The ecotoxicological effects of metformin and its metabolite, guanylurea, on Japanese medaka (*Oryzias latipes*)

By

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Abstract

Human pharmaceutical waste threatens many natural processes of non-target aquatic organisms, through the introduction of such contaminants into the environment. Pharmaceuticals are a large and diverse groups of medicinal compounds that are used for the diagnosis, cure, mitigation, treatment, and/or prevention of diseases in humans and animals. In recent years, the occurrence and fate of pharmaceuticals in the aquatic environment have become an emerging issue in aquatic toxicology, with one of the most prevalent contaminants being the type-2 diabetic drug, metformin, and its metabolite guanylurea. Metformin and guanylurea have been measured in surface waters in the ng-$\mu g \cdot L^{-1}$ range. This study aimed to investigate the toxicological effects of metformin and guanylurea on Japanese medaka (*Oryzias medaka*).

A first experimental step was to determine the potential for uptake, bioaccumulation, and depuration of metformin in early-life stage (ELS) medaka using ^{14}C-metformin. Results from this study showed that both embryo and larval medaka can take up, bioaccumulate, and successfully depurate metformin from the aquatic environment. Furthermore, egg chorion hardening (~6 hpf) prevented the uptake of metformin from by developing embryos.

Additionally, we show that waterborne exposure to metformin and guanylurea significantly impacted the growth metrics in ELS fish, showing that guanylurea caused growth retardation at exposure concentrations an order of magnitude lower that those required by the parent compound to exert similar effects. We sought to explain these growth effects by investigating potential alterations in the metabolome and gene expression of these exposed fish. Several metabolite abundances were altered by the exposure of

medaka to 3.2 $\mu g \cdot L^{-1}$ metformin and 1.0 $ng \cdot L^{-1}$ guanylurea, which included metabolites associated with cellular energetics, fatty acid synthesis and metabolism, and polyamine synthesis. Metabolomic results were applied to select genes of interest for RT-qPCR analyses, and the expressions of critical genes involved in lipid metabolism were shown to be significantly affected.

Furthermore, a full 165 day life-cycle study assessing the effects of metformin and guanylurea alone, and in combination, was performed to determine potential population-level impacts of the compounds on Japanese medaka. While exposure to metformin and guanylurea alone, and in combination, did not significantly stunt the growth of medaka, exposure to the compounds did alter the production of important sex steroid hormones in both male and female medaka. However experimental treatments did not affect the production of the egg yolk precursor protein, vitellogenin. Interestingly, no significant effect on reproductive output was seen. Collectively, these results underscore the need for additional studies examining the effects of metformin and guanylurea on aquatic ecosystems. To our knowledge, this research is the first to investigate the effects of environmentally relevant concentrations of these compounds in this manner.

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List of Abbreviations

11KT	11-keto testosterone
ABC	ATP-binding cassette transporter
ACC	Acetyl-CoA carboxylase
AMDIS	Automated Mass Spectral Deconvolution & Identification System
ANOVA	Analysis of variance
AMP	Adenosin-5-monophosphate
AMPK	5' AMP-activated protein kinase
ATP	Adenosine triphosphate
BAIBA	β-aminoisobutyric acid
bp	Base pairs
˚C	Celsius
Ci	Curies
CNS	Central nervous system
cpm	Counts per minute
CoA	Coenzyme-A
COX	Cyclooxygenase
CYP2C8	Cytochrome P4502C8
d	Day

DEPC	Diethyl pyrocarbate
DF	Degrees of freedom
DI	Deionized
DNA	Deoxyribonucleic acid
dpf	Days post fertilization
dph	Days post hatch
dpm	Disintegrations per minute
E2	17β-estradiol
EC_{50}	Effective time to reach 50% of desired effect (in this dissertation it is depuration)
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EE2	17α-ethinylestradiol
ELA	Experimental Lakes Area
ELS	Early-life stage
FADH2	Flavin adenine dinucleotide
FHM	Fathead minnow
FLFII	Female leucophore-free
FSHβ	Follicle stimulation hormone

GDM	Gestational diabetes mellitus
GLP-1	Glucagon-like peptide-1
GnRH	Gonadotropin releasing hormone
g	Gram
g	G-force, or relative centrifugal force
GC	Gas chromatography
GSH	Glutathione
h	Hour
H_0	Null hypothesis
hpf	Hours post fertilization
hs	Hours
IS	Internal standard
L	Liter
LH	Luteinizing hormone
Log Kow	N-octanol/water partition coefficient (expressed in log form)
LOEC	Lowest observed effect concentration
min	Minute
µCi	Microcuries

µg	Microgram
µL	Microliter
µm	Micrometer
µM	Micromolar
mCi	Millicuries
mg	Milligram
mL	Milliliter
mm	Millimeter
mM	Millimolar
MNA	1-methylnicotinamide
MOA	Mechanism of action
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
MSTF	N-methyl-N-trimethylsilyltri-fluoroacetamide
mTOR	Mammalian target of rapamycin
NADH	nicotinamide adenine dinucleotide
NADP+	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)

NCBI	National Center for Biotechnology Information
ng	Nanogram
NSAIDS	Non-steroidal anti-inflammatory drugs
OCT1	Organic cation transporter-1
OECD	Organization for Economic Cooperation and Development
PCOS	Polycystic ovary syndrome
pKA	Acid dissociation constant
PCR	Polymerase chain reaction
PPCPs	Pharmaceuticals and personal care products
POX	Proline oxidation
RNA	Ribonucleic acid
rpm	Revolutions per minute
ROS	Reactive oxygen species
RQI	RNA quality indicator
RT-qPCR	Quantitative reverse transcription polymerase chain reaction
SAH	S−Adenosyl homocysteine
SAMe	S-Adenosyl-L-methionine
SD	Standard deviation

SE	Standard error
SLC22A1	Solute Carrier Family 22 Member 1
SL1	Sex-linked 1
TCA	Tricarboxylic acid
TE	Tris-EDTA buffer
TMCS	Trimethylchlorosilane
UNT	University of North Texas
UOIT	University of Ontario Institute of Technology
UV	Ultraviolet
VLCFA	Very long chain fatty acid
VTG	Vitellogenin
WHO	World Health Organization
WWTP	Wastewater treatment plant

Chapter 1: Introduction and literature review

1.1 Introduction

Pharmaceuticals are a large and diverse group of medicinal compounds, used for the diagnosis, cure, mitigation, treatment, and/or prevention of diseases in both humans and animals (Corcoran *et al*., 2010). The World Health Organization (WHO) estimated the global pharmaceuticals market to be worth $300 billion (USD) yearly, a figure expected to rise to US$400 billion within three years. There are substantial regional differences in the use of pharmaceuticals internationally, influenced by economic status, health requirements, capacity for local manufacture and legal restrictions (Carius and Grüttner, 2014). Developed countries dominate global pharmaceutical sales, with North America accounting for 45%, Europe 13%, Japan 10%, and Australia 1% of recorded sales (IMS Health, 2016).

Most of the common pharmaceuticals sold in North America are in drug classes used to treat maladies considered to be associated with westernized society, such as maintaining cholesterol balance, treating stress, asthma, pain relief, chest pain, bacterial infections, and many more. The pharmaceuticals used to treat these maladies include specific lipid regulators, beta blockers, corticosteroids, analgesics, antianginal drugs, and antibiotics (Jones *et al.*, 2002). In addition to human use, pharmaceuticals are commonly used in veterinary practices across North America with antibiotics and antifungals being the most common (Corcoran *et al.*, 2010).

There is a positive correlation between frequently used classes of pharmaceuticals and their detection in the aquatic environment for a variety of reasons, and an inevitable consequence of the increase in sales of pharmaceuticals is an increase in consumption, leading to an even higher level of discharge into the environment (Corcoran *et al*., 2010).

Pharmaceuticals commonly enter the aquatic environment through human consumption, elimination, and disposal, and these pharmaceutical residues are found in the environment globally. Based on a review of over 1,000 international publications, pharmaceutical residues have been detected in wastewater treatment plant (WWTP) effluent and surface waters in 71 countries worldwide ranging from the ng · L^{-1} to µg · L^{-1} range in surface waters and µg · L^{-1} range in WWTP effluent (Carius and Grüttner, 2014). Pharmaceuticals have also been detected in groundwater, soil and even in drinking water at very low ng · L^{-1} concentrations.

The most significant and concerning characteristic of pharmaceuticals in the aquatic environment is that these compounds are designed to alter and affect physiological functions in target organisms, however, they might adversely affect non-target organisms (Carius and Grüttner, 2014). The field of aquatic toxicology investigates the effects of anthropogenic and natural compounds on aquatic organisms from the individual through to entire communities and ecosystems, aiming to address potential critical issues. Fish share many biochemical pathways and physiological similarities with mammals, thus they are likely to be susceptible to effects caused by water-borne pharmaceuticals (Corcoran *et al.*, 2010).

This study investigated the ecotoxicological effects of metformin and its bacterial metabolite, guanylurea on Japanese medaka (*Oryzias latipes*) through a multidisciplinary approach combining analytical, molecular, and whole organism laboratory methods to analyze uptake, growth, reproductive, genetic, and metabolomic endpoints. This work illustrates the necessity for researchers to examine the potential effects of anthropogenic compounds like pharmaceuticals on aquatic organisms.

1.2 Pharmaceuticals in the environment

The fate and occurrence of pharmaceuticals in the aquatic environment has been recognized as an emerging issue in environmental science, specifically aquatic toxicology (Boxall *et al*., 2012). A global review of pharmaceuticals in the environment found that of the 713 pharmaceuticals tested for, 631 were found above their detection limits in surface waters, ground water and even at minute amounts in drinking water (Carius and Grüttner, 2014). The WHO estimated the pharmaceutical market will increase by 25% over the next three years (WHO, 2016), which is concerning, as an inevitable consequence of the increase in sales of pharmaceuticals equates to an increase in consumption and consequent increases in discharges into the environment.

The majority of human pharmaceuticals are taken orally, with gut absorption ranging from 0-80% (Holford, 2001). As such, some of these compounds pass directly through the patient and are excreted completely unchanged, while other compounds will be absorbed by the body, undergo metabolism, and the resultant metabolites excreted via the urine and feces. The metabolism of drugs in the human body generally involves transformation into more polar and soluble forms to help facilitate drug elimination. The process occurs in two phases: phase I, involving oxidation, reduction, or hydrolysis, and phase II, involving conjugation of the parent compound to increase solubility (Ardrey, 2003; Jones *et al.,* 2002; Khetan and Collins, 2007; Testa and Jenner, 1978). Typically, the bioavailability of a drug is modulated by cytochrome p450, a superfamily of enzymes used to metabolize compounds in the body by converting lipophilic organic molecules to more water soluble and reactive compounds by introducing functional groups such as –OH, -SH, $-NH_2$, or –COOH during phase I transformations (Celiz *et al.,* 2009; Danielson, 2002).

During phase II transformations, these hydrophilic metabolic products then conjugate with *in vivo* molecules to form *O*- and *N*-glucuronides, sulfates, acetate esters, carboximides, and glutathionyl adducts (Celiz *et al.*, 2009). Since these molecules are now more hydrophilic, they can be readily excreted via the kidneys in the urine.

Once excreted, such pharmaceuticals and their metabolites generally make their way into municipal WWTP. The pharmaceuticals and metabolites entering WWTPs may or may not undergo biodegradation or biofiltration via activated sludge, followed by chlorine, ozonation, oxidation, or activated carbon in order to remove these compounds from the water (WHO, 2016). In general, WWTPs were designed to aid in the removal of biodegradable carbon, nitrogen, and phosphorus compounds that usually arrive in the plant in the high mg/L concentration (Verlicchi *et al.*, 2012).

The removal of pharmaceuticals through WWTPs has been shown to depend on the compound's chemical characteristics, the treatment processes used, and the concentration found in the influent, however other variables may also influence the removal efficiencies (Blair *et al.*, 2013; Le-Minh *et al.*, 2010; Oulton *et al.*, 2010; USEPA, 2012; Verlicchi *et al.*, 2012). WWTPs have three main mechanisms of removing or degrading pharmaceutical compounds: sorption to sludge, biological degradation, and volatilization (Blair *et al.*, 2013; Khan and Ongerth, 2002). These processes have demonstrated removal rates up to 90%, however, even with a 90% removal rate, significant amounts of these compounds enter the environment daily (WHO, 2016).

Aside from human consumption and veterinary use, it should be noted that a small portion of prescribed drugs are not ingested or administered, and are disposed of directly into landfills or flushed down the toilet to be deposited into wastewaters. It is estimated

that in the US alone, $1 billion (USD) of prescription drugs are discarded each year from hospitals, care facilities, pharmacies, and in household waste (Strom, 2005). Other sources also exist, including emissions and waste from manufacturing sites (Arsson, 2009), however the principal route of entry into the environment is a direct result of human consumption (Overturf *et al.*, 2015). Pharmaceutical routes of entry are depicted in Figure 1.1.

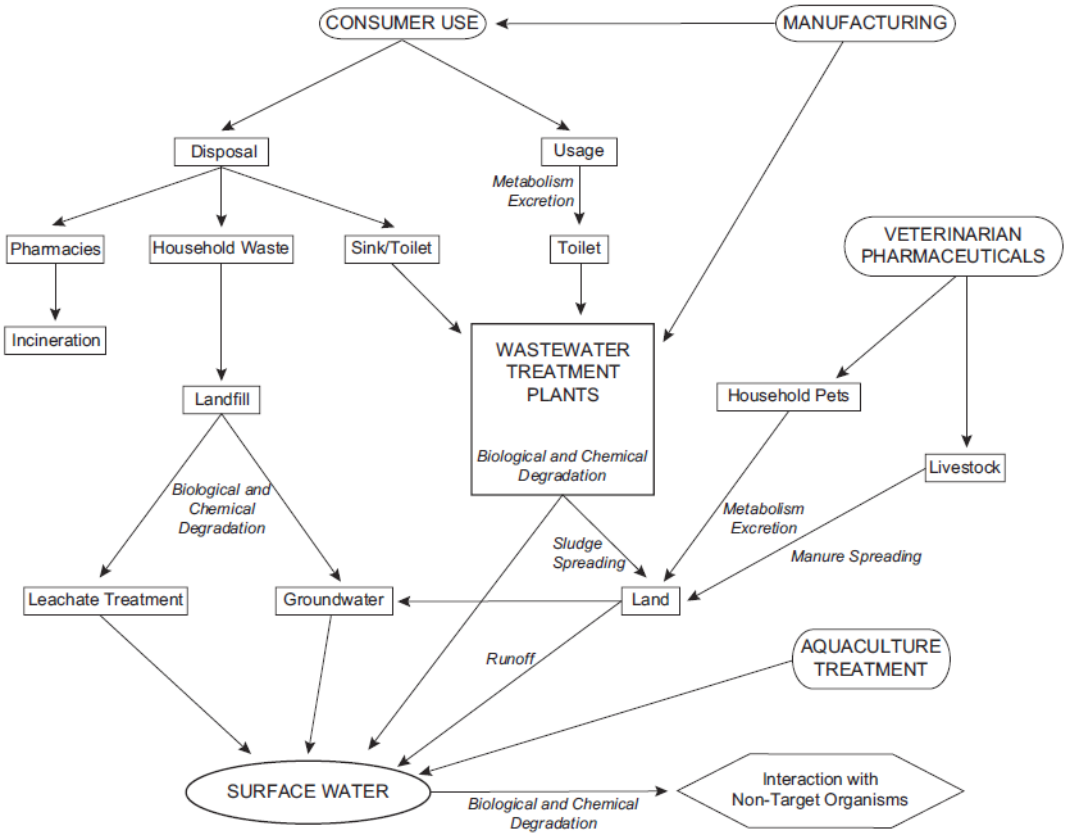


Figure 1.1: Major routes of entry of pharmaceuticals and personal care products (PPCPs) into the environment (Overturf *et al.*, 2015).

1.3 Pharmaceutical metabolites

The majority of studies investigating the amount of pharmaceuticals in the aquatic environment have focused on the parent compound with little to no attention, let alone quantification, of the transformation products formed during (a) water treatment, (b) human metabolic/excretion, and (c) decomposition of the compound. It is easy to recognize that pharmaceuticals and their metabolites discharged into the environment are ecologically significant, however it is a challenge to assess individual and collective toxicities of parent compounds and their metabolites because many metabolites have not yet been identified (Celiz *et al.,* 2009). A 2009 review reported that approximately 160 pharmaceutical products (both human and veterinary use) and only 30 by-products (biotic and abiotic) have been included in environmental occurrence, fate, and ecotoxicological investigations (Mompelat *et al.*, 2009).

Compound degradation does not always equate to detoxification, as many pharmaceutical metabolites bind to proteins and other cellular constituents often causing increased cellular function disruption than their parent compound.. For example, the glucuronidation of gemfibrozil, a common lipid lowering drug, results in a metabolite that is a more potent inhibitor of CYP2C8 (a major drug metabolizing cytochrome P450 enzyme) than the unconjugated parent drug, which can lead to accumulation and thus toxic effects by the inhibition of the metabolism activity of cytochrome P450 enzymes (Celiz *et al.,* 2009; Ogilvie *et al.,* 2006). In addition to the potential increase in toxicity of metabolites from their parent compounds, the indirect contribution of conjugated metabolites should also be considered. Conjugated drug metabolites have the potential to undergo deconjugation and transform back to the original form of the parent drug, as

commonly seen with both natural and synthetic estrogens (Celiz *et al.*, 2009; Ternes *et al.*, 1999). The glucuronide forms of estrone and 17β-estradiol are transformed back into their active parent estrogens in the environment by *Escherichia coli* bacteria which possess enzymes that facilitate deconjugation of glucuronic acid, such as β-glucuronidase (Celiz *et al.*, 2009).

1.4 Effects of pharmaceuticals on aquatic organisms

Pharmaceuticals are biologically active compounds that specifically aim to affect control mechanisms in living organisms, for example by influencing hormonal balance, alleviating signal transmission between cells, or regulating metabolism in target organisms (Carius and Grüttner, 2014). In this sense, target organisms are those receiving the pharmaceutical to achieve a desired result. When pharmaceuticals are released into the environment, this biological activity may adversely affect non-target organisms such as wildlife and impair ecosystem health. Some of these adverse effects have been reported in both field and laboratory studies, however many of the effects are still unknown.

Both the natural estrogen, 17β-estradiol (E2), and the synthetic estrogen, 17β-ethinylestradiol (EE2), the latter used in common birth control pills, are examples of well-researched compounds known to have serious adverse effects in fish. In humans, a combination of EE2 and progestin are used in the combined oral contraceptive, working to prevent ovulation (the release of an egg from an ovary), while also causing changes in the cervical mucus and uterine lining, making it harder for sperm to reach the uterus and harder for a fertilized egg to attach to the uterus (MedlinePlus Drug Information, 2015: Estrogen and Progestin). EE2 has been found in the aquatic environment at low parts per trillion (<5 ng $\cdot$ L^{-1}) (Kolpin *et al.*, 2002). Male zebrafish (*Danio rerio*) exposed in the laboratory to

0.5-5 ng · L^{-1} EE2 were shown to produce vitellogenin (VTG) mRNA and protein, early stage eggs, as well as decreased spermatozoa in their testes, ultimately leading to increased infertility and decreased reproduction (Nash *et al.,* 2004). VTG is normally synthesized by female fish and is associated with oocyte maturation as the egg yolk precursor protein; therefore increased expression of the protein in males is indicative of endocrine disruption.

In addition to laboratory studies showing decreased reproductive success in fish exposed to EE2, a 7 year whole lake study, using fathead minnows (*Pimephales promelas*) at the Experimental Lakes Area (ELA) in northwestern Ontario, showed that chronic exposure to low concentrations (~ 5 ng · L^{-1}) of the synthetic estrogen led to: feminization of males, with the production of VTG mRNA and protein, impacts on gonadal development (including intersex in males), altered oogenesis in females, and ultimately, a near extirpation of the species from the lake (Kidd *et al.,* 2007). The concentrations used in both of the above studies were comparable to concentrations observed in treated wastewater effluent and in surface waters, shown to be > 5 ng · L^{-1} in many United States streams (Kolpin *et al.,* 2002). Both field and lab studies demonstrate that concentrations of EE2 observed in freshwaters can cause adverse impacts on both individual and population levels of biological organization.

Aside from synthetic estrogens, hundreds of other pharmaceuticals enter the aquatic environment daily. For example, nonsteroidal anti-inflammatory drugs (NSAIDs), such as ibuprofen, are found in surface waters in concentrations up to 2.7 µg · L^{-1}. These compounds have a therapeutic role to reduce inflammation and pain by inhibiting cyclooxygenases (COXs) (Corcoran *et al.,* 2010). COX enzymes are well conserved across vertebrate species. One such species, the Japanese medaka (*Oryzias latipes*), was found to

have an altered pattern of spawning and reproduction after being exposed ibuprofen (1-100 $\mu g \cdot L^{-1}$) (Flippin *et al.,* 2007). In the same species, the common anti-depressant fluoxetine (surface water concentrations up to 0.030 $\mu g \cdot L^{-1}$), commonly known as Prozac, caused an increase in estradiol levels at the concentrations 0.1 and 0.5 $\mu g \cdot L^{-1}$ (Brooks *et al.*, 2003). Similarly, egg production and hatching success were both reduced in Japanese medaka exposed to 5 $\mu g \cdot L^{-1}$ of the beta-blocker, propranolol, commonly used to treat cardiac conditions, and found in surface waters at concentrations up to 0.59 $\mu g \cdot L^{-1}$ (Huggett *et al.*, 2002).

The early life stages of fish are developmentally vulnerable, as shown by researchers. The survival of fathead minnow larvae exposed to levonorgestrel from embryo through 28 days post hatch was negatively impacted, while growth was also significantly reduced compared to controls (Overturf and Huggett, 2015; Overturf *et al.*, 2014). As well, levonorgestrel down-regulates natural progestins, a critically responsible component of gamete maturation (Overturf *et al.,* 2014). Natural progestin also acts as a pheromone to indicate reproductive status, so any perturbations in progestin function can ultimately affect successful reproduction of fish (Miura *et al.,* 2006; Overturf *et al.,* 2014; Sorensen *et al.,* 1987). The early life stages of Japanese medaka are also negatively affected by pharmaceuticals such as the widely used dissociative anesthetic, ketamine and the common psychedelic, methamphetamine (Liao *et al.,* 2015). Embryonic exposure to environmentally relevant levels of ketamine and methamphetamine suppressed the heart rate, delayed hatching time and increased the incidence of developmental abnormalities of Japanese medaka (Liao *et al.,* 2015). Such results involve only the few compounds tested

among the hundreds that enter the environment daily. Thus, additional research on such emerging aquatic contaminants is of critical importance for the health of our environment.

1.5 Metformin and guanylurea

The anti-diabetic drug metformin is now thought to be the highest drug by weight released into the aquatic environment with up to 6 tons per year released from individual urban area WWTPs (Crago *et al.*, 2016). In 2013, 72.8 million prescriptions were granted, making metformin the 7th most prescribed drug in the United States (IMS Health, 2016). Recent studies have found metformin in WWTP effluent at concentrations ranging from 1 $\mu g \cdot L^{-1}$ to 47 $\mu g \cdot L^{-1}$, and in surface waters at concentrations ranging from 0.06 $\mu g \cdot L^{-1}$ to 3 $\mu g \cdot L^{-1}$ (Niemuth *et al.*, 2015; Oosterhuis *et al.*, 2013; Scheurer *et al.*, 2012).

The primary synthesis of metformin in 1922 can be attributed to the work of Werner and Bell from Trinity College in Dublin, Ireland (Marić, 2010). In humans, metformin is the first line pharmaceutical prescribed to treat patients with type-2 diabetes, and is also indicated as a treatment for various cancers as well as for polycystic ovary syndrome (PCOS), an endocrine disorder affecting 5-15% of reproductive-age women (Tang *et al.*, 2012). Despite being one of the most prescribed pharmaceuticals on the market, the exact mechanism of metformin action has not been fully elucidated.

Metformin does not readily cross cell membranes due to its low lipophilicity; therefore, the organic cation transporter 1 (OCT1) has been shown to facilitate cellular uptake of metformin (Shu *et al.*, 2007). Deletion of the OCT1 gene in mice dramatically reduced metformin uptake in hepatocytes; in addition, human patients carrying polymorphisms of the gene (*SLC22A1*) display an impaired effect of metformin in lowering blood glucose levels (Shu *et al.*, 2007). In humans, the *SLC22A1* gene is associated with

the transport of organic cations across the plasma membrane in both directions, and has important functions in pathways related to metabolism and the transport of glucose and other sugars (Becker *et al.,* 2009). In diabetes patients, metformin is used to regulate glucose levels by the activation of adenosine monophosphate kinase (AMPK), involved in regulating metabolism in cells (Crago *et al.,* 2016; Hawley *et al.,* 2010). The activation of AMPK then inhibits the target rapamycin (mTOR) signaling pathway that regulates energy utilization, thus down-regulating gluconeogenesis in the liver (Crago *et al.,* 2016; Goodyear *et al.,* 2001; Kahn *et al.,* 2005; Viollet *et al.,* 2012). In this way, AMPK and mTOR play an important role in the metabolism of glucose and fats, whole body energy balance and insulin signaling (Crago *et al.,* 2016; Goodyear *et al.,* 2001; Kahn *et al.,* 2005).

The metformin induced activation of AMPK, resulting in a decrease in mTOR mRNA and protein expression, is associated with a decrease in lipid and sterol biosynthesis (Goodyear *et al.,* 2001), stimulation of fatty acid oxidation with the inhibition of cholesterol and triglyceride synthesis (Li Gonga *et al.,* 2013), increased insulin sensitivity (Galardo *et al.,* 2009b), decreased intestinal glucose absorption and inhibition of gluconeogenesis, leading to less glucose available for storage in adipose tissue (Igel *et al.,* 2016; Seifarth *et al.,* 2013), decreased insulin growth factor (Berker *et al.,* 2004; Crago *et al., 2016*), decreased ghrelin secretion (Gagnon *et al.,* 2013) and increased GLP-1 (glucagon-like peptide-1) levels, leading to an anorectic effect (Mannucci *et al.,* 2001). Ghrelin is an orexigenic hormone, produced primarily in the endocrine cells of the stomach, which acts to increase appetite and promote energy storage (Gagnon *et al.,* 2013; Horvath *et al.,* 2016). GLP-1 is an incretin hormone, secreted from endocrine L cells of the intestine in response to nutrients in the gut lumen. GLP-1 conveys an insulinotropic effect via GLP-

1 receptors on beta cells of the pancreas, inhibiting the secretion of glucagon from the alpha cells of the pancreas, which together have a sustained effect on glycemic control (Fink-Jensen and Vilsbøll, 2016). The increased sensitivity hypothesized to be associated with metformin treatment lessens the postprandial hypoglycemia that occurs in insulin resistant states, decreasing hypoglycemia induced hunger and carbohydrate craving (Igel *et al.*, 2016). These effects ultimately drive the weight loss observed in diabetes patients treated with metformin (Crago *et al.*, 2016; Goodyear *et al.*, 2001; Kahn *et al.*, 2005).

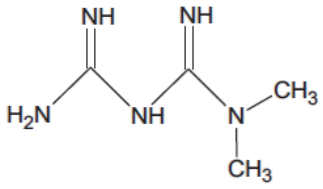
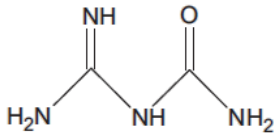
Aside from energy balance, AMPK and mTOR can affect a variety of other important pathways like those associated with sexual development, puberty, and thus reproduction. By activating AMPK and inhibiting mTOR, metformin is associated with a decrease in gonadotropin-releasing hormone (GnRH) secretion, thus decreased follicle stimulating hormone subunit beta (FSHβ) (Tosca *et al.*, 2011) and luteinizing hormone (LH) and decreased plasma 17β-estradiol levels in rat pituitary cells (Roa and Tena-Sempere, 2010). Decreases in these hormones have been shown to cause delayed puberty in rats (Roa and Tena-Sempere, 2010). In humans, metformin reduces hyperandrogenism through its effects on both the ovary and adrenal gland, suppressing their androgen production, reducing pituitary LH and increases the production of sex hormone binding globulin by the liver (Earle, 2000; Lashen, 2010). In male rats, the decrease in FSHβ through the regulation of AMPK and mTOR has been shown to down regulate the proliferation of Sertoli cells and the production of sperm (Galardo *et al.*, 2009a).

