Developmental Health Effects of Metformin and Guanylurea on Larval Zebrafish (*Danio rerio*)

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TITLE: **Developmental Health Effects of Metformin and Guanylurea on Larval Zebrafish (*Danio rerio*)**

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Lay Abstract

Pharmaceuticals have been detected at the ng to μg L^{-1} range in aqueous environments for decades. These compounds are designed to be biologically active at low concentrations and can cause elicit adverse effects in non-target species. Among the more recently detected compounds are the antihyperglycemic drug metformin and its biotransformation product (guanylurea), which have been the focus of few studies in fish. This thesis addresses multiple knowledge gaps by examining the potential impacts of metformin and guanylurea during the embryonic and early larval zebrafish period (3-120 hours post-fertilization). Exposure to metformin resulted in increased mortality and abnormalities. Guanylurea exposure increased mortality at one dose. We suggest that metformin and guanylurea cause modest effects in developing larval zebrafish.

Abstract

Metformin is the most common first-line oral therapeutic agent used in the treatment of type-2 diabetes. Because of its widespread use, metformin has been increasingly detected in wastewater effluent. It is partially bio-transformed into guanylurea is subsequently released into aquatic environments. Since the literature concerning the effect of metformin and guanyl urea on early life stage of fish is scant, the aim of this research was to understand the potential influence of metformin and guanylurea on developmental, cardiometabolic and behavioral responses in zebrafish embryos, from the 4 cell stage (3 hours post fertilization, hpf) to first feed (120 hpf). To this end, embryos were exposed to environmentally relevant (0.4, 4, 40 $\mu g \cdot L^{-1}$) and supra-environmental (400 and 4000 $\mu g \cdot L^{-1}$) concentrations of the two chemicals. Metformin caused an increased mortality and spinal abnormalities in all concentrations compared to controls. and increased pericardial and yolk sac edema at the highest tested concentration. Metformin did not cause alterations in hatch or heart rate over the examined developmental stages. In addition, metformin did not cause alterations in general swimming, light-dark movement, startle response or thigmotaxis, irrespective of exposure concentration. Exposure to guanylurea over the same developmental stages caused a significant difference in mortality at 40 $\mu g \cdot L^{-1}$ only. Guanylurea did not cause alterations to any of the other tested endpoints. Our data suggests that metformin and guanylurea caused modest impacts to embryonic development of zebrafish at these concentrations.

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Last, but certainly not least, I would like to thank my parents and family.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

ANOVA	Analysis of variance
˚C	Celsius
dpf	Days post fertilization
ELS	Early-life stage
g	Gram
hpf	Hour's post fertilization
H0	Null hypothesis
Log Kow	N-octanol/water partition coefficient (expressed in log form)
min	Minute
µg	Microgram
µL	Microliter
µm	Micrometer
µM	Micromolar
mg	Milligram
mL	Milliliter

ng	Nanogram
NSAIDS	Non-steroidal anti-inflammatory drugs
OCT1	Organic cation transporter 1
O_2	Oxygen
P-crit	Critical O2 tension
PO_2	Partial pressure of O_2
S	Seconds
SD	Standard deviation
SE	Standard error
SEM	Standard error of the mean
SLC22A1	Solute Carrier Family 22 Member 1
TL	Tupfel-Long fin
WT	Wild-type
WHO	World Health Organization
WWTP	Wastewater treatment plant

Declaration of Academic Achievement

This thesis consists of three chapters and was written with the aid and supervision of Dr. Joanna Wilson. Experimental design of laboratory exposures and supplementary support are attributed to Dr. Oana Birceanu. Images for morphometric analysis was performed by Jessica Qiu (Wilson lab, McMaster University). Experiments, data collection and analysis were completed by Shemar G. Williams with supervisory committee members.

I declare this thesis to be an original composition of our work, save for information that is indicating via referencing.

Chapter 1: General Introduction

Chapter 2: Modest Developmental Effects in Larval Zebrafish (*Danio rerio*) Exposed to Metformin and Guanylurea

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Chapter 3: General Summary and Conclusion

Chapter One: General Introduction

1.1 Medicating the Environment

Pharmaceuticals are a diverse class of therapeutic compounds designed to mitigate or prevent illness in humans and or domestic animals. The use of pharmaceuticals has become a staple in many aspects of modern life which is evidenced by global pharma revenues exceeding 1 trillion United States dollars in 2014 (Dossier, 2018). Since then, the market has been steadily increasing by 5.8% annually and is estimated to reach 1.462 trillion in 2021 (Dossier, 2018). Higher socioeconomic countries comprise the largest percentage of these sales with North America, the European Union and Japan representing 45, 13 and 10% of sales, respectively (IMS Health, 2016). In more recent years, countries such Brazil, Russia, Indian and China, have shown considerable growth in their respective pharmaceutical markets which has been attributed to their growing population, and increased availability of biosimilars and generic drugs (Tannoury and Atteih 2017).

Unsurprisingly, pharmaceutical consumption rates vary highly from region to region. Attaining accurate estimate of global pharmaceuticals consumption patterns is hampered by differences in legislature, regional health, and other socioeconomic factors. Data concerning pharmaceutical use are not readily available in all countries, making prediction of pharmaceutical use by region difficult. However, the available data suggests that there are between 3500 (Caldwell et al. 2019) and 4000 (Boxall et al., 2012) pharmaceuticals on the market for use. These numbers are projected to increase in tandem with the 5-7 % annual incline in pharmaceutical sales (IMS Health 2016) because of the developments in

medicine and increased access by low and middle-income countries (Tannoury and Atteih 2017).

The near ubiquitous growth of the pharmaceutical industry is driven predominantly by the increased consumption worldwide. This positive trend has been attributed to the growing prevalence of age-related and chronic illnesses chiefly in higher socioeconomic countries (García-peña et al., 2021). Of the available drugs on the market for medical use, patented therapeutics for the treatment of cancer (lenalidomide), glucose-intolerance (metformin), anxiety (diazepam), and antidepressants (fluoxetine; Corcoran et al., 2010) rank among the mostly commonly used. Pharmaceuticals are used in domesticated animals for preventive medicine, genetic selection, and improved nutrition and management and consists of (1) vaccines and prophylactic medication to prevent or minimize infection; (2) antimicrobials (oxytetracycline, erythromycin) and parasiticides (metronidazole, tinidazole) to treat active infection or prevent disease onset in situations that induce high susceptibility; and (3) hormones used in growth promotion (somatotrophin ; Otto and Short, 1998). Most of these compounds (~90%) are administered to patients orally due to factors including sustained and controllable delivery, ease of administration, patient compliance and access to a large surface area for absorption (Daughton et al., 2016). Ingested compounds are brought to the liver, as a parent compound, through hepato-portal circulation where they may be enzymatically inactivated or converted into an active metabolite. Whether parent compound or metabolite, the excreted compounds may be biologically active and become dispersed into the wastewater system through urine and feces (Daughton et al., 2016).

As an inevitable consequence of both the increased pharmaceutical consumption and associated market expansion, there has been an increase in the number of pharmaceuticals and their transformation products that enter the environment. The contamination of aquatic systems by pharmaceuticals has garnered increased attention as these compounds and their transformation products have been increasingly detected in various surface water, groundwater, and occasionally in drinking water (Beek et al., 2016).

The presence of pharmaceuticals in the environment is of particular concern because of their inherent characteristics. Pharmaceuticals are designed to target specific components of molecular pathways at low concentrations, with the aim of altering their function to elicit a precise desired pharmacological response in biological organisms. The nature of their physical structure (stereochemistry) determines the type of targets they will interact with; the most common pharmacological targets include receptors, kinases, ion channels, structural and membrane proteins. Many of these targets are evolutionally well-conserved across both vertebrate and invertebrate taxa. A study conducted by Gunnarsson et al (2008) used an OrthoMCL algorithm to predict whether 1318 human drug targets were present in 16 species relevant to ecotoxicity testing. Amongst the aquatic vertebrates, western clawed frog (*X. tropicalis*), zebrafish (*D. rerio*), and three-spined stickleback (*G. aculeatus*), had 1137, 1136, and 1160 orthologs, respectively, which were predicted with a similarity above 60%. As a result of these conserved molecular targets, non- target aquatic organisms are likely to suffer from pharmacologically induced impacts associated with drug exposure. For example, non-steroidal anti-inflammatory drugs (NSAIDs) such as

ibuprofen and naproxen are compounds used to alleviate symptoms of inflammation (i.e., swelling and redness). Their effects have been attributed to the inhibition of the enzyme cyclooxygenase (COX) which converts arachidonic acids to pro-inflammatory compounds (thromboxanes, prostaglandins, prostacyclins; Hawkey and Langman, 2003). COX genes have been identified in various fish lineages including lamprey and hagfish (Havird et al., 2008), sculpin (Lau et al., 2018), and zebrafish (Ishikawa, et al., 2007).

Largely, the potential adverse outcomes associated with the presence of pharmaceuticals in the environment is unknown (Daughton, 2016). Currently, only 1% of the ~4000 human-derived compounds in aqueous systems have been studied (Boxall et al., 2012). Due to his scant amount of ecotoxicological data, extrapolations to the remaining compounds cannot be made, encouraging further investigation. Well validated research into the fate and impact of pharmaceuticals in the environment is essential for prioritizing compounds in terms environmental monitoring and risk assessment practices. This thesis focused on the impacts of metformin and guanylurea in embryo and larval zebrafish (3-120 hours post fertilization, hpf).

1.2 Metformin and Guanylurea

Metformin is a potent antihyperglycemic insulin-sensitizing agent, primarily prescribed to patients with non-insulin-dependent diabetes mellitus. Metformin can be taken as a mono-therapeutic drug but is often prescribed in combination with sitagliptin or sulfonylurea as hyperglycemic symptoms seemed to be more improved when combination therapy is used (Haupt et al., 2009). Metformin has quickly become one of the most widely consumed pharmaceuticals worldwide and was added to the World Health Organizations

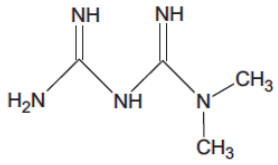
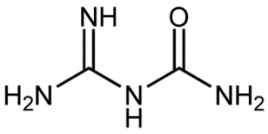
list of essential medicines in 2013 (Oosterius et al., 2013; Scheurer et al., 2009). From 2000-2015, over 550 million metformin prescriptions were dispensed in the US alone, with prescription rates increasing from 2.27 per 1000 persons in 2000 to 235 per 1000 people (Lee, 2019).

Metformin and other guanidine derivatives (i.e., phenformin and buformin compounds containing a biguanide core, composed of the coupling of two guanidine units: $HNC(NH_2)_2$. For metformin, one of the two guanidine subunits has two attached methyl groups which confer polar basic properties and high stability. Metformin has a low $logK_{ow}$ (octanal-water coefficient, which indicates the solubility of the compound in fat or water compartments; -2.63) and in tandem with its high polarity, is freely soluble in polar solvents such as water. Thus, it is unsurprising that metformin is among the most abundant pharmaceuticals detected in waters receiving wastewater effluent. Surface water concentrations range between 0.1-40 $\mu g \cdot L^{-1}$ (Niemuth et al., 2015; Oosterhuis et al., 2013).

Under aerobic wastewater treatment conditions, metformin can become bacterially degraded into the transformation product guanylurea (Blair et al., 2015). The process is purportedly the result of the removal of methyl groups from the terminal nitrogen (Markiewics et al.2017a) at a varied rates of 48-98% (Blair et al., 2015). Guanylurea is resistant to common wastewater treatment practices, like UV light radiation and ozonation and is the major end-product (Trautwein and Kummerer, 2011) in metformin biodegradation. Guanylurea has been detected in surface waters, at higher concentrations than its parent compound (Scheurer et al., 2012). Both compounds are polar compounds and are expected to be highly mobile in aquatic environments (Scheurer *et al.,* 2012). The

structure and physicochemical properties of metformin and its transformation product guanylurea are summarized in Table 1.1

Table1. 1. Molecular structure of metformin and guanylurea. Adapted from (Scheurer et al., 2012).

Compound Name	Metformin	Guanylurea
Structure		
Molecular formula	$C_4H_{11}N_5$	$C_2H_6N_4O$
Molecular weight (g/mol)	129.2	102.1

Metformin cannot passively diffuse across the lipid bilayer due largely to its dimethyl-substituted terminal amino groups and as such, requires the aid of membrane transport proteins. A plethora of studies have implicated several cation transporters in the absorption of metformin including, OCT1 (SLC22A1), OCT2 (SLC22A2), OCT3 (SLC22A3), MATE1 (SLC47A1), MATE2 (SLC47A2), PMAT (SLC29A4), OCTN1 (SLC22A4) and SERT (SLC6A4) (Li et al. 2011; Choi et al.2007; Chen et al. 2015; Chen et al 2009; Masuda et al. 2006; Zhou et al. 2007; Nakamichi et al. 2013; Han et al. 2015).Interestingly metformin is

not well metabolized by the liver and is largely excreted unaltered (Hardie, 2007)in urine and feces in mammals.

Metformin is a pleiotropic drug having multiple targets and is thought to induce numerous molecular mechanisms. In diabetic patients, it regulates glucose metabolism principally by reducing hepatic gluconeogenesis and glycogenolysis, thereby suppressing both basal and postprandial plasma glucose levels (Scarpello and Howlett, 2008; Viollet et al., 2012). Metformin has also been implicated in related antihyperglycemic mechanisms including increased peripheral glucose uptake, insulin signaling, and increased fatty acid β-oxidation (Gong et al., 2012).

Despite its use in the treatment of diabetic symptoms for nearly a century, the underlying mechanisms of action for metformin have yet to be fully elucidated. The available evidence suggests that metformin can affect both AMP-activated protein kinase (AMPK)-dependent and AMPK-independent mechanisms primarily in hepatic mitochondria (Herzig et al., 2018). In the AMPK-dependent mechanism metformin causes a decrease in ATP production through the transient inhibition of complex I in the mitochondrial electron transport chain (El-Mir et al., 2000). This action causes a reduction in NADH oxidation, decreasing the proton gradient across the inner mitochondrial membrane and a subsequent decrease in the proton driven synthesis of ATP. A decrease in ATP production results in a shift in ATP:ADP: AMP equilibrium towards increased AMP synthesis. This change in energy homeostasis drives the inhibition of glucagon-induced cAMP synthesis and the activation of 5'-AMPK. AMPK is a potent energy sensing enzyme involved in regulatory signaling cascades that aim to restore the balance between ATP

supply and demand. The activation of AMPK promotes catabolic pathways (i.e., fatty acid oxidation) to meet the ATP needs of the organism, while subsequently decreasing anabolic pathways (cholesterol and triglycerides biosynthesis and gluconeogenesis; Li Gong et al., 2012). The AMPK independent mechanisms are less explored but may involve metformin-induced inhibition of gluconeogenic enzymes such as fructose-1,6-bisphosphatase phosphoenolpyruvate carboxykinase and glucose-6-phosphatas by lowering hepatic intracellular cAMP concentration (Johanns et al., 2016).

1.3. Ecological Effects of Metformin in Fish

The effects of metformin in mammalian systems are well documented (Dowling et al., 2007; Yimmer et al., 2019) while its impacts in aquatic organisms, specifically fish, are largely unknown. However, there are six principal studies that have examined the effects of metformin and have shown adverse effects at environmentally relevant concentrations. Since each of these studies use distinct life stages and endpoints, it is not yet possible to assess whether metformin has similar effects across fish species.

1.3.1. *Pimephales promelas*

The potential endocrine disrupting effects of metformin were assessed in adult male fathead minnow (*Pimphales promelas)* after chronic exposure to 40 $\mu g \cdot L^{-1}$ (Niemuth et al., 2015). They showed that the expression of egg yolk precursor protein vitellogenin (VTG) was significantly induced in male fathead minnow liver after 4 weeks exposure to metformin, indicating endocrine disruption. In a follow-up study, Niemuth and Klaper (2015) assessed male fathead minnow testicular tissue following a chronic exposure to metformin. Their results showed that exposed males had a high presence of oocytes in

testes or spermatogonium in ovaries) suggesting that metformin increases the intersex condition in males and may also reduce fertility by decreasing sperm and milt volume. In 2018, Niemuth and Klaper examined the expression of genes related to steroid hormone (3b-HSD, 17b-HSD, CYP19A1) and xenobiotic metabolism (SULT2A1) and found that all genes examined were upregulated in the testis of exposed males.

1.3.2. *Oryzias latipes*

Lee (2019) used acute and chronic toxicity tests to document the endocrine disrupting impacts of metformin in fish models. They corroborated the findings of Niemuth et al., 2015: and Niemuth and Klaper 2015 by showing that male Oryzias showed an upregulation in VTG gene transcription. In one of the few early life stage studies (ELS-study) centered around the impact of metformin in fish, Ussery et al., (2018) documented decreased growth parameters (length and weight) in juvenile fish and showed increases levels of fatty acids, steric acid, palmitic acid and arachidonic acid in larvae. Ussery et al., also showed decreases in several metabolites such as L-lysine and L-proline and DL-3-aminoisobutyric acid. These results suggest that metformin has adverse impacts to larval fitness which may reduce survival in wild fish populations.

1.3.3. *Danio rerio*

In a study conducted by Godoy et al. (2018), that looked at increasing concentrations of metformin (0; 100; 180; 330; 600; 1100; 1500 and 2000 mg L) on 120h post fertilization zebrafish showed increased incidence of scoliosis and abnormal pigmentation in larva exposed to metformin, at concentrations of 1100 mg L-1. In the same study, Godoy et al., (2018) looked at locomotor activity in 120h post fertilization zebrafish

and showed no impairment oflarval zebrafish behavior. One of the newest published studies on metformin exposure in fish was conducted by Elizalde-Velazquez et al., (2021), which looked at mortality, malformation rate, hatch rate and oxidative stress (upregulated catalase, superoxide dismutase, and glutathione peroxidase) in 4 dpf zebrafish exposed to 1, 10, 20, 30, 40, 50, 75 and 100 μg L^- of metformin. They found that metformin induced increased incidences of scoliosis, pericardial edema, and yolk deformation. With respect to oxidative stress, metformin caused a significant induction of the activity of antioxidant enzymes and the levels of oxidative damage biomarkers.

1.3.4. *Betta splendens*

MacLaren et al., (2018) examined aggressive behavior in adult male Siamese fighting fish *Betta splendens*. Individuals in the exposure group were subjected to 40 or 80 $\mu g \cdot L^{-1}$ metformin for just over 4.5 months. Researchers used a faux male stimulus to test aggressive behavior in male *B. splendens* and found that subjects in both treatment groups displayed less aggressive behavior to the faux male stimulus, such as decreased gill flaring and fin spreading, which are used to intimidate rival conspecific males and would-be predators (MacLaren et al., 2018). These results may have long-term implications pertaining to survival of fish species, since aggressive behavior is linked to the ability of a fish to mate, defend its offspring, and maintain its nesting territory.

1.4. Ecological Effects of Guanylurea Exposure in Fish

Guanylurea exposure in fish has received comparatively less attention than that of metformin with two studies representing the bulk of our current knowledge on its effects in aquatic species. *Oryzias* larvae exposed to increasing concentrations of guanylurea (1.0,

3.2, 10, 32 and 100 μg · L^{-1}) for 28 days had decreased lengths and weights compared to control fish (Ussery et al 2018). Decreased body size is a notable detriment to larval survival as smaller fish are more readily prey upon by larger fish thereby decreasing the likelihood of surviving to sexual maturity (Crowder et al. 1992). A related study increased the duration of the exposure from 28 days to 165 days post-fertilization but did not show an effect on adult (165 dph) medaka body size (length, wet weight, or condition factor; Ussery et al., 2018). Yet, additional life cycle studies are needed to confirm these findings as the sample size used in each sampling time point (n=3/treatment on days 55, 110 and 155) was small.

In two experiments conducted by Jacob et al., (2019), embryos and nine-month-old juvenile brown trout (*Salmo trutta*) were exposed to increasing concentrations of guanylurea (0, 100 and 1,000 μg · L^{-1} for three weeks. Guanylurea exposure did not alter endpoints in larvae (heart rate and hatch time) or juveniles (body weight, length, stress proteins and lipid peroxidases). The conflicting results with regards to larval health after guanylurea exposure may be the result of differences between species responses. Yet, the available data are very limited, particularly for guanylurea, and there is need for additional research because studies illustrate a potential impact of either metformin or guanylurea in several fish species.

