, I., Schwartson, R. & Dam, M. (2016). A first screening and risk assessment of pharmaceuticals and additives in personal care products in waste water, sludge, recipient water and sediment from Faroe Islands, Iceland and Greenland. Science of the Total Environment, 562, 13-25.

Hundal, R. S., & Inzucchi, S. E. (2003). Metformin. Drugs, 63(18), 1879-1894.

Ighodaro, O. M., & Akinloye, O. A. (2018). First line defence antioxidants-superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX): Their fundamental role in the entire antioxidant defence grid. Alexandria journal of medicine, 54(4), 287-293.

International Diabetes Federation, (2019). IDF diabetes Atlas Ninth. Dunia IDF.

IPCC, 2018: Summary for Policymakers. In: Global Warming of 1.5°C. An IPCC Special Report on the impacts of global warming of 1.5°C above pre-industrial levels and related global greenhouse gas emission pathways, in the context of strengthening the global response to the threat of climate change, sustainable development, and efforts to eradicate poverty.

Jacob, S., Dötsch, A., Knoll, S., Köhler, H. R., Rogall, E., Stoll, D., Tisler, S., Huhn, C., Schwartz, T., Zwiener, C. & Triebskorn, R. (2018). Does the antidiabetic drug metformin affect embryo development and the health of brown trout (*Salmo trutta f. fario*)?. Environmental Sciences Europe, 30(1), 1-16.

Kasznicki, J., Sliwinska, A., & Drzewoski, J. (2014). Metformin in cancer prevention and therapy. Annals of translational medicine, 2(6).

Kats, L. B., & Ferrer, R. P. (2003). Alien predators and amphibian declines: review of two decades of science and the transition to conservation. Diversity and distributions, 9(2), 99-110.

Kim, J. H., Park, H. J., Kim, K. W., Hwang, I. K., Kim, D. H., Oh, C. W., Lee, J.S. & Kang, J. C. (2017). Growth performance, oxidative stress, and non-specific immune responses in juvenile sablefish, *Anoplopoma fimbria*, by changes of water temperature and salinity. Fish physiology and biochemistry, 43(5), 1421-1431.

Kolpin, D. W., Furlong, E. T., Meyer, M. T., Thurman, E. M., Zaugg, S. D., Barber, L. B., & Buxton, H. T. (2002). Pharmaceuticals, hormones, and other organic

wastewater contaminants in US streams, 1999−2000: A national reconnaissance. Environmental Science & Technology, 36(6), 1202-1211.

Kot-Wasik, A., Jakimska, A., & Śliwka-Kaszyńska, M. (2016). Occurrence and seasonal variations of 25 pharmaceutical residues in wastewater and drinking water treatment plants. Environmental monitoring and assessment, 188(12), 1-13.

Krumholz, L. A. (1948). Reproduction in the western mosquitofish, *Gambusia affinis affinis* (Baird & Girard), and its use in mosquito control. Ecological Monographs, 18(1), 1-43.

Kweku, D. W., Bismark, O., Maxwell, A., Desmond, K. A., Danso, K. B., Oti-Mensah, E. A., Quachie, A.T. & Adormaa, B. B. (2017). Greenhouse effect: Greenhouse gases and their impact on global warming. Journal of Scientific research and reports, 1-9.

Lee, J. W., Shin, Y. J., Kim, H., Kim, H., Kim, J., Min, S. A., Kim, P., Do Yu, S. & Park, K. (2019). Metformin-induced endocrine disruption and oxidative stress of *Oryzias latipes* on two-generational condition. Journal of hazardous materials, 367, 171-181.

Li, Z., Lu, G., Yang, X., & Wang, C. (2012). Single and combined effects of selected pharmaceuticals at sublethal concentrations on multiple biomarkers in *Carassius auratus*. Ecotoxicology, 21(2), 353-361.

Lionetto, M. G., Caricato, R., Calisi, A., Giordano, M. E., & Schettino, T. (2013). Acetylcholinesterase as a biomarker in environmental and occupational medicine: new insights and future perspectives. BioMed research international, 2013, 321213.