In principle, metformin works in the treatment of polycystic ovary syndrome (PCOS) by lowering the circulating insulin levels (Lashen, 2010). Some of the common effects reported in relation to metformin in PCOS patients are restoring ovulation, reducing

the risk of miscarriage (Lashen, 2010), reducing circulating androgen levels (Earle, 2000), and reducing the risk of gestational diabetes mellitus (GDM) (Glueck *et al.,* 2004), suggesting that metformin in PCOS pregnancies has a positive effect. Intuitively, the multiple impacts on parts of the reproduction pathway listed above could potentially have detrimental effects on the reproductive process of non-target organisms, such as fish.

Metformin has an average individual daily dose of 2 grams per day (WHO, 2016) which is orally administered. Metformin is not metabolized in the human body, thus it is excreted unchanged through the urine (about 70%) and the feces (Li Gonga *et al.,* 2012; Pentikäinen *et al.*, 1979). Once excreted, such pharmaceuticals and their metabolites generally make their way to municipal WWTPs, changed into their metabolite(s), or passed through via effluent. To date, guanylurea is the only known recalcitrant, aerobic, bacterial degradation product of metformin (Trautwein *et al.*, 2014). Both metformin and guanylurea are very polar compounds (Table 1.1) and should be extremely mobile in the aquatic environment (Scheurer *et al.*, 2012); thus there is potentially significant surface water relevance.

Table 1.1: Chemical structure, molecular formula and weight, and pKa of metformin and guanylurea.

	Metformin	Guanylurea
Chemical structure		
Molecular formula	$C_4H_{11}N_5$	$C_2H_6N_4O$
Molecular weight (g/mol)	129.2	102.1
pKa	2.8[a] and 12.3	8.0 and 13.5
logKow	-2.64	---

Hernández *et al.*, 2015 and Scheurer *et al.*, 2012

Sampling of five German WWTPs showed a removal rate of metformin of 90% or greater (Scheurer *et al.,* 2012). However, due to the extremely high usage of metformin and thus high influent concentrations, the effluent levels seen in this study are still between the 1.2 µg · L^{-1} and 10 µg · L^{-1} range, which are comparable with concentrations of other pharmaceuticals that are known to be poorly removed (Scheurer *et al.*, 2012). The removal of metformin in WWTPs was directly related to the formation of guanylurea (Trautwein *et al.,* 2014). Mean values for metformin and guanylurea found in major rivers in Germany are 0.22-1.2 µg · L^{-1} and 0.1-28 µg · L^{-1} (Scheurer *et al.,* 2012). Fortunately in this study, metformin and guanylurea are shown to be completely removed from raw water treated for drinking, explained by aerobic biological degradation, however the products are unknown (Scheurer *et al.,* 2012).

1.5.1 Effects of metformin and guanylurea on fish

Metformin's medicinal use in mammals has been well researched, however the effects it has on non-target aquatic organisms, specifically aquatic organisms are still unclear. However, some research shows that metformin is known to act in a similar manner in fish as it does in mammals. For instance, studies in zebrafish (*Danio rerio*) show that metformin increases insulin induced metabolic rate (Renquist *et al.,* 2013), while the drug induced AMPK expression and stimulated glucose uptake in trout muscle (Magnoni *et al.,* 2012) and liver (Polakof *et al.,* 2011).

A study conducted by Niemuth *et al*. (2015), exposed adult fathead minnows (*Pimephales promelas*) (FHM) to µg · L^{-1} of metformin for 28 days and found that the compound significantly increased the egg yolk precursor vitellogenin (VTG) mRNA expression, showing that the compound has potential endocrine disruption properties.

Another experiment found that male FHM exposed to 40 µg · L^{-1} metformin from fry (30 days post hatch) until adulthood (about 1 year) had significantly reduced weight and condition factor in metformin exposed fish compared to control fish (Niemuth and Klaper, 2016). Size can pose a population reproduction problem, as mating pairs of FHM with larger males have been shown to be more reproductively successful (Pollock *et al.*, 2008). Significant differences were found for cumulative clutches laid per mating pair over time, with metformin-treated FHM pairs laying fewer eggs when compared to control pairs (Niemuth and Klaper, 2016). Mean clutch size per pair also showed significant differences between control and metformin-treated fish, with significantly smaller clutches produced for treated fish (Niemuth and Klaper, 2016). In addition, metformin-exposed male FHMs also had a significant incidence of intersexuality shown by the occurrence/formation of oocytes in males exposed to the compound (Niemuth and Klaper, 2016).

A recent study shows significant upregulation in gonadal gene targets in male FHM exposed to 40 µg · L^{-1} when compared to control males, specifically genes involved in steroid synthesis and metabolism such as: an increase in the androgen receptor, 3β-hydroxysteroid dehydrogenase (HSD), 17β-HSD, a cytochrome p450 oxidase (CYP19A1), and the sulfanotransferase (SULT2A1) (Niemuth and Klaper, 2018). Interestingly, the authors observed significant correlations between the expression of 3β-HSD, 17β-HSD, and CYP19A1 and the degree of intersex in male FHM gonads represented by the frequent perinucleolar follicles and oocyte clumping throughout the testes (Niemuth and Klaper, 2018).

To the best of our knowledge, there is no information in the literature regarding the effects of guanylurea on aquatic organisms. These findings from a very limited number of

studies involving metformin and fish illustrate that the need for more research on the effects of metformin, and guanylurea, on aquatic organisms.

1.6 Japanese medaka research organism

Japanese medaka (*Oryzias latipes*) or rice fish, are small, egg-laying, freshwater fish inhabiting water from India throughout Southeast Asia and Japan, and are a common teleost in laboratory research settings because of their fully sequenced genome (Iwamatsu, 2004). At sexual maturity, medaka are about 2.5 to 3 cm in length which is reached within 3-4 months post hatch (Shima and Shimada, 1994). Medaka are widely used research organisms in experimental biology due to many favorable laboratory features: small size, easy to maintain, wide availability, multiple strains, daily spawning of reproductive adults, noticeable reproductive behavior, visible sexual dimorphism, large and clear embryos, and idea development time of embryos (8-10 days), a fully sequenced genome, and the ability to effectively observe sexual dimorphisms 3 days post fertilization specifically with the female leucophore-free (FLFII) strain of Japanese medaka (Balch *et al.*, 2004; Parenti, 2008). There are many different strains of medaka, each with valuable characteristics. Japanese medaka are very useful for embryo development research. Iwamatsu (2004) published a detailed paper outlining the staging of normal development of medaka embryos. Because medaka embryos are clear, it allows their development to be monitored fully monitored through hatch.

The research in this dissertation was performed using the female leucophore-free (FLFII) strain, obtained from the National Institute for Basic Biology (Okazaki, Japan), and originally introduced by Wakamatsu *et al.*, (2003). The FLFII strain has three observable sexual dimorphisms: two markers for body color and a male/female specific

DNA marker, *SL1*. Females of this strain do not produce the leucophore pigment cells, while males are wild type for leucophores. This allows for gender identification, and thus separation, as early as 3 days post fertilization under a fluorescence microscope. Juvenile medaka can be identified by the presence of orange-red xanthophores which are present in the males and absent in the females, which become increasingly apparent as the fish age. Lastly, definitive gender confirmation can be determined by analyzing the expression of the *SL1* marker via polymerase chain reaction (PCR). The ability to sexually differentiate between males and females, with both phenotypic and genetic methods, is useful for tests in which endocrine-disrupting compounds may masculinize or feminize fish (Balch *et al.*, 2004), a possible effect of metformin. Adult male and female medaka of the FLFII strain can be seen in Figure 1.2 and male and female embryo differentiation under a fluorescence microscope (Leica DM 2000) can be seen in Figure 1.3.

Figure 1.2: Sexually mature adult medaka of the female leucophore-free strain cultured in the aquatic toxicology laboratory at the University of Ontario Institute of Technology. (A) male and (B) female with a clutch of eggs.

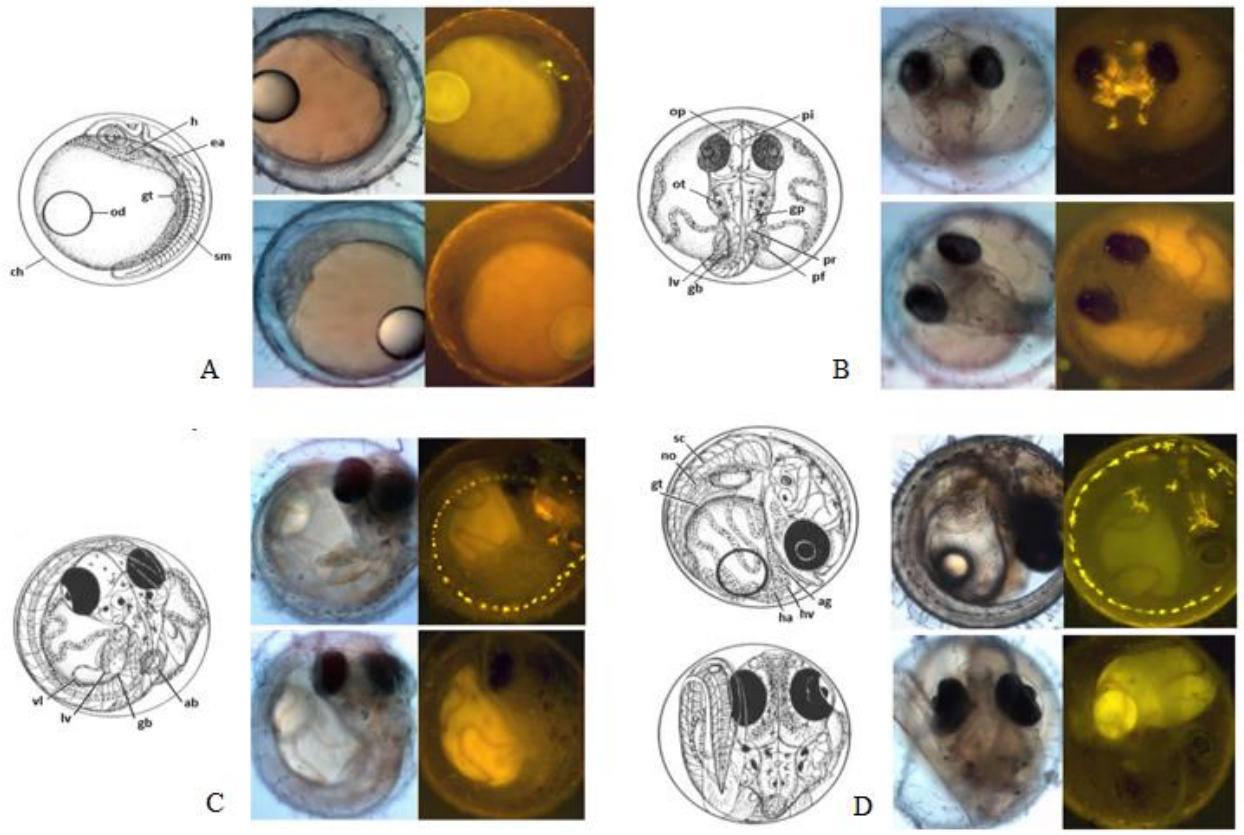


Figure 1.3: Schematics of Japanese medaka developing embryos from Iwamatsu (2004) with corresponding embryo photos taken using florescence microscope (Leica DM 2000, 25X magnification; filter cube: I3; excitation range: blue; filter: BP 450-490; dichromatic mirror (green): 510; suppression filter (red): LP 515) both under fluorescence and not. Microscope photos were taken in the aquatic toxicology laboratory at the University of Ontario Institute of Technology. Leucophores can be seen fluorescing bright yellow in males, but absent in females. (A) Stage 23-24 (~48 hpf). (B) 28-29 (~72 hpf). (C) Stage 34-35 (~118 hpf). (D) Stage 37-38 (~168 hpf).

1.7 Overall rationale and aim

In recent years, there is a growing concern that human pharmaceutical waste threatens to disrupt many natural processes of living organisms by the introduction of stressors and unnatural contaminants into the environment. One of the most common introduced stressors, metformin, is found at relatively high concentrations in the aquatic environment, thus the compound's effects on aquatic life is of the upmost importance. Although recently published research has demonstrated effects of metformin on adult fish, the experiments only focused on a narrow, specific life stage of the organisms. Additionally, the research focused on a single exposure concentration, 40 $\mu g \cdot L^{-1}$, which is an accurate representation of WWTP effluent, but is higher than the maximum reported surface water concentration of 10 $\mu g \cdot L^{-1}$ (Niemuth *et al.*, 2015; Scheurer *et al.*, 2012). This leaves large gaps in the data, as juvenile effects have not yet been examined, the effects of a full life cycle of fish are unknown, and scientists have yet to prove the uptake of metformin into fish. Of additional concern is metformin's bacterial metabolite, guanylurea, found in even higher concentrations than its parent compound. To my knowledge, there is no information in the literature defining the effects of guanylurea on aquatic organisms, leaving a large and concerning gap. This study aims to address these gaps by assessing the effects metformin, and its metabolite guanylurea, have on Japanese medaka (*Oryzias latipes*) growth, development, reproduction and sexual differentiation over the entire life cycle.

Chapter 2: Uptake and early-life stage growth studies of metformin and guanylurea in Japanese medaka (*Oryzias latipes*)

2.1 Abstract

The present study sought to determine the potential for uptake, bioaccumulation, and depuration of metformin in early life stage (ELS) Japanese medaka (*Oryzias latipes*) using ^{14}C-metformin. We also exposed fertilized embryos to environmentally relevant, waterborne concentrations of metformin, or guanylurea for 28-days post hatch, to examine effects of developmental exposure on growth endpoints. Results demonstrated that both embryo and larval stages of medaka were taken up, bioaccumulated, and depurated metformin at environmentally relevant concentrations. Furthermore, we showed that chorion hardening (~ 6 hpf), prevented the uptake of metformin by developing embryos. We also demonstrated that exposure to both metformin, and guanylurea significantly impacted growth metrics in ELS fish. Additionally, we showed that exposure to the primary metabolite of metformin, guanylurea, and also caused developmental impacts at exposure concentrations an order of magnitude lower than those required by the parent compound to exert similar effects. These results underscore the need for additional studies examining the effects of metformin, and its metabolite guanylurea, on aquatic ecosystems.

2.2 Introduction

In recent years, the occurrence and fate of pharmaceuticals in the aquatic environment has become an emerging issue in aquatic toxicology (Magi *et al.*, 2016). As mentioned in section *1.2 Pharmaceuticals in the environment*, the majority of human pharmaceuticals are taken orally, with gut absorption efficiency ranging from 0-80%, however some pass through the body without being absorbed or changed in any way (Holford, 2001). Recent studies have found metformin in WWTP effluents at concentrations varying from 1 to 47 $\mu g \cdot L^{-1}$, and in surface waters at concentrations varying from 0.06 to 3 $\mu g \cdot L^{-1}$ and is thought to be the highest drug by weight released into the aquatic environment with up to 6 tons per year released from individual urban area WWTPs (Blair *et al.*, 2013; Crago *et al.*, 2013; Ghoshdastidar *et al.*, 2014; Oosterhuis *et al.*, 2013; Scheurer *et al.*, 2012).

In humans, metformin is the first-line pharmaceutical prescribed to patients with type-2 diabetes, polycystic ovary syndrome, and is indicated as a treatment for various cancers where insulin resistance is a factor (Tang *et al.*, 2012). Both metformin and guanylurea are polar compounds (Table 1.1) and should be extremely mobile in the aquatic environment, concerning to organisms living in these aquatic habitats (Scheurer *et al.*, 2012). Despite the prevalence of metformin in receiving waters, its ecotoxicological impacts are largely unknown, including whether or not it is taken up, whether it is stored in fish tissue, and the rate at which metformin is depurated. These characteristics are important as they can be considered the first step, of many, in improving our understanding of the metformin uptake pathway and mode-of-action (MOA) in fish. There has been some controversy on whether metformin can partition into tissues, as its log Kow is low at -2.64.

To our knowledge, no one has shown whether metformin can be found inside exposed aquatic organisms. Proving that metformin does enter the fish would defend the effects researchers in the literature have seen metformin elicit on exposed fish thus far.

At the present time, it is simply not practical to monitor/test the effects of every single human and veterinary pharmaceutical (> 5,000) in use or currently in production on non-target aquatic organisms. An optimal research study would include testing the effects of both pharmaceuticals and their metabolites on whole life cycles and across multiple life cycles. While extremely valuable, these tests are not practical on a large scale because they are extremely costly, require a large number of animals, generate large amounts of aquatic waste, and take a considerable amount of time (months to years). Fish early-life stage (ELS) tests were developed in order to alleviate the monetary, waste, and time concerns, while still providing valuable information for compound effects on aquatic organisms.

The Organization for Economic Cooperation and Development (OECD) 210 outlines the value of ELS testing, while laying out a method for conducting an acceptable test. These tests are intended to define the lethal and sub-lethal effects of chemicals on the sensitive early life stages of the species being tested (OECD 210, 2013). Such tests produce valuable information that can be used to the estimation of more chronic effects of the chemical on organisms. ELS testing is currently the most frequently used bioassay for predicting chronic effects (Volz *et al.*, 2011). These tests typically last 1-3 months, in this case 28 days, and focus on the survival and growth of the organism. However, tests may be supplemented with behavioral and developmental monitoring, and coupled with endpoints analyzing genetic and metabolomic changes may be used to investigate the chemical mode of action of various compounds. For clarity, the research in this portion of

the dissertation used the OECD 210 as a basic framework for the experimental methods, however some parameters were altered and additional endpoints were investigated.

2.2.1 Objectives

The objectives of this experiment were to:

- Determine if metformin can be taken up, bio-accumulated, and depurated by exposed Japanese medaka (*Oryzias latipes*) at embryonic and larval stages.
- Asses the effects (length, wet weight, and condition factor) of a range of environmentally relevant concentrations of metformin and guanylurea on developing medaka via a 28 day ELS test.
- Assess if male and female medaka exposed to a range of environmentally relevant concentrations of metformin and guanylurea are effected (length, wet weight and condition factor) differently via a 28 day ELS.

2.2.2 Hypotheses

H_{o1}: Embryonic and larval medaka exposed to C^{14}-metformin for any amount of time will not take up C^{14}-metformin, and thus will not accumulate or depurate out the compound.

H_{o2}: Medaka exposed to various concentrations of metformin from embryo through 28 days will not have significant differences in length, wet weight, or condition factor when compared to control fish.

H_{o3}: There are no differences in length and weight of female and male medaka exposed to metformin from embryo through 28 days.

H_{o4}: Medaka exposed to various concentrations of guanylurea from embryo through 28 days will not have significant differences in length or weight when compared to control fish.

H_{o5}: There are no differences in length and weight of female and male medaka exposed to guanylurea from embryo through 28 days.

2.3 Materials and methods

2.3.1 Chemicals

Metformin hydrochloride (1,1-dimethylbiguanideine hydrochloride; CAS# 1115-70-4) was purchased from Toronto Research Chemicals (Toronto, Ontario, Canada). Guanylurea (CAS# 141-83-3) was purchased from Sigma-Aldrich (Oakville, Ontario, Canada) and methanol (CAS# 67-56-1) from ACP Chemicals Inc. (Montreal, Quebec, Canada). ^{14}C-Metformin (Metformin [biguanido-^{14}C] HCL ARC 1738A; specific activity: 100 mCi/mmol) was purchased from American Radiolabeled Chemicals Inc. (St. Louis, Missouri, USA) and scintillation fluid (Ultima Gold F, Part# 601371) was purchased from Perkin Elmer (Akron, Ohio, USA).

2.3.2 Animal care

Adult Japanese medaka (FLFII strain) were obtained from the National Institute for Basic Biology (Okazaki, Japan), and housed at the University of Ontario Institute Of Technology (Oshawa, ON, Canada). Animals were housed in three, 70 liter tanks, each containing 40 adult fish in optimal breeding ratios of two males to three females. All embryos used in the present study were obtained by gently stripping eggs from each gravid female with a gloved finger. Debris was removed from embryos by gently rolling them on clean paper towel. Embryos were pooled, placed in rearing solution (0.1% NaCl, 0.0163% $MgSO_4 \cdot 7H_2O$, 0.004% $CaCl_2 \cdot H_2O$, 0.003% KCl, and 0.0001% methylene blue, prepared in de-ionized (DI) water), and kept in a climate controlled room (27°C) for 1-hour to assess viability. As methylene blue is unable to penetrate viable embryos, a lack of color change indicated viability.

2.3.3 Radiolabeled metformin assays

2.3.3a Embryo/chorion preparation

Japanese medaka embryos were collected from the laboratory culture of Japanese medaka as previously mentioned in *2.3.2 Animal care*. Viable embryos were split into 2 groups: (1) pre-chorion hardening and (2) post-chorion hardening. There were 3 replicates per treatment, with 60 embryos per replicate. Both treatments were exposed to 10 $\mu g \cdot L^{-1}$ (6.05 E^{-5} mM) ^{14}C-metformin for 24-h, with exposure beginning either pre-chorion hardening (< 6 hours post fertilization (hpf)), or post-chorion hardening (24-hpf). Embryos were housed in 6-well plates containing 5-mL of test solution per well. In order to prepare the 6.05 E^{-5} mM ^{14}C-metformin treatment solution, a stock of 0.01 mM was prepared by mixing 10 µl of the 1-mM master stock with 990 µl of clean lab water. From this stock, the treatment solutions were prepared by mixing 544.5 µl of the 0.01 mM stock with 89.45 mL of clean lab water.

Each treatment group was exposed for 24 h. After this exposure period, embryos were rinsed 5 times with 5 ml of clean lab water. Each replicate was then transferred to a separate scintillation vial containing 5-ml of scintillation fluid (in which the embryos were quickly sacrificed). A scintillation counter (Perkin Elmer Tri-Carb 2800TR) was used to quantify ^{14}C-metformin in the embryos using the methods described in the *2.3.4d Metformin determination* section below.

2.3.3b Larval preparation

For the larval portion of the study, embryos were collected in the same manner as previously described in *2.3.2 Animal care*. Embryos were separated by sex using a fluorescent (Leica DM 2000) microscope 118 hpf and were kept separate for the remainder

of the experiment. Only male medaka of the FLFII strain contain leucophores, which fluoresce under a fluorescent microscope. Male and female embryos 118 hpf are shown in Figure 2.1. Embryos were then kept separate for the remainder of the experiment to assess if there was a difference in metformin uptake between sexes. Embryos were monitored using a dissecting scope in the same manner as the ELS studies, and once hatched, were exposed to 10 $\mu g \cdot L^{-1}$ (6.05 E^{-5} mM) ^{14}C-metformin for 24 h to compare sex based uptake differences. To determine uptake steady state, an additional study was run without sex separation. Similar to the previous study, larvae were exposed to 10 $\mu g \cdot L^{-1}$ (6.05 E^{-5} mM) ^{14}C-metformin for 24 h or 168 h (7 days) to analyze accumulation. Each treatment had 4 replicates with 20 larvae per replicate for both experiments. After the exposure periods, larvae were rinsed 5 times with 5 mL of clean lab water before being placed in a vial of scintillation fluid and analyzed for radioactivity on the scintillation counter; larvae were pooled to ensure an accurate reading.

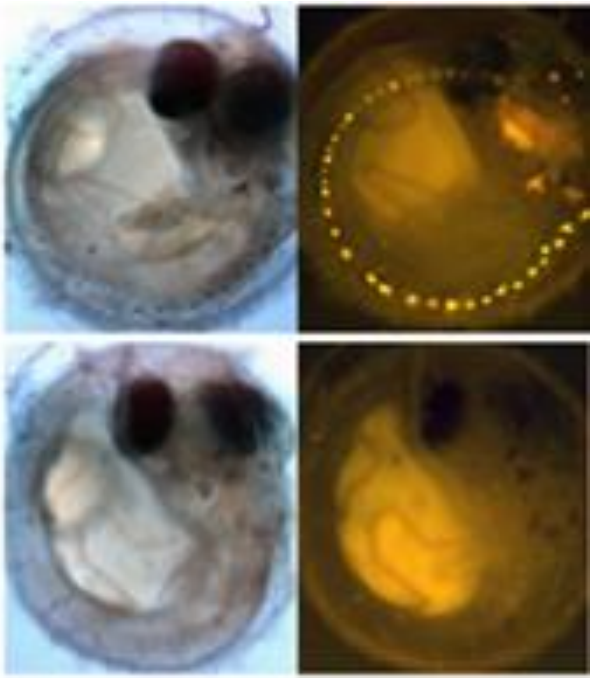


Figure 2.1. Embryo (~118 hpf) photos taken using florescence microscope (Leica DM 2000, 25X magnification; filter cube: I3; excitation range: blue; filter: BP 450-490; dichromatic mirror (green): 510; suppression filter (red): LP 515) both under fluorescence and normal light. Microscope photos were taken in the aquatic toxicology laboratory at the University of Ontario Institute of Technology. Leucophores can be seen fluorescing bright yellow in males, but are absent in females.

2.3.3c Depuration study

To examine the rate at which larval medaka can depurate metformin, we exposed embryos (< 6 hpf) to 10 µg · L^{-1} ^{4}C-metformin for 24 hours, with a sub-sampling time-point at test hour 8. Each treatment consisted of three replicates, containing 60 embryos per replicate. Following the metformin exposures, embryos were rinsed 5 times with 5 mL of clean lab water before being transferred to clean water for the remainder of the assay. The remaining body burden of ^{14}C-metformin was characterized via scintillation counter (again, methods described in more detail in *2.3.4d Metformin Determination* section below).

The assay was repeated with newly hatched larval medaka, using the same concentrations of ^{14}C-metformin, and sub-sampling/quantification methods described in the embryo assay. An additional sub-sampling time-point at 4-hours was added to improve the resolution of the assay. The 24-hour exposure to metformin was initiated immediately following hatch with, once again, three replicates per treatment, each containing 15 larvae.

2.3.4 Metformin and guanylurea exposure assays

2.3.4a Early-life stage studies

Medaka embryos were collected as previously described in *2.3.2 Animal care*. Embryos were separated by sex 118 h post-fertilization and were then kept separate for the remainder of the experiment to determine whether or not effects of exposure were dependent on sex. Embryos were randomly assigned to a treatment containing one of five concentrations of waterborne metformin (1.0, 3.2, 10, 32 and 100 µg · L^{-1}), or a solvent control treatment. Embryos were maintained in plastic petri dishes (Fisher Scientific, 60x15 mm), each containing 20-mL of the assigned treatment solution. Four replicates per

concentration (per sex) were used, with each replicate containing 30 eggs. Test solutions were renewed daily, and were prepared using a 10,000 mg · L^{-1} metformin master stock. As the master stock concentration exceeded the solubility of metformin in water, it was prepared using methanol (MeOH) as the initial carrier solvent, and then further diluted in rearing solution to achieve target concentrations. The MeOH carrier was kept at 0.01% for all solutions (including the solvent control) for the remainder of the study. For guanylurea exposures, a 100 mg · L^{-1} master stock (in deionized (DI) water) was used to prepare treatment solutions of 1.0, 3.2, 10, 32 and 100 ng · L^{-1} in rearing solution. The master stock did not exceed guanylurea's solubility limits in water, and therefore no exposure to MeOH occurred in any of the guanylurea studies.

All embryos were exposed to their assigned treatment solutions in a temperature controlled room (27 ˚C) and a 16:8 (light: dark) period with 0.5 hours each of dawn and dusk included in the light phase. Embryos were monitored under a dissecting stereomicroscope LEICA EZ4D (20x magnification) daily, using the developmental staging methods described by Iwamatsu (2004) and Wakamatsu (2003). Occurrence of developmental abnormalities, mortality, hatch success, and time-to-hatch were recorded daily.

Upon hatch, larval fish were transferred to 1-L plastic trays that corresponded with their exposure concentration and replicate number for the remainder of the 28-day study. Target waterborne concentrations of metformin (1.0, 3.2, 10, 32 and 100 µg · L^{-1}) and guanylurea (1.0, 3.2, 10, 32 and 100 ng · L^{-1}) were achieved using a flow-through system (Watson-Marlow 200 Series 16-channel peristaltic pump) that delivered stock concentrations from 1-L brown bottles in the appropriate volumes. Stocks of metformin

were prepared in clean water from a 10,000 mg · L^{-1} master stock prepared in MeOH (MeOH was 0.01% in all solutions), and a 1,000 mg · L^{-1} master stock (prepared in DI) for guanylurea. Exposure solution volume exchange insured 95% molecular turnover in 24 hours in each exposure tank. Larval medaka were fed 15 mL of live concentrated Premium Grade Brine Shrimp (Brine Shrimp Direct, San Francisco, California) twice per day for the entirety of the 28-day exposure. The bottom of the trays were vacuumed once per day to rid them of waste and leftover brine shrimp.