1.5 The Use of Zebrafish in Ecotoxicological Studies

The genus *Danio* is comprised of small (~ 3 cm long) schooling freshwater minnows (family Cyprinidae) native to regions throughout Southeastern Asia (Timor to Luzon) and Japan. At sexual maturity, males tend to be slender, with black straight longitudinal lines and a more gold coloration on the ventral and dorsal sides in addition their fins, while

females tend to be larger in size with a protruding underside and a bluish-white cast. Alongside fathead minnow (*Pimephales promelas*) and Japanese medaka (*Oryzias latipes*), zebrafish are convenient model in developmental biology due to numerous desirable traits: ease of maintenance, continuous spawning, and short developmental time.

For this thesis specifically, zebrafish were useful due to their clear chorion which allowed for direct observation of endpoints such as heart rate and spontaneous movement. Their small size allows them to be placed in petri dishes or well-plates where experimental endpoints can be easily manipulated through the administration of chemical compounds directly into the test medium which subsequently freely diffuse through the chorion. Moreover, zebrafish also display a plethora of complex behaviors (i.e., thigmotaxis, visual motor, and startle response) they exhibit early on in development (4 dpf). These behaviors represent well studied (Schnorr et al., 2012; Huang et al., 2019; Beppi et al., 2021) developmental targets that can be easily quantified through specialized equipment (ie., Danio vision).

1.6. Study Rationale and Objectives

The detection of metformin in receiving waters of WWTP effluent is relatively novel, and as such there is relatively little data concerning its impact on aquatic organisms. The available literature suggests that acute and chronic exposure to metformin can induce the intersex condition in adult male fish (Niemuth 2015; Niemuth and Klaper, 2015), upregulation in genes involved with steroid hormone production (Niemuth and Klaper, 2018), growth metrics (Ussery et al., 2018) and behavior (Maclaren et al., 2018). These studies however have focused almost exclusively on juvenile and adult life stages.

Additionally, the impact of guanylurea on aquatic organisms has received even less attention (Jacob et al., 2019; Ussery et al., 2018). As metformin and guanylurea are contaminants of concern due to their frequent detection in surface waters (Elizalde-Velazquez, 2021) and high mobility in water (Scheurer et al., 2009), more exploration into the effect of these compounds on aquatic organisms is warranted.

The use of embryo-toxicity tests on early-life stage fish models in ecotoxicological research is a standard practice used to understand the effects of contaminants on various parameters of fish health (Fent et al., 2006, Vestel et al., 2016). Embryonic and larval fish stages represent critical developmental time points as these stages are particularly sensitive to chemical stressors (Catalán et al., 2020). Studying the impacts of fish through early life stage studies provides information on both individual and population level dynamics, which can be applied to aquaculture and fisheries management. The selected compounds (metformin and guanylurea) and the test concentrations used are based our limited understanding of the potential adverse outcomes these compounds have on aquatic species. and their growing presence in surface waters (Scheurer et al., 2009).

Moreover, we chose to investigate the potential cardio-metabolic effects (heart rate and oxygen consumption) of direct contaminant exposure due to the published mechanistic literature of metformin in mammalian systems. In several studies (El-Mir et al., 2000), metformin has been shown to inhibit complex 1 of the respiratory chain resulting in decreased NADH oxidation, associated decreases in the inter mitochondrial membrane proton gradient, and ultimately decreased oxygen consumption rate. Its effects on behavioral endpoints (thigmotaxis, general swim performance, and startle response) were

analyzed in view of the inhibitory effects of metformin on adult *betta splendens* (Maclaren et al., 2019. My objectives were to expose TL zebrafish embryos (from 3-120 hpf) to environmentally relevant and supra-environmental concentrations of metformin or guanylurea. Embryos were observed every 24 hours for mortality and hatching; heart rate was determined at 24, 48, and 72 hours post-fertilization (hpf). Samples were collected at 72 hpf for morphological measures of larval structures and to determine the rate of developmental abnormalities. Behavioral assays (general swim parameters, thigmotaxis, visual motor response and startle response) and respiration rates were assessed after 120 hours of exposure.

1.7. Hypotheses

In this thesis, I hypothesized that single contaminant exposures of metformin or guanylurea would result in impairments to larval zebrafish survival, hatch, cardio-metabolic and behavioral responses. Specific hypotheses, with respect to unexposed control fish, are as follows:

H_{o1}: Embryonic and larval zebrafish exposed to metformin will not show significant differences in survival to 120hpf, time to hatch, morphology and developmental abnormalities at 72 hpf.

H_{o2}: Embryonic and larval zebrafish exposed to metformin will not have significant differences in thigmotaxis, general swim performance, and startle response at 120 hpf.

H_{o3}: Embryonic and larval zebrafish exposed to metformin will not have significant differences in cardio-metabolic end points (heart rate and respirometry) at 72 and 120hpf.

H_{o4}: Embryonic and larval zebrafish exposed to guanylurea will not show significant differences in survival to 120hpf, time to hatch, morphology and developmental abnormalities at 72 hpf.

H_{o5}: Embryonic and larval zebrafish exposed to guanylurea will not have significant differences in thigmotaxis, general swim performance, and startle response at 120 hpf.

H_{o6}: Embryonic and larval zebrafish exposed to guanylurea will not have significant differences in cardio-metabolic end points (heart rate and respirometry) at 72 and 120hpf.

1.8. References

aus der Beek, T., Weber, F.-A., Bergmann, A., Hickmann, S., Ebert, I., Hein, A., & Küster, A. (2016). Pharmaceuticals in the environment-Global occurrences and perspectives. *Environmental Toxicology and Chemistry*, *35*(4), 823–835. https://doi.org/10.1002/etc.3339

Beppi, C., Beringer, G., Straumann, D., & Bögli, S. Y. (2021). Light-stimulus intensity modulates startle reflex habituation in larval zebrafish. *Scientific Reports*, *11*(1), 22410. https://doi.org/10.1038/s41598-021-00535-9

Blair, B. D., Crago, J. P., Hedman, C. J., & Klaper, R. D. (2015). Pharmaceuticals and personal care products found in the Great Lakes above concentrations of environmental concern. *Chemosphere*, *93*(9), 2116–2123. https://doi.org/10.1016/j.chemosphere.2013.07.057

Boxall, A. B. A., Rudd, M. A., Brooks, B. W., Caldwell, D. J., Choi, K., Hickmann, S., Innes, E., Ostapyk, K., Staveley, J. P., Verslycke, T., Ankley, G. T., Beazley, K. F., Belanger, S. E., Berninger, J. P., Carriquiriborde, P., Coors, A., DeLeo, P. C., Dyer, S. D., Ericson, J. F., … Van Der Kraak, G. (2012). Pharmaceuticals and Personal Care Products in the Environment: What Are the Big Questions? *Environmental Health Perspectives*, *120*(9), 1221–1229. https://doi.org/10.1289/ehp.1104477

Caldwell, D. J., D'Aco, V., Davidson, T., Kappler, K., Murray-Smith, R. J., Owen, S. F., Robinson, P. F., Simon-Hettich, B., Straub, J. O., & Tell, J. (2019). Environmental risk assessment of metformin and its transformation product guanylurea: II. Occurrence in surface waters of Europe and the United States and derivation of predicted no-effect concentrations. *Chemosphere*, *216*, 855–865. https://doi.org/10.1016/j.chemosphere.2018.10.038

Catalán, I., Reglero, P., & Álvarez, I. (2020). Research on early life stages of fish: a lively field. *Marine Ecology Progress Series*, *650*, 1–5. https://doi.org/10.3354/meps13491

Chen, E. C., Liang, X., Yee, S. W., Geier, E. G., Stocker, S. L., Chen, L., & Giacomini, K. M. (2015). Targeted Disruption of Organic Cation Transporter 3 Attenuates the Pharmacologic Response to Metformin. *Molecular Pharmacology*, *88*(1), 75–83. https://doi.org/10.1124/mol.114.096776

Chen, Y., Li, S., Brown, C., Cheatham, S., Castro, R. A., Leabman, M. K., Urban, T. J., Chen, L., Yee, S. W., Choi, J. H., Huang, Y., Brett, C. M., Burchard, E. G., & Giacomini, K. M. (2009). Effect of genetic variation in the organic cation transporter 2 on the renal elimination of metformin. *Pharmacogenetics and Genomics*, *19*(7), 497–504. https://doi.org/10.1097/FPC.0b013e32832cc7e9

Chen, Y., Li, S., Brown, C., Cheatham, S., Castro, R. A., Leabman, M. K., Urban, T. J., Chen, L., Yee, S. W., Choi, J. H., Huang, Y., Brett, C. M., Burchard, E. G., & Giacomini, K. M. (2009). Effect of genetic variation in the organic cation transporter 2 on the renal elimination of metformin. *Pharmacogenetics and Genomics*, *19*(7), 497–504. https://doi.org/10.1097/FPC.0b013e32832cc7e9

Choi, Y. K., & Park, K.-G. (2013). Metabolic roles of AMPK and metformin in cancer cells. *Molecules and Cells*, *36*(4), 279–287. https://doi.org/10.1007/s10059-013-0169-8

Corcoran, J., Winter, M. J., & Tyler, C. R. (2010). Pharmaceuticals in the aquatic environment: A critical review of the evidence for health effects in fish. *Critical Reviews in Toxicology*, *40*(4), 287–304. https://doi.org/10.3109/10408440903373590

Crowder, LB., Rice, J.A., Miller, T.J. et al. (1992) Empirical and theoretical approaches to size-based interactions and recruitment variability in fishes, in Individual-based Models and Approaches in Ecology (eds D.L. De Angelis and L.J. Gross), Chapman and Hall, New York, pp. 237-255

Daughton, C. G. (2016). Pharmaceuticals and the Environment (PiE): Evolution and impact of the published literature revealed by bibliometric analysis. *Science of The Total Environment*, *562*, 391–426. https://doi.org/10.1016/j.scitotenv.2016.03.109

Dossier. Global Pharmaceutical Industry; Statista: Hamburg, Germany, 2018.

Dowling, R. J. O., Zakikhani, M., Fantus, I. G., Pollak, M., & Sonenberg, N. (2007). Metformin Inhibits Mammalian Target of Rapamycin–Dependent Translation Initiation in Breast Cancer Cells. *Cancer Research*, *67*(22), 10804–10812. https://doi.org/10.1158/0008-5472.CAN-07-2310

Elizalde-Velázquez, G. A., Gómez-Oliván, L. M., Islas-Flores, H., Hernández-Navarro, M. D., García-Medina, S., & Galar-Martínez, M. (2021). Oxidative stress as a potential mechanism by which guanylurea disrupts the embryogenesis of *Danio rerio*. *Science of The Total Environment*, *799*, 149432. https://doi.org/10.1016/j.scitotenv.2021.149432

El-Mir, M.-Y., Nogueira, V., Fontaine, E., Avéret, N., Rigoulet, M., & Leverve, X. (2000). Dimethylbiguanide Inhibits Cell Respiration via an Indirect Effect Targeted on the Respiratory Chain Complex I. *Journal of Biological Chemistry*, *275*(1), 223–228. https://doi.org/10.1074/jbc.275.1.223

Fent, K., Weston, A., & Caminada, D. (2006). Ecotoxicology of human pharmaceuticals. *Aquatic Toxicology*, *76*(2), 122–159. https://doi.org/10.1016/j.aquatox.2005.09.009

García-peña, G. E., Rubio, A. V, Mendoza, H., Fernández, M., Milholland, M. T., Aguirre, A. A., Suzán, G., Zambrana-torrelio, C., & Zambrana-torrelio, C. (2021). *Land-use change and rodent-borne diseases : hazards on the shared socioeconomic pathways*. Philosophical Transactions B. 376: 20200362.https://doi.org/10.15468/dl.pqwhfw

Godoy, A. A., Domingues, I., Arsénia Nogueira, A. J., & Kummrow, F. (2018). Ecotoxicological effects, water quality standards and risk assessment for the anti-diabetic metformin. *Environmental Pollution*, *243*, 534–542. https://doi.org/10.1016/j.envpol.2018.09.031

Gong, L., Goswami, S., Giacomini, K. M., Altman, R. B., & Klein, T. E. (2012). Metformin pathways. *Pharmacogenetics and Genomics*, *22*(11), 820–827. https://doi.org/10.1097/FPC.0b013e3283559b22

Gunnarsson, L., Jauhiainen, A., Kristiansson, E., Nerman, O., & Larsson, D. G. J. (2008). Evolutionary Conservation of Human Drug Targets in Organisms used for Environmental Risk Assessments. *Environmental Science & Technology*, *42*(15), 5807–5813. https://doi.org/10.1021/es8005173

Han, T. (Kevin), Proctor, W. R., Costales, C. L., Cai, H., Everett, R. S., & Thakker, D. R. (2015). Four Cation-Selective Transporters Contribute to Apical Uptake and Accumulation of Metformin in Caco-2 Cell Monolayers. *Journal of Pharmacology and Experimental Therapeutics*, *352*(3), 519–528. https://doi.org/10.1124/jpet.114.220350

Haupt, D., Weitoft, G. R., & Nilsson, J. L. G. (2009). Refill adherence to oral antihyperglycaemic drugs in Sweden. *Acta Diabetologica*, *46*(3), 203–208. https://doi.org/10.1007/s00592-008-0076-1

Hardie, DG.(2007).AMP-activated protein kinase as a drug target. *Annual Review Pharmacology Toxicology. 47*:185–210. https://doi.org/11/0002-0081/

Havird, J. C., Miyamoto, M. M., Choe, K. P., & Evans, D. H. (2008). Gene Duplications and Losses within the Cyclooxygenase Family of Teleosts and Other Chordates. *Molecular Biology and Evolution*, *25*(11), 2349–2359. https://doi.org/10.1093/molbev/msn183

Hawkey CJ, Langman MJ. Non-steroidal anti-inflammatory drugs: overall risks and management. Complementary roles for COX-2 inhibitors and proton pump inhibitors. Gut. 2003 Apr;52(4):600-8. doi: 10.1136/gut.52.4.600. PMID: 12631678; PMCID: PMC1773617.

Herzig, S., & Shaw, R. J. (2018). AMPK: guardian of metabolism and mitochondrial homeostasis. *Nature Reviews Molecular Cell Biology*, *19*(2), 121–135. https://doi.org/10.1038/nrm.2017.95

Huang, K.-H., Rupprecht, P., Frank, T., Kawakami, K., Bouwmeester, T., & Friedrich, R. W. (2020). A virtual reality system to analyze neural activity and behavior in adult zebrafish. *Nature Methods*, *17*(3), 343–351. https://doi.org/10.1038/s41592-020-0759-2

IMS Health. www.imshealth.com. Last accessed October 2016.

Ishikawa, T., Griffin, K. J. P., Banerjee, U., & Herschman, H. R. (2007). The zebrafish genome contains two inducible, functional cyclooxygenase-2 genes. *Biochemical and Biophysical Research Communications*, *352*(1), 181–187. https://doi.org/10.1016/j.bbrc.2006.11.007

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), 48. https://doi.org/10.1186/s12302-018-0179-4

Jacob, S., Knoll, S., Huhn, C., Köhler, H.-R., Tisler, S., Zwiener, C., & Triebskorn, R. (2019). Effects of guanylurea, the transformation product of the antidiabetic drug metformin, on the health of brown trout (*Salmo trutta f. fario*). *PeerJ*, *7*, e7289. https://doi.org/10.7717/peerj.7289

Johanns, M., Lai, Y.-C., Hsu, M.-F., Jacobs, R., Vertommen, D., Van Sande, J., Dumont, J. E., Woods, A., Carling, D., Hue, L., Viollet, B., Foretz, M., & Rider, M. H. (2016). AMPK antagonizes hepatic glucagon-stimulated cyclic AMP signalling via phosphorylation-induced activation of cyclic nucleotide phosphodiesterase 4B. *Nature Communications*, *7*(1), 10856. https://doi.org/10.1038/ncomms10856

Johnson et al. (2014). Metformin use in women with polycystic ovary syndrome. *Annual Translational Medicine.* 2(6):56

Lau, G. Y., & Richards, J. G. (2018). Interspecific variation in brain mitochondrial complex I and II capacity and ROS emission in marine sculpins. *Journal of Experimental Biology*. https://doi.org/10.1242/jeb.189407

Lee, J. W., Shin, Y.-J., Kim, H., Kim, H., Kim, J., Min, S.-A., Kim, P., Yu, S. Do, & Park, K. (2019). Metformin-induced endocrine disruption and oxidative stress of *Oryzias latipes* on two-generational condition. *Journal of Hazardous Materials*, *367*, 171–181. https://doi.org/10.1016/j.jhazmat.2018.12.084

Li, S.; Chen, Y.; Zhang, S.; More, S. S.; Huang, X.; Giacomini, K. M. Role of Organic Cation Transporter 1, OCT1 in the Pharmacokinetics and Toxicity of Cis-Diammine(Pyridine)-Chloroplatinum(II) and Oxaliplatin in Mice. Pharmaceutical Research. 2011, 28, 610−625.

MacLaren, R. D., Wisniewski, K., & MacLaren, C. (2018). Environmental concentrations of metformin exposure affect aggressive behavior in the Siamese fighting fish, *Betta splendens*. *PLOS ONE*, *13*(5), e0197259. https://doi.org/10.1371/journal.pone.0197259

Markiewicz, M., Jungnickel, C., Stolte, S., & Białk-bieli, A. (2017). *Ultimate biodegradability and ecotoxicity of orally administered antidiabetic drugs*. *333*, 154–161. Journal of Hazardous Materials 333, 154-161.https://doi.org/10.1016/j.jhazmat.2017.03.030

Masuda, S., Hospital, W. N., Yonezawa, A., & Inui, K. (2006). *Identification and Functional Characterization of a New Human Kidney- Specific H+/Organic Cation Antiporter*, *Kidney-Specific Multidrug and Toxin Extrusion 2*. *Journal of the American Society Nephrology* 17: 2127–2135. https://doi.org/10.1681/ASN.2006030205

Nakamichi, N., Shima, H., Asano, S., Ishimoto, T., Sugiura, T., Matsubara, K., Kusuhara, H., Sugiyama, Y., Sai, Y., Miyamoto, K., Tsuji, A., & Kato, Y. (2013). Involvement of Carnitine/Organic Cation Transporter OCTN1/SLC22A4 in Gastrointestinal Absorption of Metformin. *Journal of Pharmaceutical Sciences*, *102*(9), 3407–3417. https://doi.org/10.1002/jps.23595

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. https://doi.org/10.1002/etc.2793

Niemuth, N. J., & Klaper, R. D. (2015). Emerging wastewater contaminant metformin causes intersex and reduced fecundity in fish. *Chemosphere*, *135*, 38–45. https://doi.org/10.1016/j.chemosphere.2015.03.060

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. https://doi.org/10.1016/j.scitotenv.2012.10.046

Otto, K. A., & Short, C. E. (1998). Pharmaceutical control of pain in large animals. *Applied Animal Behaviour Science*, *59*(1–3), 157–169. https://doi.org/10.1016/S0168-1591(98)00130-0

Scarpello, J. H., & Howlett, H. C. (2008). Metformin therapy and clinical uses. *Diabetes and Vascular Disease Research*, *5*(3), 157–167. https://doi.org/10.3132/dvdr.2008.027

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. https://doi.org/10.1016/j.watres.2012.06.019

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. https://doi.org/10.1039/b909311g

Schnörr, S. J., Steenbergen, P. J., Richardson, M. K., & Champagne, D. L. (2012). Measuring thigmotaxis in larval zebrafish. *Behavioural Brain Research*, *228*(2), 367–374. https://doi.org/10.1016/j.bbr.2011.12.016

Tannoury, M., & Attieh, Z. (2017). The Influence of Emerging Markets on the Pharmaceutical Industry. *Current Therapeutic Research*, *86*, 19–22. https://doi.org/10.1016/j.curtheres.2017.04.005

Toyama, K., Yonezawa, A., Masuda, S., Osawa, R., Hosokawa, M., Fujimoto, S., Inagaki, N., Inui, K., & Katsura, T. (2012). Loss of multidrug and toxin extrusion 1 (MATE1) is associated with metformin-induced lactic acidosis. *British Journal of Pharmacology*, *166*(3), 1183–1191. https://doi.org/10.1111/j.1476-5381.2012.01853.x