Liu, Y., Wang, W. N., Wang, A. L., Wang, J. M., & Sun, R. Y. (2007). Effects of dietary vitamin E supplementation on antioxidant enzyme activities in *Litopenaeus vannamei* (Boone, 1931) exposed to acute salinity changes. Aquaculture, 265(1-4), 351-358.

Lo, S., Russell, J., & Taylor, A. (1970). Determination of glycogen in small tissue samples. Journal of applied physiology, 28(2), 234-236.

Mallik, R., & Chowdhury, T. A. (2018). Metformin in cancer. Diabetes research and clinical practice, 143, 409-419.

Mhawej, M., Fadel, A., & Faour, G. (2020). Evaporation rates in a vital lake: a 34-year assessment for the Karaoun Lake. International Journal of Remote Sensing, 41(14), 5321-5337.

Maulvault, A. L., Barbosa, V., Alves, R., Anacleto, P., Camacho, C., Cunha, S., Fernandes, J.O., Ferreira, P.P., Rosa, R., Marques, A. & Diniz, M. (2018). Integrated multi-biomarker responses of juvenile seabass to diclofenac, warming and acidification co-exposure. Aquatic Toxicology, 202, 65-79.

Monteiro, J. N., Pinto, M., Crespo, D., Pardal, M. A., & Martinho, F. (2021). Effects of climate variability on an estuarine green crab *Carcinus maenas* population. Marine Environmental Research, 169, 105404.

Morales, D. R., & Morris, A. D. (2015). Metformin in cancer treatment and prevention. Annual Review of Medicine, 66, 17-29.

Nathan, D. M. (2015). Diabetes: advances in diagnosis and treatment. Jama, 314(10), 1052-1062.

Nathan, D. M. (1993). Long-term complications of diabetes mellitus. New England journal of medicine, 328(23), 1676-1685.

Nicholls, R. J. Impacts of and responses to sea-level rise. in Understanding Sea-Level Rise and Variability (eds. Church, J. A., Woodworth, P. L., Aarup, T. & Wilson, W. S.) 17–51.

Nie, X. P., Liu, B. Y., Yu, H. J., Liu, W. Q., & Yang, Y. F. (2013). Toxic effects of erythromycin, ciprofloxacin and sulfamethoxazole exposure to the antioxidant system in *Pseudokirchneriella subcapitata*. Environmental Pollution, 172, 23-32.

Nielsen, D. L., Brock, M. A., Rees, G. N., & Baldwin, D. S. (2003). Effects of increasing salinity on freshwater ecosystems in Australia. Australian Journal of Botany, 51(6), 655-665.

Niemuth, N. J., Jordan, R., Crago, J., Blanksma, C., Johnson, R., & Klaper, R. D. (2015). Metformin exposure at environmentally relevant concentrations causes potential endocrine disruption in adult male fish. Environmental Toxicology and Chemistry, 34(2), 291-296.

Niemuth, N. J., & Klaper, R. D. (2018). Low-dose metformin exposure causes changes in expression of endocrine disruption-associated genes. Aquatic Toxicology, 195, 33-40.

Niemuth, N. J., & Klaper, R. D. (2015). Emerging wastewater contaminant metformin causes intersex and reduced fecundity in fish. Chemosphere, 135, 38-45.

Nunes, B., Carvalho, F., & Guilhermino, L. (2006). Effects of widely used pharmaceuticals and a detergent on oxidative stress biomarkers of the crustacean *Artemia parthenogenetica*. Chemosphere, 62(4), 581-594.

Nunes, B. (2011). The use of cholinesterases in ecotoxicology. Reviews of Environmental Contamination and Toxicology, 212, 29-59.

Nunes, B., Gaio, A. R., Carvalho, F., & Guilhermino, L. (2008). Behaviour and biomarkers of oxidative stress in *Gambusia holbrooki* after acute exposure to widely used pharmaceuticals and a detergent. Ecotoxicology and Environmental Safety, 71(2), 341-354.