2.3.4b Biological endpoints

Three sub-sampling events occurred during the 28-day growth study; on days 7, 14, and 28 (test termination). On day 7, four fish were sub-sampled from each of the four replicate trays (per sex) maintained in the flow-through system. Five fish per replicate (per sex) were sub-sampled on day 14, with all remaining fish were collected and sampled at test-termination on day 28. At each sampling point (day 7, 14 and 28), larval fish were euthanized in water containing tricaine methane-sulphonate (200 mg · L^{-1}), buffered with sodium bicarbonate (400 mg · L^{-1}). Individual fish length was measured using a digital caliper, and wet weight was measured using a Metler Toledo MX5 microbalance. At each time point, the individual lengths and weights of subsampled fish were averaged to find a replicate mean. Replicate means were used for all statistical analyses regarding growth endpoints (n = 4 trays). Condition factor was calculated via the following medaka established equation, weight (g) x 10^5/length $(mm)^3$ (Brooks *et al.*, 2003).

2.3.4c Statistical analysis

All data were analyzed using SigmaPlot (Systat Software Inc.) unless otherwise specified. Normality of all data was confirmed using a Shapiro-Wilk test prior to

subsequent statistical analysis. The effects of chorion hardening on ^{14}C-metformin uptake were determined using a 1-way ANOVA. Effects of sex on size (length, weight, and condition factor) of 28-dph fish were assessed using a 2-factor ANOVA, followed by a Bonferroni *post hoc*. Similarly, a 2-factor ANOVA was used to determine the effects of sex on larval uptake of ^{14}C-metformin over time. The time required for larvae to depurate 50% of ^{14}C-metformin (ET50; with a 95% confidence interval) was calculated by performing an inverse prediction (50%) of the exponential depuration curve, with JMP software (Version 13, SAS Institute). Treatment effects on growth (using both length, and weight) over time (dph) were determined using a 2-factor ANOVA (with exposure concentration and time as factors), followed by a Tukey's *post hoc* test. An α of 0.05 was used to determine statistical significance for all tests.

2.3.4d Metformin determination

The concentration of ^{14}C-metformin in tissue samples was determined via scintillation counter, which detects ionizing radiation emitted from ^{14}C-metformin via excitation of incident radiation in the scintillation fluid (Perkin Elmer, Akron, Ohio, USA). A 10-point quench curve was performed to determine counting efficiency, with light pulses measured in counts per minute (cpm). The established quench curve used the relationship between counting efficiency and quench-indicating parameters to convert the measured counts per minute (cpm) to disintegrations per minute (dpm). Dpm were then divided by the rate constant (2.22×10^{12} dpm/Ci) to obtain curies (Ci). The calculated Ci was then divided by the specific activity of ^{14}C-metformin (1 Ci/mmol) to convert Ci to mmoles (all radiochemical equations found on Perkin Elmer Radiochemical Calculations, www.perkinelmer.com). Background readings from the scintillation counter (determined

as the average of 3 blanks containing only scintillation fluid) were subtracted from the radio-labeled sample readings.

Determinations of waterborne metformin and guanylurea concentrations in the ELS studies were performed using a Shimadzu 10A liquid chromatograph coupled with an AB Sciex Qtrap 5500 mass spectrometer, at the Water Quality Centre at Trent University (Peterborough, Ontario, CA). Metformin and guanylurea were below the method detection limit (MDL; 0.25 $\mu g \cdot L^{-1}$) in all control samples, and all guanylurea treatment samples (Table 2.2). Water samples were collected twice for chemical analysis during each ELS study, once at the beginning and once at the end, by collecting 50 ml of solution from each tank into 60 mL polypropylene copolymer bottles and storing at 4˚C in the dark until transport to Trent University.

Table 2.1. Nominal and mean concentrations (± standard error) of metformin as measured by liquid chromatography-mass spectrometry.

Nominal metformin ($\mu g \cdot L^{-1}$)	Mean measured metformin Males ($\mu g \cdot L^{-1}$; SE)	Mean measured metformin females ($\mu g \cdot L^{-1}$; SE)
Solvent control	< 0.25	<0.25
1.0	0.94 (0.131)	0.96 (0.062)
3.2	3.18 (0.441)	3.53 (0.491)
10	11.9 (1.35)	11.1 (1.38)
32	36.5 (3.59)	34.1 (1.87)
100	108 (8.29)	112 (6.07)

2.4 Results

2.4.1 Metformin uptake and depuration

Embryos exposed to 10 µg · L^{-1} (6.05 E^{-5} mM) ^{14}C-metformin prior to chorion hardening (< 6 h post-fertilization) had significantly (DF = 5, F = 267.13, $p < 0.001$) more ^{14}C-metformin compared to embryos exposed post-chorion hardening (24-hpf), which did not appear to take up any ^{14}C-metformin (concentrations were below the MDL; Figure 2.2). Consequently, all instances where embryos are discussed in the uptake and depuration results and discussion sections will hereafter refer to embryos exposed < 6-hpf. The mean (± 1 SE) amount of ^{14}C-metformin measured in medaka embryos was 0.08 ± 0.01 µg/egg. Following transfer to clean water medaka embryos rapidly excreted ^{14}C-metformin, with body-burdens of ^{14}C-metformin returning to background levels (below the MDL) within the 24-hour depuration period (Figure 2.3). The calculated ET50 for embryos is 8.79 hours (95% Confidence Intervals: 3.91, 13.68). The embryos depurated much more rapidly than expected, resulting in a very wide confidence interval, thus we were not able to generate a reliable ET50. Larval methods were adjusted accordingly.

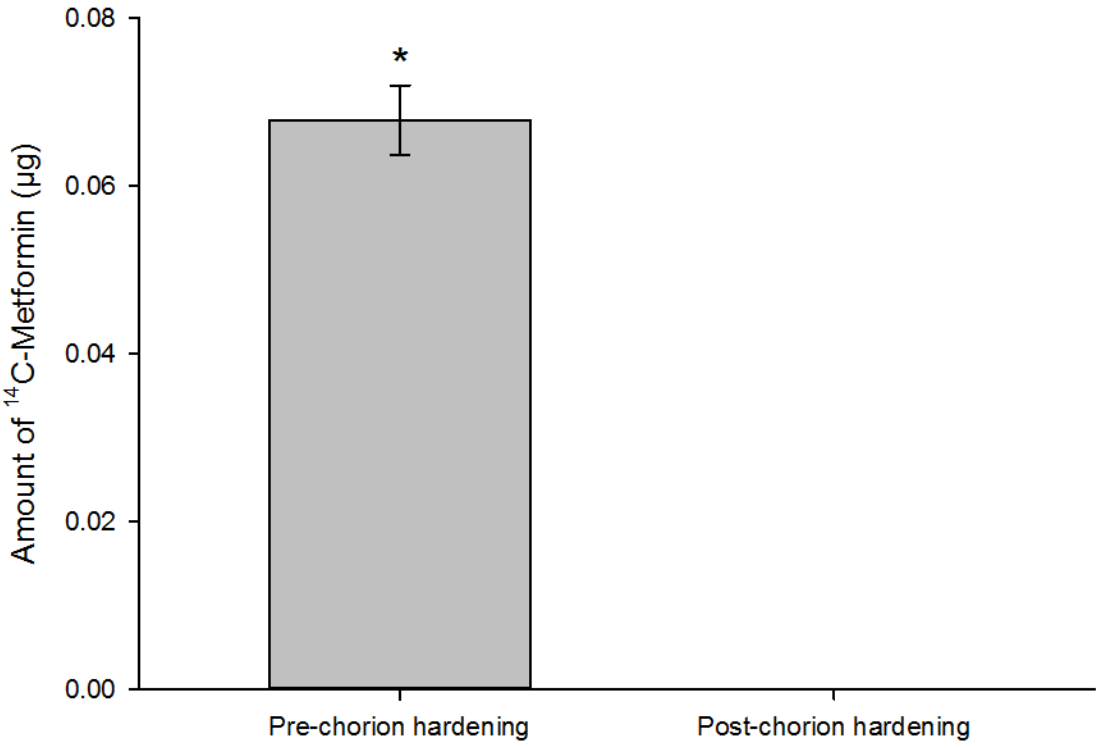


Figure 2.2: Amount of ^{14}C-metformin uptake by Japanese medaka embryos exposed to 10 µg · L^{-1} (6.05 E^{-5} mM) ^{14}C-metformin for 24 hours. Pre-chorion hardening represents embryos exposed within 6 hours of fertilization, and hardened chorion hardening represents embryos exposed 24 hours post fertilization (n = 3, with 60 embryos/replicate). Bars represent mean, ± standard error. * Indicates a significant difference from hardened chorion.

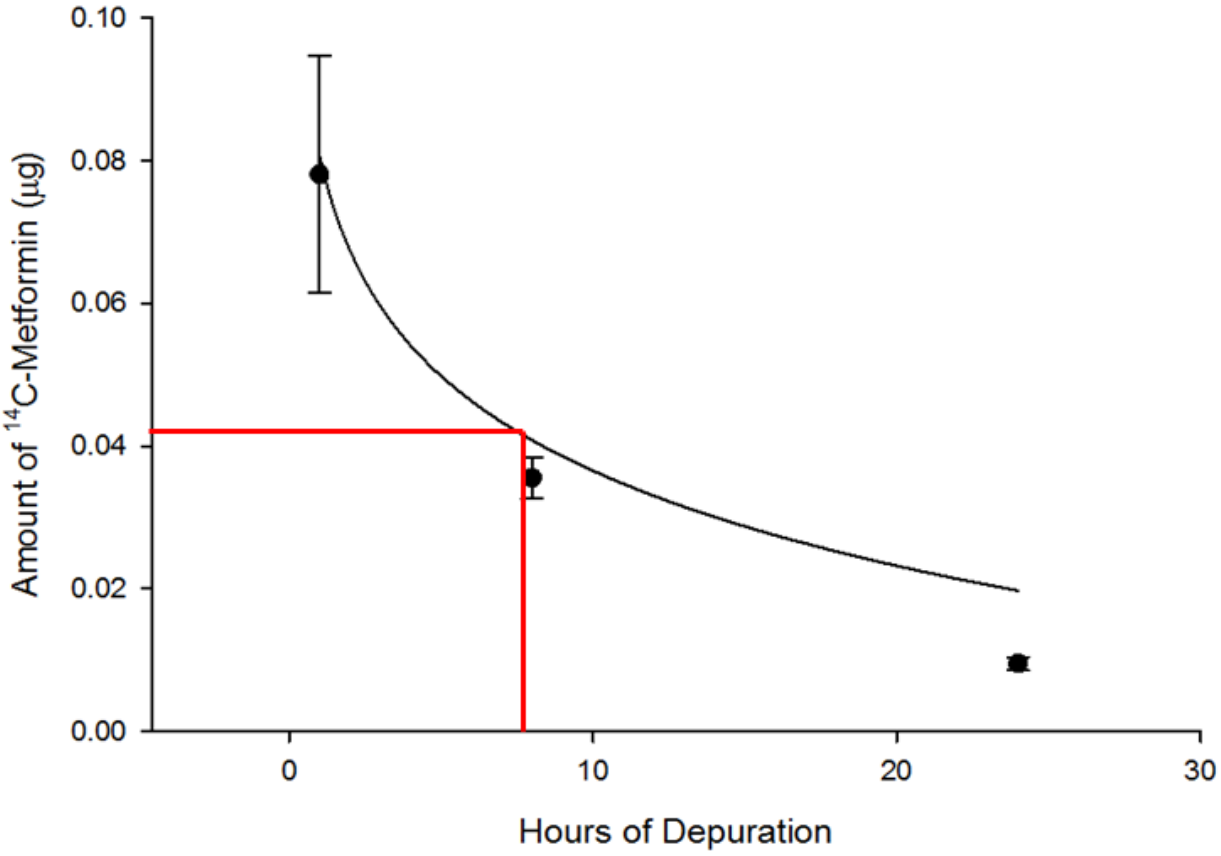


Figure 2.3: Depuration curve representing the amount of ^{14}C-metformin in Japanese medaka embryos exposed to 10 µg · L^{-1} ^{14}C-metformin for 24 hours, followed by clean lab water depuration periods for up to 24 hours (n=60 embryos/day, r^2 = 0.98). Solid circles represent mean ^{14}C-metformin, ± standard error bars.

Body-burdens of ^{14}C-metformin measured in larvae exposed to 10 µg · L^{-1} ^{14}C-metformin for 24-h, and 168-h were not significantly different from one another (DF = 1, F = 1.66, p = 0.267, Figure 2.4), indicating larvae reached steady-state rather quickly. On average, each larval fish contained 0.78 ± 0.03 µg of ^{14}C-metformin/larvae, with no significant differences indicated between sexes (ANOVA DF = 1, F = 0.843, p = 0.410), corresponding to an approximate body burden of 3.12 µg/mg body weight . Therefore, sexes were not separated for the remainder of the analyses. Following transfer to clean water, larval fish rapidly excreted ^{14}C-metformin, with body-burdens of ^{14}C-metformin returning to background levels (below the MDL) within the 24-hour depuration period (Figure 2.6). The calculated ET50 for larval fish was 4.88 hours (95% Confidence Intervals: 4.39, 5.37, Figure 2.5).

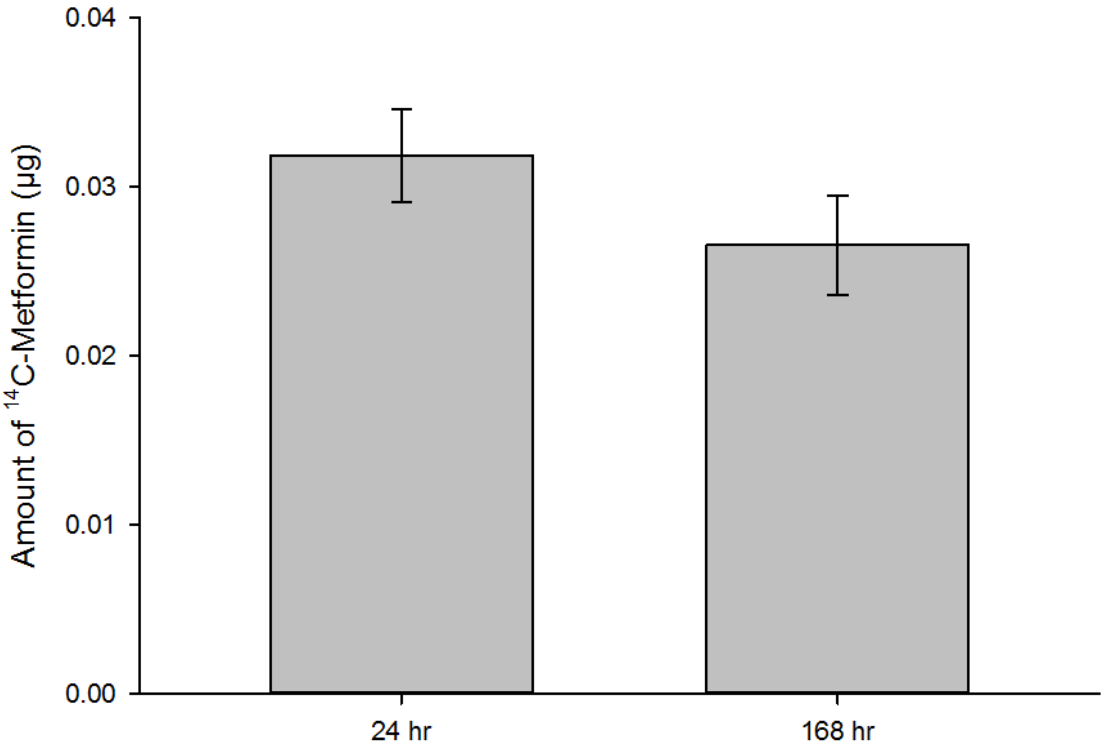


Figure 2.4: Comparison in the amount of ^{14}C-metformin found in 24 hr post hatch Japanese medaka larvae exposed to 10 µg · L^{-1} ^{14}C-metformin for 24 hr and 168 hr (n = 20 larvae/treatment). Bars represent mean, ± standard error.

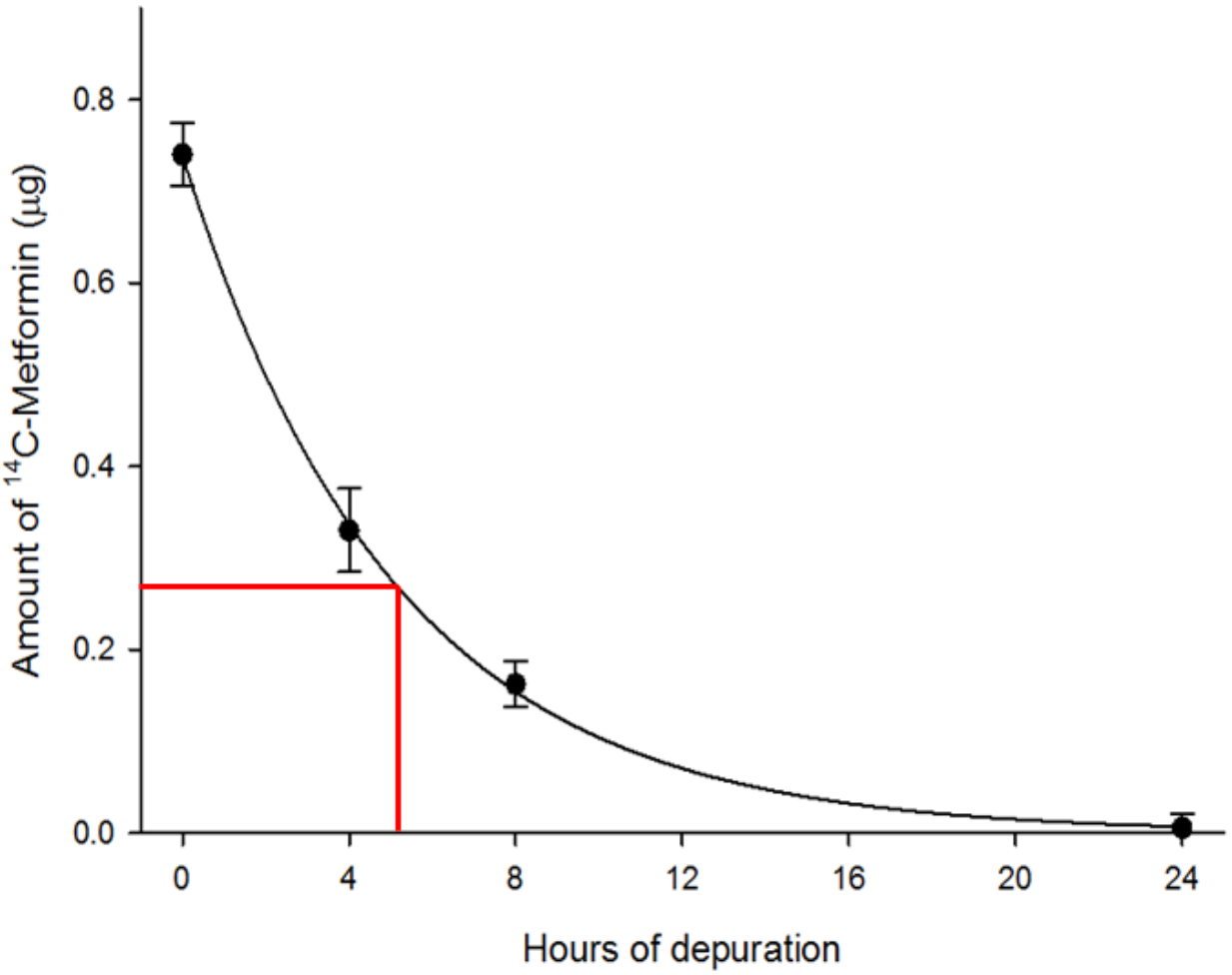


Figure 2.5: Depuration curve representing the amount of ^{14}C-metformin in Japanese medaka larvae exposed to 10 µg · L^{-1} ^{14}C-metformin for 24 hours, followed by clean lab water depuration periods for up to 24 hours (n = 15 fish/day, r^2 = 0.99). Solid circles represent mean ^{14}C-metformin, ± standard error bars.

2.4.2 Effects of metformin on larval growth

There were no significant differences in percent mortality (ANOVA DF = 5, F = 0.951, p = 0.458), hatch success (ANOVA DF = 5, F = 0.591, p = 0.707) or time-to-hatch (ANOVA DF = 5, F = 1.78, p = 0.114) in medaka embryos exposed to metformin, relative to controls (Table 2.4). Similarly, no developmental abnormalities were seen in any control or exposed embryos (apart from reduced size). Sex did not significantly affect the size (weight or length) of 28-dph fish in the present study (length: 2-way ANOVA DF = 5, F = 0.915, p = 0.471; length: 2-way ANOVA DF = 5, F = 1.45, p = 0.206), and therefore growth metrics between sexes were pooled for the remainder of the analyses.

Metformin exposure significantly affected fish weight over the course of the 28-day study, with significantly reduced mean wet weights of larvae observed at the first sampling time-point (7-dph) in the 100 µg · L-1 metformin (Figure 2.7A) treatment. Similarly, the length of developing larvae was affected by exposure to metformin. No significant effects of treatment on fish length were indicated at the 7-dph sampling time-point (Figure 2.7B). However, significant treatment effects on both mean wet weight and length were observed by 14-dph (Figure 2.7). At 28-dph, mean wet weight was significantly reduced in all exposure concentrations (DF = 17, F = 322, $p < 0.001$; Figure 2.7B, Table 2.4), while exposure to metformin concentrations ≥ 3.2 µg · L-1 significantly decreased the mean length of medaka (DF = 17, F = 902, $p < 0.001$; Figure 2.7B; Table 2.4) when compared with controls. However, condition factor (weight (g) x 10^5/length $(mm)^3$) was unaffected by metformin exposure (ANOVA DF = 5, F = 1.62, p = 0.152).

Table 2.2. Percent larval mortality, percent hatch success, and mean time to hatch (± standard error) for medaka exposed to metformin for a 28 day early life stage study.

Concentration ($\mu g \cdot L^{-1}$)	Larval mortality (%,±SE)	Hatch success (%, ±SE)	Time to hatch (days, ±SE)
Solvent control	11.7 (0.111)	87.5 (0.921)	8.91 (0.041)
1.0	11.7 (0.167)	85.8 (0.921)	8.78 (0.042)
3.2	13.3 (0.143)	87.9 (0.962)	8.63 (0.043)
10	11.7 (0.167)	92.5 (0.674)	8.78 (0.042)
32	16.7 (0.164)	89.6 (0.989)	8.83 (0.042)
100	11.7 (0.167	89.6 (0.854)	8.78 (0.037)

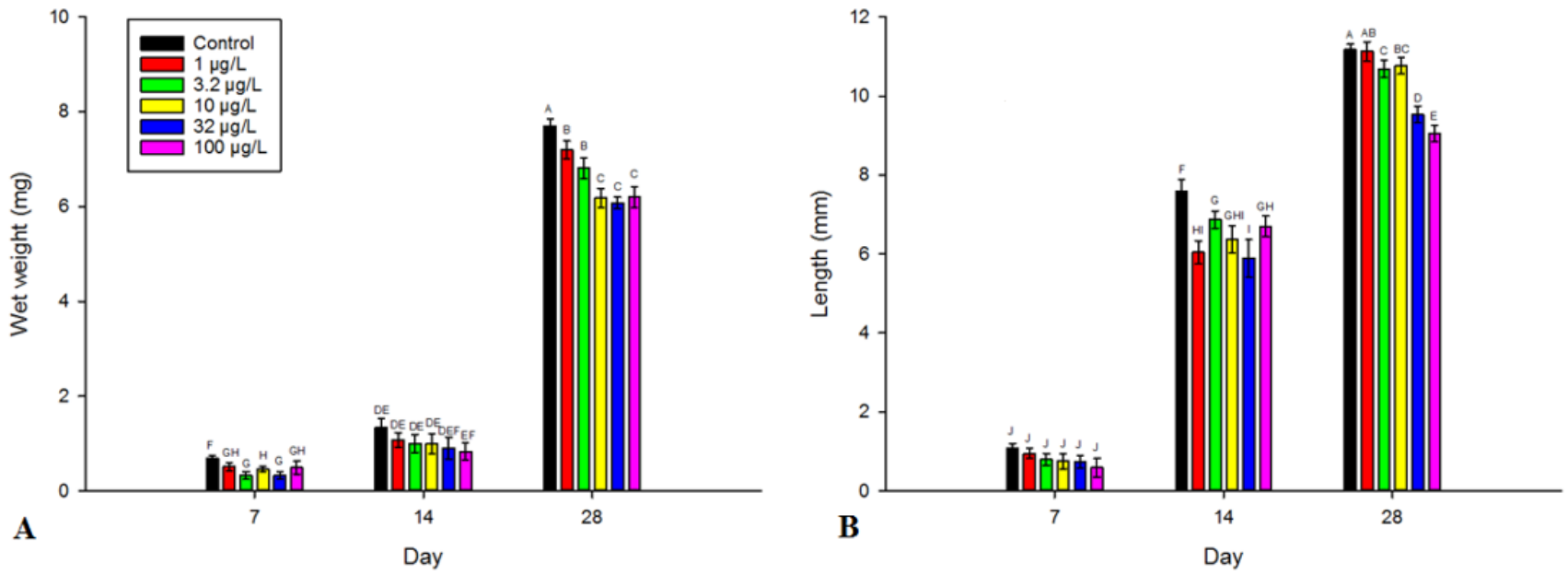


Figure 2.6: Mean wet weight (A) and length (B) of 28 day old Japanese medaka (± SE) by day and metformin treatment concentration (day 7 n = 4 (with 8 fish/replicate), day 14 n = 4 (with 10 fish/replicate), day 28 n = 4 (with 40 fish/replicate). Letters indicate significantly different groups (Tukey HSD, $\alpha = 0.05$).

Table 2.3. Japanese medaka wet weight and length as percent control for each metformin exposure concentration. Values reflect means and (± standard error). Values with an alphabetical superscript in common are not significantly different $p < 0.05$.

	Metformin ($\mu g \cdot L^{-1}$)				
	1	3.2	10	32	100
% control					
Wet weight	77.6(0.017)^{a}	73.4(0.001)^{a}	66.6(0.008)^{b}	65.5(0.013)^{b}	64.5(0.013)^{b}
Length	99.6(0.009)^{a}	95.6(0.024)^{b}	96.4(0.007)^{ab}	85.3(0.016)^{c}	81.0(0.005)^{d}

2.4.3 Effects of guanylurea on larval growth

No significant differences in embryo mortality (ANOVA DF = 5, F = 0.423, p = 0.831), hatch success (ANOVA DF = 5, F = 0.401,p = 0.846), or time-to-hatch (ANOVA DF = 5, F = 1.914, p = 0.089) were observed in any of the guanylurea treatment concentrations, when compared with controls (Table 2.5). Similarly, no developmental abnormalities were observed in control or guanylurea-exposed embryos (apart from reduced size). Again, sex did not significantly affect the size (weight or length) of 28-dph fish in the present study (weight: 2-way ANOVA DF = 5, F = 0.464, p = 0.803; length: 2-way ANOVA DF = 5, F = 0.266, p = 0.931), and therefore growth metrics were not separated by sex for the remainder of the analyses.

Developmental exposure to guanylurea (through 28-dph) led to significant differences in wet weight and length of medaka (Figure 2.10). Specifically, significant differences in fish size (both wet weight and length) were not apparent at the 7, or 14-dph time points ($p > 0.05$). However, 28-dph fish from all guanylurea exposure concentrations weighed significantly less (DF = 17, F = 1626, p = < 0.001); Figure 2.10A; Table 2.5), and were significantly shorter (DF = 17, F = 316, $p < 0.001$); Figure 2.10B; Table 2.5), when compared with control fish. However, condition factor (weight (g) x 10^5/length $(mm)^3$) was unaffected by metformin exposure (ANOVA DF = 5, F = 1.03, p = 0.410).

Table 2.4. Percent larval mortality, percent hatch success, and mean time to hatch (± standard error) for medaka exposed to guanylurea for a 28 day early life stage study.