Trautwein, C., Berset, J.-D., Wolschke, H., & Kümmerer, K. (2014). Occurrence of the antidiabetic drug Metformin and its ultimate transformation product Guanylurea in several compartments of the aquatic cycle. *Environment International*, *70*, 203–212. https://doi.org/10.1016/j.envint.2014.05.008

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. https://doi.org/10.1016/j.aquatox.2018.10.003

Ussery, E., Bridges, K. N., Pandelides, Z., Kirkwood, A. E., Guchardi, J., & Holdway, D. (2019). Developmental and Full-Life Cycle Exposures to Guanylurea and Guanylurea–Metformin Mixtures Results in Adverse Effects on Japanese Medaka (*Oryzias latipes). Environmental Toxicology and Chemistry*, *38*(5), 1023–1028. https://doi.org/10.1002/etc.4403

Vestel, J., Caldwell, D. J., Constantine, L., D'Aco, V. J., Davidson, T., Dolan, D. G., Millard, S. P., Murray-Smith, R., Parke, N. J., Ryan, J. J., Straub, J. O., & Wilson, P. (2016). Use of acute and chronic ecotoxicity data in environmental risk assessment of pharmaceuticals. *Environmental Toxicology and Chemistry*, *35*(5), 1201–1212. https://doi.org/10.1002/etc.3260

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. https://doi.org/10.1042/CS20110386

Yimer, E. M., Surur, A., Wondafrash, D. Z., & Gebre, A. K. (2019). The Effect of Metformin in Experimentally Induced Animal Models of Epileptic Seizure. *Behavioural Neurology*, *2019*, 1–13. https://doi.org/10.1155/2019/6234758

Zhou, M., Xia, L., & Wang, J. (2007). Metformin Transport by a Newly Cloned Proton-Stimulated Organic Cation Transporter (Plasma Membrane Monoamine Transporter) Expressed in Human Intestine. *Drug Metabolism and Disposition*, *35*(10), 1956–1962. https://doi.org/10.1124/dmd.107.015495

Chapter Two: Modest Developmental Effects in Larval Zebrafish (*Danio rerio*) Exposed to Metformin and Guanylurea

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2.1. Abstract

Metformin is the most common first-line oral therapeutic agent used in the treatment of type-2 diabetes. It is excreted by humans predominantly through urine and is transformed into guanylurea (> 98 %) in wastewater treatment plants. Both compounds have been frequently detected in surface waters globally. Studies on the effect of metformin and guanylurea on early-life stage fish are scant. The aim of this study was to examine the potential impact of metformin and guanylurea on zebrafish development (mortality, hatching, abnormalities, and growth), and cardiometabolic (heart rate and oxygen consumption) and behavioral responses. Embryos were exposed from 3 hours post-fertilization to 5 days post-fertilization at environmentally relevant (0.4, 4, 40 $\mu g \cdot L^{-1}$) and supra-environmental (400, 4000 $\mu g \cdot L^{-1}$) concentrations. Metformin ($\geq$0.4 $\mu g \cdot L^{-1}$) and guanylurea (40 $\mu g \cdot L^{-1}$) exposure increased mortality. Metformin increased spinal abnormalities at all concentrations and increased edema (yolk and pericardial edema) at the highest concentration; certain morphometric parameters (head diameter, eye diameter, snout-vent length) were decreased in the highest concentration tested. Exposure to guanylurea over the same developmental stages caused a significant increase in mortality at 40 $\mu g \cdot L^{-1}$ only. Neither metformin and guanylurea exposure altered hatchability, time to hatch, thigmotaxis, startle response, general swim metrics, visual motor response, heart rate or oxygen consumption, suggesting that effects on zebrafish development are modest, even at supra-environmental concentrations.

2.2. Introduction

An ever-expanding number of pharmaceuticals have been detected in freshwater systems worldwide (Daughton, 2016). Pharmaceuticals mainly enter aquatic systems through patient excretion and subsequent discharge via municipal wastewater treatment plants. These compounds have become a concern due to their propensity to cause physiological alterations in non-target organisms at low concentrations (Daughton, 2016) particularly during early-life stages. Among the most common pharmaceuticals found in surface waters is metformin due to its broad medical application in treating conditions such as, type 2 diabetes mellitus (Rojas and Gomes, 2013), and polycyclic ovarian syndrome (Johnson, 2014).

Metformin (MW 129.16, CAS #657-24-9, Table 1.1) is an orally administered dimethyl biguanide that reduced basal and post prandial glucose levels through the inhibition of excessive hepatic gluconeogenesis and glycogenolysis (Hundal et al., 2000; Gong et al., 2012). Metformin is not readily metabolized and is largely excreted as the parent compound through urine (70-90%; Li Gonga et al., 2012). Once excreted, it is transported to wastewater treatment plants where it is transformed into guanylurea (MW 102.10, CAS # G844495) via aerobic bacteria (~98%; Cadwell et al., 2019). Metformin has been detected in effluent and surface waters at 1 to 47 μg · L and 0.06 to 3 μg · L, respectively (Trautwein et al., 2014). Guanylurea has been detected in surface waters at concentrations that range 0.1-28 μg · L^{-1} (Scheurer et al.,2009; Scheurer et al.,2012). The potential impacts of metformin exposure on aquatic organisms are largely unknown as the available literature is scant. To date, six studies have examined the effects of metformin on

adult fish. Male fathead minnow displayed increased levels of VTG mRNA following 4 weeks of exposure to 40 µg · L (Niemuth et al., 2015). Another study documented decreased weights, presence of oocytes in the testes and reduced fecundity (Niemuth and Klaper, 2015) following a 305d exposure to 40 µg · L. MacLaren et al. (2018) examined aggressive behavior in adult male Siamese fighting fish *Betta splendens* with ~4.5 months of exposure to 40 or 80 µg·L^{-1} metformin. Aggressive behaviors towards a faux male stimulus, such as decreased gill flaring and fin spreading, was less with both doses; these behaviors are used to intimidate rival conspecific males and would-be predators. Decreased aggressive behaviors are linked to the inability of a fish to mate, defend its offspring, and maintain its nesting territory (Bronstein, 1982).

The impacts of metformin on early life stages have been examined in a few studies. Growth (length and weight) was decreased in juvenile medaka exposed to increasing concentrations of metformin (1.0, 3.2, 10, 32, and 100 µg·L^{-1}; Ussery et al., 2018) for 165 days. However, other chronic exposures have shown no impacts of metformin on growth in fathead minnow (Parrot et al., 2021:2022) and Japanese medaka (Lee et al., 2019). Metformin exposed juveniles had increased levels of the fatty acids, steric acid, palmitic acid and arachidonic acid and decreased L-lysine, L-proline, and DL-3-aminoisobutyric acid (Ussery et al., 2018). In zebrafish, embryos exposed to 1, 10, 20, 30, 40, 50, 75 and 100 µgL^{-1} of metformin, had increased mortality, hatch rate and incidences of scoliosis, pericardial edema, and yolk deformation at 4 days post fertilization (dpf) for all tested concentrations (Philips et al., 2021; Elizalde-Velazquez et al., 2021).

Exposure to guanylurea induced antioxidant enzyme activity (catalase, superoxide dismutase, and glutathione peroxidase) and levels of oxidative damage biomarkers (Elizalde-Velazquez et al., 2022). Similarly, metformin caused moderate metabolic effects in brown trout (*Salmo trutta*). Larval exposed to metformin (1 $\mu g \cdot L^{-1}$) had an increase in hepatic glycogen content (Jacob et al., 2018). Conversely, in two chronic exposures of fathead minnow embryos to metformin (0.020 - 269 $\mu g \cdot L^{-1}$) there were no alterations in survival, hatch, or growth metrics in larval fish (Parrot et al., 2021, 2022). Given the current research, fish at early and adult life stages seem to be sensitive to metformin at environmentally relevant concentrations.

Only five studies have examined the effects of guanylurea on early-life stages of fish. Ussery et al. (2018) exposed larval Japanese medaka (*Oryzias latipes*) to increasing concentrations of guanylurea (1.0, 3.2, 10, 32 and 100 $\mu g \cdot L^{-1}$) for 28 days. While growth metrics such as length and weight did not differ between control and treatment groups after 7-and 14 -days. Decreased length and weight were observed by day 28, suggesting impairment with prolonged exposure (Ussery et al., 2019). Jacob et al. (2019) exposed larval brown trout (*Salmo trutta*) to increasing concentrations of guanylurea (0, 100 and 1,000 $\mu g \cdot L^{-1}$) for three weeks. Guanylurea exposure did not alter endpoints in larvae (percent mortality, time to hatch, heart rate, body length, or lipid peroxide levels; Jacob et al., 2019). It is not clear why medaka, but not brown trout, early life stages appear to be sensitive to guanylurea, but the dose and exposure length were similar across studies, suggesting species sensitivity were likely different. Guanylurea exposure can cause oxidative stress in larval stage fish like metformin. Exposure to environmentally relevant concentrations of guanylurea (25 $\mu g \cdot L^{-1}$) for 96 hours post fertilization (hpf) elevated

levels of superoxide dismutase, catalase, and glutathione peroxidase in zebrafish embryos (Elizalde-Velazquez et al. 2021). Exposed larva also had increased lipid peroxidation, protein carbonyl and hydroperoxide content suggesting that guanylurea may induce alterations in cell signaling, DNA damage and cytotoxicity after prolonged exposure (Elizalde-Velazquez et al. 2021).

The objective of this study was to investigate the effects of environmental and supra-environmental concentrations of metformin and guanylurea during embryogenesis in zebrafish (*Danio rerio*). Static renewal exposures over 5 days were used to assess the effects of metformin and guanylurea on cumulative mortality, hatching (48 hours post fertilization, hpf), morphology and percent abnormality (metformin only,72 hpf), heart rate (24, 48 and 72 hpf), oxygen consumption (5 dpf) and behavior (4-5dpf). Behavioral assessments included general swimming, visual motor response, thigmotaxis and startle response. The results of this study provide important data for understanding the effects of metformin and guanylurea on developing fish and may be useful for risk assessment of pharmaceuticals in aquatic environments.

2.3. Materials and Methods

2.3.1. Test Chemicals

Metformin hydrochloride (1,1-dimethylbiguanideine hydrochloride; CAS# 1115-70-4) was purchased from Toronto Research Chemicals (Toronto, Ontario, Canada). Guanylurea (CAS# G844495) was purchased from Sigma-Aldrich (Oakville, Ontario, Canada).

2.3.2. Fish Care and Breeding

Breeding stocks of mature wild-type zebrafish, age 4-12 months, were maintained in a semi-recirculation rack housing systems with 15% daily distilled water replacement and automated sodium bicarbonate and salt dosing (Instant Ocean, SpectrumBrands, USA). The system was maintained at 28.5°C, pH of 7-8, and conductivity of 450 -470 µS with a light-dark cycle of 14:10 hours. The fish were fed three times a day (weekdays, two times on weekends) with Tetramin Topical Flakes (Tetra, USA) and live artemia (GSL Brine Shrimp, US). All fish care was provided in accordance with McMaster University's animal ethics research board using approved protocols under Animal Use Protocol #20-06-23.

2.3.3. Embryo Collection and Rearing

Embryo traps were inserted into a tank the night prior to embryo collection. Traps with embryos were removed 1.5 hours after first light, carefully rinsed to remove all debris, and placed in petri dishes (100 individuals per dish) with E3 embryo media (5mM NaCl, 0.17mM KCl, 0.33 mM $MgSO_4$, and 0.33mM $Cacl_2$; pH 7.0-7.5). Embryos were observed under light microscopy to ensure only 12-32 cell stage embryos were selected for the exposures. All exposures started by 3hpf. Embryos were kept at 28.5 °C and monitored daily under light microscopy for survival and developmental milestones for the duration of the experiment with periodic removal for endpoint collection. Chorions (post-hatching) and dead embryos were removed daily; water change outs were 50% daily.

2.3.4. Exposures, Mortality and Hatching

Fish embryo acute toxicity tests with *D. rerio* were based off the OECD guideline 236 (OECD, 2013). 100 newly fertilized embryos (3 hpf) were transferred to petri dishes (100

x100 mm) filled with 100 ml of metformin or guanylurea dissolved in E3 medium. Metformin and guanylurea solutions were diluted from one of two stock solutions (40 mg L^{-1} for the 0.4, 4 ug L^{-1} treatment groups and 4 g L^{-1} for the 400, 4000 ug L^{-1} treatment groups). Control plates containing E3 medium were used for negative controls. A total of three independent exposure experiments were carried out and each experiment had 3 replicate petri dishes for each concentration. Control and test solutions were renewed daily after observation of embryos under stereomicroscopy (Zeiss, model Axio Zoom. V16); at least 50% of the volume of each well was replaced with freshly prepared test solutions. Mortality and number hatched were observed every 24 hours. The general hardness (GH), carbonate hardness (KH), pH, nitrite (NO_2^-) Nitrate (NO_3) were evaluated at the end of each test at 120 hpf (Table 2).

Table 2.1. Water quality parameters of test mediums during experiments (n=3).

Compound	Treatment	GH ppm	KH	pH	NO_2^- ppm	NO_3 ppm
Metformin	Control	160 ± 0.0	40 ± 0.0	7 ± 0.0	0 ± 0.0	0 ± 0.0
	0.4	160 ± 0.0	20 ± 12.0	7 ± 0.0	0 ± 0.0	0 ± 0.2
	4	160 ± 23.0	20 ± 23.0	7.5 ± 4.2	0 ± 0.0	0.5 ± 0.2
	40	200 ± 0.0	40 ± 2.3	7 ± 3.5	0 ± 0.0	0.5 ± 0.0
	400	180 ± 0.0	40 ± 0.0	7 .5 ± 0	0 ± 0.0	0.5 ± 0.
	4000	160 ± 23.0	20 ± 23.0	7.5 ± 4.2	0 ± 0.0	0.5 ± 0.2
Guanylurea	Control	110 ± 4.3	40 ± 0	7 ± 0	0 ± 0.0	0 ± 0.0
	0.4	180 ± 0.0	40 ± 0	7 .5 ± 0	20 ± 0.0	0 ± 0.0
	4	140 ± 34.6	45 ± 6	7.5 ± 0.0	0 ± 0.0	0 ± 0.2
	40	110 ± 4.3	40 ± 0	7 ± 0	0 ± 0.0	0 ± 0.0
	400	120 ± 34.6	40 ± 0	7 .5 ± 0	0 ± 0.0	0 ± 0.0
	400	180 ± 4.3	40 ± 0	7 ± 0	0 ± 0.0	0 ± 0.0

GH-general hardness; KH-carbonate hardness; NO_2^- -nitrite; NO_3^- -nitrate

2.3.5. Larval fixation and morphological and abnormality assessment

Zebrafish larvae (N=60; 20 per treatment for each triplicate experiment) were euthanized at 72 hours post fertilization with tricaine (300 mg L^{-1}) for 10 minutes. Tricaine was replaced with 1mL neutral buffered saline for 1 hour for larval fixation. Post fixation, larvae were washed with 2 mL phosphate buffered saline (PBS), dehydrated with 1 ml 70% ethanol, and stored at 4°C for storage. An upright digital microscope (Zeiss, model Axio Zoom. V16) was used to acquire lateral images of the embryos (Fig.2.1). Seven morphological parameters were measured using the free graphical image analysis software ImageJ (National Institutes of Health, Bethesda, MD, USA): Standard length (SL), snout-vent length (SVL), height at anterior of anal fin (HAA), head depth (HD), and eye diameter (ED), yolk sac depth and yolk sac length (Figure 2.2). The calculation of yolk sac volume was done using the following formula according to Subhan et al., 2020:

$$V = \frac{\pi}{6} x\ LH^2$$

V = Yolk sac volume (mm^3) L = Yolk sac length (mm), H = Yolk sac depth (mm)

Images of larvae were also used to assess deformities of the spine (scoliosis, bends, or kinks), yolk sac edema, and pericardial edema.

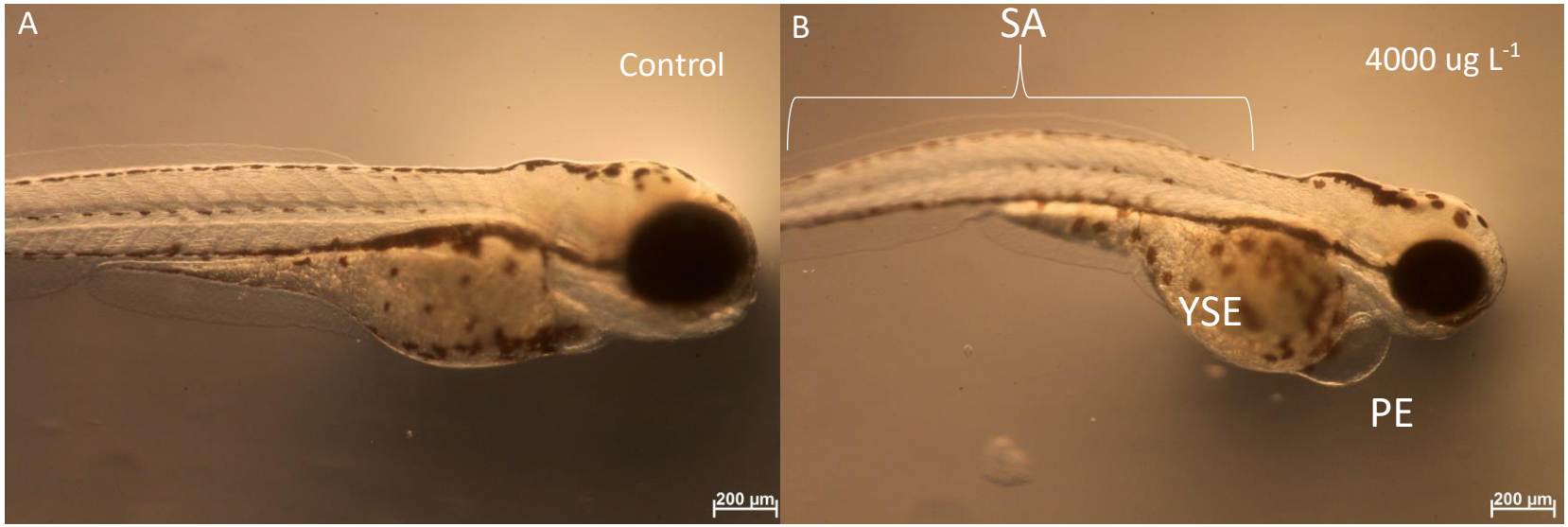

Figure 2. 1. Representative images of developmental abnormalities in 72 hours post fertilization (hpf) zebrafish treated with metformin. A) Control zebrafish, B) Zebrafish from 4000 ug L^{-1} treatment with deformities. Spinal abnormalities (SA, scoliosis), yolk-sac edema (YSE), spinal curvature (SC), pericardial edema (PE) are present in this fish. Scale bar is 200 um

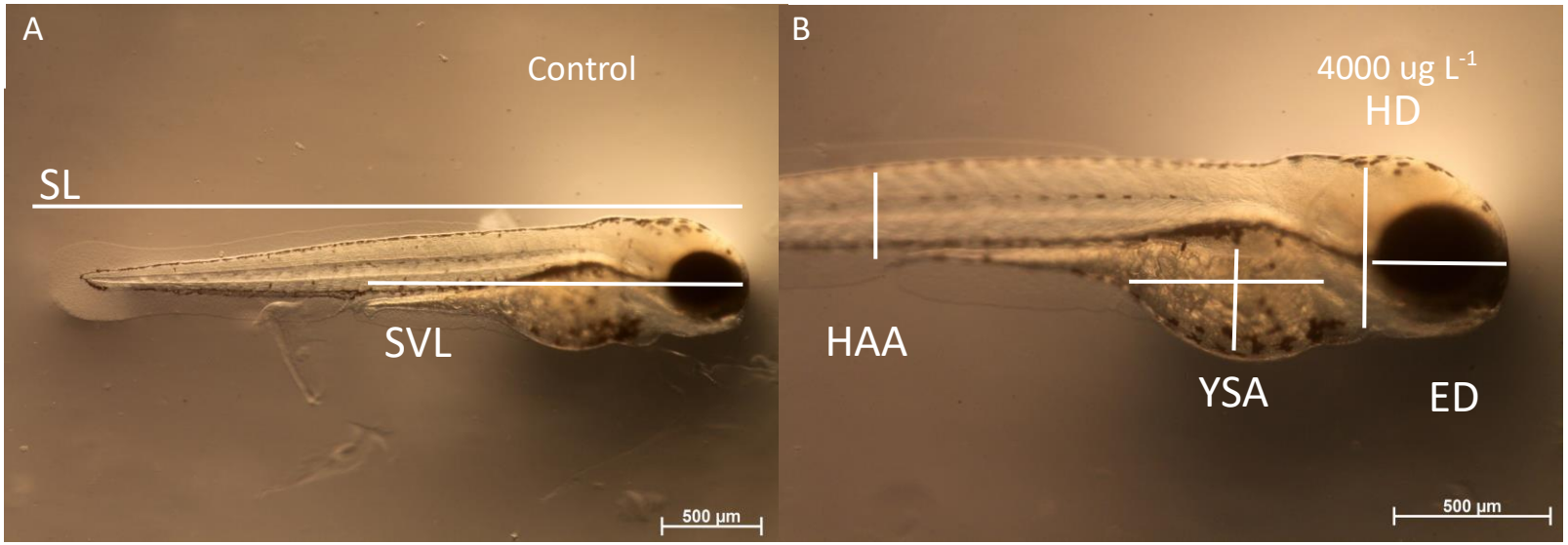

Figure 2. 2. Morphological measurements of larval zebrafish. Representative images of larvae at 72 hpf with labeled morphological measured from lateral images. Measured traits included in control zebrafish (A) standard length (SL), snout-vent length (SVL), and in (B) fish exposed to 4000 ug L^{-1} metformin, height at anterior of anal fin (HAA), yolk sac area (YSA) which is calculated by the yolk sac length and depth (see 2.3.5 for formula), head depth (HD), eye diameter (ED). Scale bar is 500 um.