Nyitrai, D., Martinho, F., Dolbeth, M., Rito, J., & Pardal, M. A. (2013). Effects of local and large-scale climate patterns on estuarine resident fishes: the example of *Pomatoschistus microps* and *Pomatoschistus minutus*. Estuarine, Coastal and Shelf Science, 135, 260-268.

OECD (1992). Test no. 203: fish, acute toxicity test. OECD Guidelines for the Testing of Chemicals, Section 2. OECD Publishing.

OECD (2000). Test no. 215: fish, juvenile growth test. OECD Guidelines for the testing of Chemicals. OECD.

Oosterhuis, M., Sacher, F., & Ter Laak, T. L. (2013). Prediction of concentration levels of metformin and other high consumption pharmaceuticals in wastewater and regional surface water based on sales data. Science of the Total Environment, 442, 380-388.

Ouslimani, N., Peynet, J., Bonnefont-Rousselot, D., Thérond, P., Legrand, A., & Beaudeux, J. L. (2005). Metformin decreases intracellular production of reactive oxygen species in aortic endothelial cells. Metabolism, 54(6), 829-834.

Paital, B., & Chainy, G. B. N. (2010). Antioxidant defenses and oxidative stress parameters in tissues of mud crab (*Scylla serrata*) with reference to changing salinity. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology, 151(1), 142-151.

Pandey, S., Parvez, S., Sayeed, I., Haque, R., Bin-Hafeez, B., & Raisuddin, S. (2003). Biomarkers of oxidative stress: a comparative study of river Yamuna fish

Wallago attu (Bl. & Schn.). Science of the Total Environment, 309(1-3), 105-115.

Pereira, P., de Pablo, H., Vale, C., & Pacheco, M. (2010). Combined use of environmental data and biomarkers in fish (*Liza aurata*) inhabiting a eutrophic and metal-contaminated coastal system–Gills reflect environmental contamination. Marine Environmental Research, 69(2), 53-62.

Pfeifer, S., Schiedek, D., & Dippner, J. W. (2005). Effect of temperature and salinity on acetylcholinesterase activity, a common pollution biomarker, in *Mytilus sp.* from the south-western Baltic Sea. Journal of Experimental Marine Biology and Ecology, 320(1), 93-103.

Pistocchi, A., Udias, A., Grizzetti, B., Gelati, E., Koundouri, P., Ludwig, R., Papandreou, A. & Souliotis, I. (2017). An integrated assessment framework for the analysis of multiple pressures in aquatic ecosystems and the appraisal of management options. Science of the Total Environment, 575, 1477-1488.

Pyke, G. H. (2005). A review of the biology of *Gambusia affinis* and *G. holbrooki*. Reviews in Fish Biology and Fisheries, 15(4), 339-365.

Queiroz, E. A., Puukila, S., Eichler, R., Sampaio, S. C., Forsyth, H. L., Lees, S. J., Barbosa, A. M., Dekker, R. F., Fortes, Z. B. & Khaper, N. (2014). Metformin induces apoptosis and cell cycle arrest mediated by oxidative stress, AMPK and FOXO3a in MCF-7 breast cancer cells. PloS ONE, 9(5), e98207.

Ramachandran, S. D., Sweezey, M. J., Hodson, P. V., Boudreau, M., Courtenay, S. C., Lee, K., King, T. & Dixon, J. A. (2006). Influence of salinity and fish species on PAH uptake from dispersed crude oil. Marine Pollution Bulletin, 52(10), 1182-1189.

Ramos, A. S., Correia, A. T., Antunes, S. C., Gonçalves, F., & Nunes, B. (2014). Effect of acetaminophen exposure in *Oncorhynchus mykiss* gills and liver: detoxification mechanisms, oxidative defence system and peroxidative damage. Environmental Toxicology and Pharmacology, 37(3), 1221-1228.