Concentration ($ng \cdot L^{-1}$)	Larval mortality (%,±SE)	Hatch success (%, ±SE)	Time to hatch (days, ±SE)
Control	15.0 (0.147)	87.5 (0.921)	8.92 (0.043)
1.0	16.7 (0.189)	85.8 (0.921)	8.78 (0.042)
3.2	18.3 (0.252)	87.1 (0.854)	8.77 (0.046)
10	15.0 (0.309)	90.8 (0.491)	8.81 (0.044)
32	18.3 (0.156)	87.9 (0.801)	8.77 (0.043)
100	18.3 (0.211)	88.75 (0.754)	8.78 (0.039)

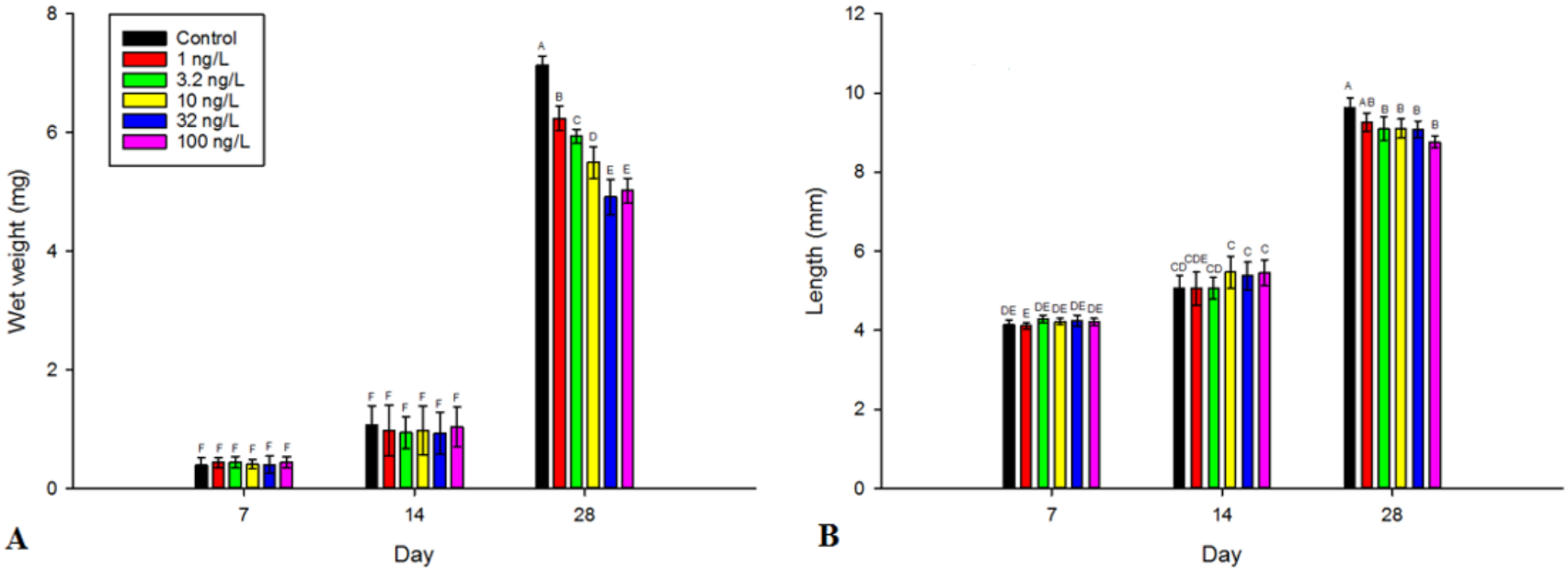


Figure 2.7: Mean wet weight (A) and length (B) of 28 day old Japanese medaka (± SE) by day and guanylurea treatment concentration (day 7 n = 4 (with 8 fish/replicate), day 14 n = 4 (with 10 fish/replicate), day 28 n = 4 (with 40 fish/replicate). Means not sharing the same letter are significantly different (Tukey HSD, $\alpha = 0.05$).

Table 2.5. Japanese medaka wet weight and length as percent control for each guanylurea exposure concentration. Values reflect means and (± standard error). Values with an alphabetical superscript in common are not significantly different $p < 0.05$.

	Guanylurea ($ng \cdot L^{-1}$)				
	1	3.2	10	32	100
% control					
Wet weight	87.4(0.010)^{a}	83.2(0.009)^{ab}	77.0(0.010)^{ab}	68.8(0.007)^{ab}	75.6(0.019)^{b}
Length	96.2(0.007)^{a}	94.5(0.008)^{b}	94.6(0.014)^{c}	94.4(0.007)^{d}	91.0(0.007)^{d}

2.5 Discussion

These results demonstrated that the common water contaminant, metformin, and its metabolite, guanylurea, have measurable impacts on the growth of early life-stage Japanese medaka. One concern not previously addressed in studies of metformin is whether or not uptake and accumulation occurs in fish. Because of metformin's low log Kow (-2.64), it has generally been thought that the compound would not readily cross the gills into the organism (US EPA, 2012). This is the first study to investigate and show that metformin can be taken up and transiently stored in the embryo and larval fish. This is an important finding, as it provides confirmation that the documented effects of metformin in fish are likely due to compound uptake. Due to the similar chemical structure of guanylurea, it is plausible to assume that it also can be taken up by fish, thus providing further insight into the effects of metformin's metabolite on larval growth.

Another interesting finding from our study was the metformin uptake and accumulation in pre-chorion hardened eggs compared to post-chorion hardened eggs. We also showed that when embryos were exposed to ^{14}C-metformin before the chorion hardened, followed by a short 24 h depuration, all of the detectable metformin was depurated out of the embryo. Similarly, this has been observed in zebrafish exposed to various xenobiotics (Fischer *et al.*, 2013). Researchers found that an ATP-binding cassette (ABC) transporter, specifically Abcb4, was successful in transporting xenobiotics out of the developing embryo, thus protecting the embryos from toxic chemicals dissolved in water (Fischer *et al.*, 2013).

Metformin and guanylurea exposure over 28-days resulted in stunted growth in both male and female medaka at environmentally relevant concentrations, with significant

concentrations seen as low as 3.2 $\mu g \cdot L^{-1}$ metformin and 1.0 $ng \cdot L^{-1}$ guanylurea. This suggests that exposure to both compounds may potentially lead to population-level effects, as insufficient body size can pose both reproductive and survival problems in fish. Pollock *et al.* (2008) observed significantly less reproductive success for smaller male fathead-minnow (FHM) mating-pairs. Additionally, significantly fewer cumulative clutches with significantly smaller clutch sizes were laid per mating pair in FHM exposed to 40 $\mu g \cdot L^{-1}$ metformin when compared to controls (Niemuth and Klaper, 2016). The implication of stunted growth in these early life stages, as seen in our study could lead to broader reproductive problems at the population scale, as seen in the aforementioned FHM studies.

To the best of our knowledge, this is the first study to address the effects of broad, environmentally relevant concentrations of metformin and guanylurea on the early life stages of fish. Although several studies have documented the occurrence of metformin in WWTP effluent at concentrations varying from 1 $\mu g \cdot L^{-1}$ to 47 $\mu g \cdot L^{-1}$, and in surface waters at concentrations varying from 0.06 $\mu g \cdot L^{-1}$ to 3 $\mu g \cdot L^{-1}$ (Blair *et al.,* 2013; Ghoshdastidar *et al.,* 2014; Niemuth *et al.,* 2015; Oosterhuis *et al.,* 2013; Scheurer *et al.,* 2012), very few have looked at guanylurea. One study specifically found that the removal of metformin from German wastewater treatment plants was correlated to the formation of guanylurea, found at concentrations of 0.22-1.2 $\mu g \cdot L^{-1}$ and 0.1-28 $\mu g \cdot L^{-1}$, respectively (Scheurer *et al.,* 2012). We are now able to confirm that the environmentally relevant concentrations of metformin and guanylurea can cause detectable effects, especially at concentrations far below the range measured for guanylurea in surface waters.

It remains unclear what underlying mechanism(s) are involved in the measured growth effects of metformin and guanylurea in Japanese medaka. However, because

metformin acts primarily by inhibiting complex I of the mitochondrial transport chain, it may lead to a decrease in available ATP, which promotes the activation of adenosine monophosphate kinase (AMPK) involved in regulating cell metabolism (Crago *et al.*, 2016; Gong *et al.*, 2012; Hawley *et al.*, 2010). The activation of AMPK then inhibits acetyl-CoA-carboxylase, which in turn inhibits fatty acid synthesis while promoting fatty acid β-oxidation (Gong *et al.*, 2012). This likely results in fatty acid dysregulation leading to disrupted energy stores, which could negatively affect growth. Unfortunately, nothing is known at this time about the mode of action of guanylurea in living organisms.

Overall findings from this chapter of the dissertation demonstrate the need for further research into the effects of metformin and its metablite guanylurea on non-target aquatic organisms. It is apparent from the research presented here that low, environmentally relevant concentrations of both compounds cause a detectable decrease in the growth of Japanese medaka. Future studies should investigate changes in gene expression and metabolism in response to metformin and guanylurea exposure, particularly during developmental windows from embryo to adulthood.

Chapter 3: Metabolomics and gene expression in early-life stage Japanese medaka (*Oryzias latipes*) exposed to metformin and guanylurea

3.1 Abstract

Environmentally relevant concentrations of metformin, and its metabolite guanylurea, have been shown to inhibit the growth of developing Japanese medaka (*Oryzias latipes*) exposed to the compounds from embryo through 28 days post hatch. In this chapter, metabolomics was used to identify cellular pathways disrupted by metformin and guanylurea exposure in medaka larvae exposed from embryo through 28 days post hatch. Results were further applied to select genes of interest for RT-qPCR analyses. Several metabolite abundances were altered by the exposure of medaka to 3.2 $\mu g \cdot L^{-1}$ metformin and 1.0 $ng \cdot L^{-1}$ guanylurea, which included metabolites associated with cellular energetics, fatty acid synthesis and metabolism, and polyamine synthesis. Expression of critical genes involved in lipid metabolism were also significantly affected. Collectively, these results suggest that environmentally relevant concentrations of metformin and guanylurea are sufficient to disrupt important cellular pathways in early life stage fish.

3.2 Introduction

Chapter 2 showed that both metformin and guanylurea have significant impacts on the growth of Japanese medaka (*Oryzias latipes*) at environmentally relevant concentrations. Medaka wet weight and length were significantly affected by exposures to metformin at concentrations as low as 3.2 µg · L^{-1} (Ussery *et al.,* 2018). Additionally, we demonstrate effects on fish size at waterborne guanylurea concentrations as low as 1.0 ng · L^{-1}, over an order of magnitude lower than the metformin concentrations required to evoke similar effects and about 30,000 times lower than the concentrations measured in German surface waters (Scheurer *et al.,* 2012; Ussery *et al.,* 2018).

As previously mentioned, ELS testing is currently the most frequently used bioassay for predicting chronic effects of compounds on fish (Volz *et al.,* 2011). ELS studies typically focus on the survival and growth of the organism, however tests may be supplemented with endpoints analyzing genetic and metabolomic changes to investigate the chemical mode of action of compounds. Metabolome analysis is used to study concentrations of organic, low molecular weight metabolites in tissues (Bridges *et al.,* 2016; Nishiumi *et al.,* 2014). To our knowledge, there is no prior information in the literature outlining the genetic or metabolomic effects of metformin and guanylurea in early-life stage fish.

The metabolome represents the final endpoint of the ‘omics cascade (genomics>transcriptomics>proteomics>metabolomics), leading it to be the closest point in the cascade related to the organism’s phenotype (Nishiumi *et al.,* 2014). Metabolite profiles are a summary of upstream ‘omics profiles, specifically the enzymatic activities of various proteins, and metabolome analysis might be able to detect subtle changes in

metabolic pathways and deviations from homeostasis before potential phenotypic changes occur (Nambiar *et al.*, 2010; Rochfort, 2005). Thus, the metabolite profile of a cell is more likely to represent the cell's current status than its DNA, RNA, or protein profile (Nishiumi *et al.*, 2014). Additionally, gene expression work is a useful tool to use in combination with metabolomics data, therefore, this study aims to address how exposure to environmentally relevant concentrations of metformin and guanylurea affect both the metabolome and gene expression of Japanese medaka, with hopes that this could help explain the stunted growth seen in Chapter 2.

3.2.2 Objectives

The objectives of this experiment were to:

- Employ metabolomics as a screening method to identify potential disruptions in normal cellular metabolism/homeostasis in Japanese medaka exposed to environmentally relevant concentrations of metformin and guanylurea after a 28 day ELS study.
- Based on metabolomics results, choose specific genes that may be differentially expressed as a result of metformin and guanylurea exposure, and verify via RT-qPCR.

3.2.3 Hypotheses

H_{o1}: The metabolome of Japanese medaka will not be altered following 28-day exposure to environmentally relevant concentrations of metformin and guanylurea.

H_{o2}: Medaka exposure to environmentally relevant concentrations of metformin and guanylurea for 28 days will not alter the expression of targeted genes in the organism.

3.3 Materials and methods

The analysis performed in Chapter 3 are a continuation of the ELS studies reported on in Chapter 2. The experimental lowest observed effect concentrations (LOEC) were used for each experimental exposure: 3.2 µg · L^{-1} metformin and 1.0 ng · L^{-1} guanylurea, and were each compared to their respective controls. Subsamples of larval fish were transferred to clean microcentrifuge tubes and flash frozen in liquid nitrogen. Eight biological replicates containing 20 mg of tissue (~2 larvae) were analyzed for each of the treatments.

3.3.1 Metabolite analysis

Larvae were homogenized in cold 2:5:2 chloroform:methanol:Mili-Q^{TM} water solution with a motorized pestle and centrifuged at 14,000 rotations per minute (rpm) at 4˚C for 8 minutes. The supernatant was removed and transferred to a clean microcentrifuge tube and spiked with 10 µL of 150 mg/mL D-27 Myristate internal standard (IS), followed by evaporation under a gentle stream of nitrogen. Evaporated samples were derivatized with 50 µL of 15 mg · L^{-1} methoxyamine in pyridine solution, vortexed, then heated at 50˚C for 30 minutes. A second derivatization step with the addition of 50 µL of N-methyl-N-trimethylsilyltri-fluoroacetamide (MSTFA) with 1% trimethylchlorosilane (TMCS), vortexed, and again heated at 70˚C for 30 minutes. After 30 minutes, samples were analyzed by gas chromatography-mass spectrometry (GC-MS; Agilent 6890 GC and 5973 MS).

To identify the metabolites, the Agilent Fiehn Retention Time Locking Library (Agilent part # G1676-90000) was used according to the manufacturer's recommendations. Chromatographic (.D) files were processed using the Automated Mass Spectral

Deconvolution & Identification System (AMDIS; National Institute of Standards and Technology, Gaithersburg, Maryland), then submitted to the Agilent Fiehn library to generate semi-quantitative relative response factors ratios against the response of the IS. A suite of fatty acid methyl ester standards were injected into every 5^{th} sample (n=3) to provide data for the AMDIS Retention Index library used to track slight variations in predicted metabolite retention times.

3.3.2 Measures of gene expression

Subsamples of two larval fish per treatment from ELS studies and transferred to clean microcentrifuge tubes and flash frozen in liquid nitrogen. Again, experimental LOECs were used for each exposure: 3.2 $\mu g \cdot L^{-1}$ metformin and 1.0 $ng \cdot L^{-1}$ guanylurea, and compared to their respective controls. Larvae were homogenized in 250 µL of TRI reagent (PureZOL^{TM} RNA Isolation Reagent, Bio-Rad) using a hand held homogenizer. Once homogenized, an additional 250 µL of TRI reagent was added to each sample. Samples were vortexed, then centrifuged at 12,000 *g* for 10 minutes at 4˚C. Post centrifugation, the clear middle layer was transferred to a new 1.5 mL RNAase/DNAase free Eppendorf tube, and was allowed to stand for 5 minutes at room temperature. Afterward, 150 µL of chloroform was added to each sample and gently inverted repeatedly to mix. The samples were left to stand at room temperature to allow for visible separation of the layers (~ 5 minutes). Once separated, samples were centrifuged at 12,000 *g* for 15 minutes at 4˚C resulting in three phases: a protein layer at the bottom, a middle interface containing DNA, and a colorless upper aqueous phase containing RNA. The upper aqueous RNA phase was transferred to a fresh 1.5 mL Eppendorf tube and 200 µL of isopropanol was added, vortexed, and left to stand for 5 minutes. After 5 minutes, the samples were

centrifuged at 12,000 *g* for 10 minutes at 4˚C. Post centrifugation, the supernatant was discarded and the RNA pellet was washed with 500 µL of 75% EtOH, vortexed and centrifuged at 7600 *g* for 5 minutes at 4˚C. The excess EtOH was removed, leaving the pellet undisturbed. The RNA pellet was then dissolved in 50 µL of Tris-EDTA (TE) buffer and stored at -20˚C until use.

RNA concentration and purity was determined using a 0.2 mm TrayCell coupled with a Cary 50 Bio UV-Visible Spectrophotometer. Samples were analyzed at 260 nm and a 2000 factor, with factor = sample specific factor x virtual dilution factor (ssRNA = 40 x 50, respectively). The Cary was zeroed using 2 µL of TE buffer. Then, 2 µL of each sample was run in duplicate, cleaning with EtOH between each sample and replicate. Samples were deemed acceptable if the purity ratio ($OD_{260/280}$) was greater than 1.7 and the RNA concentration was between 50 ng $\cdot$ μL^{-1} (min) – 500 ng $\cdot$ μL^{-1} (max).

RNA quality was determined using Experion RNA StdSens kits, following the manufacturer's protocol (BioRad). Electrodes were cleaned/rinsed by pipetting 800 µL of electrode cleaner into any middle well of the electrode cleaning chip, and placed into the Experion for 2 minutes. The chip was removed, and 800 µL of diethyl pyrocarbonate (DEPC)-H_2O was pipetted into the DEPC chip, and left in the Experion for 5 minutes. The Experion was allowed to air dry for about a minute after the chip was removed, then the cleaning procedure was repeated once more. The filtered gel was prepared by pipetting 600 µL of gel stain into the filter tube and centrifuging at 1500 *g* for 10 minutes. The gel stain was prepared by adding 1 µL of stain to 65 µL of filtered gel in a 1.5 mL tube and vortexing. RNA ladder and samples were prepared by pipetting 2 µL of ladder/samples into 0.2 mL PCR tubes, followed by a 2 minute denaturing step at 70˚C, then left for 5 minutes on ice.

The RNA StdSens chip was primed with 9 µL of gel stain into the gel priming well, then inserted into the Experion priming station and primed using setting B1. After priming, the chip was loaded in the following order: 9 µL of filtered gel, 5 µL of loading buffer into the 13 wells, and 1 µL of sample plus ladder into the wells. The chip was then placed in the Experion vortex station for 1 minute, then run on the Experion using the Experion RNA StdSens Assay. RNA Quality was reported via RNA quality indicator (RQI). Samples with a value of >7 were deemed acceptable for use in RT-qPCR work.

Genes chosen for RT-qPCR analysis were selected based on literature availability and their relevance to the metabolomics data sets. Medaka genome sequences were found in the national center for biotechnology information (NCBI) database. Primers were designed using Primer3 with a product size range of 75-200 bp (Table 3.1) and purchased in powdered form from Invitrogen. Primers were immediately suspended in TE buffer to a concentration of 100 µM/L, split into 15 µL aliquots and stored at -20˚C until use. Primers were validated through an 8-point temperature gradient to verify that each anneal at 60˚C and melt peaks were checked to ensure only one product was created. A 4-point primer concentration curve was also run using a 10 fold dilution (100, 10, 1, and 0.1 ng/µL) and tested in triplicate.

Five reference genes were tested: ribosomal protein l7 (*rpl7*), ribosomal protein l8 (*rpl8*), eukaryotic elongation fact 1 alpha (*eEF1A*), TATA Box BiTATA Box Binding Protein Ending Protein (*tbp*), Beta-2-Microgobulin (*B2m*), and Beta-actin (*ACTB*; Table 3.2). Reference gene primers were validated in the same manner as the experimental gene primers, and the reference gene with the best stabilization value was used. *rpl7* was used for all gene expression work.

Reactions were prepared using the iTaq Universal SYBR Green One-Step Kit (Bio-Rad, Mississauga, Ontario) according to the manufacturer's guidelines. Reactions were performed using RNA samples brought to a concentration of 40 ng/µL in TE buffer. A Bio-Rad CFX96 was used for RT-qPCR analysis using the following settings: SYBR/FM; Reverse Transcription Reaction, 10 minutes at 50˚C; Polymerase Activation and DNA Denaturation, 1 minutes at 95˚C; Amplification: Denaturation at 95˚C, 10 seconds; Annealing/Extension + plate read at 60˚C, 10-30 seconds; Cycles, 45; Melt-Curve Analysis, 65-95˚C with 0.5˚C increments 2-5 seconds/step. Non-template controls were run for all primers to monitor contamination and primer-dimer formation. All samples were run in triplicate, and normalized to *rpl7* transcript levels. Differences in gene expression between treatments were evaluated via statistical analysis using the $2^{-\Delta\Delta CT}$ (relative change of change).

Table 3.1 Primers used for RT-qPCR analysis. References were simply used for information on genes, not for primer sequences.

Gene Name	Primer Sequence	References	Query
β hydroxyacyl-CoA dehydrogenase (*hcd*)	F:TTGCATGAGAGAGGTCACGG R:TGTGGGGATCCTTTGCACTC	Schulz, 2008	XM_004065897.4
HMG-CoA synthesis (*hgs*)	F:TGACGTCACAGACAGGATGC R:CGCTTGGTCCACAAAATGGG	Baenke *et al.*, 2013	XM_011482022.2
glucose-6-phosphate dehydrogenase (*g6p*)	F:TGTTTTCTGCTTCGACAAGCG R:AGCCAGCAGAATACGATAGAGC	Woo *et al.*, 2011	XM_020704631.2
stearoyl-CoA desaturase (*scd*)	F:CCCAAACCTCGCAGGATGAT R:CCGTGATTCCCAGAGCACTT	Baenke *et al.*, 2013	XM_004080425
acetyl-CoA carboxylase – 2 (*ac2*)	F:ACGTGCAGTGGTTGTGTATGA R:ATCCGATCCCTGTCCTTCCA	Baenke *et al.*, 2013	XM_023958485.1
elongation of very long chain fatty acids protein 1-like (*elo*)	F:TTGATGTCTGGATGGGCCAC R:TCGAATCATCCTGAGAGCCTG	Baenke *et al.*, 2013 and Ofman *et al.*, 2010	XM_004079253.4

Table 3.2. Primers used for reference genes in RT-qPCR analysis.

Gene Name	Primer Sequence	NCBI Accession #
ribosomal protein L7 (*rpl7*)	F:CCCCAACTTGAAGTCTGTGC R:TGTTGGCAGGCTTGAAGTTC	NM_001104870
β-actin (*actb*)	F:CCAGCCTTCCTTCCTTGGTA R:GTACCTCCAGACAGCACAGT	S74868
ribosomal protein L8 (*rpl8*)	F:CCTGTTGAGCATCCATTCGG R:CCTTCTCCTGGACGGTCTTT	NM_001104909
elongation factor 1 alpha (*eef1a*)	F:CAAGAACGACCCACCAAAGG R:CAATGGCAGCATCTCCAGAC	NM_001104662
TATA-box binding protein (*tbp*)	F:GTGTTCAGTCCCATGATGCC R:AGTCTGTCCTGATGCTCCTG	NM_001278855
beta 2-microglobulin (*b2m*)	F:AGTCCAGGTGTACAGTCGTG R:GCGCTCTTTGTCAGGTGAAA	NM_001104660

3.3.3 Statistical analysis

Metabolomics reports generated using the Agilent Fiehn Retention Time Locking Library were filtered using a R (v. 2.15.2) program designed at the University of North Texas (UNT, Denton, TX, USA) which determined acceptable metabolite identification based on the similarity between sample retention indices and library retention indices, and a net mass spectral quality score of 70% or greater as calculated by AMDIS. The metabolites with results that met all filtration criteria were included in a two-tailed Welch's t-test, in order to determine statistically significant differences in RF values between the control and metformin and guanylurea treated groups. The $2^{-\Delta\Delta CT}$ values from RT-qPCR analysis were analyzed using a single factor ANOVA, by treatment. An α of 0.05 was used to determine statistical significance for all tests.

3.4 Results

3.4.1 Metabolite and gene expression analysis on larval medaka exposed to metformin

Larval medaka exposed to 3.2 $\mu g \cdot L^{-1}$ metformin from embryo through 28 days post hatch had significantly altered abundances of various metabolites (T-test, $p < 0.05$, Table 3.3). The fatty acids: stearic acid, palmitic acid, and arachidic acid, were all found to be significantly increased in metformin exposed larvae (T-test, $p < 0.05$), with RF values of 1.77, 1.54, and 2.59 respectively (Table 3.3). The amino acids L-lysine and L-proline were found to be significantly decreased in exposed larvae (T-test, $p < 0.05$) with RF values of 0.51 and 0.37, respectively (Table 3.3). The metabolite DL-3-aminoisobutyric acid was found to be significantly decreased (T-test, $p < 0.05$, RF = 0.60), while the metabolite 1-methylnicotinamide was found to be significantly increased (T-test, $p < 0.05$, RF = 1.36) in exposed medaka larvae (Table 3.3). Lastly, the metabolite phosphoric acid was found to be significantly increased (T-test, $p < 0.05$) in metformin exposed larvae with a RF value of 2.13 (Table 3.3). RT-qPCR results (Table 3.4) revealed a significant down-regulation in the expression of *hcd* in metformin exposed larvae when compared to controls with a $\log_2$ fold change of -1.60 (DF = 1, F = 31.6, $p < 0.05$). PCR analysis also showed a significant down-regulation in the expression of *hgs* with a fold change of -3.25 if exposed larvae (DF = 1, F = 17.9, $p < 0.05$). No significant difference, however, was seen in the expression any of the remaining genes (Table 3.4, Figure 3.1).

Table 3.3. Metabolites detected in larvae. An * is used to denote significance between control and 3.2 µg · L^{-1} metformin exposed larvae metabolite response factors (relative to IS).

Name	N Control	Control Mean ± 1 SD	N Met	Met Mean ± 1 SD	Met/Control (RF value)	P-value
*stearic acid	7	1340 ± 301	8	2380 ± 254	1.8	1.3E-05
*palmitic acid	7	988± 115	8	1520 ± 203	1.5	5.0E-05
*phosphoric acid	7	8890± 4800	8	18900 ±4340	2.1	1.1E-03
*L-lysine 2	7	2420 ± 677	7	1240 ±275	0.5	2.8E-03
*DL-3-aminoisobutyric acid 2	7	830 ± 213	8	500 ± 134	0.6	5.7E-03
*arachidic acid	6	6.50 ± 2.00	8	16.9 ± 8.00	2.6	5.7E-03
*L-proline 2	6	379± 135	8	142 ± 121	0.4	6.5E-03
*1-methyl nicotinamide 1	7	111± 37.0	8	151 ±33.0	1.4	4.7E-02
pyruvic acid	7	23.4 ± 9.00	6	14.0 ± 7.00	0.6	7.1E-02
L-serine 2	7	4890 ± 1290	8	6490 ± 2180	1.3	1.1E-01
putrescine	7	410± 83.0	8	328 ± 142	0.8	1.9E-01
adenine 1	7	30.3 ± 8.00	8	35.6 ± 11.0	1.2	3.1E-01
tyrosine 2	6	416± 125	8	339 ± 143	0.8	3.1E-01
glycolic acid	7	35.4 ± 19.0	8	45.3 ± 20.0	1.3	3.4E-01
D-malic acid	7	1110 ± 313	8	981 ± 228	0.9	3.7E-01
L-pyroglutamic acid	5	2254 ± 1280	5	1620 ± 812	0.7	3.8E-01
L-(+) lactic acid	7	11200± 2450	8	10100 ± 2520	0.9	4.1E-01
urea	7	575± 187	8	663 ± 217	1.2	4.1E-01
L-ornithine 2	7	274± 150	8	216 ± 133	0.8	4.4E-01
Beta- alanine 1	7	296± 144	8	247 ± 86.0	0.8	4.5E-01
L-mimosine 1	7	132 ± 55.0	8	113 ± 34.0	0.9	4.5E-01
citraconic acid 1	6	17.8 ± 10.0	8	21.7 ± 9.00	1.2	4.7E-01
DL-isoleucine 2	7	291± 133	8	340 ± 128	1.2	4.9E-01

picolonic acid	7	18.0 ± 8.00	7	16.7 ± 4.00	0.9	7.1E-01
2-hydroxypyridine	7	345± 91.0	8	360 ± 67.0	1.0	7.4E-01
oleic acid	7	125 ± 67.0	8	135 ± 58.0	1.1	7.5E-01
L-valine 2	7	605± 136	8	631 ± 200	1.0	7.7E-01
glycine	7	17800 ± 3860	8	18500 ± 4520	1.0	7.7E-01
N-acetyl-L-aspartic acid 1	7	555 ± 126	8	568 ± 68.0	1.0	8.1E-01
hypoxanthine 1	7	1370± 303	8	1340 ± 183	1.0	8.3E-01
4-guanidinobutyric acid 2	7	1290 ± 425	8	1260 ± 370	1.0	8.6E-01
fumaric acid	7	64.7 ± 31.0	8	65.3 ± 20.0	1.0	9.6E-01
L-threonine 2	7	1630 ± 357	8	1630 ± 288	1.0	9.9E-01
L-methionine 2	7	153 ± 112	8	153 ± 54.0	1.0	9.9E-01

Table 3.4 Gene expression results for 3.2 $\mu g \cdot L^{-1}$ metformin exposed larvae. An * is used to denote significant changes in gene expression between controls and medaka larvae exposed to metformin ($\alpha = 0.05$). 2 larvae were homogenized per treatment replicate (n = 3 for all groups).