2.3.6. Larval Behavior

All behavioral assays were completed using the Danio vision observation chamber (Noldus B.V., Wageningen, the Netherland) and EthoVision XT version 15 video tracking software. Analyses were performed in an isolated room and the chamber was maintained at 28.5 °C. Briefly, larvae were transferred to clear 96-well plates with lids and allowed to acclimate overnight (96 hpf at time of measurement) before analyses for visual motor and startle responses (300 μL test medium). Larvae were transferred to a 24 well plate and were allowed to acclimate overnight (120 hpf at time of measurement) for general locomotor activity and thigmotaxis (2 ml test medium).

In zebrafish, visual motor response is a method used to measure locomotion behavior in alternating periods of light (lower activity) and dark (higher activity; Best and Vijayan, 2017). This assay is used to measure both locomotor activity and behavioral response to different light conditions. Visual motor response measured the total distance moved during combined dark or light periods, respectively. The assay ran for 60 minutes over four alternating periods of light (maximum light intensity) and darkness lasting 7.5 min (450s) each. Data was collected in 3 trials (16 larvae/treatment group/plate) for a total of 48 larvae per treatment.

Startle response in zebrafish involves an evoked "fast start "behavior consisting of a rapid turn a swim away from the stimulus (Rice et al., 2011). After a 5-minute acclimation period, Larval zebrafish were subjected to 10 acoustic/vibrational stimuli (DanioVision intensity setting 6) with a 20s inter-stimulus interval. The variable of interest used as a proxy for startle response was *maximum velocity* (mm/s). and this was always after the

first acoustic tap. The response data were inspected for repeated/consistent responses in subsequent taps, but this data was excluded from the statistical analyses. General locomotor activity and thigmotaxis was collected in 3 trials (16 larvae/treatment group/plate) for a total of 48 larvae per treatment. General locomotor activity was determined over 30 minutes in 20% reduced light capacity; activity was measured as the total distance moved (mm) over the whole arena (i.e., each well) and the percent time spent active. Thigmotaxis determined the time (seconds) spent in the inner and outer areas of the well; the outer area was defined as within 4mm of the wall. The total area for inner and outer areas were equivalent. The assay was 30 minutes in duration and was run at 20% reduced light capacity. Thigmotaxis was calculated as the percent time spent in the outer zone.

2.3.7. Oxygen consumption and heart rate

Oxygen consumption rates (MO_2) were determined at 28 ± 0.1°C for larval zebrafish at 5 dpf. MO_2 was measured using REDFLASH technology (PyroScience) composed of an oxygen probe, temperature probe (TDIP15) and oxygen meter (PyroScience FireSting O2 Optical Oxygen Meter; FSPRO-4). The system was calibrated at time zero (t0) for 0 and 100% DO saturation using vails containing aerated E3 medium and sodium bicarbonate, respectively. Vials containing 100% air saturated water were measured alongside experimental groups. These vials were used for background correction to minimize contribution from non-larval sources (i.e., aerobic bacteria). Groups of larvae (5 per chamber) were placed in 1 mL glass vials containing 90-100% oxygen saturated E3 and allowed to acclimate for 15 minutes. Oxygen levels were recorded for 30 minutes post acclimation and the oxygen consumption rate was used as a proxy for metabolic rate.

Oxygen consumption rate was calculated using the slope of the decrease in oxygen content with time inside each vial. Oxygen consumption was calculated as μmol O_2/ h^{-1} and five-six fish per replicate (six per treatment group) were utilized. Heart rate was determined by counting the number of beats observed for 30s and multiplying the value by two. Data was collected in 3 trials (15 larvae/treatment group/plate) for a total of 45 larvae per treatment and reported as a mean and standard deviation.

2.3.8 Statistical Analysis

All statistical analyses were performed using R (v. 3.5.2). Normality of all data was assessed using a Shapiro-Wilk test prior to subsequent statistical analysis. For cumulative mortality, number hatched and oxygen consumption, the data were not normal, and nonparametric statistical tests (Kruskal Wallis one-way ANOVA and Dunn test) were used. P- values in the text are from chi-squared distributions of the data with 5 degrees of freedom. Data from behavioral assays and morphology were normally distributed and analysis of variance (ANOVA) tests using untransformed data were applied. Tukey HSD tests were used when there were significant differences between treatments in the ANOVA. For percent abnormality and morphometric analyses, a post-hoc Bonferroni test was used following an ANOVA. All p- values for these tests are shown in the text, figures, and tables.

2.4 Results

2.4.1. Cumulative Mortality and Hatchability

The cumulative mortality (3-120 hpf) in the control groups for metformin and guanylurea experiments were 6% and 8%, respectively (Figure 2.3). Exposure to metformin significantly increased cumulative mortality in all treatment groups (ANOVA DF $= 5$, $F = 5$, $p = 0.006$; Figure: 2.3a). Guanylurea exposure significantly increased mortality at the 40 $\mu g \cdot L^{-1}$ treatment only (ANOVA DF $= 5$, $F = 11.38$, $p = <0.001$; Figure 2.3b). There were no effects on time to hatch (data not shown) or hatchability (percent of eggs hatched; Table 2.2) in larva exposed to metformin (ANOVA DF $= 5$, $F = 1.925$, $p = <0.107$ or guanylurea (ANOVA DF $= 5$, $F = 2.01$ $p = <0.09$).

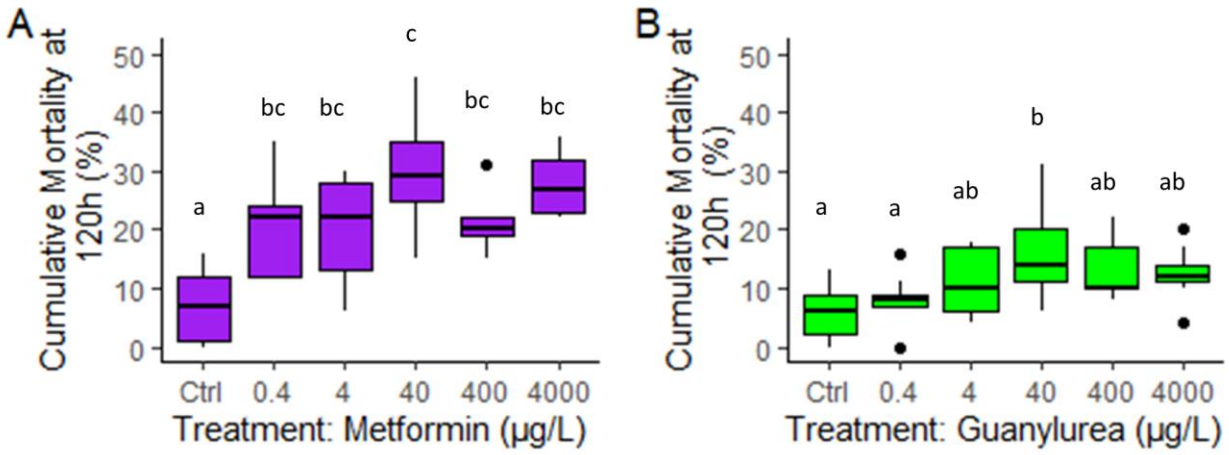

Figure 2.3. Cumulative mortality in zebrafish exposed to increasing concentrations of (A) metformin and (B) guanylurea. Mortality was assessed every 24 hours from 3 – 120 hpf by direct observation under light microscopy. Each box shows the upper, median (indicated by the central horizontal black line), and lower quartile values. Error bars indicate the standard deviation and outliers are indicated as black dots. Three repeated experiments were conducted with three replicates per experiment (100 larva in each replicate; n=9). Letters above the boxes indicate statistical differences across treatment groups for each compound.

Table 2.2. Hatching of embryo-larval zebrafish exposed to metformin and guanylurea for 48hpf. Data are presented as treatment means ± standard deviation. Each petri dish started with 100 zebrafish embryos. Hatchability is the percentage of eggs that hatched and was calculated for each dish, n=9.

Compound	Concentration (µg/L)	Hatchability (%)
Metformin	Control	86.0 ± 0.3
	0.4	77.0 ± 0.5
	4	90.0 ± 0.3
	40	90.0 ± 0.2
	400	90.0 ± 0.2
	4000	91.0 ± 0.4
Guanylurea	Control	68.0 ± 0.7
	0.4	71.0 ± 0.3
	4	63.7 ± 0.4
	40	66.0 ± 0.5
	400	76.0 ± 0.3
	4000	71.0 ± 0.3

2.4.2. Abnormalities and Morphometrics

Spinal abnormalities of control larvae were 41%. Exposure to metformin at all tested concentrations caused increased prevalence of spinal abnormalities (ANOVA DF = 5, F =4.763, p = <0.001; Table 2.3). Metformin exposure increased the prevalence in both yolk sac (ANOVA DF = 5, F =12.06, p = <0.001) and pericardia (ANOVA DF = 5, F =9.405, p = <0.001) edema at the highest tested concentration (4000 μg · L^{-1}). Metformin exposure decreased snout vent length (Chi squared=36.24, df=5, p-value=<0.001; Table 2.4), height at anterior anal fin (Chi squared=22.286, df=5, p-value=<0.001) and eye diameter (Chi squared=33.66, df=5, p-value=<0.001) at the highest tested concentration tested. Yolk sac area (Chi squared=36.43, df=5, p-value= 0.3) and head depth (Chi squared=34.1, df=5, p-value= 2,2) were not impacted by metformin exposure.

Table 2. 3. Abnormalities in larval zebrafish exposed to metformin. Embryos were exposed from 3 - 72 hpf. Values reflect percent abnormalities from n=60 larval zebrafish per treatment group; abnormalities are calculated for spinal abnormality, yolk sac edema, and pericardial edema. Values with a different alphabetical superscript are significantly different at $p < 0.05$.

Treatment ($\mu g \cdot L^{-1}$)	Spinal Abnormality (%)	Yolk Sac Edema (%)	Pericardial Edema (%)
Control	41^{a}	1^{a}	0^{a}
0.4	73^{b}	0^{a}	0^{a}
4	78^{b}	0^{a}	0^{a}
40	78^{b}	2^{a}	2^{a}
400	60^{b}	1^{a}	3^{a}
4000	75^{b}	41^{b}	28^{b}

Table 2. 4. Morphological traits in larval zebrafish exposed to metformin. Embryos were exposed from 3 - 72 hpf and fixed prior to imaging. Morphological structures were determined in lateral images, as shown in Figure 2.2. Values with a different alphabetical superscript are significantly different $p < 0.05$ (n=13-16 for standard length; n=60 for other measures). Standard lengths were not taken for fish that exhibited spinal abnormalities. Data is presented as mean and standard deviation. SL= standard length; SVL= snout-vent length; YSA= yolk- sac area; HAA= height at anterior anal fin; HD= head diameter; ED= eye diameter.

Treatment ($\mu g \cdot L^{-1}$)	SL	SVL	YSA	HAA	HD	ED
Control	$3.4^{a} \pm 0.01$	$1.99^{a} \pm 0.08$	$7.5^{a} \pm 0.1$	$0.27^{a} \pm 0.1$	$0.50^{a} \pm 0.06$	$0.33^{a} \pm 0.06$
0.4	$3.5^{a} \pm 0.03$	$1.93^{ab} \pm 0.09$	$7.4^{a} \pm 0.7$	$0.27^{a} \pm 0.09$	$0.51^{a} \pm 0.04$	$0.32^{a} \pm 0.04$
4	$3.4^{a} \pm 0.03$	$1.94^{ab} \pm 0.04$	$7.4^{a} \pm 0.7$	$0.26^{a} \pm 0.09$	$0.49^{a} \pm 0.04$	$0.32^{a} \pm 0.04$
40	$3.4^{a} \pm 0.07$	$1.95^{ab} \pm 0.1$	$7.2^{a} \pm 0.7$	$0.27^{a} \pm 0.1$	$0.5^{a} \pm 0.02$	$0.32^{a} \pm 0.01$
400	$3.4^{a} \pm 0.04$	$1.97^{a} \pm 0.1$	$7.3^{a} \pm 0.7$	$0.27^{a} \pm 0.2$	$0.51^{a} \pm 0.04$	$0.33^{a} \pm 0.02$
4000	$3.4^{a} \pm 0.01$	$1.90^{c} \pm 0.17$	$7.4^{a} \pm 0.6$	$0.25^{b} \pm 0.01$	$0.48^{a} \pm 0.34$	$0.30^{b} \pm 0.04$

2.4.3. Behavioral Responses

No significant differences in time spent active (ANOVA DF = 5, F =1.09 , p = <0.36) or total distance moved (ANOVA DF = 5, F =0.92, p = <0.0.46) were observed in any of the metformin treatment conditions (Figure 2.4 A). Similarly, there were also no significant differences in time spent active (ANOVA DF = 5, F =1.02, p = <0.92) or total distance moved (ANOVA DF = 5, F =1.9, p = <0.41) were observed in any of the guanylurea treatment conditions. Similarly, there were no significant effects of metformin (ANOVA DF = 5, F =1.79, p = <0.12) or guanylurea (ANOVA DF = 5, F =0.38, p = <0.88) exposure on thigmotaxis behavior (Figure 2.5 A, B). The percent time spent in the outer zone were 93.2 - 96.1% in the metformin exposures and 74.5 - 90.7% in the guanylurea exposures.

Zebrafish, regardless of treatment group, had higher activity in the dark in the visual motor response for both metformin and guanylurea (Figure 2.6 A, B versus C). Metformin exposure did not affect total distance moved during light (ANOVA DF = 5, F =9.09, p = <0.31) or dark periods (ANOVA DF = 5, F =0.36, p = <0.93; Figure 2.6). The mean distance travelled ranged from 1781 to 2042 mm in the dark. Likewise, guanylurea exposure did not affect total distance moved during light (ANOVA DF = 5, F =9.82, p = <0.21) or dark periods (ANOVA DF = 5, F =9.53, p = <0.42). The mean distance travelled ranged from 1849 to 1988 mm in the dark periods.

All control larvae responded to each acoustic stimulus and habituation to stimulus was seen in the control and treatment groups (Figure 2.7 A, B). The first acoustic stimulus always produced the largest response. There were no differences in maximum velocity (Vmax) with metformin exposure (ANOVA DF = 5, F =9.53, p = <0.42; Figure 2.7C). Vmax

ranged from 83-96 mm s^{-1}. Likewise, guanylurea exposed larvae did not have alter maximum velocity, compared to controls (ANOVA DF = 5, F =7.23, p = <0.63; Figure 2.7 D).

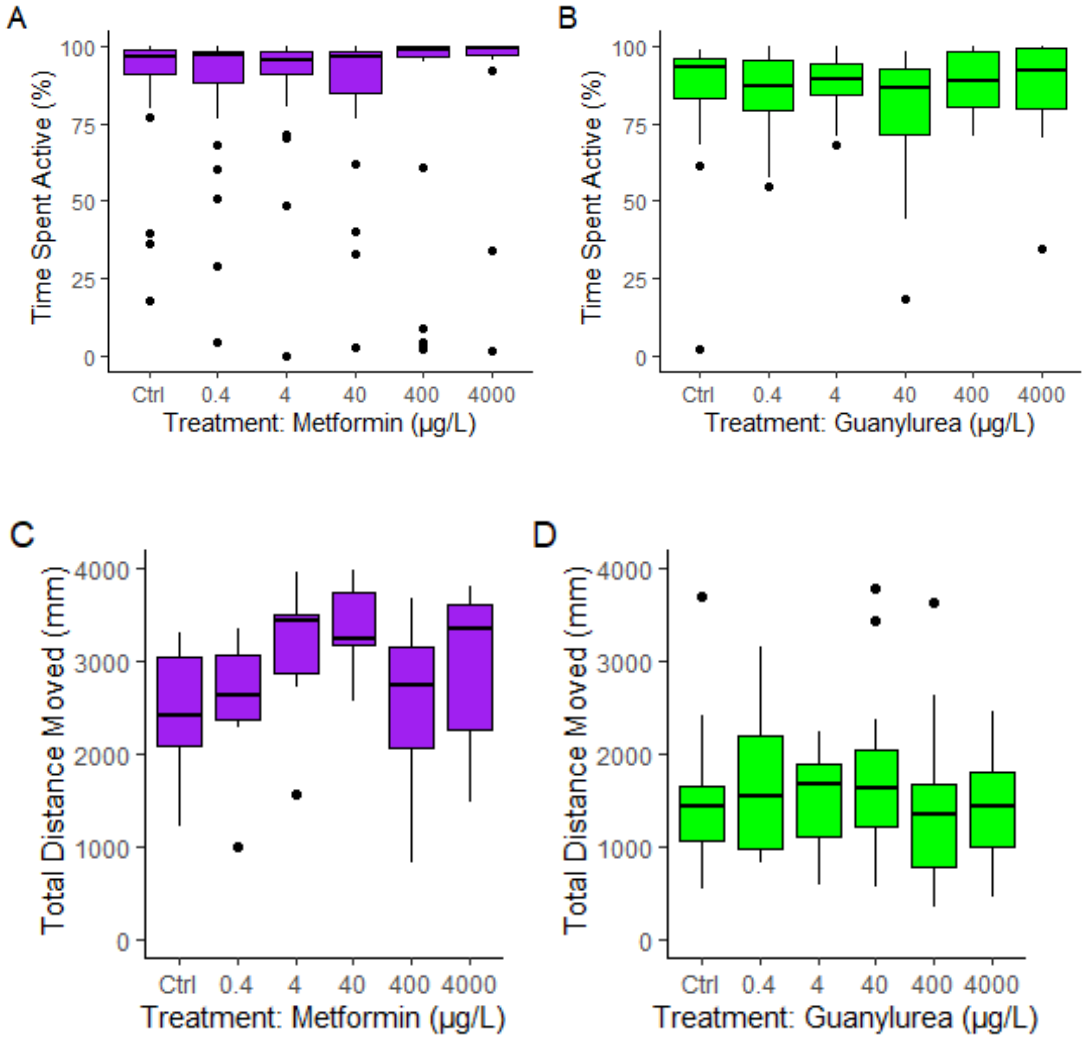

Figure 2. 4. General swimming behavior in larval zebrafish exposed to metformin and guanylurea. Boxplots depicting the percent time spent active for metformin (A) and guanylurea (B) exposed larvae over a 30-minute period. Data is represented as mean and standard deviation. Boxplots depicting total distance moved in metformin (C) and guanylurea (D) exposed larvae. Each box shows the upper, median (indicated via the central horizontal black line), and lower quartile values. Outliers are indicated as black

dots. Three replicate experiments were conducted with 16 larvae per control or treatment group for each experiment (n=48).