Regoli, F., Giuliani, M. E., Benedetti, M., & Arukwe, A. (2011). Molecular and biochemical biomarkers in environmental monitoring: a comparison of biotransformation and antioxidant defense systems in multiple tissues. Aquatic Toxicology, 105(3-4), 56-66.

Regoli, F., & Giuliani, M. E. (2014). Oxidative pathways of chemical toxicity and oxidative stress biomarkers in marine organisms. Marine Environmental Research, 93, 106-117.

Rhee, J. S., Kim, B. M., Jeong, C. B., Park, H. G., Leung, K. M. Y., Lee, Y. M., & Lee, J. S. (2013). Effect of pharmaceuticals exposure on acetylcholinesterase (AchE) activity and on the expression of AchE gene in the monogonont rotifer, *Brachionus koreanus*. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology, 158(4), 216-224.

Santos, L. H., Gros, M., Rodriguez-Mozaz, S., Delerue-Matos, C., Pena, A., Barceló, D., & Montenegro, M. C. B. (2013). Contribution of hospital effluents to the load of pharmaceuticals in urban wastewaters: identification of ecologically relevant pharmaceuticals. Science of the Total Environment, 461, 302-316.

Scheurer, M., Michel, A., Brauch, H. J., Ruck, W., & Sacher, F. (2012). Occurrence and fate of the antidiabetic drug metformin and its metabolite guanylurea in the environment and during drinking water treatment. Water Research, 46(15), 4790-4802.

Scheurer, M., Sacher, F., & Brauch, H. J. (2009). Occurrence of the antidiabetic drug metformin in sewage and surface waters in Germany. Journal of Environmental Monitoring, 11(9), 1608-1613.

Serra-Compte, A., Maulvault, A. L., Camacho, C., Alvarez-Munoz, D., Barceló, D., Rodriguez-Mozaz, S., & Marques, A. (2018). Effects of water warming and acidification on bioconcentration, metabolization and depuration of pharmaceuticals and endocrine disrupting compounds in marine mussels (*Mytilus galloprovincialis*). Environmental Pollution, 236, 824-834.

Sarma, K., Prabakaran, K., Krishnan, P., Grinson, G., & Kumar, A. A. (2013). Response of a freshwater air-breathing fish, *Clarias batrachus* to salinity stress: an experimental case for their farming in brackishwater areas in Andaman, India. Aquaculture International, 21(1), 183-196.

Sharma, V. K., Nautiyal, V., Goel, K. K., & Sharma, A. (2010). Assessment of thermal stability of metformin hydrochloride. Asian Journal of Chemistry, 22(5), 3561.

Smolders, R., Bervoets, L., De Coen, W., & Blust, R. (2004). Cellular energy allocation in zebra mussels exposed along a pollution gradient: linking cellular effects to higher levels of biological organization. Environmental Pollution, 129(1), 99-112.

Smolders, R., De Boeck, G., & Blust, R. (2003). Changes in cellular energy budget as a measure of whole effluent toxicity in zebrafish (*Danio rerio*). Environmental Toxicology and Chemistry: An International Journal, 22(4), 890-899.

Solé, M., Shaw, J. P., Frickers, P. E., Readman, J. W., & Hutchinson, T. H. (2010). Effects on feeding rate and biomarker responses of marine mussels experimentally exposed to propranolol and acetaminophen. Analytical and Bioanalytical Chemistry, 396(2), 649-656.

Tahara, K., Yonemoto, A., Yoshiyama, Y., Nakamura, T., Aizawa, M., Fujita, Y., & Nishikawa, T. (2006). Determination of antihyperglycemic biguanides in serum and urine using an ion-pair solid-phase extraction technique followed by HPLC-UV on a pentafluorophenylpropyl column and on an octadecyl column. Biomedical Chromatography, 20(11), 1200-1205.

Tian, L., Tan, P., Yang, L., Zhu, W., & Xu, D. (2020). Effects of salinity on the growth, plasma ion concentrations, osmoregulation, non-specific immunity, and intestinal microbiota of the yellow drum (*Nibea albiflora*). Aquaculture, 528, 735470.