Gene name	Log_2 (fold Δ)	p-value
*β hydroxyacyl-CoA dehydrogenase (*hcd*)	-1.60	5.0E-3
*HMG-CoA synthesis (*hgs*)	-3.25	0.02
glucose-6-phosphate dehydrogenase (*g6p*)	0.41	0.11
stearoyl-CoA desaturase (*scd*)	-0.53	0.29
acetyl-CoA carboxylase – 2 (*ac2*)	-0.09	0.82
elongation of very long chain fatty acids protein 1-like (*elo*)	-0.14	0.84

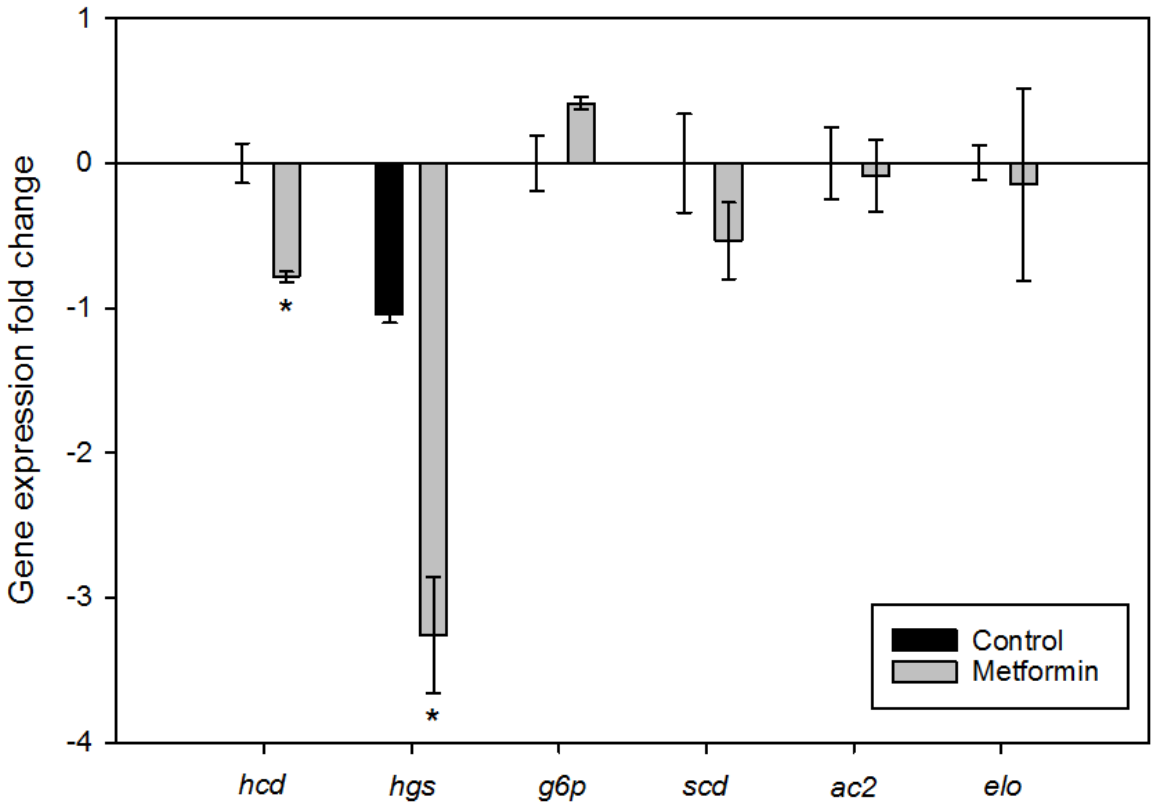


Figure 3.1: Mean fold change (± 1 SE) in gene expression between metformin exposed and control larvae. An asterisk * denotes significantly altered gene expression relative to controls ($p < 0.05$). 2 larvae were homogenized per treatment replicate ($n = 3$ for all groups).

3.4.2 Metabolite and gene expression analysis on larval medaka exposed to guanylurea

Similar to metformin exposed larvae, medaka exposed to 1.0 ng · L^{-1} guanylurea from embryo through 28 days post hatch had significantly altered abundances of various metabolites (T-test, $p < 0.05$, Table 3.5). All 7 altered metabolites in guanylurea treated fish were found to be significantly increased in abundance (T-test, $p < 0.05$, Table 3.5) when compared to control fish. Specifically, the metabolites glucose-6-phosphate, panthothenic acid, putrescine, O-phosphocholamine, adenosine-5-monophosphate, and stearic acid were all shown to be increased when compared to control fish with RF values of 1.4, 1.6, 1.3, 1.1, 1,2, 1.8, and 1,8 respectively. The results of the RT-qPCR analysis on guanylurea exposed medaka larvae (Table 3.6) revealed a significant down-regulation of *ac2* in guanylurea exposed larvae when compared to controls with a $\log_2$ fold change of -0.88 ($DF = 1$, $F = 10.25$, $p < 0.05$). Additionally, a significant down-regulation in *elo* was seen in exposed larvae compared with controls with a fold change of -1.53 ($DF = 1$, $F = 9.84$, $p < 0.05$). However, no significant differences were seen in the expression of the remaining genes of interest (Table 3.6, Figure 3.2).

Table 3.5. Metabolites detected in larvae exposed to 1.0 ng · L^{-1} guanylurea. An * is used to denote significance between control and guanylurea exposed larvae metabolite response factors (relative to IS).

Name	N control	Control Mean ± 1 SD	N Guan	Guan Mean ± 1 SD	Guan/Control (RF value)	P-value
*D-glucose-6-phosphate 1	8	865 ± 161	8	1200 ± 179	1.4	1.5E-03
*pantothenic acid 2	8	227 ± 47.8	8	305 ± 55.3	1.3	9.1E-03
*putrescine	8	274 ± 31.8	8	307 ± 22.0	1.1	3.1E-02
*O-phosphocolamine	8	968 ± 139	8	1160 ± 192	1.2	4.3E-02
*adenosine-5-monophosphate 1	8	192 ± 125	8	347 ± 158	1.8	4.8E-02
*stearic acid	8	1120 ± 176	8	1990 ± 1030	1.8	4.8E-02
2,3-dihydroxypyridine	8	25.4 ± 18.5	8	11.1 ± 3.80	0.4	6.8E-02
palmitic acid	8	754 ± 109	8	1170 ± 546	1.6	6.8E-02
5-aminovaleric acid 1	8	41.1 ± 15.6	8	28.2 ± 11.3	0.7	8.0E-02
guanosine 1	8	145 ± 42.0	8	192 ± 59.3	1.3	8.7E-02
L-methionine 2	8	374 ± 162	5	257 ± 40.5	0.7	8.8E-02
L-histidine 3	8	2690± 655	8	3200 ± 414	1.2	8.8E-02
uric acid 1	6	4060 ± 751	8	5180 ± 1500	1.3	9.4E-02
tyrosine 2	8	779 ± 267	8	581 ± 159	0.7	9.7E-02
N-methyl-DL-glutamic acid 3	8	66.2 ± 9.30	6	75.5 ± 9.70	1.1	1.0E-01
hypotaurine	7	101 ± 57.6	7	58.8 ± 11.4	0.6	1.0E-01
N-acetyl-L-aspartic acid 1	8	602 ± 69.1	8	702 ± 149	1.2	1.2E-01
glycerol 1-phosphate	8	2650 ± 362	8	3050 ± 591	1.2	1.3E-01
D-malic acid	8	1340 ± 193	8	1190 ± 176	0.9	1.4E-01
dihydroxyacetone phosphate 2	8	154 ± 22.4	8	185 ± 50.6	1.2	1.4E-01
methyl-beta-D-galactopyranoside	7	250 ± 147	8	155 ± 72.4	0.6	1.6E-01
L-valine 2	8	717 ± 369	8	514 ± 122	0.7	1.8E-01
iminodiacetic acid 2	7	118 ± 49.7	7	219 ± 174	1.9	1.8E-01
glycine	8	8390 ± 1710	8	10000 ± 2840	1.2	1.9E-01
L-ornithine 2	7	571 ± 212	8	449.0 ± 128	0.8	2.2E-01
urea	8	770 ± 231	5	467 ± 467	0.6	2.3E-01
N-acetyl-D-mannosamine 1	8	80.4 ± 21.3	8	104 ± 48.8	1.3	2.4E-01

allo-inositol	8	2650 ± 416	8	3390 ± 1600	1.3	2.4E-01
L-proline 2	8	566 ± 509	7	331 ± 102	0.6	2.4E-01
aspartic acid 2	8	1660 ± 328	7	2040 ± 733	1.2	2.4E-01
beta-glycerolphosphate	8	19.7 ± 4.40	8	22.8 ± 5.70	1.2	2.4E-01
arachidic acid	7	9.30 ± 5.60	7	13.1 ± 7.20	1.4	3.0E-01
hypoxanthine 1	8	2210 ± 578	8	1930 ± 442	0.9	3.0E-01
3-phosphoglyceric acid	7	387 ± 29.5	8	424 ± 89.5	1.1	3.0E-01
D-lyxose 2	8	5670 ± 1430	8	5060 ± 699	0.9	3.0E-01
isomaltose 1	8	82.9 ± 52.9	8	61.7 ± 18.0	0.7	3.1E-01
4-aminobenzoic acid	5	29.6 ± 4.20	7	33.6 ± 8.60	1.1	3.2E-01
N-acetyl-L-histidine 2	8	1390 ± 634	8	1670 ± 410	1.2	3.3E-01
uridine 5'-monophosphate 2	7	90.3 ± 57.7	8	125 ± 77.9	1.4	3.4E-01
pyruvic acid	8	57.5 ± 14.7	7	63.0 ± 6.30	1.1	3.6E-01
inosine	8	3960 ± 811	8	3580 ± 801	0.9	3.6E-01
N-methylalanine	6	98.8 ± 37.2	8	79.7 ± 38.6	0.8	3.7E-01
tagatose 2	5	76.2 ± 8.20	5	101 ± 53.3	1.3	3.7E-01
trans-4-hydroxy-L-proline 2	8	1100 ± 585	8	854 ± 501	0.8	3.8E-01
D-allose 1	8	3300 ± 693	7	3580 ± 508	1.1	3.9E-01
cholesterol	8	1130 ± 366	8	1350 ± 585	1.2	3.9E-01
picolonic acid	8	14.7 ± 4.50	8	17.2 ± 6.04	1.2	3.9E-01
L-tryptophan 2	6	128 ± 76.5	6	98.7 ± 19.4	0.8	4.0E-01
2-hydroxypyridine	8	145 ± 18.1	7	155 ± 28.2	1.1	4.1E-01
dehydroascorbic acid 4	8	76.2 ± 21.8	6	91.8 ± 40.0	1.2	4.2E-01
xylitol	8	23.7 ± 5.20	8	26.3 ± 7.50	1.1	4.4E-01
1-methyl nicotinamide 1	8	97.7 ± 33.3	8	114 ± 48.4	1.2	4.4E-01
L-threonine 2	8	1790 ± 713	8	1570 ± 374	0.9	4.7E-01
L-cysteine 2	8	155 ± 69.7	8	134 ± 35.1	0.9	4.7E-01
adenine 1	8	27.8 ± 7.50	8	30.2 ± 5.50	1.1	4.8E-01
urea	8	770 ± 231	8	643 ± 431	0.8	4.8E-01
D-ribose-5-phosphate 1	8	161 ± 65.9	8	226 ± 248	1.4	4.9E-01
DL-glyceraldehyde 3-phosphate 2	7	24.7 ± 3.80	8	26.8 ± 7.30	1.1	5.0E-01
cellobiose 2	7	178 ± 156	5	125 ± 104	0.7	5.0E-01
dioctyl phthalate	5	5.30 ± 7.00	7	3.00 ± 2.50	0.6	5.1E-01
ribulose-5-phosphate 3	7	253 ± 84.6	7	280 ± 59.6	1.1	5.1E-01

L-glutamine 2	8	1360 ± 699	6	1190 ± 229	0.9	5.4E-01
phosphoric acid	8	10600 ± 4000	8	11800 ± 4220	1.1	5.7E-01
L-serine 2	8	4650 ± 1720	6	4950 ± 245	1.1	6.4E-01
porphine 1	6	6.50 ± 2.70	6	7.30 ± 3.40	1.1	6.4E-01
4-guanidinobutyric acid 2	8	1360 ± 567	8	1250 ± 328	0.9	6.5E-01
glyceric acid	8	24.6 ± 9.40	6	22.8 ± 5.50	0.9	6.7E-01
oleic acid	7	179 ± 63.2	8	164 ± 75.7	0.9	6.8E-01
Creatinine	8	2900 ± 596	8	2730 ± 1110	0.9	7.1E-01
L-asparagine 2	7	956 ± 459	8	846 ± 820	0.9	7.5E-01
xanthine	8	248 ± 84.5	8	259 ± 37.3	1.0	7.6E-01
maltose 2	5	1640 ± 713	6	1540 ± 394	0.9	7.7E-01
isopropyl beta-D-1-thiogalactopyranoside	8	81.8 ± 27.6	6	77.7 ± 31.2	0.9	8.0E-01
6-phosphogluconic acid	7	107 ± 36.5	7	112 ± 39.6	1.0	8.1E-01
fumaric acid	8	147 ± 32.0	8	142. ± 42.6	1.0	8.2E-01
D-glucose 1	8	9130 ± 4420	8	8730 ± 2080	1.0	8.2E-01
L-(+) lactic acid	8	6880 ± 1160	8	6980 ± 702	1.0	8.3E-01
DL-3-aminoisobutyric acid 2	8	766 ± 187	8	750 ± 156	1.0	8.6E-01
Beta- alanine 1	8	250 ± 24.4	8	248 ± 25.7	1.0	8.8E-01
glycolic acid	7	33.5 ± 29.8	7	35.3 ± 11.7	1.1	8.8E-01
L-mimosine 1	8	73.3 ± 16.9	8	73.4 ± 25.9	1.0	9.9E-01
L-lysine 2	8	2120 ± 714	8	2120 ± 526	1.0	9.9E-01

Table 3.6 Gene expression results for larvae exposed to ng · L^{-1} guanylurea. An * is used to denote significant changes in gene expression between controls and medaka larvae exposed to guanylurea ($\alpha = 0.05$). 2 larvae were homogenized per treatment replicate (n = 3 for all groups).

Gene name	Log_2 (fold Δ)	p-value
*acetyl-CoA carboxylase – 2 (*ac2*)	-0.88	0.03
*elongation of very long chain fatty acids protein 1-like (*elo*)	-1.53	0.04
β hydroxyacyl-CoA dehydrogenase (*hcd*)	-0.27	0.25
stearoyl-CoA desaturase (*scd*)	-0.35	0.43
HMG-CoA synthesis (*hgs*)	0.78	0.52
glucose-6-phosphate dehydrogenase (*g6p*)	0.10	0.71

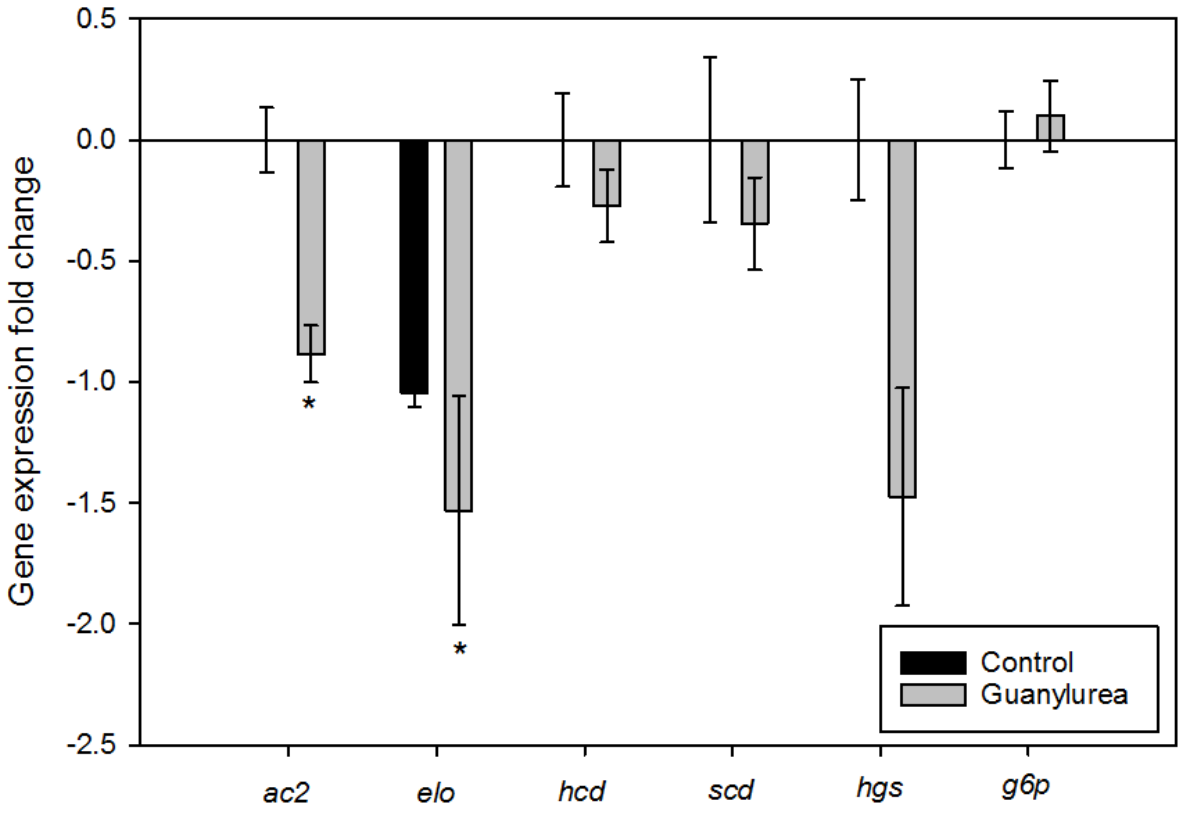


Figure 3.2: Mean fold change (± 1 SE) in gene expression between guanylurea exposed and control larvae. An asterisk * denotes significantly altered gene expression relative to controls ($p < 0.05$). 2 larvae were homogenized per treatment replicate ($n = 3$ for all groups).

3.5 Discussion

Changes in metabolite response factors and gene expression offer information about cellular pathways potentially affected by the presence of aquatic contaminants, such as metformin and its metabolite guanylurea. The diminished size of larval medaka seen in Chapter 2 may be attributed to disruptions in cellular pathways, indicated by the significant alterations seen in specific metabolites, as shown in the present chapter.

One such metabolite, L-lysine, is an essential amino acid and is part of the intermediatory metabolism in the tricarboxylic acid (TCA) cycle (Kokushi *et al.,* 2012). L-lysine is metabolized to produce acetyl-CoA (Khan *et al.,* 2016), which is a necessary molecule entering the TCA cycle, as it is oxidized by the mitochondria for energy for the cell (Reece and Niel, 2011). The decrease seen in L-lysine in metformin exposed fish could be due to this amino acid being converted to ketone bodies. Ketone bodies are typically produced from fatty acids, however the production of ketone bodies by L-lysine has been shown to occur under instances where metabolic conditions may be disrupted (Fasulo *et al.,* 2012) such as what is potentially happening with our metformin exposed larvae, as we see fatty acid dysregulation. Similar to other amino acids, L-lysine plays an important role in building muscle proteins (Xu *et al.,* 2012), thus the deficiency in L-lysine seen in metformin exposed fish could explain the stunted growth reported in Chapter 2. HMG-CoA synthase (*hgs*) catalyzes the formation of HMG-CoA from acetoacetyl-CoA and acetyl-CoA in the sterol biosynthetic pathway (Ercal and Crawford, 2017). Recent studies have demonstrated that *hgs* is regulated by lysine acetylation and succinylation, which increases *hgs* activity (Ercal and Crawford, 2017). A significant down-regulation in *hgs*

expression was seen in metformin exposed larvae when compared to controls, likely caused by the decrease in L-lysine present in the exposed fish. HMG-CoA synthase is important for regulating the flux through the isoprenoid/mevalonate pathway and therefore important in controlling cell growth and the synthesis of cholesterol (Harris *et al.*, 2000), thus altered expression of *hgs* can lead to a dysregulation of such endpoints.

Another explanation for the stunted growth seen in metformin exposed larvae is the significant decrease in the amino acid, L-proline. Arginine is taken up by cells and metabolized into L-proline, which is then used as a precursor to collagen formation (Endo *et al.*, 2003). One of the proposed mechanisms of actions (MOA) of metformin in patients with type-2 diabetes is through the activation of adenosine monophosphate kinase (AMPK), which inhibits the target rapamycin (mTOR) signaling pathway that regulates energy utilization, thus down-regulating gluconeogenesis in the liver (Crago *et al.*, 2016; Goodyear *et al.*, 2001; Kahn *et al.*, 2005; Viollet *et al.*, 2012). The inhibition of mTOR in combination with nutrient stress (such as the dysregulation in lipids and fatty acids as seen in metformin exposed larvae), however, also leads to the induction of proline oxidase (POX) activity (Pandhare *et al.*, 2009), resulting in the metabolism of proline. Proline metabolism is important during times of nutrient stress, as proline is readily available from the breakdown of the extracellular matrix (ECM) with its degradation resulting in the generation of adenosine triphosphate (ATP; Pengfei *et al.*, 2011). The ECM is composed predominantly of collagen, with the major contributor to the production of collagen being proline (Pandhare *et al.*, 2009). The ECM is a highly dynamic structure, and its remodeling is an effective mechanism whereby diverse cellular behaviors such as cell proliferation,

adhesion and migration, cell differentiation, and cell death can be regulated (Pengfei *et al.*, 2011). However, prolonged degradation of the ECM is concerning, as it can lead to abnormal ECM dynamics such as deregulated cell proliferation and invasion, failure of cell death, and loss of cell differentiation (Pandhare *et al.*, 2009). This decrease in L-proline is corroborated by the increase seen in phosphoric acid in metformin exposed larvae when compared to control fish, as phosphoric acid is a direct by product of the degradation of proline (Abdullah *et al.*, 2017).

A common mechanism of action that has been proposed for metformin is through AMPK, as AMPK has also been defined as the upstream kinase for the critical metabolic enzymes acetyl-CoA carboxylase (ACC) and HMG-CoA reductase (HGR), which function in the rate limiting steps for fatty acid and sterol synthesis (Bjorklund *et al.*, 2010; Zagorska *et al.*, 2010; Zhang *et al.*, 1999). In this sense, the activation of AMPK phosphorylates ACC, causing it to be deactivated. When acetyl-CoA carboxylase is active, melanyl-CoA is produced, which is the building block for new fatty acids (Mihaylova and Shaw, 2011). With the proposed activation of AMPK by metformin, AMPK will acutely control lipid metabolism through phosphorylation of ACC (Li *et al.*, 2011) leading to a decrease in the synthesis of fatty acids (Mihaylova and Shaw, 2011). In order to rule this mechanism of action out, we sought to investigate the expression of acetyl-CoA carboxylase-2 (*ac2*) in metformin and guanylurea treated larvae. No difference in the expression of *ac2* was seen between the treatments for metformin, however a significant down-regulation was seen in guanylurea exposed larvae when compared to controls, which could explain the stunted growth seen in guanylurea exposed larvae.

The increase seen in important fatty acids such as stearic and palmitic acid may also help explain the stunted growth seen in metformin and guanylurea exposed fish. Palmitic acid is the most common saturated fatty acid which is elongated to stearic acid (Malgorzata *et al.*, 2017). Stearic acid is a saturated fatty acid that is metabolized to oleic acid via steroyl-CoA desaturase (*scd*), and the ratio of stearic acid to oleic acid has been implicated in the regulation of cell growth and differentiation (Zhang *et al.,* 1999). Although the levels of oleic acid were not high enough to be considered significantly different between the metformin and guanylurea exposed larvae and their respective controls, we sought to rule out this decrease in the stearic acid conversion by investigating the expression of *scd* as the possible mechanism by which stearic acid RFs increased. The RT-qPCR results, however revealed no significant changes in the expression of *scd* between treatments (Tables 3.4 and 3.6). Additionally, elongation of very long chain fatty acid protein like-1 (*elo*) can be found numerous times throughout the fatty acid synthase pathway. The protein *elo* functions as an elongase catalyzing the synthesis of both saturated very long-chain fatty acids (VLCFA) and mono-unsaturated VLCFA (Ofman *et al.,* 2010). Additionally, *elo* knockdown in humans has been shown to reduce the elongation of C22:0 and C26:0 fatty acids (Ofman *et al.,* 2010). With this in mind, we sought to investigate if *elo* expression is altered in metformin and guanylurea exposed fish, which could explain the accumulation of fatty acids, such as palmitic and stearic, seen in exposed larvae. Interestingly, RT-qPCR results revealed no significant changes in the expression of *elo* between metformin exposed and control larvae. However, results show a significant decrease in the expression of *elo* in guanylurea exposed fish. Maintaining proper ratios of

fatty acids is important for cell growth and differentiation, as they participate in cell membrane fluidity and cell signaling (Zhang *et al.,* 1999).

The β-oxidation pathway is an important pathway in which fatty acids are broken down to produce energy (Lopaschuk *et al.,* 2010). Specifically, β-oxidation produces acetyl-CoA, nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FADH2), where the acetyl-CoA produced enters the TCA cycle and the NADH and FADH2 produced are used in the electron transport chain to produce ATP (Schulz, 2008). The NADH produced during β-oxidation is catalyzed by β hydroxyacyl-CoA dehydrogenase (*hcd*), and is thus an important rate limiting step in energy production (Schulz, 2008). Interestingly, no significant difference was seen in the expression of *hcd* in guanylurea treated fish, while a significant decrease was seen in the expression of *hcd* in metformin exposed fish when compared to controls, potentially leading to a decrease in β-oxidation output. This could be the reason we are seeing an increase in the metabolism of L-lysine in metformin exposed fish, as the cell is desperate for acetyl-CoA to enter the TCA cycle.

Pantothenic acid is a component of coenzyme A (CoA) molecule along with adenosine-5-monophosphate (AMP) and cysteine, and is a necessary nutrient that must be obtained through dietary sources (Leonardi and Jackowski, 2007). Pantothenic acid and AMP, in their functions as cofactors for CoA, are necessary for the synthesis of many compounds including fatty acids, cholesterol, steroid hormones, neurotransmitters, amino acids, and are necessary for energy extraction during the β-oxidation of fatty acids and the

oxidation of amino acids (Leonardi and Jackowski, 2007). Pantothenic acid and CoA play a central role in the acylation of proteins with fatty acids, mainly myristic and palmitic acids (Leonardi and Jackowski, 2007). Both pantothenic acid and AMP were found to be significantly increased in guanylurea exposed larvae when compared to controls, potentially in an attempt to increase the biosynthesis of fatty acids.