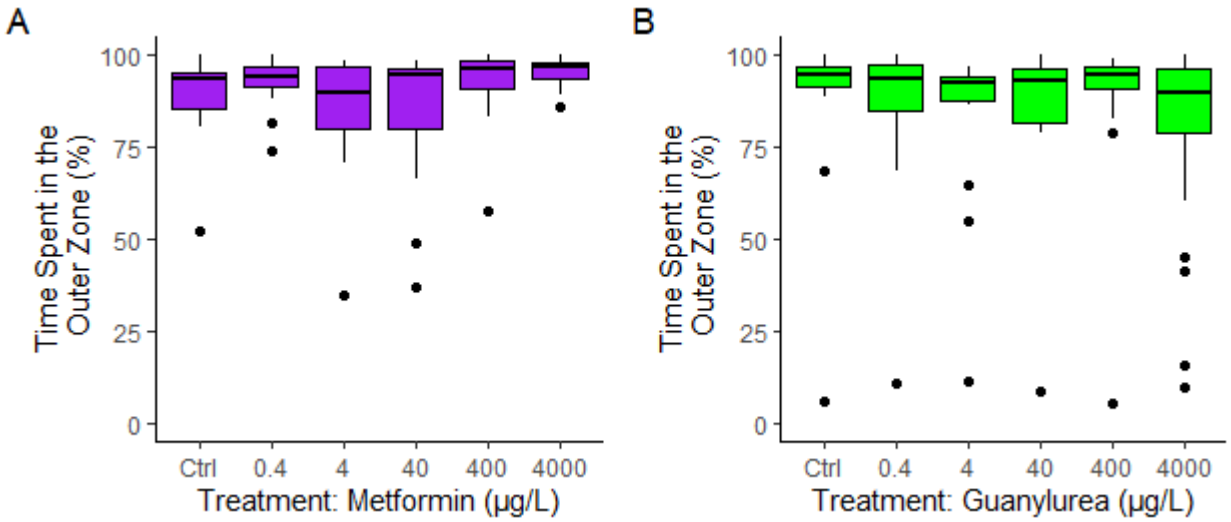

Figure 2. 5. Thigmotaxis behavior in larval zebrafish exposed to metformin and guanylurea. The testing apparatus consisted of wells (diameter 16.2 mm) with the width of the outer zone set at 4 mm relative to the border of the well; the inner and outer zones cover equivalent spatial area. Thigmotaxis was measured as the percent time spent in the outer zone for larva exposed to metformin (A) and guanylurea (B). Each box shows the upper, median (indicated via the central horizontal black line), and lower quartile values. Outliers are indicated as black dots. Three replicate experiments were conducted with 16 larvae per control and treatment group for each experiment(n=48).

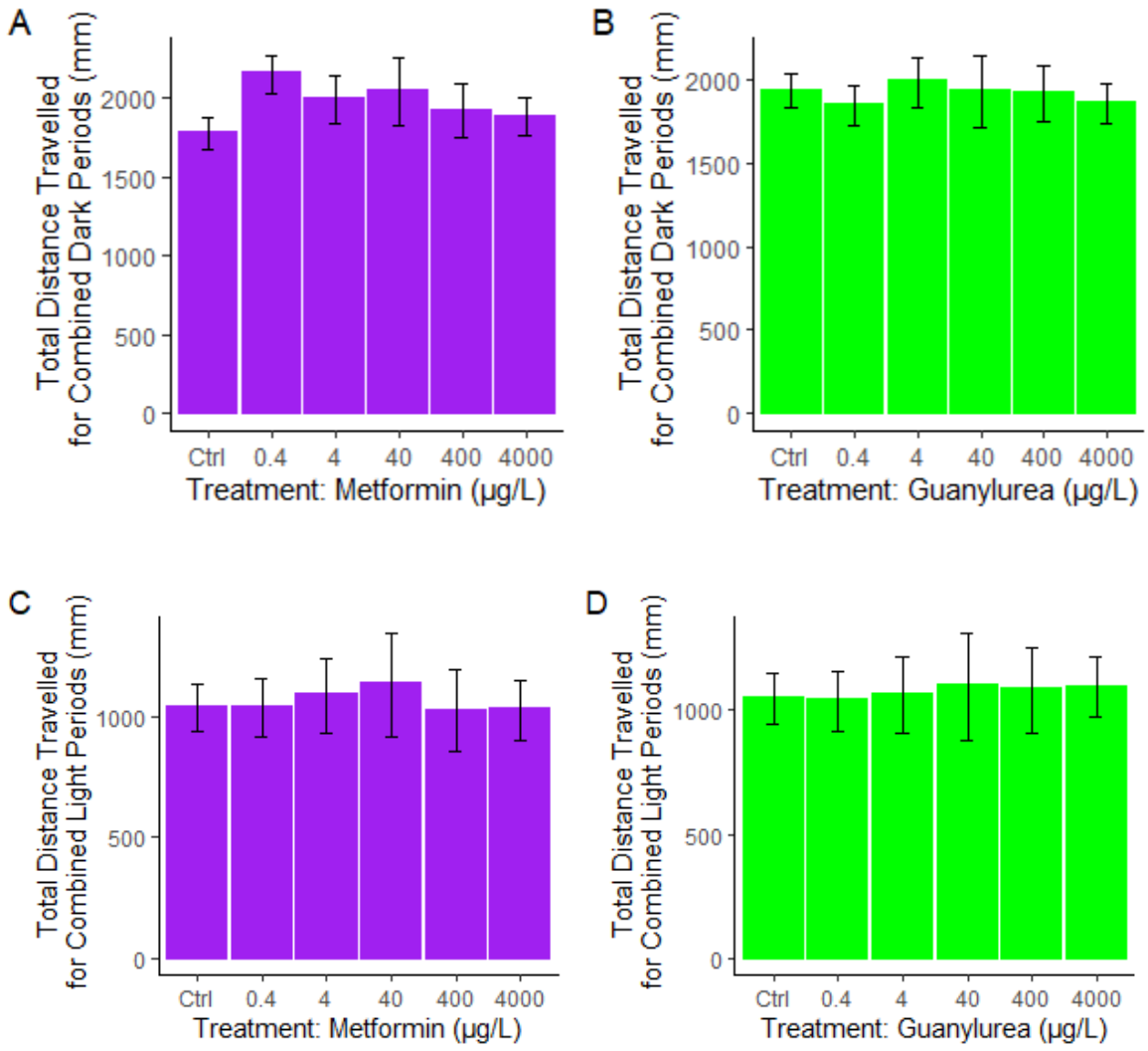

Figure 2. 6. Visual motor responses in larval zebrafish exposed to metformin and guanylurea. Total swimming distance moved during combined dark (7.5 min, metformin: A; guanylurea: B) and light (7.5 min, metformin: C; guanylurea: D) cycles. Data are presented as mean and standard deviation. Three replicate experiments were conducted with 16 larvae per control and treatment group for each experiment (n=48).

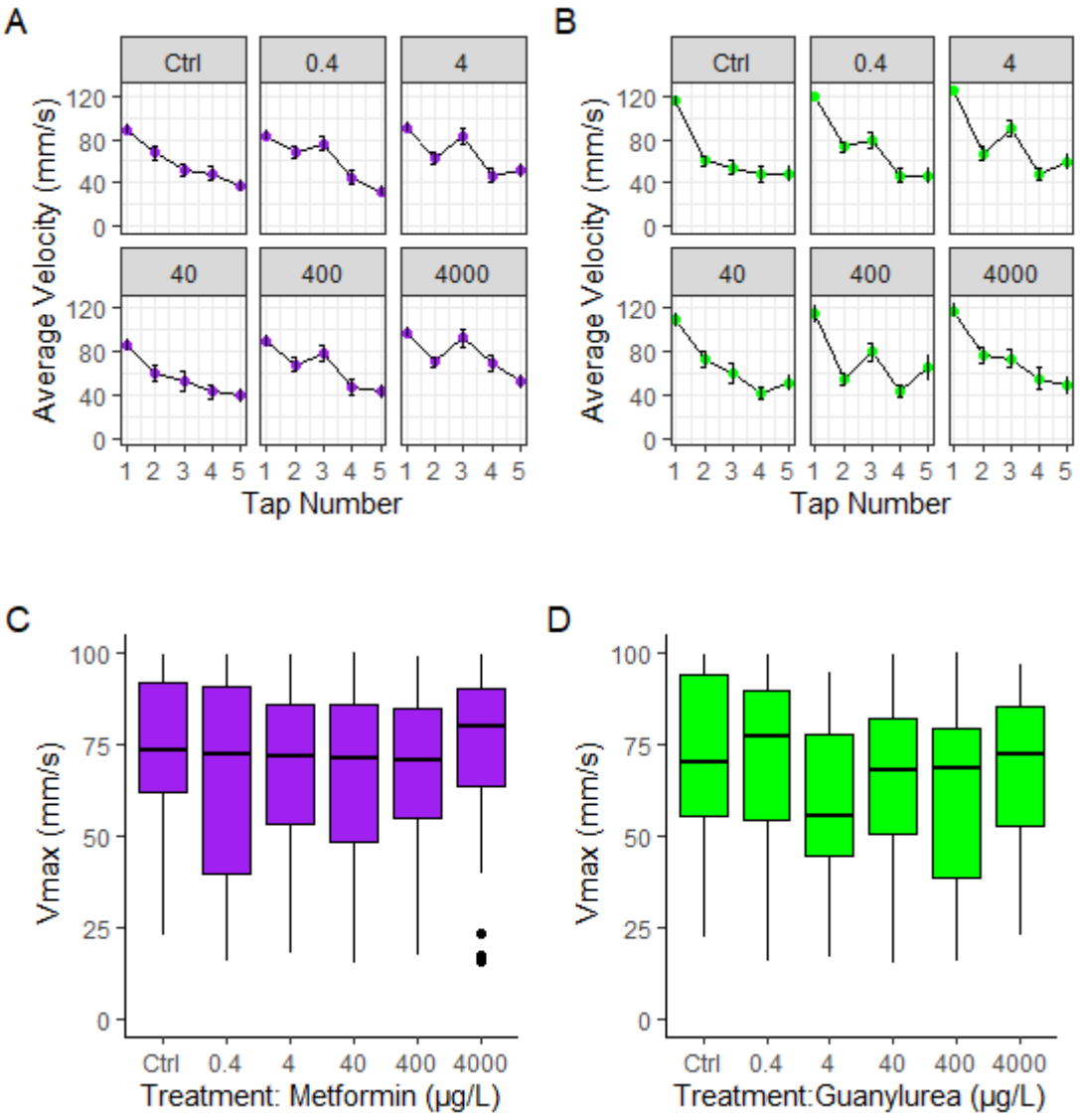

Figure 2.7. Startle response to acoustic stimuli in larval zebrafish exposed to metformin and guanylurea. Representative normal behavioral profile of larval zebrafish to 5 acoustic stimuli with an inter stimulus interval of 20 seconds for fish from metformin (A) and guanylurea (B) exposure experiments. Maximum swim velocity (Vmax) of larval zebrafish exposed to metformin (C) and guanylurea (D). Each box shows the upper, median (indicated via the central horizontal black line), and lower quartile values. Outliers are indicated as black dots. Three replicate experiments were conducted with 16 larvae per control and treatment group for each experiment (n=48).

2.4.4. Heart rate and Oxygen Consumption

Mean values for heart rate at 24, 48, and 72 hpf are shown in Table 2.5. The average heart rate of control larva during metformin exposures was of 119, 156 and 160 bpm at 24, 48 and 72 hpf, respectively. For the guanylurea experiments, larvae in the control group had a mean heart rate of 134, 144 and 148 bpm at the three developmental time points. There was a trend for heart rate to increase over developmental time in all groups under both exposure experiments, but it was not statistically significant. Larval heart rate was unaffected by metformin (ANOVA $p \geq = 0.151, 0.171, 0.266$) or guanylurea (ANOVA $p \geq = 0.67, 0.33, 0.438$) exposure, regardless of the dose.

For the metformin treatments, oxygen consumption ranged from approximately 0.42-0.99 µl O_2/µmol/ h^{-1} (Figure 2.8 A). Metformin did not cause significant effects on larval oxygen consumption (ANOVA $DF = 5, F = 2.13, p = < 0.37$). Oxygen consumption rates in guanylurea exposed embryos ranged from approximately 0.48 to 1.1 $\mu g \cdot L^{-1}$ O_2 hr^{-1} (Figure 2.8B). Likewise, oxygen consumption did not differ between controls and guanylurea exposed larvae (ANOVA $DF = 5, F = 1.46, p = < 0.23$).

Table 2. 5. Heart rate of larval zebrafish exposed to metformin and guanylurea. Heart rate was determined by direct observation under light microscopy at 24, 48 and 76 hpf. Data are provided as mean and standard deviation in beats per minute. 15 individuals were examined in each of triplicate experiments (n = 45).

Chemical	Concentration ($\mu g\ L^{-1}$)	24 (bpm, $\pm$SD)	48 (bpm, $\pm$SD)	72 (bpm, $\pm$SD)
Metformin	Control	119 ± 4	156 ± 4	160 ± 4
	0.4	119 ± 1	142 ± 3	173 ± 3
	4	116 ± 3	146 ± 2	171 ± 4
	40	113 ± 0	156 ± 2	165 ± 6
	400	116 ± 2	155 ± 3	159 ± 5
	4000	117 ± 2	163 ± 5	169 ± 2
Guanylurea	Control	134 ± 2	159 ± 2	169 ± 2
	0.4	141 ± 2	148 ± 2	168 ± 2
	4	143 ± 2	154 ± 2	167 ± 2
	40	141 ± 1	155 ± 2	162 ± 2
	400	144 ± 3	149 ± 5	166 ± 2
	4000	145 ± 1	154 ± 2	161 ± 2

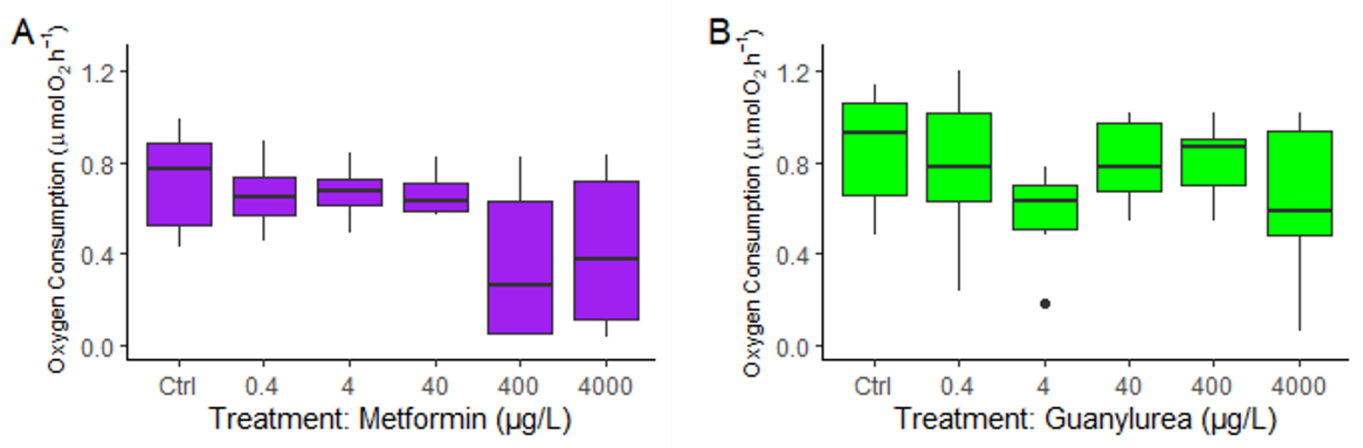

Figure 2. 8. Oxygen consumption rates in 120 hpf larval zebrafish exposed to metformin (A) and guanylurea (B). Two replicated experiments were conducted with duplicate pools of 3 larva per vial (n=6).

2.5. Discussion

The anti-diabetic drug metformin and its biotransformation product guanylurea are emerging contaminants of concern due to their near ubiquitous presence in aquatic ecosystems (Ambrosio-Albuquerque et al., 2021). As both compounds are polar and largely non-biodegradable, they are likely to move freely through aqueous environments and encounter a variety of aquatic organisms (Reemtsma et al. 2006; Tassoulas et al., 2021). In this experiment, zebrafish larvae were exposed to metformin and guanylurea for 5 d at environmentally relevant (0.4-40 $\mu g \cdot L^{-1}$) and supra-environmental concentrations (400 and 4000 $\mu g \cdot L^{-}$). Effects on mortality, hatching, abnormalities, morphology, behavior, and cardio-metabolic parameters were assessed under daily static renewal conditions. Exposure experiments were typically run-in triplicate, with replicated dishes in each exposure (3-18 replicate petri dishes containing 100 embryos/larvae), an experimental design with high biological replication for most endpoints (n=6 for oxygen consumption, n= 9 for hatching and mortality; n=45 for heart rate, n=48 for behavioral, n=60 for abnormalities and morphology.

2.5.1. Mortality and Morphology

Metformin exposure resulted in a concentration dependent elevation in mortality at environmentally relevant concentrations as low as 0.4 $\mu g \cdot L^{-1,}$ in agreement with prior studies in zebrafish which have shown that embryos exposed to metformin from 4-96 hpf showed decreased survival rates at concentrations under 100 $\mu g \cdot L^{-1}$ (Elizalde-Velazquez, et al 2021; Philips et al 2021). Interestingly, studies in brown trout (Jacob et al., 2018), Japanese medaka (Ussery et al., 2018) and fathead minnow (Parrott et al., 2021;2022), did not show increased mortality in any of the treatments tested ($\sim$100 $\mu g \cdot L^{-1}$). Guanylurea

exposure also elevated mortality at environmental concentrations; the lowest dose with elevated mortality was 40 μg · L^{-1}. This agrees with other studies in zebrafish where embryos exposed from 4-96 hpf showed decreased survival rates at concentrations under 25 and 50 μg · L^{-1} (Elizalde-Velazquez, et al 2021). Like metformin, studies in brown trout (Jacob et al., 2018) and Japanese medaka (Ussery et al., 2018) did not show increased mortality in any of the treatments tested (~100 μg·L^{-1}). While differences in species sensitivity are a probable consideration as to why zebrafish, but not other species, have increased mortality due to metformin and guanylurea, temperature may play a role for those species reared at lower temperatures. Metformin exposures carried out in brown trout were conducted at 7 ◦C and 11 ◦C and metformin tissue concentrations were higher at 11◦C compared to 7◦C at the highest concentration tested (Jacob et al. 2018), suggesting that uptake may be enhanced at elevated temperatures. Thus, we might expect the tropical species zebrafish to have higher uptake of at least metformin than most other species studied to date. Yet, medaka have similar rearing temperatures to zebrafish and thus experimental temperature alone does not fully explain why metformin and guanylurea induced mortality in zebrafish but not in any other species tested to date.

Metformin exposure increased spinal abnormalities in larval zebrafish at all concentrations. While the rate of spinal abnormalities was unexpectedly high in the control group; this did not match notes from direct observation of live embryos, and we believe this is caused by fixation and the use of fixed embryos for this endpoint. This will need to be explicitly tested. Edemas (yolk-sac and pericardial) were increased at the highest concentrations of metformin tested (4000 μg · L^{-1}). Metformin exposure in zebrafish embryos has previously caused the appearance of multiple abnormalities, including

scoliosis, pericardial edema, yolk deformation, hypopigmentation, eye absent, and craniofacial malformation (Philips et al., 2021; Velazquez et al., 2021). While we did not see indications of hypopigmentation, eye absent or craniofacial malformations in our study, spinal abnormalities were among the most documented impairment in both this study and Elizalde-Velazquez et al (2021). While the number of edemas (pericardial and yolk sac edema) found in larva increased slightly with exposure to the highest dose (Table 2.3), we found no change in the rate for edemas at environmentally relevant concentrations. Interestingly, metformin did not induce developmental abnormalities in Japanese medaka (Ussery et al, 2019), suggesting that zebrafish are perhaps more sensitive than medaka to metformin for both mortality and deformations.