Trautwein, C., & Kümmerer, K. (2011). Incomplete aerobic degradation of the antidiabetic drug Metformin and identification of the bacterial dead-end transformation product Guanylurea. Chemosphere, 85(5), 765-773.

Tseng, Y. C., & Hwang, P. P. (2008). Some insights into energy metabolism for osmoregulation in fish. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology, 148(4), 419-429.

Ussery, E., Bridges, K. N., Pandelides, Z., Kirkwood, A. E., Bonetta, D., Venables, B. J., Guchardi, J. & Holdway, D. (2018). Effects of environmentally relevant metformin exposure on Japanese medaka (*Oryzias latipes*). Aquatic Toxicology, 205, 58-65.

Vargas, C. I., Vaz, N., & Dias, J. M. (2017). An evaluation of climate change effects in estuarine salinity patterns: Application to Ria de Aveiro shallow water system. Estuarine, Coastal and Shelf Science, 189, 33-45.

Venâncio, C., Castro, B. B., Ribeiro, R., Antunes, S. C., & Lopes, I. (2019). Sensitivity to salinization and acclimation potential of amphibian (*Pelophylax perezi*) and fish (*Lepomis gibbosus*) models. Ecotoxicology and Environmental Safety, 172, 348-355.

Vial, G., Detaille, D., & Guigas, B. (2019). Role of mitochondria in the mechanism (s) of action of metformin. Frontiers in endocrinology, 10, 294.

Viollet, B., Guigas, B., Garcia, N. S., Leclerc, J., Foretz, M., & Andreelli, F. (2012). Cellular and molecular mechanisms of metformin: an overview. Clinical science, 122(6), 253-270.

Vörösmarty, C. J., McIntyre, P. B., Gessner, M. O., Dudgeon, D., Prusevich, A., Green, P., Glidden, S., Bunn, S.E., Sullivan, C.A., Liermann, C.R. & Davies, P. M. (2010). Global threats to human water security and river biodiversity. Nature, 467(7315), 555-561.

Wheaton, W. W., Weinberg, S. E., Hamanaka, R. B., Soberanes, S., Sullivan, L. B., Anso, E., Glasauer, A., Dufour, E., Mutlu, G.M., Budigner, G.S. & Chandel, N. S. (2014). Metformin inhibits mitochondrial complex I of cancer cells to reduce tumorigenesis. Elife, 3, e02242.

World Health Organization. (2019). Classification of diabetes mellitus.

World Health Organization. (2020). Insulin and associated devices: access for everybody: WHO stakeholder workshop, 21 and 23–25 September 2020.

Wiles, S. C., Bertram, M. G., Martin, J. M., Tan, H., Lehtonen, T. K., & Wong, B. B. (2020). Long-term pharmaceutical contamination and temperature stress disrupt fish behavior. Environmental Science & Technology, 54(13), 8072-8082.

Wilson, J. M., Bunte, R. M., & Carty, A. J. (2009). Evaluation of rapid cooling and tricaine methanesulfonate (MS222) as methods of euthanasia in zebrafish (*Danio rerio*). Journal of the American Association for Laboratory Animal Science, 48(6), 785-789.

Wood, C. M., McDonald, M. D., Walker, P., Grosell, M., Barimo, J. F., Playle, R. C., & Walsh, P. J. (2004). Bioavailability of silver and its relationship to

ionoregulation and silver speciation across a range of salinities in the gulf toadfish (*Opsanus beta*). Aquatic Toxicology, 70(2), 137-157.

Yang, P. K., Hsu, C. Y., Chen, M. J., Lai, M. Y., Li, Z. R., Chen, C. H., Chen, S.U. & Ho, H. N. (2018). The efficacy of 24-month metformin for improving menses, hormones, and metabolic profiles in polycystic ovary syndrome. The Journal of Clinical Endocrinology & Metabolism, 103(3), 890-899.

Yoshikawa, T., & Naito, Y. (2002). What is oxidative stress? Japan Medical Association Journal, 45(7), 271-276.