The pentose phosphate pathway is an important metabolic pathway supplying energy to cells by maintaining the level of the co-enzyme nicotinamide adenine dinucleotide phosphate (NADPH) which is important for tissues involved in the biosynthesis of fatty acids or isoprenoids (Thomas *et al.*, 1991). An important enzyme in this pathway is glucose-6-phosphate dehydrogenase (*g6p*) which reduces NADP+ to NADPH while oxidizing glucose 6-phosphate (Aster *et al.*, 2010). Because of the significant increase in glucose-6-phosphate in guanylurea exposed fish, we sought to investigate whether *g6p* expression was altered, which could assist in the explanation of the increased metabolite. However, no significant difference in the expression of *g6p* was seen between the treatments, thus another explanation for the increased glucose-6-phosphate should be investigated.

The abundance of palmitic acid seen in metformin exposed fish may also explain the increased amount of arachidic acid found in exposed larvae when compared to controls, as palmitic acid is first converted to stearic acid via elongase, which is then converted to oleic acid via steroyl-CoA desaturase, then converted to linoleic acid via Δ12-desaturase (Akoh and Min, 2002). Linoleic acid is then converted to gamma-linolenic acid in the

reaction catalyzed by Δ6-desaturase, then it is elongated to form dihomo-gamma-linolenic acid, which in turn is desaturated in the reaction catalyzed by Δ5-desaturase to form arachidonic acid (Akoh and Min, 2002). Arachidonic acid is the precursor of many eicosanoids such as prostaglandins, thromboxanes, and leukotrienes, however it can also be hydrogenated to arachidic acid (Akoh and Min, 2002). Thus, the increased levels of arachidic acid seen in metformin exposed fish might be due to a rapid increase in plasma arachidonic acid levels (Valeem, 2012). The hydrogenation of arachidonic acid inhibits the production of arachidonic acid related eicosanoids such as prostaglandins, thromboxanes, and leukotrienes (Akoh and Min, 2002; Ruan *et al.,* 2010; Yui *et al.,* 2016) potentially leading to the growth retardation seen in metformin and guanylurea exposed larvae.

Putrescine is the simplest of the polyamines, serving as a precursor to all higher PAs and is found in all eukaryotes (Bridges *et al.,* 2018). An early response to central nervous system (CNS) injury is the induction of PA metabolism, resulting in increased levels of putrescine (Adibhatla *et al.,* 2002; Muller *et al.,* 2007), which we see in guanylurea exposed larvae. O-phosphocolamine was also found to be significantly increased in guanylurea exposed larvae when compared to controls. O-phosphocolamine is an ethanolamine derivative that is used to construct the phospholipids, glycerophospholipid and sphingomyelin (Toda *et al.,* 2017). Sphingomyelin is an important sphingolipid which are of great importance in the nervous system, playing a role in cell signaling cascades which mediate cellular apoptosis, proliferation, stress response, necrosis, and differentiation (Bridges *et al.,* 2018; Perry and Hannun, 1998). The increase in both putrescine and O-phosphocolamine is indicative of potential CNS damage, however

a more targeted investigative approach such as whole brain metabolomics could better investigate the potential neurotoxic behavior of guanylurea. Interestingly, and what should be of great concern is that FHM larvae exposed via maternal transfer to MeHg, a compound known to cause CNS damage, showed a similar metabolic profile with the elevation of putrescine and O-phosphocolamine (Bridges *et al.*, 2018).

The significant increase in methyl-nicatinamide (MNA) seen in metformin exposed larvae can also be attributed to the reduction in size of the metformin exposed medaka seen in Chapter 2. An increase in MNA indicates peroxisome proliferation related to inflammation, obesity, and metabolic syndrome, and has been shown to be a byproduct which is formed during the conversion of S-adenosylmethionine (SAMe) to S-adenosylhomocysteine (SAH) (Tyagi *et al.*, 2014). The SAH produced is utilized in the trans-sulfuration pathway to regenerate glutathione (GSH; Tyagi *et al.*, 2014). It has been suggested that the increase in MNA found in organisms might be due to the upregulation of enzymes involved in the regeneration of GSH via SAH and other intermediates which results in increased produced of GSH and its utilization due to more production of reactive oxygen species (ROS) to cope with potential oxidative stress (Tyagi *et al.*, 2014).

β-aminoisobutyric acid (BAIBA) is a small molecule metabolite, which has been recently identified as potential novel myokine and has been inversely associated with cardiometabolic risk including fasting glucose and insulin (Roberts *et al.*, 2014). BAIBA induces white fat browning, with its plasma level increasing with exercise, whereas the low plasma concentration is associated with the metabolic risk factors such as high cholesterol, triacylglycerol and body mass index (Roberts *et al.*, 2014). The decrease in BAIBA seen

in metformin exposed larvae could be a result of the lack of movement of the exposed fish due to the lipid/amino acid dysregulation potentially leading to a disruption of energy stores, and thus a lack a movement in the energy deprived fish.

Overall, these findings demonstrate the need for even further research into the biochemical effects of metformin and its metabolite guanylurea on non-target aquatic organisms, such as fish. It is apparent from the research presented thus far that the environmentally relevant concentrations of both compounds is able to cause detectable changes in the metabolome of exposed Japanese medaka, as well as changes in expression of associated genes. From this research, it is reasonable to conclude that current concentrations of both compounds in surface waters may lead to adverse consequences for individual fitness, likely leading to reduced survival of wild larval fish.

Chapter 4: Effects of metformin and guanylurea on the full life-cycle of Japanese medaka (*Oryzias latipes*)

4.1 Abstract

While fish ELS studies generate a multitude of valuable data, they are not reliable in determining how emerging/novel compounds affect the full life-cycle of fish. Such studies help to determine how compounds, such as metformin and guanylurea, affect aquatic organisms over all developmental and reproductive stages. Japanese medaka (*Oryzias latipes*) were chronically exposed to environmentally relevant concentrations of metformin and guanylurea alone and in mixture from embryo through sexual maturity (165 days). The growth, steroid hormone and vitellogenin production, and reproductive output were examined. While metformin and guanylurea treatment alone, and in combination, did not significantly stunt the growth of medaka, exposure to the compounds did significantly alter the production of important sex steroid hormones in both male and female medaka. However, exposure to metformin and guanylurea alone, or in mixture, did not affect the production of the egg yolk precursor protein, vitellogenin, in male medaka. Interestingly, no significant effects on reproductive output were seen in the present study. To our knowledge, this research is the first to study the exposure effects of environmentally relevant concentrations of metformin and guanylurea alone and in combination over the full life-cycle of Japanese medaka.

4.2 Introduction

Chapter 2 demonstrated that exposure to low concentrations of metformin and guanylurea caused growth retardation in larval Japanese medaka (*Oryzias latipes*), while Chapter 3 outlined the potential mechanisms of action with which metformin and guanylurea elicits the aforementioned growth effects. Development, especially early development, is a sensitive period in the life-cycle of an organism, which includes (but is not limited to) important pathways for sexual differentiation (Johns *et al.,* 2011). While it is too early to show sexual differentiation and reproductive effects in 28 day old medaka, the dysregulation of arachidic acid seen in metformin treated fish (*3.4.1 Metabolite and gene expression analysis on larval medaka exposed to metformin*) indicates that the hormonal processes of adult medaka could potentially be effected.

In short, the therapeutic path of metformin in patients with type-2 diabetes and PCOS is to act on metabolic pathways to promote catabolism and glucose uptake, causing a sensitization of cells to insulin, thereby affecting pathways which are regulated by insulin, such as steroidogenic pathways (Niemuth *et al.,* 2015). Metformin has been shown to affect the expression of cytochrome p450 11A and 3β-hydroxysteroid dehydrogenase and the cholesterol transporter steroid acute regulatory protein, all steroidogenic enzymes, in mammals at concentrations ranging from 10 mg/kg/d to 250 mg/kg/d (Viollet *et al.*, 2012). Metformin exposure (40 $\mu g \cdot L^{-1}$) caused a more than 30-fold overexpression of the egg yolk precursor, vitellogenin (VTG) mRNA, with a significant induction of tests-ova in male FHM (Niemuth *et al.*, 2015; Niemuth and Klaper, 2016). Additionally, FHM mating pairs exposed to metformin from 30 days post hatch until adulthood, produced significantly

fewer egg clutches over time when compared to control mating pairs (Niemuth *et al.*, 2015). At this time, no experiment found in the literature has investigated the effects of both metformin and guanylurea on the full life-cycle of fish, being sure to capture the sensitive early life-stage from embryo to 28 days. To the best of our knowledge, the only experiment in the literature started experimental treatments at 30 days post hatch (Niemuth and Klaper, 2016). Additionally, the effects of guanylurea exposure from any window of development through adulthood have yet to be determined. Furthermore, reports on the effects of metformin and its metabolite guanylurea on the gonadosomatic index (GSI) and hepatosomatic index (HIS) index of exposed fish have not been shown. GSI and HSI are common analyses that can offer the information on the general health of aquatic organisms. Altered GSI and HSI are common endpoints that can offer the information on the general health of aquatic organisms (Environmental Protection Department of Hong Kong, 2005).

The work presented thus far in the dissertation, combined with the limited research found in the literature, demonstrate the need to investigate the effects of both metformin and its metabolite guanylurea on the full life cycle of fish. Full life-cycle experiments are important to aid in assessing both lethal and sublethal responses to aquatic contaminants, as they follow fish from the egg stage through all stages of development (Parrott and Metcalfe, 2017). This chapter aimed to address a number of gaps in the literature by investigating the exposure effects of metformin and guanylurea on the full life cycle (embryo through sexual maturity), with specific aims targeted at growth, steroid hormone production, vitellogenin production in males, and reproductive output of Japanese medaka.

4.2.1 Objectives

The objectives of this experiment were to:

- Assess the growth effects (length, wet weight, and condition factor) of metformin and guanylurea alone, and in combination, on the full life-cycle of Japanese medaka.
- Assess the gonadosomatic and hepatosomatic index effects of metformin and guanylurea alone, and in combinations, on the full life-cycle of Japanese medaka.
- Determine if Japanese medaka exposed to metformin and guanylurea alone, and in combination, cause altered production of the steroid hormones: estradiol and 11-keto Testosterone.
- Determine if male Japanese medaka exposed to metformin and guanylurea alone, and in combination, cause altered concentration of the protein: vitellogenin.
- Assess the reproductive (daily egg output) effects of Japanese medaka exposed to metformin and guanylurea alone, and in combination.

4.2.2 Hypotheses

H_{o1}: Exposure of adult medaka to metformin and guanylurea alone, and in combination, will not cause a significant difference in the growth (length, wet weight, and condition factor) when compared to control fish.

H_{o2}: Exposure of adult medaka to metformin and guanylurea alone, and in combination, will not cause a significant effect in the gonadosomatic and hepatosomatic index when compared to control fish.

H_{o3}: Steroid hormone production in adult medaka exposed to metformin and guanylurea alone, and in combination, will not be significantly altered when compared to control fish.

H_{o4}: Vitellogenin concentration in adult male medaka exposed to metformin and guanylurea alone, and in combination, will not be significantly different when compared to control fish.

H_{o5}: Exposure to metformin and guanylurea alone, and in combination, will not affect the reproductive (daily egg output) success of medaka when compared to control fish.

4.3 Materials and methods

4.3.1 Chemicals and analysis kits

Metformin hydrochloride (1,1-dimethylbiguanideine hydrochloride; CAS# 1115-70-4) was purchased from Toronto Research Chemicals (Toronto, Ontario, Canada). Guanylurea (CAS# 141-83-3) was purchased from Sigma-Aldrich (Oakville, Ontario, Canada) and methanol (CAS# 67-56-1) from ACP Chemicals Inc. (Montreal, Quebec, Canada). Formic acid (CAS# F0507) and sulfuric acid (CAS# 339741) was purchased from Sigma Aldrich (St. Louis, Missouri, United States). Cayman Chemical Estradiol (item # 58221) and 11-keto Testosterone (item # 582751) ELISA kits were purchased from Cayman Chemical (Ann Arbor, Michigan, United States). Medaka vitellogenin ELISA kit (product # V01013403), produced by Biosence Laboratories, and was also purchased from Cayman Chemical.

4.3.2 Animal care

The culturing and housing of Japanese medaka was performed in the same manner as described in *2.3.2 Animal care*.

4.3.3 Metformin and guanylurea exposure assays

Medaka embryos were collected as previously described in *2.3.2 Animal care*. Embryos were once again separated by sex 118 h post-fertilization and were then kept separate until fish were arranged into respective exposure breeding harems. Viable embryos were randomly assigned to a waterborne treatment containing either 1.0 ng $\cdot$ L^{-1} guanylurea, 7.5 µg $\cdot$ L^{-1} guanylurea, 3.2 µg $\cdot$ L^{-1} metformin, a mixture (3.2 µg $\cdot$ L^{-1} metformin + 7.5 µg $\cdot$ L^{-1} guanylurea), or a control treatment. Embryos were maintained in

plastic petri dishes (Fisher Scientific, 60x15 mm), each containing 20-mL of the treatment solution. Four replicates per treatment (per sex) were used, with each replicate containing 30 embryos. Exposure solutions were renewed daily, and were prepared using a 100,000 $\mu g \cdot L^{-1}$ metformin or guanylurea master stock. The master stock did not exceed metformin or guanylurea's solubility limits in water, and therefore no solvent was needed for the life-cycle studies.

All embryos were exposed to their assigned treatment solutions in a temperature controlled room (27 ˚C) and a 16:8 (light: dark) photoperiod with 0.5 hours each of dawn and dusk included in the light phase. Embryos were monitored under a dissecting stereomicroscope LEICA EZ4D (20x magnification) daily, using the developmental staging methods described by Iwamatsu (2004) and Wakamatsu (2003). Occurrence of developmental abnormalities, mortality, hatch success, and time-to-hatch were recorded daily.

Upon hatch, larval medaka were transferred to 1-L plastic trays corresponding to their exposure concentration and replicate number for 30 days before being transferred to 10-L tanks each containing 10 fish in breeding ratios of two males to three females, still in replicates of four. Extra fish were euthanized in water containing tricaine methane-sulphonate ($200 mg \cdot L^{-1}$), buffered with sodium bicarbonate ($400 mg \cdot L^{-1}$) and flash frozen in liquid nitrogen. Fish were to be left in breeding replicates until age of successful breeding, however medaka males in control tanks began exhibiting aggressive behavior, some wounding each other to the point of mortality. Interestingly, metformin and guanylurea exposed fish were not exhibiting as much aggressive behavior, which could be

a ‘side-effect’ of compound exposure. At this point, the four replicates were mixed and divided into three large replicates and housed in 70-L tanks filled to a volume of 45-L. Medaka were housed in the 70-L tanks in their treatments (3 replicates/treatment) for the remainder of the study. Target waterborne concentrations of metformin (3.2 µg · L^{-1}), guanylurea (1.0 ng · L^{-1} and 7.5 µg · L^{-1}), and the mixture treatment (3.2 µg · L^{-1} metformin + 7.5 µg · L^{-1} guanylurea) were achieved using a Watson-Marlow 200 Series 16-channel peristaltic pump, which delivered stock concentrations from 1-L brown bottles in the appropriate volumes. Stocks of both metformin and guanylurea were prepared in clean water from a 100,000 µg· L^{-1} master stock prepared in DI water. Exposure solution volume exchange insured ~95% molecular turnover in 24 hours in each exposure tank. Fish were fed 15 mL of Premium Grade Brine Shrimp (Brine Shrimp Direct, San Francisco, California) twice per day for the first 28-days of the exposure, with the volume of brine increasing by 5 mL every other week until total brine volume reached 80 mL, which was held constant for the reminder of the exposure.

4.3.4 Biological endpoints

Once egg production in each control tank reached steady state (roughly 10 eggs/reproductive female/day) eggs were collected and counted for a total of 10 days. Eggs were collected from each replicate tank by gently stripping eggs from each gravid female with a gloved finger and placed into 50 mL of corresponding treatment solution and transported to the temperature control room to be counted. Debris was removed from embryos by gently rolling them on a clean paper towel. Embryos from each replicate were

kept in separate in their corresponding exposure solutions. Eggs from each treatment replicate were counted for 10 consecutive days.

At the close of the study (165 days), the remaining fish from the study were collected and euthanized in water containing tricaine methane-sulphonate (200 mg · L^{-1}), buffered with sodium bicarbonate (400 mg · L^{-1}). Individual fish length was measured using a digital caliper, and wet weight was measured using a Metler Toledo balance (AB204-S). Fish were dissected for liver and gonads. Each male and female liver was weight using a Metler Toledo balance (AB204-S), flash frozen and stored for steroid hormone and VTG (male only) analysis. Additionally, female gonads were weighed, flash frozen, and stored for future analyses. Condition factor was calculated via the following medaka established equation, weight (g) x 10^5/length $(mm)^3$ (Foran *et al.,* 2003). Hepatosomatic index was calculated using the equation, liver weight (mg)/total body wet weight (mg). Gonadosomatic index was calculated using the equation, gonad weight (mg)/total body wet weight (mg).

4.3.5 Steroid analysis

4.3.5a Steroid extraction

One liver was used for per sample for female steroid analysis, while 2 livers were pooled per male sample (n = 3) per treatment. Livers were homogenized using a hand held homogenizer in 400 µL of phosphate buffered saline (PBS) in a 1.5 mL microcentrifuge tube. Once homogenized, samples were centrifuged at 10,000 *g* for 10 minutes. After centrifugation, the supernatant was collected into a new 1.5 mL tube. 600 µL of methanol was added to the supernatant, then samples were centrifuged at 5000 *g* for 5 minutes. The

supernatant was transferred to a fresh 2 mL microcentrifuge tube, 1 mL of DI water with 1% formic acid was added, then samples were vortexed.

Solid phase extraction (SPE, Oasis® HLB 3cc (60 mg)) cartridges arranged on a vacuum manifold were conditioned using 2 mL of methanol (this methanol is discarded as waste), followed by an equilibration step with 2 mL of DI water with 0.1% formic acid, again discarded as waste. Samples were then loaded into the SPE cartridges, followed by a second wash with 2 mL of DI water with 0.1% formic acid, then a final was with 2 mL of 1:1 methanol:DI water with 0.1% formic acid; discarded as waste. Clean and labeled 1.5 mL microcentrifuge tubes were now placed under each SPE cartridge. Cartridges were eluted with 500 µL of methanol, which is collected into the clean 1.5 mL tube. Samples were evaporated in a 65˚C bead bath in a fume hood over-night. Once evaporated, samples were reconstituted with 500 µL of ELISA buffer (made up from Cayman Chemical kits).

4.3.5b Analysis of steroids using ELISA kits

Estradiol, 11KT, and VTG kits were used according to the manufacturer's guidelines. Briefly, an 8-point standard curve was prepared separately for the estradiol and 11KT assays, while an 11-point standard curve was used for VTG assays. A standard curve with a r^2 of 0.99 or greater was deemed acceptable for use in the assay. Three biological replicates with three technical replicates each were run per treatment, per sex. An example of plate setup for estradiol and 11kT plates can be found in Figure 4.1, with an example of VTG plate setup found in Figure 4.2. Once all necessary reagents were added to ELISA plates, the plates were incubated. Estradiol ELISA plates were incubated for one hour on an orbital shaker, set at low speed, at room temperature (20-25˚C). After incubation,

estradiol plates were rinsed 5 times with Wash Buffer, then 200 µL of Ellman's Reagent was added to each well. Finally 5 µL of tracer was added to the TA well, then plates were covered with plastic film, wrapped in foil, and allowed to develop for 90 minutes using an orbital shaker, set at low speed. For the 11KT ELISA assays, after all reagents were added, the plate was incubated at 4˚C for 18 hours. After the 18 hours, the wells were emptied and rinsed 5 times with Wash Buffer, then 200 µL of Ellman's Reagent was added to each well. Finally 5 µL of tracer was added to the TA well, then plates were covered with plastic film, wrapped in foil, and allowed to develop for 120 minutes using an orbital shaker. For both the estradiol and 11KT assays, once development of the plate was finished, the bottom of the plate was wiped off using a Kimwipe®, the plastic film was carefully removed from the plate, and the plate was read on a Synergy™ HT Multi-Detection Microplate Reader by Bio-Tek®, at a wavelength of 412 nm. Estradiol and 11KT analysis was performed using Cayman Chemical data reduction software from the Cayman Chemical website.

For the VTG analysis, only male samples were run to determine if metformin and guanylurea caused endocrine disrupting effects in male medaka. After all reagents were added to the plate (pictured in Figure 4.2), plates were sealed with plastic film and incubated at room temperature for 1 hour. After incubation, wells were emptied and washed three times with Wash Buffer (from VTG kits), then 100 µL of diluted detecting antibody was added to all wells. Plates were again sealed with plastic film and allowed to incubate at room temperature for 1 hour. To develop plates, the plastic film was removed and plates were washed five times with Wash Buffer, then 100 µL of TMB Substrate (from VTG kits) was added to each well. Plates were wrapped in foil and incubated at room temperature for

an additional 15 minutes. After 15 minutes, the reaction was stopped by adding 100 µL of 0.3M H_2SO_4 to each well. Once the reaction was stopped, the bottom of the plate was wiped off using a Kimwipe®, the plastic film was carefully removed from the plate, and the plate was read on a Synergy™ HT Multi-Detection Microplate Reader by Bio-Tek®, at a wavelength of 450 nm. VTG results were analyzed by first calculating the NSB-corrected absorbance by subtracting the mean of the NSB wells from each of the samples, including the standard curve. The absorbance values for the standards were plotted against the VTG concentration, and a regression analysis was performed by a linear curve fit, where an r^2 value of 0.99 or higher was deemed acceptable. The mean of the NSB-corrected absorbance values for each set of sample duplicates was calculated. Here, only samples that fall within the standard curve working range were used. The mean NSB-corrected absorbance values were divided by the weight of each liver to get the amount of VTG in ng/mg of liver. Directions for equations can be found in section *K. Calculation of Results* on page 11 of the Medaka VTG ELISA Kit pamphlet.

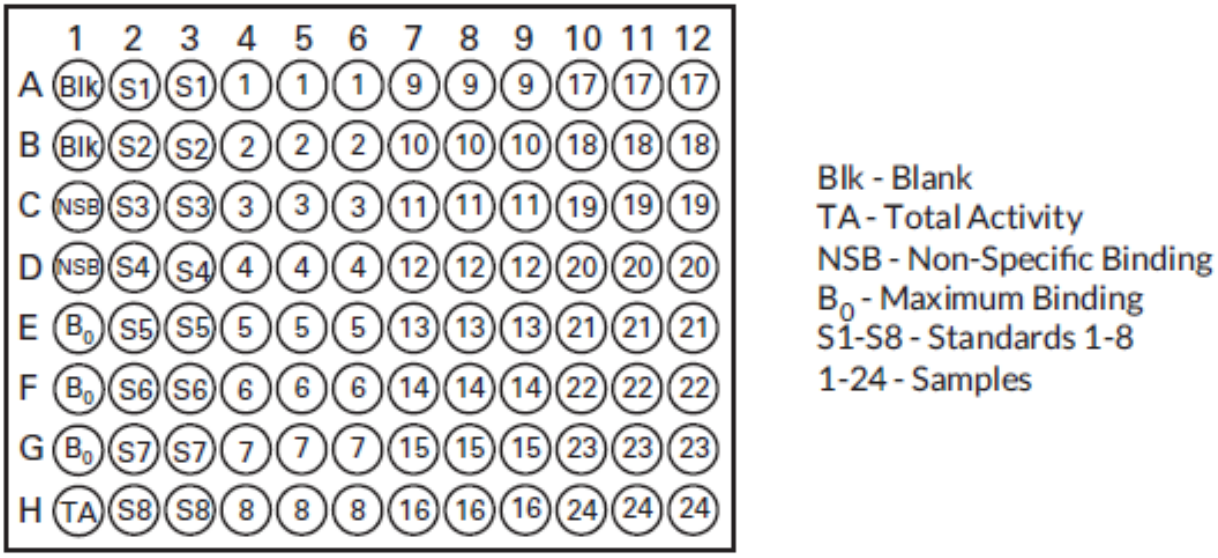


Figure 4.1: Estradiol and 11keto-Testosterone example plate setup from Cayman Chemical Estradiol ELISA online protocol. (https://www.caymanchem.com/product/582251).

	1	2	3	4	5	6	7	8	9	10	11	12
A	NSB	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11
B	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
C	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12
D	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
E	P13	P14	P15	P16	P17	P18	P19	P20	P21	P22	P23	P24
F	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
G	P25	P26	P27	P28	P29	P30	P31	P32	P33	P34	P35	P36
H	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓

NSB= NON-SPECIFIC BINDING WELLS

S1-S11 = STANDARDS 1-11 (0.05-50 NG/ML VTG)
P1-P36 = SAMPLES

Figure 4.2: Vitellogenin example plate setup from Cayman Chemical Vitellogenin ELISA online protocol (https://www.caymanchem.com/product/10009223).

4.3.6 Statistical analysis

All data were analyzed using SigmaPlot (Systat Software Inc.). Normality of all data was confirmed using a Shapiro-Wilk test prior to subsequent statistical analysis. Treatment effects on growth (length, wet weight, condition factor, HSI, and GSI), and steroid expression in livers of treated and untreated males and females were determined using a 1-way ANOVA, followed by a Dunnett *post-hoc* test. Average growth parameters were used to generate a replicate mean, and replicate means were further averaged to generate treatment means to give an n of 3. Cumulative egg output data was first normalized based on reproductive females, then a one-way analysis of covariance (ANCOVA) followed by a Bonferroni *post hoc* analysis to determine significance. An α of 0.05 was used to determine statistical significance for all tests. Determination of waterborne metformin and guanylurea concentrations in the life-cycle study were again performed at the Water Quality Center at Trent University (Peterborough, Ontario, CA) in the same manner mentioned in *2.3.2d Metformin determination*. Measured waterborne concentrations are shown in Table 4.1. Because levels of 1.0 $ng \cdot L^{-1}$ guanylurea are below the machine's limit of detection, contents of the brown stock bottle were analyzed for concentration which have a nominal concentration of 1.0 $\mu g \cdot L^{-1}$ guanylurea.

Table 4.1 Nominal and mean concentrations (± standard error) of metformin and guanylurea as measured by liquid chromatography-mass spectrometry. Mixture = 3.2 µg·L^{-1} metformin $_+$ 7.5 µg·L^{-1} guanylurea.

Nominal concentration µg·L^{-1}	Mean measured metformin (µg·L^{-1}; SE)	Mean measured guanylurea (µg·L^{-1}; SE)
Control	< 0.25	<0.25
0.001 Guanylurea	< 0.25	1.25 (0.115)*(stock solution)
7.5 Guanylurea	< 0.25	6.85 (0.239)
3.2 Metformin	3.22 (0.237)	< 0.25
Mixture	3.27 (0.261)	6.69 (0.341)

4.4 Results

4.4.1 Percent mortality, hatch success, and time-to-hatch

There was no significant difference observed in percent mortality (ANOVA DF = 4, $F = 1.20$, $p > 0.05$), hatch success (ANOVA DF = 4, $F = 0.64$, $p > 0.05$), or time-to-hatch (ANOVA DF = 4, $F = 1.66$, $p > 0.05$) in medaka embryos exposed to any of the experimental treatments, relative to controls (Table 4.2). Similarly, no developmental abnormalities were seen in any control or exposed embryos.

Table 4.2 Percent larval mortality, percent hatch success, and mean time to hatch (± standard mean) for medaka exposed to metformin and guanylurea for 165 days. Mixture = 3.2 µg·L^{-1} metformin + 7.5 µg·L^{-1} guanylurea.

Concentration	Larval mortality (%,±SE)	Hatch success (%, ±SE)	Time to hatch (days, ±SE)
Control	15.0 (0.180)	86.9 (0.840)	8.89 (0.042)
1.0 ng ·L^{-1} guanylurea	12.5 (0.338)	85.0 (0.926)	8.77 (0.043)
7.5 µg·L^{-1} guanylurea	12.7 (0.236)	86.2 (1.40)	8.77 (0.044)
3.2 µg·L^{-1} metformin	15.0 (0.077)	91.3 (0.596)	8.75 (0.043)
Mixture	11.7 (0.310)	88.3 (0.964)	8.81 (0.044)

4.4.2 Size and condition factor

No significant growth differences were seen in male Japanese medaka exposed to any treatment concentration compared to control males (weight: DF = 4, F = 1.81, p = 0.204, Figure 4.3A; length: DF = 4, F = 2.75, p = 0.088, Figure 4.3B; condition factor: DF = 4, F = 0.681, p = 0.621, Figure 4.4). Male medaka mean weight, length, and condition factor can be found in Table 4.3. Similarly, no significant difference in female medaka growth was seen when compared to control females (weight: DF = 4, F = 1.76, p = 0.213, Figure 4.5A; length: DF = 4, F = 1.53, p = 0.266, Figure 4.5B; condition factor: DF = 4, F = 0.751, p = 0.581, Figure 4.6). Mean weight, length, and condition factor for female medaka can be found in Table 4.4.