2.5.2. Metformin and Guanylurea Did Not Impact Hatching, Cardio-metabolic Parameters, or Behavior

Metformin did not have a measurable impact on hatching zebrafish, as was reported for fathead minnow (Parrott et al. 2020), Japanese medaka (Ussery et al. 2018) and brown trout (Jacob et al. 2021), exposed to environmentally relevant (<40 μg · L^{-1}) and supra-environmental ($>$ 40 μg · L^{-1}) concentrations of metformin. Likewise, guanylurea had no measurable impact on hatching, like brown trout exposed to 1-1000 μg · L^{-} (Jacob et al. 2019) and medaka exposed to1-100 μg · L^{-1} (Ussery et al. 2019). Yet, a significant increase in the percent of larval hatched has been previously reported for in zebrafish exposed to 25-75,000 μg · L^{-1} guanylurea and metformin concentrations as low as 1 μg · L^{-1} (Elizalde-Valezquez et al., 2021), a concentration which overlaps with this study. The discrepancies between the zebrafish studies are difficult to reconcile considering that the exposure

concentrations, length, and developmental stages are similar. Yet, there may be differences in exposure apparatus and test medium replacement practices. The current study used petri dishes (100 ml of test medium) with 100 embryo-larva per dish and renewed 50% of the test medium daily. In Elizalde-Valezquez et al (2021), embryo-larvae were kept in 24 well-plates (no volume given) with 1 embryo larva per well, but the study did not state if the test medium was renewed during the exposure.

It has been reported that metformin can alter behavior in adult fish (MacLaren et al., 2018). Our study found no significant effects of metformin or guanylurea exposure on any behavioral parameter assessed in larval zebrafish at environmental or supra-environmental concentrations. To our knowledge, this is the first study to look at metformin and guanylurea exposure on thigmotaxis in any larval fish species. However, the effects of these compounds on visual motor and startle response have been documented in the literature (Godoy et al. 2018; Jacob et al. 2018). Metformin exposure at supra-environmental concentrations (1000 $\mu g \cdot L^{-1}$) did not show differences in total distance moved during either dark or light periods (Godoy et al. 2018) in 4 dpf zebrafish. Likewise, visual motor responses were not altered in juvenile brown trout (*Salmo trutta*) exposed to metformin (Jacob et al. 2018). Yet, larval zebrafish exposed to metformin (1 and 1000 $\mu g \cdot L^{-1}$) for 5 days increased total distance moved during dark and light periods (Phillips et al., 2021). These differences may be explained by different durations of the assay in each study. Light and dark periods lasted for 3.5 minutes in Phillips et al., (2021), while the present study used periods lasting 7.5 minutes per cycle.

Currently, most studies have found no treatment effects of metformin (this study, Godoy et al., 2018, Jacobs et al., 2018) or guanylurea (this study, Jacobs et al., 2019) on larval fish behavior, but some work is needed to resolve why the visual motor responses in zebrafish exposed to metformin are conflicting (Phillips et al., 2021 and this study). Neither metformin nor guanylurea had an impact on heart rate or oxygen consumption in this study. These results agree with the literature; heart rate was not impacted in larval and juvenile brown trout exposed to 1-1000 $\mu g \cdot L^{-1}$ metformin and guanylurea for 48d (Jacob et al., 2018;2019). However, it is important to note that the current study had a relatively small sample size (n=6) for oxygen consumption and higher replication may be needed to confirm that cardiometabolic parameters were not impacted by either compound. Cardiometabolic parameters were of interest because of the mode of action of metformin. After uptake, metformin accumulates in the hepatic inner mitochondrial membrane where it inhibits NADH-ubiquinone reductase (mitochondrial complex I), preventing the production of mitochondrial ATP, leading to increased ADP: ATP and AMP: ATP ratios, and enzymes involved with glucose metabolism (e.g., AMP-activated protein kinase; Sharma et al., 2009). Complex 1 inhibition decreases NADH oxidation, which inevitably results in a decreased proton gradient across the mitochondrial membrane and associated decrease in oxygen consumption in mammalian systems (El-Mir et al., 2000). Furthermore, metformin has been shown to elevate genes such as abraa, (100 nM), smdt1a, atp2a2a, tnnilc, and hsd11b2(10,000 $\mu g \cdot L^{-1}$) which have been implicated in cardiovascular development and function (Philips et al.2021). Guanylurea impairs GTP-related protein and, guanylate cyclase soluble subunit alpha in medaka, which is involved with heart dysfunction (Ussery et al., 2018). Given the conflicting results between molecular/cellular response which align

with the mechanism of action, and whole organism responses, future research may be needed to integrate metabolomics/ transcriptomics with whole organism responses to gain greater insight on the effects of metformin and guanylurea on fish.

2.5.3. Conclusion

This study documented the effects of metformin and guanylurea on early life stages of zebrafish, due to the high frequency of detection and concentrations found in surface waters (Ambrosio-Albuquerque et al., 2021). Metformin is a critical human pharmaceutical in wide use and an assessment of the environmental effects of this drug are needed. There were no measurable impacts on most endpoints with either compound. Larval zebrafish exposed to environmentally relevant (0.4-40 $\mu g \cdot L^{-1}$) and supra-environmental (400 and 4000 $\mu g \cdot L^{-1}$) concentrations of metformin and guanylurea showed no significant effect on hatching, behaviors (general swimming, thigmotaxis, visual motor responses), heart rate or oxygen consumption. However, early embryonic exposure to metformin increased mortality and spinal abnormalities at environmentally relevant concentrations (0.4-40 $\mu g \cdot L^{-1}$) and edema (pericardial and yolk sac) at supra-environmental concentrations (4000 $\mu g \cdot L^{-1}$). Early exposure to metformin also resulted in small reductions in morphology (snout-vent length, height at anterior of anal fin, eye diameter) at supra-environmental concentrations (4000 $\mu g \cdot L^{-1}$).

Overall, the present results show that exposures to metformin and guanylurea at environmentally relevant and supra-environmental concentrations cause modest impairment in larval zebrafish. The larval fish data concerning the impact of metformin on larval fish is mixed with studies in Japanese medaka (Ussery et al., 2018;2019) and other

zebrafish studies (Philips et al., 2021, Elizalde-Valezquez et al., 2021;2022) showing some effects while virtually no effects were in fathead minnow (Parrot et al., 2021; 2022) and brown trout (Jacobs et al., 2018). Clear influences of metformin on gene expression, steroid hormone synthesis and reproductive tissue have been seen in adult fishes (Niemuth et al., 2015, Niemuth and Klaper, 2015), suggesting that the influences of metformin are varied and may be more sensitive to adult life stages. A caveat here is that most of the research in adult fish have used $> 40\ \mu g \cdot L^{-1}$ meaning there is a lack of information on whether these impairments occur at lower concentrations. Future metformin exposures should focus on incorporating more adult research with metformin concentrations lower than $40\ \mu g \cdot L^{-1}$ to gain a full understanding of the full range of environmentally relevant concentrations. In the case of guanylurea, more research is required as there are very few studies for this compound.

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2.7. References

Ambrosio-Albuquerque, E. P., Cusioli, L. F., Bergamasco, R., Sinópolis Gigliolli, A. A., Lupepsa, L., Paupitz, B. R., Barbieri, P. A., Borin-Carvalho, L. A., & de Brito Portela-Castro, A. L. (2021). Metformin environmental exposure: A systematic review. *Environmental Toxicology and Pharmacology*, *83*, 103588. https://doi.org/10.1016/j.etap.2021.103588

Best C, Vijayan MM. Cortisol elevation post-hatch affects behavioural performance in zebrafish larvae. General and Comparative Endocrinology. (2018). 1; 257:220-226. doi: 10.1016/j.ygcen.2017.07.009.

Bronstein, P.M. Breeding, paternal behavior, and their interruption in *Betta splendens*. *Animal Learning & Behavior* 10, 145–151 (1982). https://doi.org/10.3758/BF03212262

Caldwell, D. J., Mastrocco, F., Margiotta-Casaluci, L., & Brooks, B. W. (2019). An integrated approach for prioritizing pharmaceuticals found in the environment for risk assessment, monitoring and advanced research. *Chemosphere*, *115*, 4–12. https://doi.org/10.1016/j.chemosphere.2014.01.021

Catalán, I., Reglero, P., & Álvarez, I. (2020). Research on early life stages of fish: a lively field. *Marine Ecology Progress Series*, *650*, 1–5. https://doi.org/10.3354/meps13491

Chen, E. C., Liang, X., Yee, S. W., Geier, E. G., Stocker, S. L., Chen, L., & Giacomini, K. M. (2015). Targeted Disruption of Organic Cation Transporter 3 Attenuates the Pharmacologic Response to Metformin. *Molecular Pharmacology*, *88*(1), 75–83. https://doi.org/10.1124/mol.114.096776

Choi, J. H., Yee, S. W., Ramirez, A. H., Morrissey, K. M., Jang, G. H., Joski, P. J., Mefford, J. A., Hesselson, S. E., Schlessinger, A., Jenkins, G., Castro, R. A., Johns, S. J., Stryke, D., Sali, A., Ferrin, T. E., Witte, J. S., Kwok, P.-Y., Roden, D. M., Wilke, R. A. Giacomini, K. M. (2011). A common 5′-UTR variant in MATE2-K is associated with poor response to metformin. *Clinical Pharmacology & Therapeutics*, *90*(5), 674–684. https://doi.org/10.1038/clpt.2011.165

Chen, Y., Li, S., Brown, C., Cheatham, S., Castro, R. A., Leabman, M. K., Urban, T. J., Chen, L., Yee, S. W., Choi, J. H., Huang, Y., Brett, C. M., Burchard, E. G., & Giacomini, K. M. (2009). Effect of genetic variation in the organic cation transporter 2 on the renal elimination of metformin. *Pharmacogenetics and Genomics*, *19*(7), 497–504. https://doi.org/10.1097/FPC.0b013e32832cc7e9

Corcoran, J., Winter, M. J., & Tyler, C. R. (2010). Pharmaceuticals in the aquatic environment: A critical review of the evidence for health effects in fish. *Critical Reviews in Toxicology*, *40*(4), 287–304. https://doi.org/10.3109/10408440903373590

Daughton, C. G. (2016). Pharmaceuticals and the Environment (PiE): Evolution and impact of the published literature revealed by bibliometric analysis. *Science of The Total Environment*, *562*, 391–426. https://doi.org/10.1016/j.scitotenv.2016.03.109

Dowling, R. J. O., Zakikhani, M., Fantus, I. G., Pollak, M., & Sonenberg, N. (2007). Metformin Inhibits Mammalian Target of Rapamycin–Dependent Translation Initiation in Breast Cancer Cells. *Cancer Research*, *67*(22), 10804–10812. https://doi.org/10.1158/0008-5472.CAN-07-2310

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. https://doi.org/10.1016/j.chemosphere.2021.131213

Elizalde-Velázquez, G. A., Gómez-Oliván, L. M., Islas-Flores, H., Hernández-Navarro, M. D., García-Medina, S., & Galar-Martínez, M. (2022). Oxidative stress as a potential mechanism by which guanylurea disrupts the embryogenesis of *Danio rerio*. *Science of The Total Environment*, *799*, 149432. https://doi.org/10.1016/j.scitotenv.2021.149432

Godoy, A. A., Domingues, I., Arsénia Nogueira, A. J., & Kummrow, F. (2018). Ecotoxicological effects, water quality standards and risk assessment for the anti-diabetic metformin. *Environmental Pollution*, *243*, 534–542. https://doi.org/10.1016/j.envpol.2018.09.031

Hundal, R. S., Krssak, M., Dufour, S., Laurent, D., Lebon, V., Chandramouli, V., Shulman, G. I. (2010). Mechanism by which metformin reduces glucose production in type 2 diabetes.*NIH Public Access*. *49*(12), 2063–2069.

Jacob, S., Dötsch, A., Knoll, S., Köhler, H. R., Rogall, E., Stoll, D., Triebskorn, R. (2018). Does the antidiabetic drug metformin affect embryo development and the health of brown trout (*Salmo trutta f. fario*)? *Environmental Sciences Europe*. https://doi.org/10.1186

Jacob, S., Knoll, S., Huhn, C., Köhler, H., Tisler, S., Zwiener, C., & Triebskorn, R. (2019). Effects of guanylurea , the transformation product of the antidiabetic drug metformin , on the health of brown trout (*Salmo trutta f . fario*). *PeerJ*. 1–21. https://doi.org/10.7717

Johnson, N.P. Metformin use in women with polycystic ovary syndrome. Annals of Translational Medicine. (2014) 2(6):56. doi: 10.3978/j.issn.2305-5839.2014.04.15.

Li, Gong, Goswami, Srijib, Giacomini, M., Kathleen, Altman, B., Russ, Klein, E., T. (2012).Metformin pathways: pharmacokinetics and pharmacodynamics. NIH Public Access, *22*(11), 820–827. https://doi.org/10.1097

Lee, J. W., Shin, Y.-J., Kim, H., Kim, H., Kim, J., Min, S.-A., Kim, P., Yu, S. Do, & Park, K. (2019). Metformin-induced endocrine disruption and oxidative stress of *Oryzias latipes* on

two-generational condition. *Journal of Hazardous Materials*, *367*, 171–181. https://doi.org/10.1016/j.jhazmat.2018.12.084

Maclaren, R. D., Wisniewski, K., & Maclaren, C. (2018). Environmental concentrations of metformin exposure affect aggressive behavior in the Siamese fighting fish, *Betta splendens*. *PLoS One*. 13-(5).

Niemuth, N. J., & Klaper, R. D. (2015). Chemosphere Emerging wastewater contaminant metformin causes intersex and reduced fecundity in fish. *Chemosphere*, *135*, 38–45. https://doi.org/10.1016

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. https://doi.org/10.1002/etc.2793

Oosterhuis, M., Sacher, F., & Thomas, L. (2013). Science of the Total Environment Prediction of concentration levels of metformin and other high consumption pharmaceuticals in wastewater and regional surface water based on sales data. *The Science of the Total Environment, 442*, 380–388. https://doi.org/10.1016

Parrott, J. L., Pacepavicius, G., Shires, K., Clarence, S., Khan, H., Sullivan, C., … Canada, C. C. (2021). Fathead minnow exposed to environmentally relevant concentrations of metformin for one life cycle show no adverse effects. *FACETS*. 998–1023. https://doi.org/10.1139.

Parrott, J. L., Restivo, V. E., Kidd, K. A., Zhu, J., Shires, K., Clarence, S., & Khan, H. (2022). Chronic Embryo - Larval Exposure of Fathead Minnows to the Pharmaceutical Drug Metformin : Survival , Growth , and Microbiome Responses. *Environmental Toxicology and Chemistry*.*41*(3), 635–647. https://doi.org/10.1002.

Pentik, P. J., Neuvonen, P. J., Penttilfi, A., & Hc, I. (1979). Pharmacokinetics of Mefformin After Intravenous and Oral Administration to Man. *European Journal of Clinical Pharmacology*. *202*, 195–202.

Phillips, J., Akemann, C., Shields, J. N., Wu, C., Meyer, N., Baker, B. B., … Baker, T. R. (2021). Developmental phenotypic and transcriptomic effects of exposure to nanomolar levels of metformin in zebrafish. *Environmental Toxicology and Pharmacology*, *87*: 103716. https://doi.org/10.1016

Rice C, Ghorai JK, Zalewski K, Weber DN. Developmental lead exposure causes startle response deficits in zebrafish. Aquat Toxicol. 2011 Oct;105(3-4):600-8. doi: 10.1016/j.aquatox.2011.08.014. Epub 2011 Aug 27. PMID: 21955963; PMCID: PMC3207002.

Rogall, E. T., Jacob, S., Triebskorn, R., & Schwartz, T. (2020). The impact of the anti - diabetic drug metformin on the intestinal microbiome of larval brown trout *(Salmo trutta f. fario*). *Environmental Sciences Europe*. https://doi.org/10.1186S

Rojas L.B., Gomes MB. Metformin: an old but still the best treatment for type 2 diabetes. Diabetolology Metabolic Syndrome. (2013)15;5(1):6. doi: 10.1186/1758-5996-5-6.

Scheurer, M., Michel, A., 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. *6*, 0–12. https://doi.org/10.1016

Scheurer, M., & Sacher, F. (2009). Occurrence of the antidiabetic drug metformin in sewage and surface waters in Germany. Water Research. 1608–1613. https://doi.org/10.1039

Trautwein, C., Berset, J., Wolschke, H., & Kümmerer, K. (2014). Occurrence of the antidiabetic drug Metformin and its ultimate transformation product Guanylurea in several compartments of the aquatic cycle. *Environment International*, *70*, 203–212. https://doi.org/10.1016

Ussery, E. J., Nielsen, K. M., Simmons, D., Pandelides, Z., Mansfield, C., & Holdway, D. (2021). An ' omics approach to investigate the growth effects of environmentally relevant concentrations of guanylurea exposure on Japanese medaka (*Oryzias latipes*). *Aquatic Toxicology*, *232*, 105761. https://doi.org/10.1016

Ussery, E., Bridges, K. N., Pandelides, Z., Kirkwood, A. E., Bonetta, D., Venables, B. J., … Holdway, D. (2018). Effects of environmentally relevant metformin exposure on Japanese medaka (*Oryzias latipes*). *Aquatic Toxicology*, *205*(July), 58–65. https://doi.org/10.1016

Chapter Three: General Discussion and Conclusions

3.1. General Discussion and Main Findings

The antidiabetic drug metformin and its transformation product, guanylurea, are compounds of emerging concern due to their near ubiquitous presence in aqueous environment. These levels of metformin and guanylurea (0-40 $\mu g \cdot L^{-1}$) in surface waters are projected to increase in tandem with the rise in diabetes diagnosis (Oosterhuis et al., 2013) and broadening scope of use for other ailments including cancer (Dowling et al., 2011; Mallik et al., 2018), obesity (Masarwa et al., 2022), polycystic ovarian syndrome (Oguz et al., 2022) and mood disorders (Jones et al., 2021). Currently, data concerning the impact of these compounds on aquatic organisms are limited in fish. Some of these studies have centered around metformin exposure in juvenile and adult life stage fish (Cargo et al., 2016; MacLaren et al., 2018) while other studies have used only one environmentally concentration (Nimeuth et al., 2015; Nimeuth and Klaper 2015; MacLaren et al., 2018). As such, the present thesis investigated the potential health effects of environmentally relevant (0.4-40 $\mu g \cdot L^{-1}$) and supra-environmental concentrations (400 -4000 $\mu g \cdot L^{-1}$) of metformin and guanylurea on larval zebrafish. I hypothesized that metformin and guanylurea would cause impairments on early-developmental parameters (survival, hatch, abnormalities, morphology, behavior heart rate, oxygen consumption). Chapter 2 reported elevated mortality (metformin and guanylurea) at environmental concentrations and elevated incidences of edema and some morphometrics (metformin) at the highest concentration, providing some support for hypothesis. However, most endpoints tested were null. This suggest that these compounds cause modest impairment, even at supra-environmental concentrations.

3.2. Research Contribution and Significance

This study was the first to investigate the potential impacts of metformin and guanylurea on thigmotaxis (anxiety-like behavior) and general swim parameters. The impact of metformin at high environmental concentrations (40 $\mu g \cdot L^{-1}$) on aggressive behavior in betta splendens prompted me to investigate whether metformin could illicit neuro-modulatory effects in larval fish at a range of environmentally relevant concentrations. Studying behaviors (thigmotaxis and startle response) and general swim parameters in larval fish are important because of its links to other crucial behaviors (e.g., predator avoidance) which are linked to survival. To date, no studies have looked at the potential behavioral impacts of guanylurea on fish at any life stage. This study was also the first to investigate the potential impacts of both metformin and guanylurea on oxygen consumption. Metformin has been shown to decrease oxygen consumption in mammalian cells (El-mir et al., 2000) and thus may have the same effect in fish. Decreased oxygen consumption may limit aerobic swimming ability and disrupt growth metrics and thus may decrease physiological fitness for developing fish. Lastly, in this chapter (3.4 Power Analyses), I have calculated the statistical power for several endpoints in this thesis and in the literature to better asses the quality of the available data. Considering that much of the available data show no or small effects, the power analysis can be used to determine if null results are due to insufficient power rather than a lack of effect.

3.3. Ecotoxicological Relevance

The choice of test compounds was three-fold: 1) their pervasive presence in freshwater aquatic environments (Trautwein et al., 2014Tao et al., 2018; Ambrosio-Albuquerque et al., 2021), 2) their relatively high concentrations in surface waters compared to other pharmaceuticals, and 3) the lack information regarding their potential impacts on developing larval fish. The concentrations used for both compounds, were grouped into environmentally relevant (0.4, 4, 40 $\mu g \cdot L^{-1}$) which mirror the concentrations found in treated wastewater effluent and surface waters and supra-environmental concentrations (400 and 4000 $\mu g \cdot L^{-1}$). This range of concentrations were used to determine the concentrations where detrimental health effects may occur and better examine any dose-response relationships.

The endpoints of interest were chosen because they provide relevant information at the individual level. This study used a combination of traditional (mortality, hatch, abnormalities, morphology) and non-traditional (behavior, heart rate, and oxygen consumption) endpoints to assess individual larval fish health. Mortality, hatch, and morphological assessment are commonly used individual endpoints in larval exposures that are highly relevant to estimating population- level effects. General locomotor, visual motor response, thigmotaxis, startle responses and heart rate and metabolic rate are individual measures that can influence physiological fitness (i.e., escape, prey-capture). Individual responses (i.e., locomotion) are integrated responses that can lend insight into how these compounds may cause impairment in lower (biochemical) and higher (population) levels of biological organization.

3.4. Power Analyses

The main purpose of a toxicity test is to provide a robust ecologically relevant measure of chemical-induced impairment on a given organism. To this end, studies should seek to minimize variation during the exposure, utilize a sensitive and relevant test species and have appropriate statistical power for meaningful inferences. Power refers to the probability of rejecting the null hypothesis when it is false (i.e., detecting a difference where there is a difference The main aim of a power analysis is to determine whether the null hypothesis can be accurately rejected when the alternative hypothesis is true. The research conducted in this thesis highlighted some potential impacts of metformin and guanylurea regarding mortality, abnormalities, and morphology in larval zebrafish. However, most of the results of the study were non-significant, as had been found in other studies. For example, studies in larval fathead minnow (Parrott et al., 2021:2022) and brown trout (Jacob et al., 2018) largely do not show impairment to metformin at environmental or supra-environmental concentrations. Similarly, guanylurea exposures at environmental concentrations do not cause impairment in brown trout (Jacob et al., 2019). The question remains, are these findings the result of a non-interaction or a lack of statistical power to detect a "meaningful" difference?

Failure to make an accurate statistical inference can result in at least one or two errors. Type 1 errors (α) are false positives and occur when the null hypothesis is true but is rejected. The α value is typically set at 0.05 and indicates the rate at which the type I error will be acceptable. Conversely, type 2 errors (β) are false negatives, and occur when the null hypothesis is false but is accepted. Power is a function of the type 2 error rate (1-β). The β value describes the rate at which a type II error will be accepted and can be

predefined as a range of values. If the type 2 error rate is too high, it becomes possible to assess whether a statistical test in fact had a fair chance of rejecting an incorrect H_0. A post-hoc power analysis is recommended when a result is not statistically significant because the non-significant association may be due to having low power (Thomas and James, 1996). Low power can result from either insufficient sample sizes or high variability (>30%). In the case where a determined power is high (>80%) then the level of confidence in the statistical result can be high. To determine if the exposure scenarios in Chapter 2 had enough power to detect an effect, a post hoc power analysis with the PS: Power and Sample Size Calculation version 3.1.6 software (Dupont et al., 1990) was conducted (Table 3.3). For each selected endpoint, the analysis was performed for independent groups *t*- test comparing control groups with the treatment group. The smallest significant differences were looked at for this analysis. A critical effect size of 25% (two standard deviations) was chosen for this analysis as it been determined to be a reasonable metric for a variety of ecological monitoring endpoints (Munkittrick et al., 2009). Power analyses were likewise completed for similar endpoints in published studies with metformin and guanylurea to better understand the strength of the cumulative data. These results help us understand the effects of metformin and guanylurea on early-life stages and inform experimental design in future studies.

In this thesis, replication was sufficient to detect a medium change (<25%) for cumulative mortality. We had a power of 80% and 87% to detect a 25% difference for mortality with metformin and guanylurea exposures respectively. This study had a lower power than studies using fathead minnow (99%; Parrot et al., 2021;2022) and a higher power (12%, 64%) to studies using brown trout (Jacob et al. 2018; 2019). All studies

included in this analysis reported no impacts to mortality when larvae were exposed to metformin and guanylurea. However, two additional studies documented increased mortality rates in larval zebrafish exposed to environmentally relevant concentrations of metformin (Elizalde-Velaquez et al., 2021) and guanylurea exposure (Elizalde-Velaquez et al., 2022) respectively. Determinations regarding the reliability of these findings cannot be ascertained at this moment as statistical power was not calculated. Since there were such a small number of experiments included for guanylurea (2) I would suggest that future experiments continue to measure mortality in the case of guanylurea.

Replication (n=9) for hatchability was insufficient to detect a medium change. Despite having a high replication (n=9), we had a power of 62% (metformin) and 52% (guanylurea) to detect a 25% change with a $p > 0.05$. Power for both compounds was less than studies using fathead minnow (80% Parrott et al., 2021, Parrot et al., 2022). Since data from only two studies had sufficient power, I cannot be confident that hatchability is not impacted by metformin or guanylurea. For sufficient power in my study, I would have required a sample size of 12(metformin) and 13(guanylurea), respectively. Additional replicates for hatching are needed. I would suggest that future experiments continue to measure hatchability and possibly include other hatch related endpoints such as time-to-hatch in the case of both compounds.

For metformin, the replication for standard length was sufficient to detect a medium sized difference between treatments. For my study, power was determined to be 100% (metformin) which was similar to a study using Japanese medaka (Ussery et al., 2018) or brown trout (100%; Jacob et al., 2019). Currently, only 1/3 studies included in this analysis

(Ussery et al., 2018) reported difference in standard lengths between treatments. The exposure duration for this study was 28d suggesting that impairment to growth may occur with longer duration studies. Investigations into the potential for growth impairments of metformin and guanylurea would to be a useful pursuit in future studies. Chronic exposure regimes would be useful here as growth impairments may be more pronounced with an increased exposure duration.

For both compounds, replication (n=48) was sufficient to detect a 25% difference with a power of 84% (metformin) and 90% (guanylurea)for visual motor response. Power was lower than a similar study using brown trout (Jacobs et al., 2018;2019), the brown trout study is not directly comparable in terms of endpoints because it used free swimming eleuthero embryos in aquaria rather than larval fish in 24-well plates. The results of the power analysis suggest that the findings of the available studies are reliable.

For heart rate, we had sufficient power of 100% to detect a 25% difference. The calculated power was higher than that of the studies using brown trout 31% (metformin) and 50% (guanylurea) respectively (Jacob et al., 2019; 2020). For heart rate, only 1/3 studies were sufficiently powered however and as such, increasing the number of well-powered studies is advised. For the oxygen consumption, neither metformin (15) or guanylurea (64) had sufficient power to detect a 25% difference. As oxygen consumption has yet to be examined in larval fish (excluding this study) exposed to metformin or guanylurea, no comparisons to the literature could be made. For my oxygen consumption experiment to be sufficiently powered, I would have needed an N of 12 for metformin and

10 for guanylurea. As such, future studies should seek to ensure that their experiments are sufficiently powered for meaningful interpretations to be drawn

Table 3. 1.Analyses of the variability, size of experimental difference, and power for select endpoints. Analyses are for this thesis (data in Chapter 2) and published literature (Power calculated to detect a 25 percent difference given the standard deviation and sample size of the study and an α of 0.05; a 25% difference was based on Munkittrick et. 2019) *if no differences were significant then the largest non-significant difference was used.

Endpoint	Unit	Test organism	Life stage	Compound	Control (mean) (N)	SD of control	CV (%)	Smallest significant difference (%) *	Power (25 %)	Reference
Mortality	%	Zebrafish (*Danio rerio*)	Larval (5dpf)	Metformin	5.75 (9)	5.39	93.7	57	80	This study
				Guanylurea	8(9)	6	75	40	87	
	%	Brown trout (*Salmo trutta*)	Larval (46dpf)	Metformin	2.0(15)	1.6	80	42*	12’	Jacob et al. 2018
				Guanylurea	5.6(15)	1.6	28.6	2.6*	64’	Jacob et al. 2019

	%	Fathead Minnow (Pimephales promelas)	Larval (9dph)	Metformin	5.3°*	4	75.4	30*	99’	Parrott et al. 2021
Hatchability	%	Zebrafish (*Danio rerio*)	(3dpf)	Metformin	9	0.3	3.6	5.6*	62	This study
				Guanylurea	9	0.7	7.7	11	32	
	%	Fathead Minnow (Pimephales promelas)		Metformin	18	1.9	10.5	2	99’	Parrott et al. 2021
Standard length	mm	Zebrafish (*Danio rerio*)	Larval (3dpf)	Metformin	3.4*(35)	0.016	0.47	3*	100	This study
	mm	Japanese medaka (*Oryzias latipes*)	Larval (7dpf)	Metformin	1.1° (8)	0.1°	8.3	59*	100’	Ussery et al. 2018
				Guanylurea	4°(8)	0.2°	5	2.5*	100	Ussery et al.2019

	cm	Brown trout (*Salmo trutta*)	Larval (46 dpf)	Metformin	3.5 (15)	0.2	5.7	20*	98°	Jacob et al. 2018
				Guanylurea	3.8 (15)	0.3	8	2.7*	99	Jacob et al. 2019
Total Distance Moved	mm	Zebrafish (*Danio rerio*)	Larval (5dpf)	Metformin	3341 (48)	2050	65.1	37*	19	This study
				Guanylurea				36*	22	
	cm	Brown trout (*Salmo trutta*)	Larval	Metformin	2119.5	664.2	31.2	13*	55°	Jacob et al. 2018
Heart rate	bpm	Zebrafish (*Danio rerio*)	Larva (1,2,3 dpf)	Metformin	119.2	14	15.1	1.7*	100	This study
					157.6	17	11.0	9.4*	100	
					160	21	13.1	7.8*	100	
				Guanylurea	134	2	1.5	7.9*	100	
					159	3	1.2	6.5*	100	
					169	2	1.1	4.8	100	

	bpm	Brown trout (*Salmo trutta*)	Larval (46 dpg)	Metformin	77.4	7.0	9.0	4*	31’	Jacob et al. 2018
				Guanylurea	59.2	5.1	15	0.2*	50’	Jacob et al. 2019
Oxygen Consumption	O_2/µmol/h^{-1}	Zebrafish (*Danio rerio*)	Larval (5dpf)	Metformin	0.07	0.03	53.7	15*	13	This study
				Guanylurea	0.14	0.04	31.4	64.5*	25	

‘Data estimated from figure in publication

CV=coefficient of variation

SD=standard deviation

3.5 Knowledge Gaps and Future Directions

3.5.1. Is Guanylurea or Metformin a Higher Environmental Risk?

Investigations into the ecological risks of several pharmaceutical contaminants (Boxall et al., 2004; Kuster and Adler, 2014) have largely focused on assessing the ecotoxicity of parent compounds. This culminates in a scarcity of risk assessment data on transformation product which ignores their potential toxicity. This trend has also been seen in the metformin and guanylurea data. Currently, there are approximately 15 studies looking at the effects of metformin on fish species and only 5 for guanylurea. Given the limited number of studies, both compounds need to be studied further with respect to ecological risks to aquatic biota. However, I argue that guanylurea should be the main chemical of concern. Firstly, at concentrations >0.5 $\mu g \cdot L^{-1}$ guanylurea is present at five times higher concentrations than its parent compound (Scheuer et al., 2012; Trautwein et al., 2014). The higher environmental concentrations of guanylurea due to its transformation rates which are up to 98% in wastewater treatment plants (Cadwell et al., 2019). Secondly, in juveniles' life stages, guanylurea elicits toxic effects at lower concentrations than metformin in the studies available to date. For example, juvenile Japanese medaka (*Oryzias latipes*) exposed to guanylurea showed significantly impaired growth metrics at concentrations that were an order of magnitude lower than metformin (0.25 $\mu g \cdot L^{-1}$ versus 3.2 $\mu g \cdot L^{-1,}$ respectively, Ussery et al., 2018;2019).

It should be noted that adult fish (*Danio rerio*) exposed to guanylurea have shown impaired behavioral responses (decreased swimming distance and increased time spent frozen) at 25 $\mu g \cdot L^{-1}$ (Elizalde-Valequez et al., 2022). For metformin, impaired behavioral

responses (decreased aggression) in betta splendens were documented at a concentration of 40 $\mu g \cdot L^{-1}$ (MacLaren et al. 2018). Due to the differences in endpoints measured and species tested, comparisons between the two compounds with respect to toxicity in adults is difficult. Future studies should seek to test the same endpoints in the same species for both compounds to ensure that meaningful comparisons can be drawn.

3.5.2 Are Their Differences in Species Sensitivities to Metformin or Guanylurea?

The basis of species sensitivity is that exposure to chemicals at sufficient concentrations can result in adverse impairment to physiology and behavior. The resilience of a species to a given contaminant is contingent on life history traits (e.g., age-at maturity), behavior (e.g., feeding behavior), metabolic capacities (e.g., CYP expression patterns), and stress-response capabilities (e.g., heat-shock protein response; Barton, 2002). Determining species sensitivities to certain compounds is paramount to 1) prioritizing which compounds should be evaluated with respect to impacts on biota and 2) determining at what concentration these compounds become a concern. In the case of metformin and guanylurea, only, six species of fish (6 for metformin and 3 for guanylurea) have been used for ecotoxicity studies. From the included studies, it was apparent that some species exhibited greater resilience to both compounds.

During larval stages, fathead minnow (*Pimephales promelas*) was among the least sensitive species tested for metformin at environmental relevant concentrations. In both studies, conventional endpoints such as growth, survival and hatch were not significantly impacted by metformin exposure (Parrott et al., 2021:2022). Minor alterations to gastrointestinal bacterial community were found (Parrot et al. 2022), however it was

unclear if these changes were solely due to metformin exposure or were the result of the euthanization method (MS222). Brown trout (*Salmo trutta*) also appear to be tolerant to metformin and guanylurea. In the case of metformin, all endpoints, other than gut microbiome composition and muscle glycogen content did not show alterations with exposure to at environmentally relevant concentrations (1, 10 $\mu g \cdot L^{-1}$; Jacob et al. 2018). It should be noted however that, the larval brown trout were exposed to metformin at 48 days post fertilization so it remains unclear whether exposure at an earlier time point would lead to severe health effects. Guanylurea exposures in brown trout likewise did not result in significant alterations in the tested health parameters (Jacob et al., 2019).

Larval zebrafish appear to be more sensitive to metformin and guanylurea relative to other species. For metformin, increased mortality rates (Elizalde-Velaquez et al., 2021; this study), increased incidence of abnormalities (Elizalde-Velaquez et al., 2021; this study), and increased markers of oxidative stress (Elizalde-Velaquez et al., 2021, Jacobs et al., 2021) have been shown at environmentally relevant concentrations. Other impairments have been shown including impaired visual motor response (Philips et al., 2021). The lack of tested behavioral endpoints in larval fish in other species makes direct comparisons difficult. Future studies should aim to address these gaps in knowledge by incorporating more behavioral endpoints (e.g., visual motor response, startle response) to allow for more direct comparisons. For guanylurea, only two studies in larval zebrafish are available (Elizalde-Velaquez etal., 2022; this study) and showed increased mortality rates mortality rates at environmentally relevant concentrations. Like metformin, accelerated hatch rate, increased incidence of abnormalities, and increased oxidative stress have been documented in with exposure to environmentally relevant concentrations of guanylurea

(Elizalde-Velaquez et al., 2021). This study and Elizalde-Velaquez et al. (2021) are the only two studies documenting the impacts of guanylurea in larval zebrafish. As such, future studies should seek to replicate these studies in zebrafish to corroborate these findings.

3.6. Conclusion and Limitations

This thesis aimed to investigate the effects of metformin and guanylurea on larval zebrafish. The results of this study address some of the knowledge gaps associated with the literature however additional questions remain. Metformin has been shown to be a neuroactive compound in mammals, with studies suggesting influences on molecular targets involved with mood disorders (Ying et al., 2014;), anxiety (Zemedegs et al., 2019), and memory (Piasecka-Markowicz et al., 2017). It is currently unknown if guanylurea has these same effects in humans. However, there was one study in adult zebrafish that showed increased anxiety-like behavior to environmentally relevant concentrations of guanylurea (25 $\mu g \cdot L^{-1}$) for 120 days (Elizalde-Velazquez, 2022). Beyond the study in adult Japanese fighting fish (Maclaren et al., 2018) there exists limited data on the potential impairment of these compounds on fish behavior and this may be a fruitful area of research. Behavioral impairment in fish may cause detrimental effects (e.g., predator avoidance, prey capture) and may ultimately result in population-level consequences. As these behavioral impairments are likely to be present at later life stages, the use of juvenile and adults under a chronic exposure regime would be appropriate.

My study documented some modest decreases in morphology (snout-vent length, height at anterior anal fin and eye diameter) associated with exposure to metformin at supra -environmentally relevant concentrations. However, the data concerning the impact

metformin on fish size is mixed. Some studies in Japanese medaka show decreased lengths when fish are exposed to environmentally relevant concentrations of metformin (Ussery et al., 2018) while other studies in fathead minnow do not (Parrot et al., 2021; 2022). Conversely, there is a scant amount of data addressing the potential of guanylurea to cause growth impairment. Thus, future studies may want to explore how these compound impacts fish energetics and subsequently growth. In addition to inhibiting glycogenolysis and gluconeogenesis in the liver (Pernicova et al., 2014), metformin has been shown to inhibit transepithelial transport of glucose transport in the intestine of rodents (Horakova, et al., 2019). It is unknown if this response occurs in fish as well. However, decreased hepatic and intestinal glucose availability for prolonged periods may impair growth through an elevation of cortisol through increased levels of glucocorticoids. Determining plasma cortisol levels in fish and tissue and plasma glucose levels would be beneficial in understanding if metformin or guanylurea induces the stress response and subsequently reduced growth in fish.

For my study, there are limitations that should be addressed. 1) Some of the endpoints analyzed in this study (hatchability and oxygen consumption) did not have sufficient replication. To resolve this, additional experiments will need to be performed to increase the sample size for these endpoints. 2) While 20 ml samples of the test medium were collected for chemical analysis and confirmation of nominal dose, they have not yet been analyzed. As such, I do not have confirmation that the concentrations in the well are close to nominal. The collected samples will need to be analyzed in the future. 3) Morphometrics and abnormalities were measured after metformin exposure but not yet for guanylurea. The samples have been collected and imaged, but the data are still being

analyzed and were not included in the thesis. 4) Spinal abnormalities were unusually high in the control group, and I have proposed that this is because abnormalities were determined in fixed samples. The effects of fixatives on spinal bends needs to be directly assessed. It will be important to address these limitations in the future.

3.7.References

Ambrosio-Albuquerque, E. P., Cusioli, L. F., Bergamasco, R., Sinópolis Gigliolli, A. A., Lupepsa, L., Paupitz, B. R., Barbieri, P. A., Borin-Carvalho, L. A., & de Brito Portela-Castro, A. L. (2021). Metformin environmental exposure: A systematic review. *Environmental Toxicology and Pharmacology*, *83*, 103588. https://doi.org/10.1016/j.etap.2021.103588

Boxall, A. B. A., Rudd, M. A., Brooks, B. W., Caldwell, D. J., Choi, K., Hickmann, S., Innes, E., Ostapyk, K., Staveley, J. P., Verslycke, T., Ankley, G. T., Beazley, K. F., Belanger, S. E., Berninger, J. P., Carriquiriborde, P., Coors, A., DeLeo, P. C., Dyer, S. D., Ericson, J. F., … Van Der Kraak, G. (2012). Pharmaceuticals and Personal Care Products in the Environment: What Are the Big Questions? *Environmental Health Perspectives*, *120*(9), 1221–1229. https://doi.org/10.1289/ehp.1104477

Caldwell, D. J., Mastrocco, F., Margiotta-Casaluci, L., & Brooks, B. W. (2019). An integrated approach for prioritizing pharmaceuticals found in the environment for risk assessment, monitoring and advanced research. *Chemosphere*, *115*, 4–12. https://doi.org/10.1016/j.chemosphere.2014.01.021

Crago, J., Bui, C., Grewal, S., & Schlenk, D. (2016). Age-dependent effects in fathead minnows from the anti-diabetic drug metformin. *General and Comparative Endocrinology*, *232*, 185–190. https://doi.org/10.1016/j.ygcen.2015.12.030

Dowling, R. J., Goodwin, P. J., & Stambolic, V. (2011). Understanding the benefit of metformin use in cancer treatment. *BMC Medicine*, *9*(1), 33. https://doi.org/10.1186/1741-7015-9-33

Dupont, W. D. (2017). *Power and Sample Size Calculations .* A Review and Computer Program. American Psychological association. 11, 116128.https://doi.org/10.1016/0197-2456(90)90005.