Table 4.3: Mean weight, length, and condition factor (K) for adult male medaka exposure to metformin and guanylurea for 165 days. Mixture = 3.2 µg· L^{-1} metformin + 7.5 µg · L^{-1} guanylurea (n = 3).

Concentration	Mean weight (mg, ±SE)	Mean length (mm, ±SE)	Mean K (±SE)
Control	412.0 (26.7)	33.5 (0.454)	1.08 (0.029)
1.0 ng ·L^{-1} guanylurea	389.0 (8.71)	33.3 (0.225)	1.04 (0.006)
7.5 µg·L^{-1} guanylurea	375.0 (6.21)	32.7 (0.270)	1.07 (0.014)
3.2 µg·L^{-1} metformin	364.0 (14.8)	32.5 (0.190)	1.08 (0.011)
Mixture	368.0 (9.82)	32.5 (0.304)	1.06 (0.021)

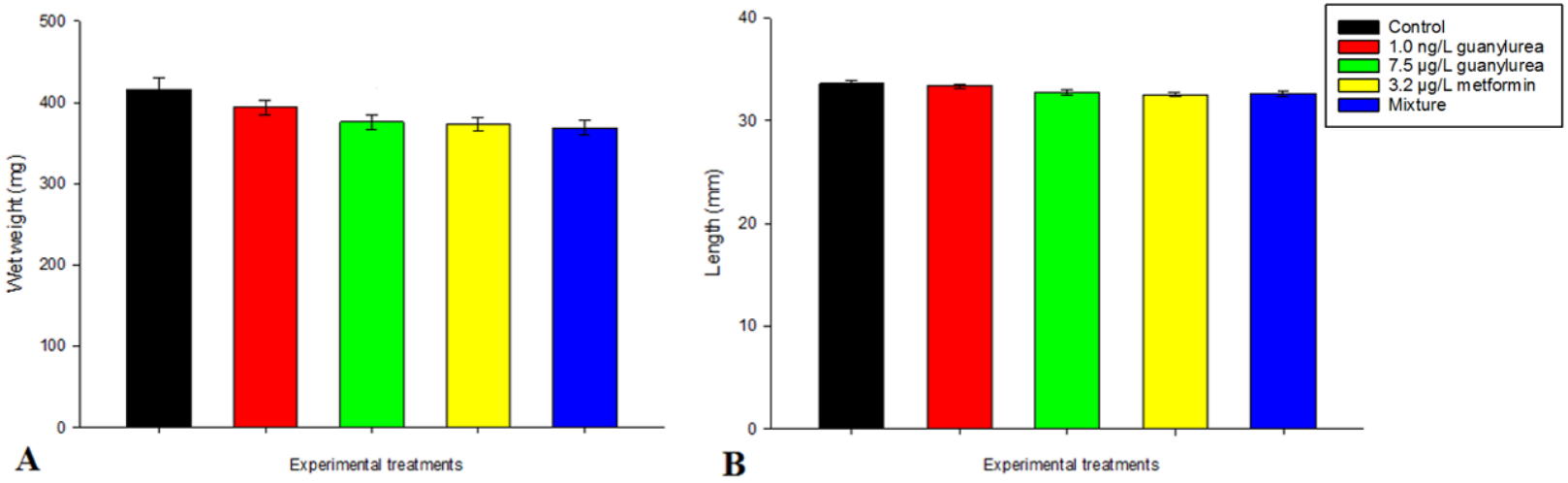


Figure 4.3: Mean male wet weight (A) and length (B) of 165 day old adult Japanese medaka (± SE) by treatment concentration (n = 3). Mixture = 3.2 µg· L^{-1} metformin + 7.5 µg · L^{-1} guanylurea.

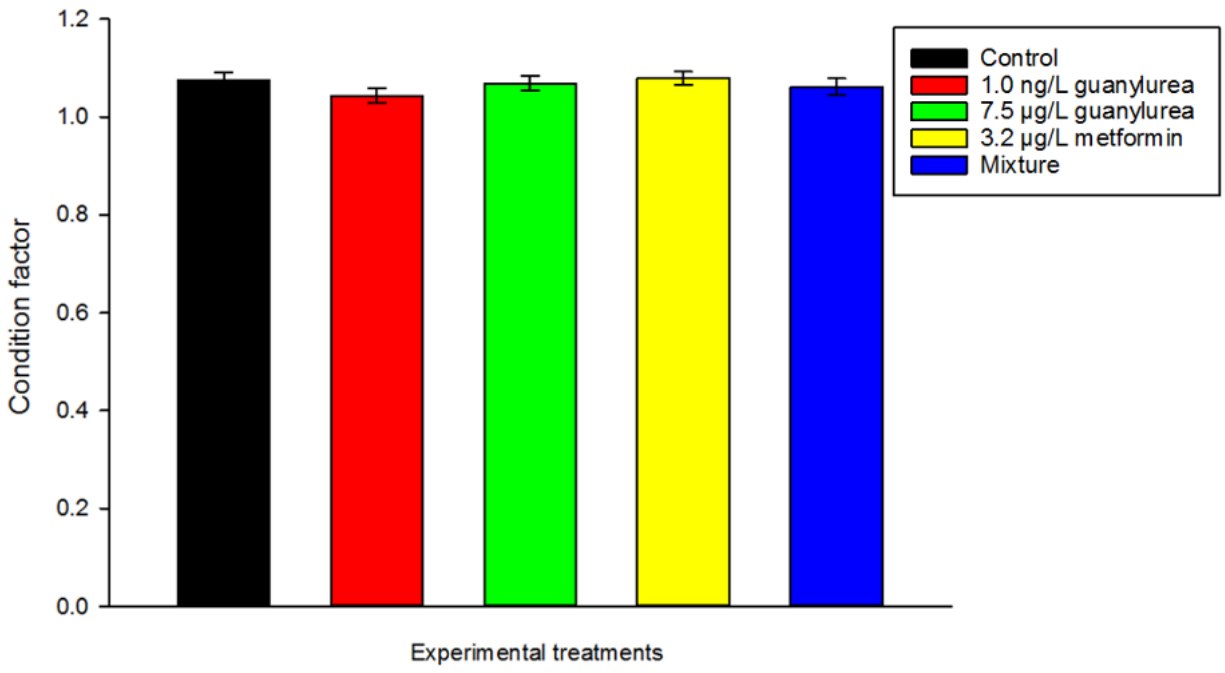


Figure 4.4: Mean condition factor of 165 day old adult Japanese medaka (± SE) by treatment concentration (n = 3). Mixture = 3.2 µg· L^{-1} metformin + 7.5 µg · L^{-1} guanylurea.

Table 4.4: Mean weight, length, and condition factor (K) for adult female medaka exposure to metformin and guanylurea for 165 days. Mixture = 3.2 µg· L^{-1} metformin + 7.5 µg · L^{-1} guanylurea (n = 3).

Concentration	Mean weight (mg, ±SE)	Mean length (mm, ±SE)	Mean K (±SE)
Control	449.0 (5.31)	33.9 (0.398)	1.16 (0.022)
1.0 ng ·L^{-1} guanylurea	472.0 (12.0)	34.6 (0.124)	1.14 (0.021)
7.5 µg·L^{-1} guanylurea	415.0 (10.8)	33.2 (0.312)	1.14 (0.012)
3.2 µg·L^{-1} metformin	411.0 (25.5)	33.6 (0.647)	1.08 (0.052)
Mixture	427.0 (30.2)	33.7 (0.412)	1.13 (0.041)

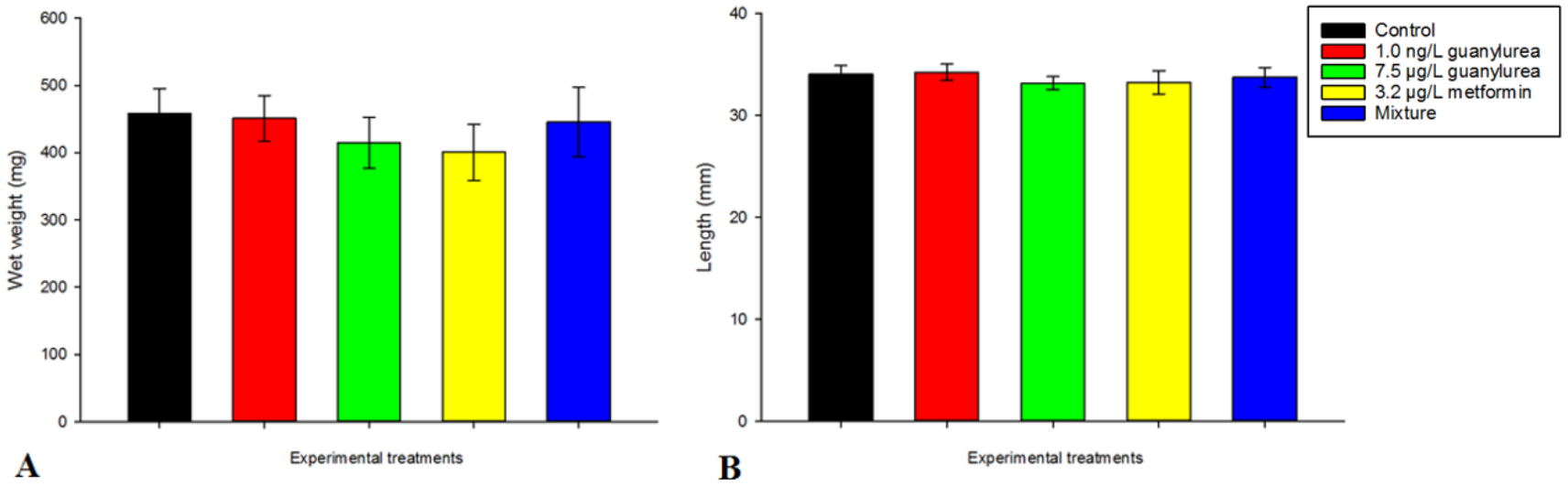


Figure 4.5: Mean female wet weight (A) and length (B) of 165 day old adult Japanese medaka (± SE) by treatment concentration (n = 3). Mixture = 3.2 µg· L^{-1} metformin + 7.5 µg · L^{-1} guanylurea.

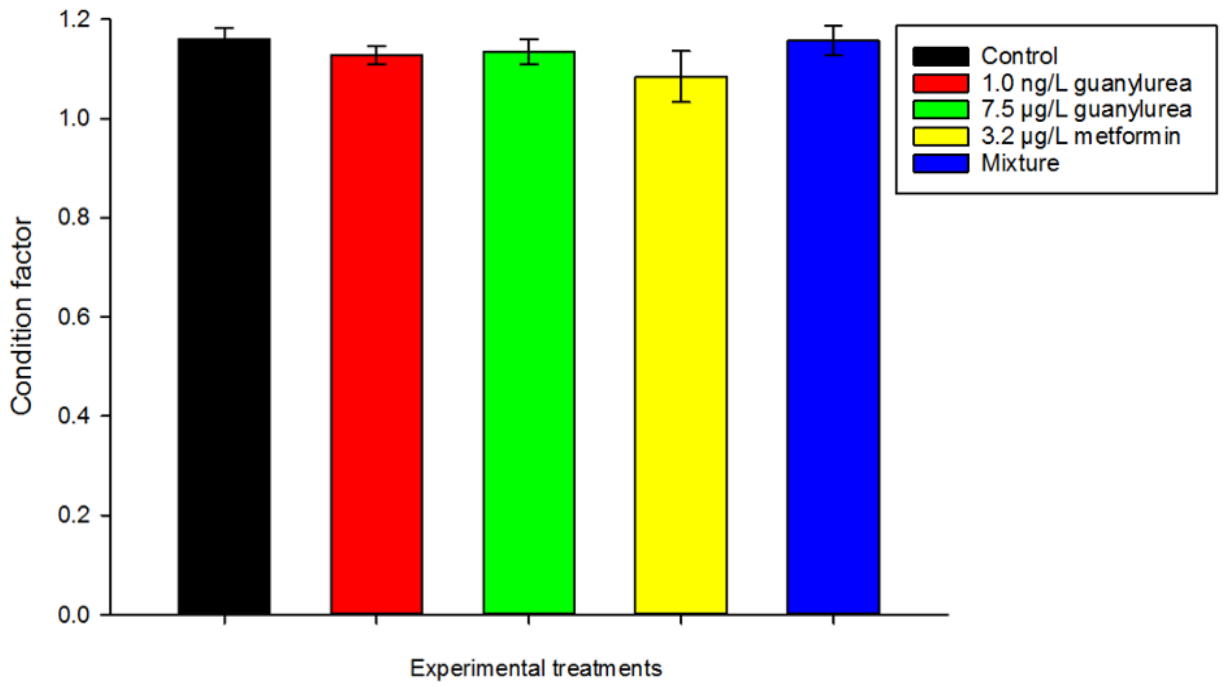


Figure 4.6: Mean female condition factor of 165 day old adult Japanese medaka (± SE) by treatment concentration (n = 3). Mixture = 3.2 µg· L^{-1} metformin + 7.5 µg · L^{-1} guanylurea.

4.4.3 Hepatosomatic index and gonadosomatic index

No significant effects of any treatment on male fish HSI was observed (DF = 4, F = 1.86, p = 0.197, Figure 4.7). Similarly, no significant effects of exposure treatments were seen on female HSI (DF = 4, F = 0.781, p = 0.563, Figure 4.8A) or GSI (DF = 4, F = 2.39, p = 0.120, Figure 4.8B) were observed.

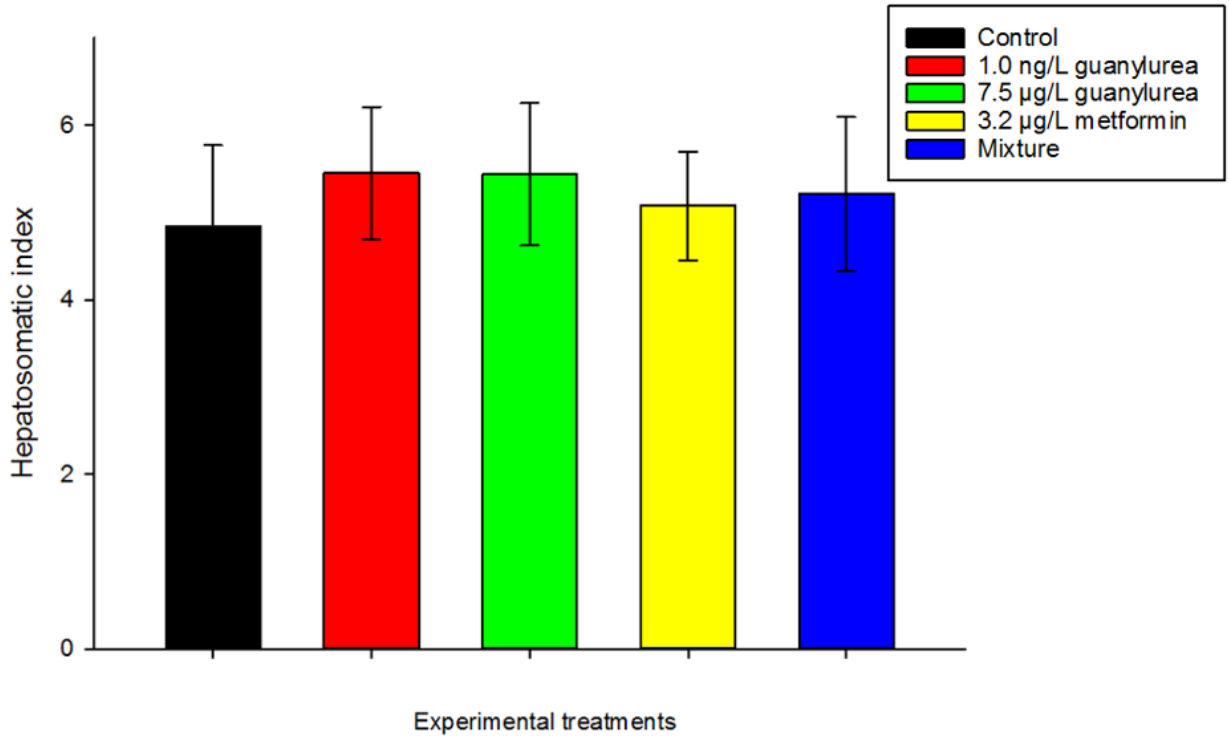


Figure 4.7: Mean male hepatosomatic index of 165 day old adult Japanese medaka (± SE) by treatment concentration (n = 3). Mixture = 3.2 µg· L^{-1} metformin + 7.5 µg · L^{-1} guanylurea.

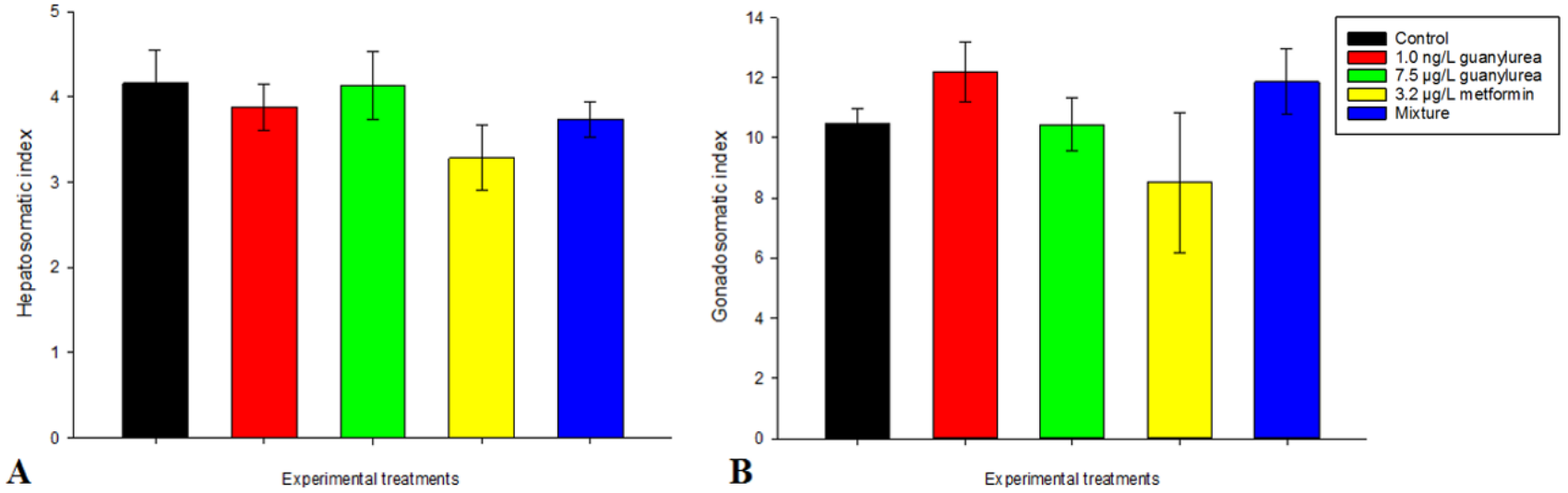


Figure 4.8: Mean female hepatosomatic index (A) and gonadosomatic index (B) of 165 day old adult Japanese medaka (± SE) by treatment concentration (n = 3). Mixture = 3.2 µg· L^{-1} metformin + 7.5 µg · L^{-1} guanylurea.

4.4.4 Steroid hormone analysis

A significantly higher production of estradiol was seen in the liver of male medaka exposed to 7.5 $\mu g \cdot L^{-1}$ guanylurea ($DF = 4$, $F = 6.20$, $p = 0.011$, Figure 4.9) when compared to control males, however no other experimental treatment caused a significant effect in estradiol production. Additionally, livers of male medaka exposed to the mixture treatment showed a significant increase in the production of 11-KT ($DF = 4$, $F = 5.76$, $p = 0.014$, Figure 4.10) when compared to control males, while no significant effect of treatment on the concentration of VTG was seen ($DF = 4$, $F = 4.02$, $p = 0.069$, Figure 4.11). No significant difference in the production of estradiol was found in the liver of adult female medaka at any experimental treatment ($DF = 4$, $F = 2.71$, $p = 0.057$, Figure 4.12), while a significant increase in the production of 11KT was seen in the liver of females exposed to 3.2 $\mu g \cdot L^{-1}$ metformin when compared to control females ($DF = 4$, $F = 31.3$, $p = 0.005$, Figure 4.13).

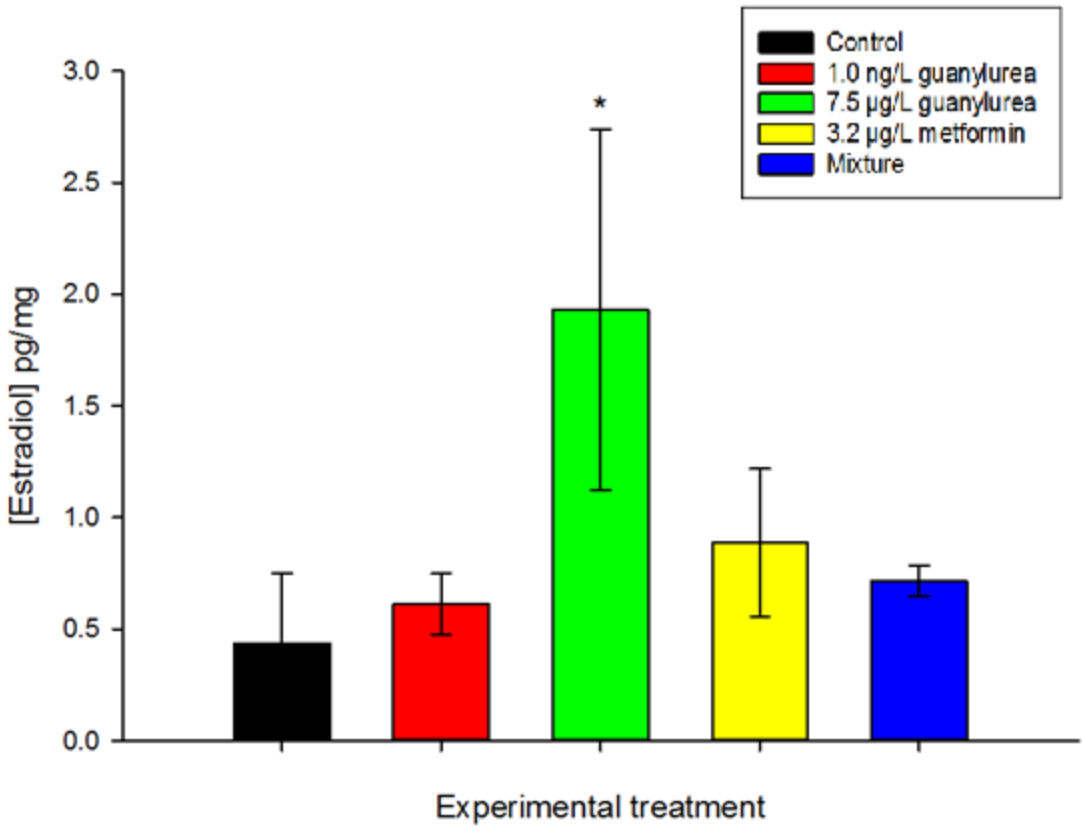


Figure 4.9: Mean estradiol production in adult male Japanese medaka livers (± SE) by treatment concentration (n = 3). Mixture = 3.2 µg· L-1 metformin + 7.5 µg · L-1 guanylurea. An asterisk * denotes significantly different groups ($p < 0.05$).

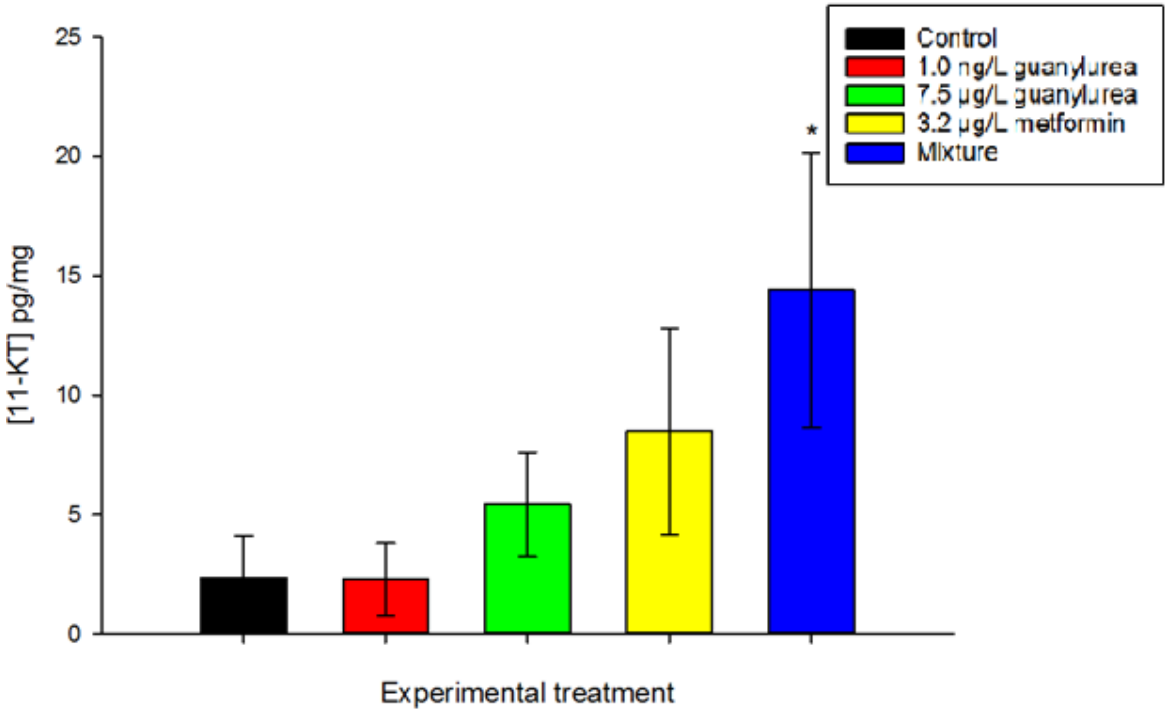


Figure 4.10: Mean 11-keto testosterone (11KT) production in adult male Japanese medaka livers (± SE) by treatment concentration (n = 3). Mixture = 3.2 µg· L-1 metformin + 7.5 µg · L-1 guanylurea. An asterisk * denotes significantly different groups ($p < 0.05$).

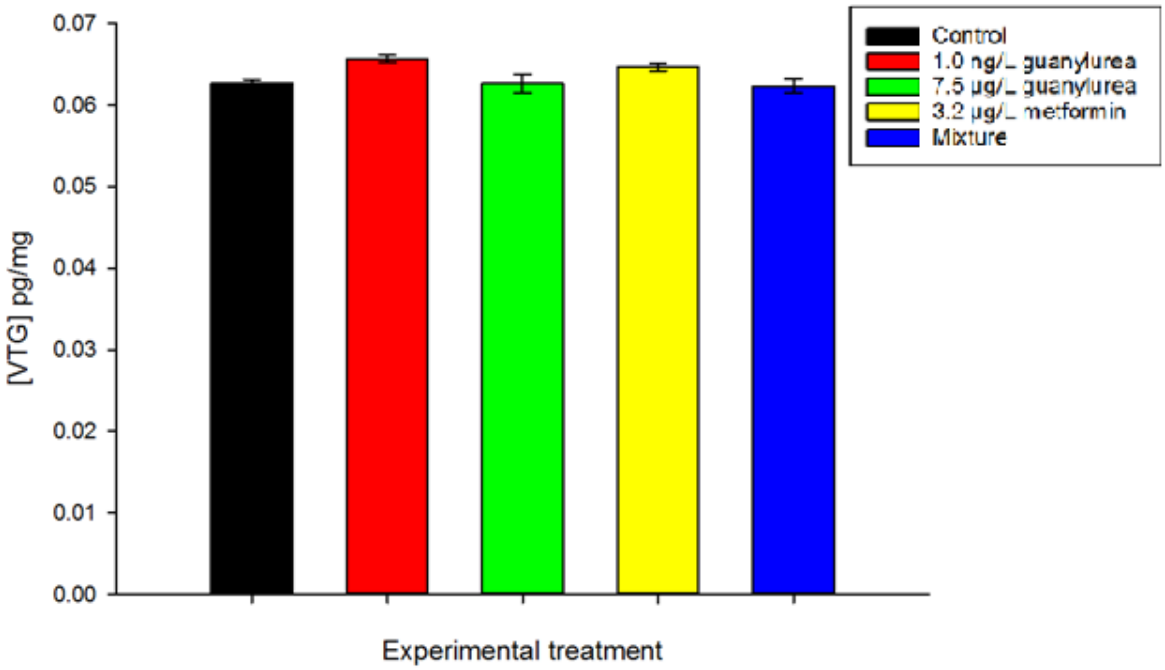


Figure 4.11: Mean vitellogenin (VTG) concentration in adult male Japanese medaka livers (± SE) by treatment concentration (n = 3). Mixture = 3.2 µg· L-1 metformin + 7.5 µg · L-1 guanylurea. An asterisk * denotes significantly different groups ($p < 0.05$).