El-Mir, M.-Y., Nogueira, V., Fontaine, E., Avéret, N., Rigoulet, M., & Leverve, X. (2000). Dimethylbiguanide Inhibits Cell Respiration via an Indirect Effect Targeted on the Respiratory Chain Complex I. *Journal of Biological Chemistry*, *275*(1), 223–228. https://doi.org/10.1074/jbc.275.1.223

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. https://doi.org/10.1016/j.chemosphere.2021.131213

Elizalde-Velázquez, G. A., Gómez-Oliván, L. M., Islas-Flores, H., Hernández-Navarro, M. D., García-Medina, S., & Galar-Martínez, M. (2021). Oxidative stress as a potential mechanism by which guanylurea disrupts the embryogenesis of *Danio rerio*. *Science of The Total Environment*, *799*, 149432. https://doi.org/10.1016/j.scitotenv.2021.149432

Ford, J. L., Gerhart, J. G., Edginton, A. N., Yanovski, J. A., Hon, Y. Y., & Gonzalez, D. (2022). Physiologically Based Pharmacokinetic Modeling of Metformin in Children and Adolescents With Obesity. *The Journal of Clinical Pharmacology*. https://doi.org/10.1002/jcph.2034

Horakova, O., Kroupova, P., Bardova, K. *et al.* Metformin acutely lowers blood glucose levels by inhibition of intestinal glucose transport. *Scientific Report* 9, 6156 (2019). https://doi.org/10.1038/s41598-019-42531-0

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), 48. https://doi.org/10.1186/s12302-018-0179-4

Jacob, S., Knoll, S., Huhn, C., Köhler, H.-R., Tisler, S., Zwiener, C., & Triebskorn, R. (2019). Effects of guanylurea, the transformation product of the antidiabetic drug metformin, on the health of brown trout (Salmo trutta f. fario). *PeerJ*, *7*, e7289. https://doi.org/10.7717/peerj.7289

Jones, B. D. M., Farooqui, S., Kloiber, S., Husain, M. O., Mulsant, B. H., & Husain, M. I. (2021). Targeting Metabolic Dysfunction for the Treatment of Mood Disorders: Review of the Evidence. *Life*, *11*(8), 819. https://doi.org/10.3390/life11080819

Ku¨ster A, Adler N. 2014 Pharmaceuticals in the environment: scientific evidence of risks and its regulation. Philosophical Transactions of the Royal Society B 369: 20130587.ttp://dx.doi.org/10.1098/rstb.2013.0587

Klimisch, H.-J., Andreae, M., & Tillmann, U. (1997). A Systematic Approach for Evaluating the Quality of Experimental Toxicological and Ecotoxicological Data. *Regulatory Toxicology and Pharmacology*, *25*(1), 1–5. https://doi.org/10.1006/rtph.1996.1076

MacLaren, R. D., Wisniewski, K., & MacLaren, C. (2018). Environmental concentrations of metformin exposure affect aggressive behavior in the Siamese fighting fish, *Betta splendens*. *PLOS ONE*, *13*(5), e0197259. https://doi.org/10.1371/journal.pone.0197259

Malfatti, A. de L. R., Mallmann, G. C., Oliveira Filho, L. C. I., Carniel, L. S. C., Cruz, S. P., & Klauberg-Filho, O. (2021). Ecotoxicological test to assess effects of herbicides on spore germination of *Rhizophagus clarus* and *Gigaspora albida*. *Ecotoxicology and Environmental Safety*, *207*, 111599. https://doi.org/10.1016/j.ecoenv.2020.111599

Mallik, R., & Chowdhury, T. A. (2018). Metformin in cancer. *Diabetes Research and Clinical Practice*, *143*, 409–419. https://doi.org/10.1016/j.diabres.2018.05.023

Masarwa, R., Brunetti, V. C., Aloe, S., Henderson, M., Platt, R. W., & Filion, K. B. (2021). Efficacy and Safety of Metformin for Obesity: A Systematic Review. *Pediatrics*, *147*(3). https://doi.org/10.1542/peds.2020-1610

Moermond, C. T. A., Kase, R., Korkaric, M., & Ågerstrand, M. (2016). CRED: Criteria for reporting and evaluating ecotoxicity data. *Environmental Toxicology and Chemistry*, *35*(5), 1297–1309. https://doi.org/10.1002/etc.3259

Munkittrick, K. R. (2009). A review of potential methods of determining critical effect size for designing environmental monitoring programs. 28: 1361-1371.https://doi.org/10.1897/08-376.1.

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. https://doi.org/10.1002/etc.2793

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. https://doi.org/10.1016/j.aquatox.2017.12.003

Niemuth, N. J., & Klaper, R. D. (2015). Emerging wastewater contaminant metformin causes intersex and reduced fecundity in fish. *Chemosphere*, *135*, 38–45. https://doi.org/10.1016/j.chemosphere.2015.03.060

Oguz, S. H., Sendur, S. N., Unluturk, U., & Yildiz, B. O. (2022). Targeting metabolism in the management of PCOS: Metformin and beyond. In *Polycystic Ovary Syndrome* (pp. 117–133). Elsevier. https://doi.org/10.1016/B978-0-12-823045-9.00006-7

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. https://doi.org/10.1016/j.scitotenv.2012.10.046

Parrott, J. L., Pacepavicius, G., Shires, K., Clarence, S., Khan, H., Sullivan, C., Alaee, M., Science, W., Directorate, T., & Canada, C. C. (2021). Fathead minnow exposed to environmentally relevant concentrations of metformin for one life cycle show no adverse effects. 998–1023. https://doi.org/10.1139/facets-2020-0106

Parrott, J. L., Restivo, V. E., Kidd, K. A., Zhu, J., Shires, K., Clarence, S., & Khan, H. (2022). Chronic Embryo - Larval Exposure of Fathead Minnows to the Pharmaceutical Drug Metformin : Survival , Growth , and Microbiome Responses. *41*(3), 635–647. https://doi.org/10.1002/etc.5054

Pernicova, I., Korbonits, M. Metformin—mode of action and clinical implications for diabetes and cancer. *Nature Review Endocrinology.* 10, 143–156 (2014). https://doi.org/10.1038/nrendo.2013.256

Phillips, J., Akemann, C., Shields, J. N., Wu, C., Meyer, N., Baker, B. B., … Baker, T. R. (2021). Developmental phenotypic and transcriptomic effects of exposure to nanomolar levels of metformin in zebrafish. *Environmental Toxicology and Pharmacology*, *87*: 103716. https://doi.org/10.1016

Markowicz-Piasecka M, Huttunen KM, Mateusiak L, Mikiciuk-Olasik E, Sikora J. Is Metformin a Perfect Drug? Updates in Pharmacokinetics and Pharmacodynamics. Current Pharmaceutical Design. 2017;23(17):2532-2550. doi:10.2174/1381612822666161201152941.

Rudén, C., Adams, J., Ågerstrand, M., Brock, T. C., Poulsen, V., Schlekat, C. E., Wheeler, J. R., & Henry, T. R. (2017). Assessing the relevance of ecotoxicological studies for regulatory decision making. *Integrated Environmental Assessment and Management*, *13*(4), 652–663. https://doi.org/10.1002/ieam.1846

Schneider, D., Marquardt, P., Zwahlen, M., & Jung, R. E. (2009). A systematic review on the accuracy and the clinical outcome of computer-guided template-based implant dentistry. *Clinical Oral Implants Research*, *20*, 73–86. https://doi.org/10.1111/j.1600-0501.2009.01788

Tao, Y., Chen, B., Zhang, B. (Helen), Zhu, Z. (Joy), & Cai, Q. (2018). *Occurrence, Impact, Analysis and Treatment of Metformin and Guanylurea in Coastal Aquatic Environments of Canada, USA and Europe* (pp. 23–58). https://doi.org/10.1016/bs.amb.2018.09.005

Trautwein, C., Berset, J.-D., Wolschke, H., & Kümmerer, K. (2014). Occurrence of the antidiabetic drug Metformin and its ultimate transformation product Guanylurea in several compartments of the aquatic cycle. *Environment International*, *70*, 203–212. https://doi.org/10.1016/j.envint.2014.05.008

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. https://doi.org/10.1016/j.aquatox.2018.10.003

Ussery, E., Bridges, K. N., Pandelides, Z., Kirkwood, A. E., Guchardi, J., & Holdway, D. (2019). Developmental and Full-Life Cycle Exposures to Guanylurea and Guanylurea–Metformin Mixtures Results in Adverse Effects on Japanese Medaka (Oryzias latipes). *Environmental Toxicology and Chemistry*, *38*(5), 1023–1028. https://doi.org/10.1002/etc.4403

Ying M. A., Maruschak N., Mansur R., Carvalho A. F., Cha D. S., McIntyre R. S. (2014). Metformin: repurposing opportunities for cognitive and mood dysfunction. *Frontiers in Aging Neuroscience.* 13 1836–1845. 10.2174/1871527313666141130205514

Zemdegs J, Martin H, Pintana H, Bullich S, Manta S, Marqués MA, Moro C, Layé S, Ducrocq F, Chattipakorn N, Chattipakorn SC, Rampon C, Pénicaud L, Fioramonti X, Guiard BP. Metformin Promotes Anxiolytic and Antidepressant-Like Responses in Insulin-Resistant Mice by Decreasing Circulating Branched-Chain Amino Acids. *Journal of Neuroscience*.39(30):5935-5948. doi: 10.1523/J.2904-18.2019.

Appendix

The following section contains a set of additional and figures used to append the behavioral data discussed in Chapter 2.

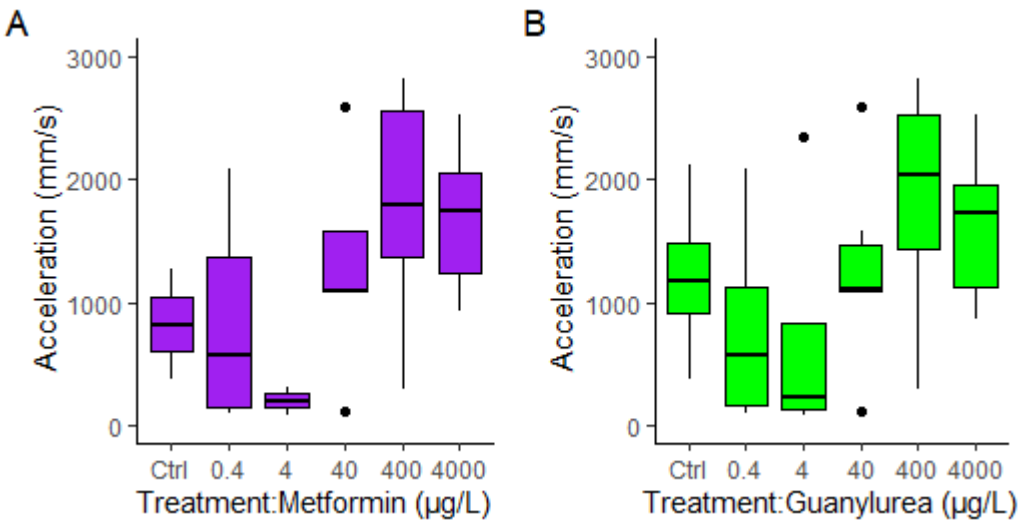

Figure A1: Total acceleration (mm/s) of 5-day old larval zebrafish exposed to various concentrations of metformin (A) and guanylurea (B). Each box shows the upper, median (indicated via the central horizontal black line), and lower quartile and outliers are indicated as black dots. Three replicate experiments were conducted with 16 larva per control and treatment group: N=48.

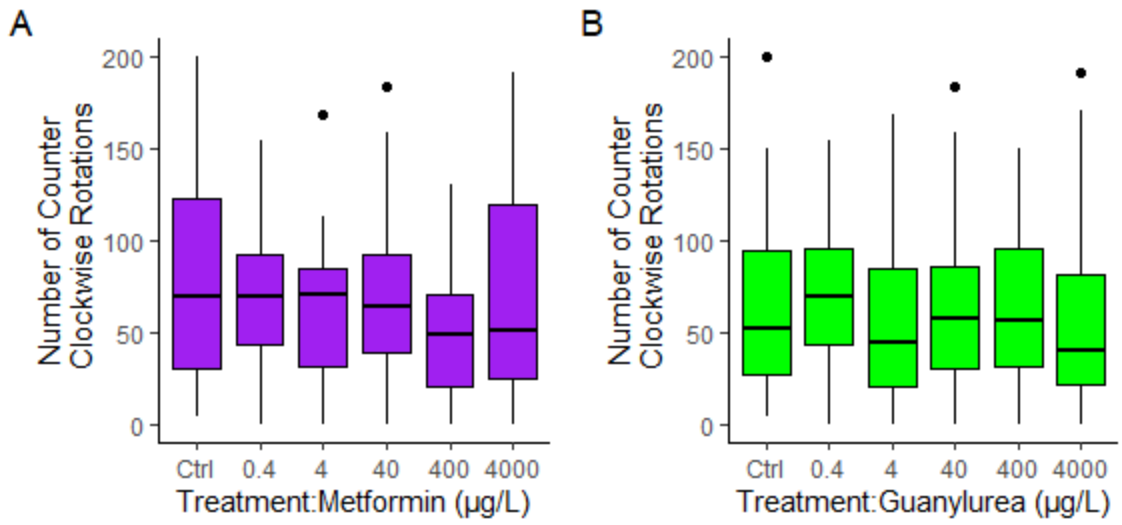

Figure A3: Number of counter clock wise rotations in 5-day old larval zebrafish exposed to various concentrations of metformin (A) and guanylurea (B). Each box shows the upper, median (indicated via the central horizontal black line), and lower quartile and outliers are indicated as black dots. Three replicate experiments were conducted with 16 larva per control and treatment group: N=48.

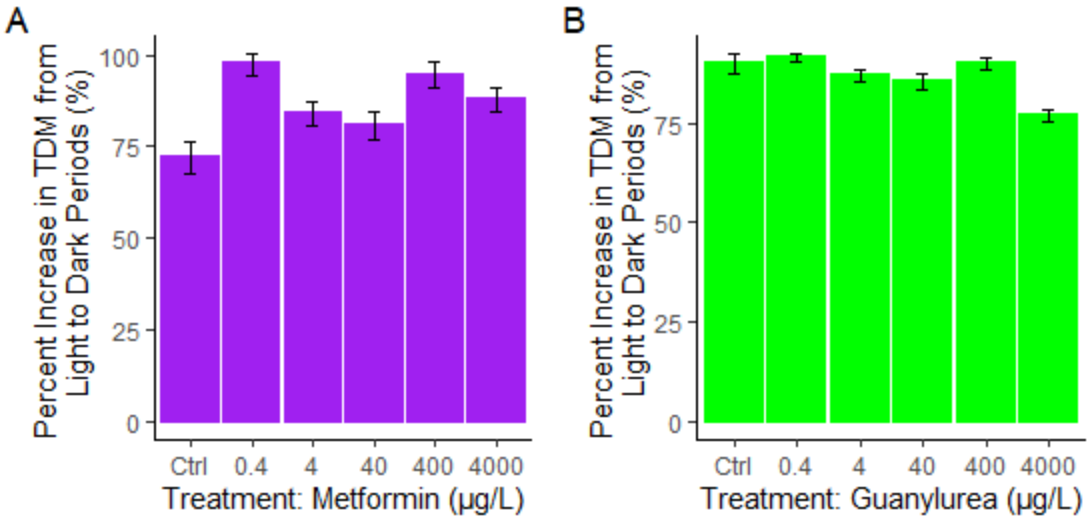

Figure A5: Latency to first zone transition (s) in 5-day old larval zebrafish exposed to various concentrations of metformin (A) and guanylurea (B). Each bar shows the upper, median (indicated via the central horizontal black line), and lower quartile and outliers are indicated as black dots.

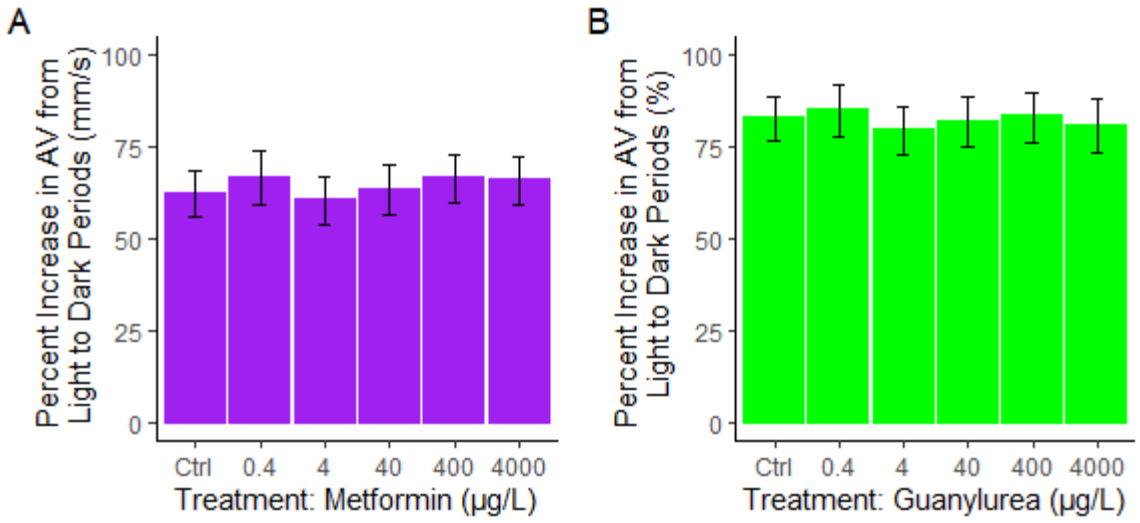

Figure A6: Latency to first zone transition (s)??? in 5-day old larval zebrafish exposed to various concentrations of metformin (A) and guanylurea (B). Each bar shows the upper, median (indicated via the central horizontal black line), and lower quartile and outliers are indicated as black dots.

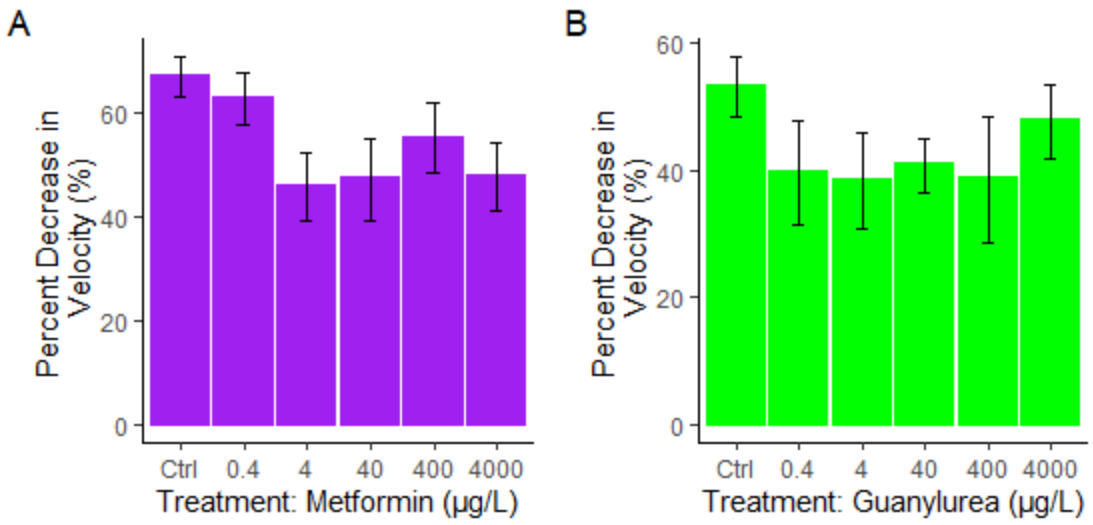

Figure A7: Latency to first zone transition (s) in 5-day old larval zebrafish exposed to various concentrations of metformin (A) and guanylurea (B). Each bar shows the upper, median (indicated via the central horizontal black line), and lower quartile and outliers are indicated as black dots.