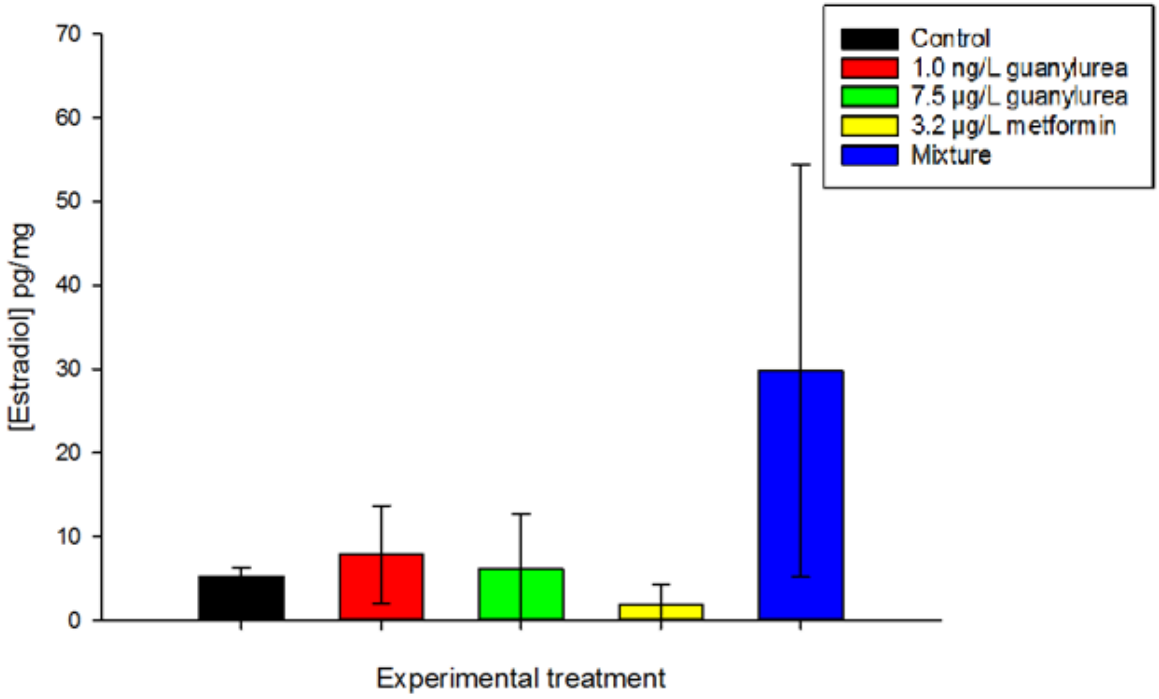


Figure 4.12: Mean estradiol production in adult female Japanese medaka livers (± SE) by treatment concentration (n = 3). Mixture = 3.2 µg· L-1 metformin + 7.5 µg · L-1 guanylurea. An asterisk * denotes significantly different groups ($p < 0.05$).

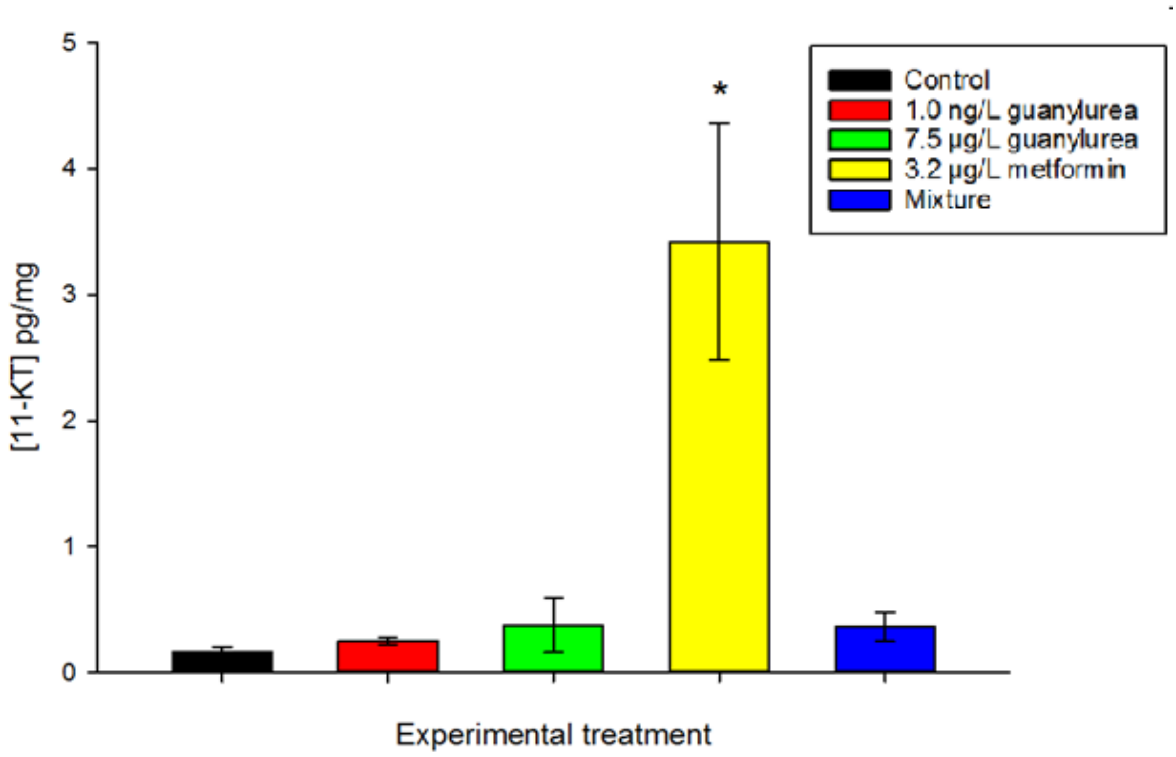


Figure 4.13: Mean 11-keto testosterone (11KT) production in adult female Japanese medaka livers (± SE) by treatment concentration (n = 3). Mixture = 3.2 µg· L-1 metformin + 7.5 µg · L-1 guanylurea. An asterisk * denotes significantly different groups ($p < 0.05$).

4.4.5 Reproductive effects

Results from a 2-way ANCOVA showed that there is no interaction between experiment treatment and egg collection per day (DF = 4, F = 0.151, p = 0.962). Similarly, and despite the significant effects seen in adult medaka growth and steroid production, no significant effects were seen in reproductive output for any of the experiment treatments (DF = 4, F = 1.10, p = 0.359, Figure 4.14). However, an analysis of the equal slopes model showed that there is a significant difference in the slopes of the 1.0 ng· L^{-1} guanylurea and 3.2 µg· L^{-1} metformin experimental treatment groups when compared to the control group (DF = 4, F = 5.34, p < 0.001), indicating that a longer collection period would potentially result in significant differences between the mentioned treatment groups and the control.

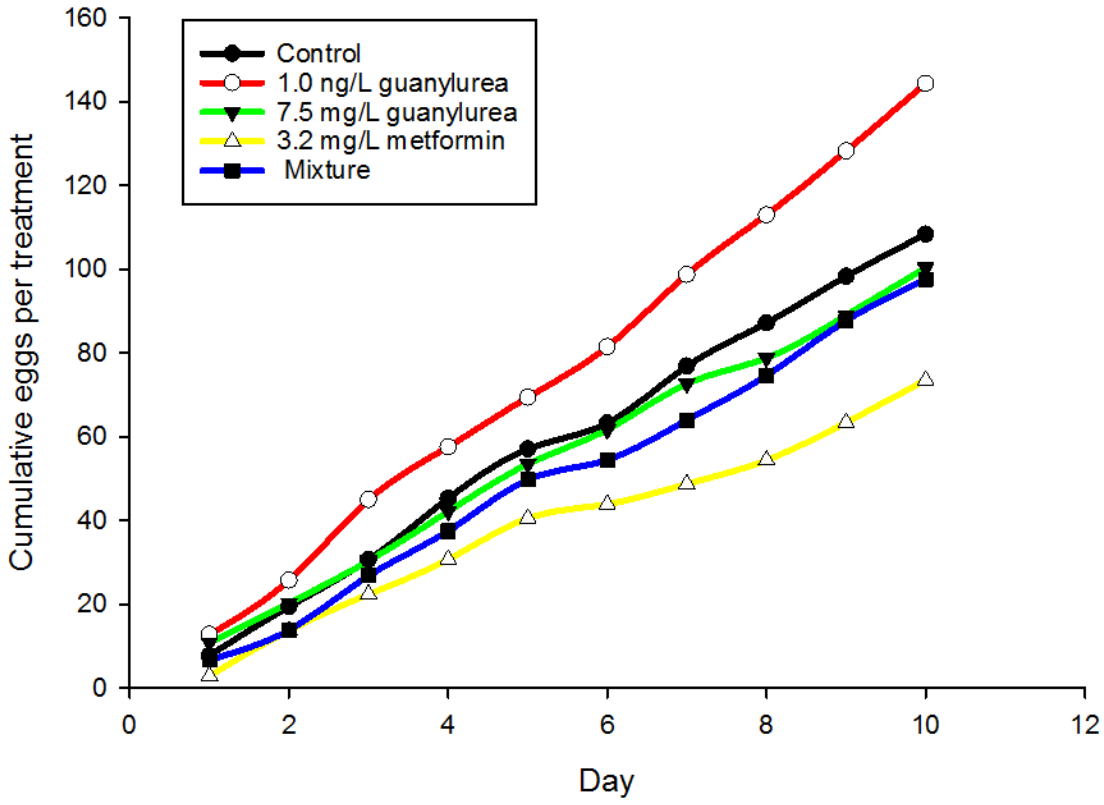


Figure 4.14: Cumulative reproductive output normalized to reproductive by treatment concentration (n = 14 control females; 3, 3.2 µg· L-1 metformin females; remainder of the treatments contained 9 reproductive females each). Cumulative egg data spans 10 collection days. Data points represent the replicate means per treatment (Data with error bars can be found in *Chapter 6: Appendices*).

4.5 Discussion

These results demonstrated that while environmentally relevant concentrations of metformin, and its metabolite, guanylurea, do not cause measurable impacts on the growth of adult Japanese medaka, they do have effects on the steroid hormone production in exposed adult fish. The results seen here for metformin exposure do not specifically corroborate the results seen by Niemuth and Klaper (2016) which showed a significant decrease in weight of metformin treated male FHM. Niemuth and Klaper (2016) also observed a significant reduction in the condition factor of metformin treated males, while no significant difference was observed in male medaka condition factor in the present study. This could be due to the relatively small sample size (n = 3) at the close of the current study. Adult male medaka are notoriously aggressive especially in response to feeding (Ruzzante and Doyle, 1991). In our study in particular, male medaka were wounding other males and females, resulting in occasional mortality. Although not significant, the growth of male medaka exposed to metformin and guanylurea appeared to be more sensitive than females, in the current study. This trend does corroborate the results seen by Niemuth and Klaper (2016), which showed a significant decrease in the weight of metformin treated male FHM, while females showed no effect. It should be pointed out that the Niemuth and Klaper (2016) used an experimental treatment of 40 $\mu g \cdot L^{-1}$, which is 12.5 times the concentration used in the present study of 3.2 $\mu g \cdot L^{-1}$ metformin. These combined results suggest that metformin may be taken up by males more readily than females. However, our metformin uptake results demonstrate that there is comparable uptake of metformin by both sexes at the larval stage. Future studies should investigate why males appear to be more sensitive to both compounds.

No significant effect was seen in the HSI of male and female medaka for any treatment concentration. Similarly, no significant effect was seen in the GSI of female medaka for any treatment concentration, however female medaka exposed to 3.2 $\mu g \cdot L^{-1}$ showed high variability in gonad weight, and thus GSI (Figure 4.7), which could explain the lack of significance. Unlike previous results in the literature, the present study did not observe effects on the concentration of the protein VTG in exposed male livers at any treatment concentration, where-as a 30-fold overexpression in VTG in exposed male FHM was seen in Niemuth *et al.* (2015). Again, this could be due to the differing exposure concentrations, or differing methods in the measurement of VTG. VTG concentration was measured in the plasma of male FHM, while the present study utilized male medaka livers because of the extremely small volume of blood that can be removed from medaka.

Interestingly, male medaka exposed to 7.5 $\mu g \cdot L^{-1}$ guanylurea produced significantly more estradiol, while males exposed to a mixture of the two compounds (3.2 $\mu g \cdot L^{-1}$ + 7.5 $\mu g \cdot L^{-1}$) produced significantly more 11KT when compared to control males. It appears that metformin and guanylurea act in an antagonistic manner on the production of estradiol in male medaka (Figure 4.8), while the compounds act in an additive manner on the production of 11KT (Figure 4.9). Although the results were not significant, metformin and guanylurea appeared to act in a synergistic manner on the production of estradiol in female medaka (Figure 4.11). Of particular interest is the significantly increased production of 11KT in female medaka exposed to 3.2 $\mu g \cdot L^{-1}$ metformin, as the pharmaceutical was shown to decrease circulating androgen levels in human females with polycystic ovary syndrome (Earle, 2000). The effect of a mixture of metformin and guanylurea on 11KT

production in female medaka also appears to be extremely antagonistic, with females exposed to the mixture producing 11KT levels similar to that of controls (Figure 4.12).

Despite significant effects seen in steroid hormone production, no significant effects were seen in reproductive output of Japanese medaka exposed to environmentally relevant concentrations of metformin and guanylurea, alone, or in combination. This differs from results found in the literature which showed a significant decrease in the number of cumulative egg clutches produced by metformin exposed mating FHM pairs when compared to controls (Niemuth and Klaper, 2016). Although the reproductive output findings in the present research were not significant, an analysis of the equal slopes model shows that there is a difference in the slope of the lines plotting the cumulative egg production per day for medaka exposed to 1.0 ng $\cdot$ L^{-1} guanylurea (increased production) and 3.2 µg $\cdot$ L^{-1} metformin (decreased production). These merely show a trend, and future research should increase the number of days allotted to assess reproductive output.

Chapter 5: General Conclusions

5.1 General conclusions

This dissertation aimed to investigate the effects associated with metformin and guanylurea exposure to two critical life stages of Japanese medaka. The results demonstrated that these common surface water contaminants have a measurable impact on the growth of early-life stage medaka, while they have a potential impact on the growth of male adult medaka. To date, this is the first study to investigate and show that metformin can be taken up and transiently stored in embryo and larval medaka. This finding is extremely important, as it provides confirmation that the effects of metformin reported both in the literature and in this thesis are likely due to compound uptake. Although the uptake of guanylurea was not addressed in this research, due to the similar chemical structure to its parent compound, it is plausible that it also can be taken up by fish, thus providing insight into the effects of guanylurea on larval growth.

Interestingly, waterborne metformin uptake and accumulation was dependent on chorion hardening in the present study, with hardening appearing to completely prevent uptake. This may indicate that windows of sensitivity exist for embryos exposed to metformin in aquatic ecosystems prior to chorion hardening. Stages 1 through 11 of medaka development fall within the first 8 hours after fertilization (pre-chorion hardening), which encompass the early through late blastula stages (Iwamatsu, 2004). Exposure during these stages may have implications for early cell division, or incomplete meiosis, leading to a failure in embryo development (Iwamatsu, 2004). This study also showed that when both embryos and larvae were exposed to ^{14}C-metformin, followed by a short 24-h depuration, all of the detectable metformin was depurated out of the embryo and larvae.

Metformin and guanylurea exposure over a 28-day period resulted in significantly stunted growth in both male and female medaka at environmentally relevant concentrations, with significant effects observed in exposure concentrations as low as 3.2 $\mu g \cdot L^{-1}$ metformin and 1.0 $ng \cdot L^{-1}$ guanylurea. Decreased size in 28 day old fish exposed to metformin and guanylurea may be attributed to disruptions in cellular pathways involved in growth and cell proliferation, as seen by significant changes in metabolite response factors and the altered gene expression in exposed larvae. It should be noted that gene expression is a snap-shot of the expression at the moment of sampling, and it is possible that metformin and guanylurea altered the expression of the genes of interest during critical growth periods that were not measured here. Additionally, the genes of interest were chosen based on metabolomics findings and feasibility, however it would be advantageous for future research to pair metabolomics with transcriptomics and proteomics to gain insight on the full organism effects of metformin and guanylurea.

While the full life-cycle exposure to environmentally relevant concentrations of metformin and guanylurea alone, and in combination, did not results in a statistically significant impact on growth, steroid hormone production in adult Japanese medaka was significantly affected. Additionally, no significant effects in HSI for male medaka was seen, and no significant effect in female HSI or GSI was seen. However, this could be explained by the large variability seen in gonad weight between females.

The combination of metformin and guanylurea act in an antagonistic manner on the production of estradiol in male medaka, while the compounds act in an additive manner on the production of 11KT. Of particular interest is the significantly increased production of 11KT in female medaka exposed to 3.2 $\mu g \cdot L^{-1}$ metformin, as the pharmaceutical is used to

decrease circulating androgen levels in human females with polycystic ovary syndrome. Additionally, combination effects of metformin and guanylurea on 11KT production appear to be extremely antagonistic in female medaka.

Despite the significant effects seen in steroid hormone production, no significant effects were seen in the reproductive output of Japanese medaka exposed to environmentally relevant concentrations of metformin and guanylurea, alone, or in combination. Additionally, no effect was seen in the production of VTG in male medaka in any of the experimental treatments. Again, it would be advantageous for future work to investigate the potential transcriptomic and proteomic effects in metformin and guanylurea exposed adult fish. This would better help explain the interesting effects seen in this research.

Overall, our findings demonstrate the need for further research into the effects of metformin and its metabolite guanylurea on non-target aquatic organisms. These results are novel, as there have been no previous studies addressing these endpoints in this manner in fish. This thesis has significantly contributed to the knowledge regarding the effects of metformin and guanylurea and has successfully filled in some of the current gaps in the literature. It is apparent from this research that environmentally relevant concentrations of both compounds cause alterations in the cellular function of Japanese medaka. Field studies should also be performed to elucidate their possible effects on wild fish populations in receiving waters. This would help to tease out which life-stage is most sensitive, and offer new insights on potential population-level impacts. Considering that surface waters will likely continue to have increasing concentrations of metformin and guanylurea, gaining new ecotoxicological information on these compounds in aquatic systems should be a priority.

Chapter 6.0 References

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Chapter 7.0 Appendices

Appendix A: 7-day metformin toxicity testing

Table A1: Percent hatch in Japanese medaka embryos exposed to various concentrations of metformin.

Conc. (µg/L)	% hatch after 7 days	% hatch after 8 days	% hatch after 9 days
Control	17.5	50	95
SC	5	70	97
8	30	55	100
40	45	45	92
200	45	40	95
1000	35	50	100
5000	17.5	67.5	92

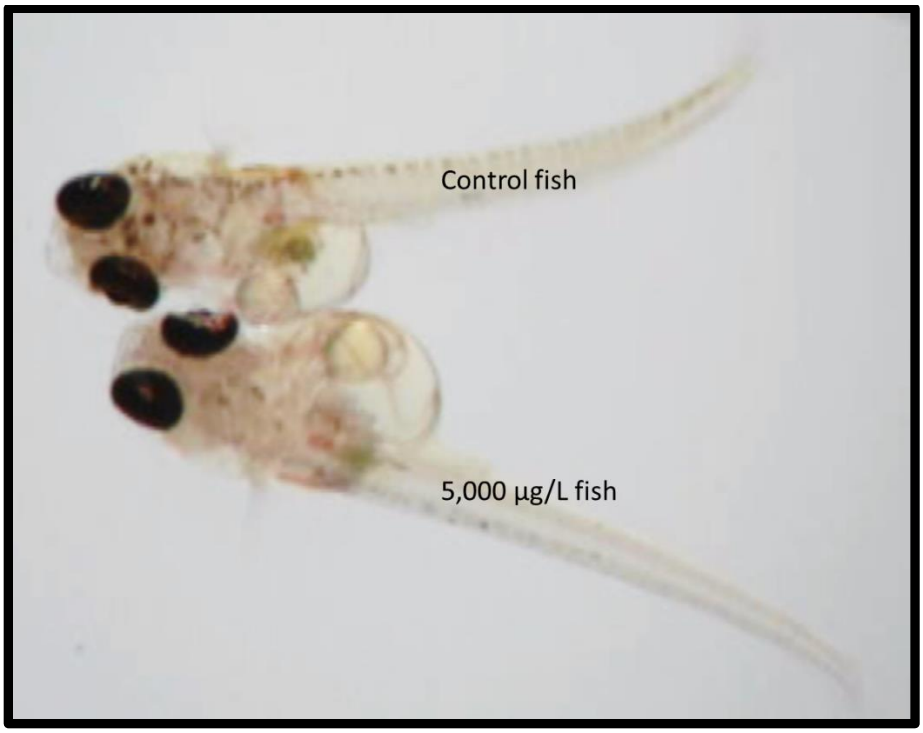

Figure A1: Larval (7 dph) medaka from control treatment and from 5,000 µg · L^{-1} treatment. Figure shows no developmental abnormalities in either treatment.

Appendix B: Embryo-larval growth study

Table B1: Percent hatchability in Japanese medaka embryos exposed to various concentrations of metformin.

Metformin Concentrations (µg/L)	% hatchability
Solvent control	93
3.2	88
10	91
32	92
100	89
320	89
1000	92

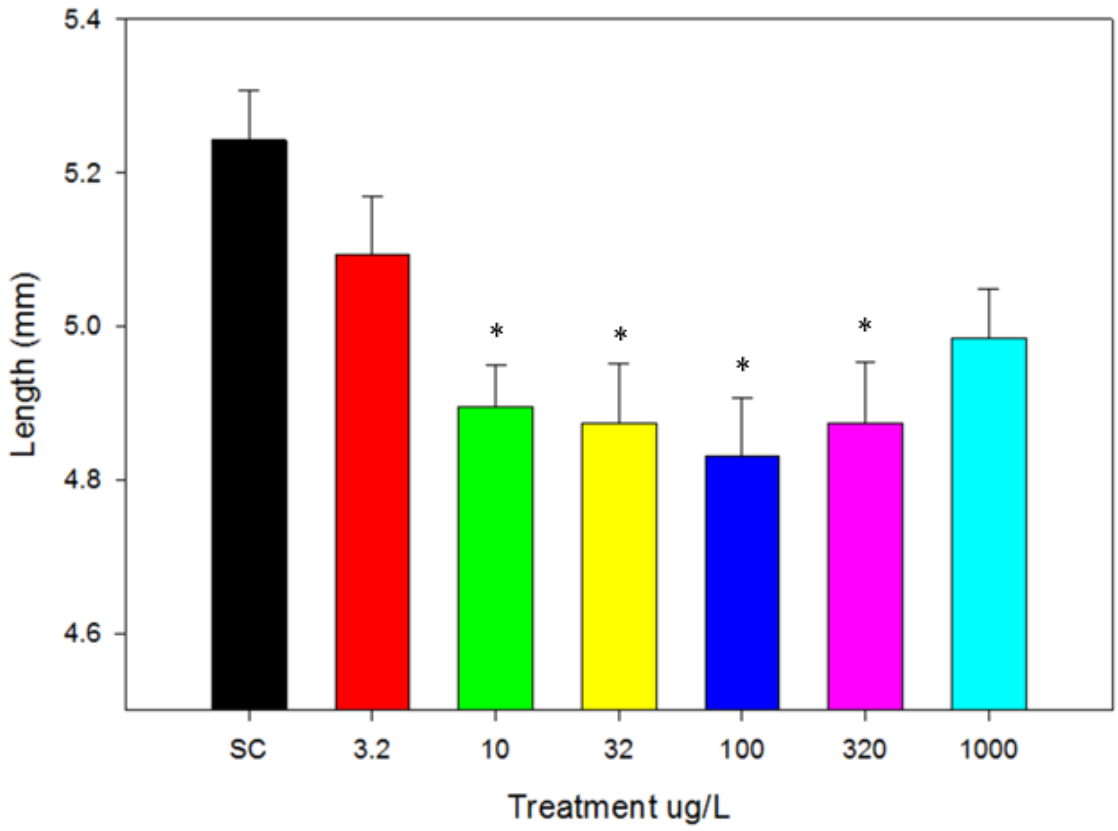

Figure B1: Mean length (mm) of 7 day old adult Japanese medaka (± SE) by treatment concentration (n = 80/treatment) An asterisk * denotes significantly different groups from control ($p < 0.05$).

Appendix C: Life-cycle reproductive results with error bars

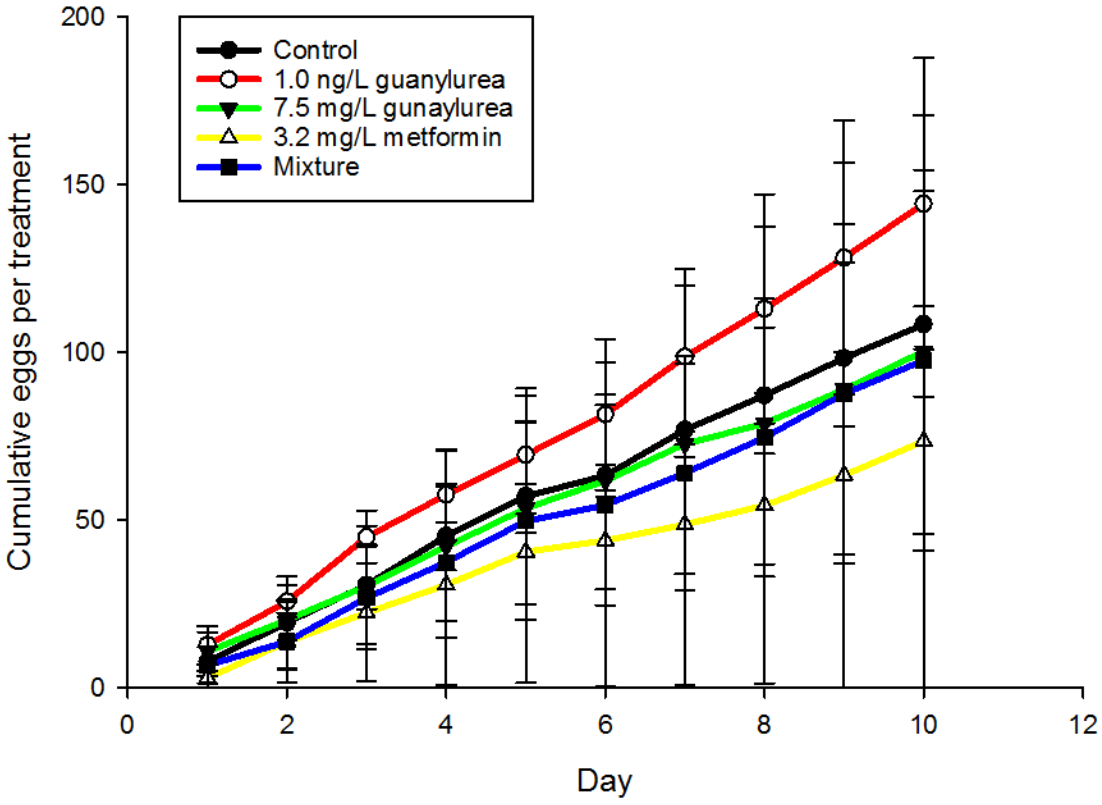

Figure C1: Cumulative reproductive output normalized to reproductive by treatment concentration (n = 14 control females; 3, 3.2 µg· L-1 metformin females; remainder of the treatments contained 9 reproductive females each). Cumulative egg data spans 10 collection days. Data points represent the replicate means per treatment (± SE).