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Developmental phenotypic and transcriptomic effects of exposure to nanomolar levels of metformin in zebrafish

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Abstract

Metformin is found in the majority of lakes and streams in the United States, leading to widespread environmental exposure. Results of the present study indicate that extended duration metformin exposure at critical developmental periods leads to decreased survival rates in zebrafish (*danio rerio),* an NIH approved human model. Significant abnormalities are seen with extended duration metformin exposure from 4 hours post fertilization up to 5 days post fertilization, although short term metformin exposure for 24 hours at 4–5 days post fertilization did not lead to any significant abnormalities. Both extended and short term duration did however have an impact on locomotor activity of zebrafish, and several genes involved in neurological and cardiovascular development were differentially expressed after exposure to metformin. The changes seen in behavior, gene expression and morphological abnormalities caused by metformin exposure should be examined further in future studies in order to assess their potential human health implications as metformin prescriptions continue to increase worldwide.

Keywords

metformin; danio rerio; zebrafish; environmental toxicity; aquatic environment; ground water chemicals

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Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

1. Introduction

Diabetes is one of the most common diseases around the world. An estimated 459 million adults will be affected worldwide by the year 2030, and in the United States (US) alone, the estimated number of individuals affected by diabetes will increase to more than 36 million Americans between 2010 and 2030 (Shaw et al., 2010). One of the first line treatments for diabetic patients is the pharmaceutical agent, metformin, due to multiple mechanisms that reduce blood glucose, specifically inhibiting hepatic glucose production, improving glucose uptake and utilization, and decreasing glucose absorption through the intestinal tract (Gong et al., 2012). In recent years, metformin has been increasingly used for a variety of other conditions that include preventing weight gain due to antipsychotic prescriptions (Prajapati 2014), treatment of polycystic ovary disease (Creanga et al., 2008), adjunct treatment in cancer (Morales and Morris, 2015), and cardiovascular disease therapy (Han et al., 2019). With this multitude of uses, metformin has become the fourth most prescribed medication in the US, with approximately 79 million prescriptions written in 2017 (Medical Expenditure Panel Survey (MEPS) 2007–2017).

This increased use is concerning as metformin is orally administered and undergoes virtually no metabolism in humans, with around 70% excreted unchanged in urine (Gong et al 2012). Thus, a large amount of bioactive metformin travels to surface waters via wastewater treatment plants (WWTPs) every year, making metformin one of the most commonly found pharmaceutical agents in aquatic environments. In a recent study investigating over 300 streams throughout the United States, metformin was found in 68% of all sample sites across all regions of the United States, with median concentrations ranging from approximately 1 ng/L to over 1000 ng/L (Bradley et al., 2020). Another study examining metformin in a diversity of aquatic environments, found metformin ranges from 10 ng/L to greater than 400 ng/L in lake water, river water, and seawater, and subsequently in tap water in concentrations up to 61 ng/L (Trautwein et al., 2014). Metformin has a high degradation rate of 70 – 90% in WWTPs but is still consistently detected in effluent and surface waters, ranging from major rivers to small streams, in concentrations of 1–3 ng/L, dependent on wastewater burden delivered to the surface water, which will only increase as metformin prescriptions continue to increase (Schreuer et al., 2012).

Despite consistent detection in WWTP effluent, surface water, and drinking water, little is known about the health impacts of persistent metformin environmental exposures on both people and wildlife, particularly following developmental exposure. However, previous research provides evidence for endocrine disruption. Though metformin exposure at 200 mg/kg body weight/day in adult obese mice led to overall increases in fertility, due to improved integrity of the blood testes barrier and repair of oxidative damage (Ye et al., 2019), most studies reveal adverse reproductive impacts due to metformin exposure. For example, a study chronically exposing adult fathead minnows (*Pimephales promelas)* to 40 μg /L metformin (approximately 5 nM), a concentration found in WWTP effluent, revealed increased intersex associated with > 30-fold upregulation of vitellogenin, an egg yolk precursor typically more prevalent in females (Niemuth et al., 2015). Additional studies in fathead minnows showed that exposure to environmentally relevant levels of metformin from fry stage through adulthood resulted in an 84% incidence of intersex compared to 13%

for control fish (Niemuth and Klaper, 2015). Fish with the intersex condition had concurrent decreases in weight and fecundity, characterized by significant reduction in the number and size of clutches. In non-obese mice, maternal metformin exposure at 300 mg/kg/day during pregnancy led to decreased testes size and Sertoli cell number in fetal and neonatal mice. Similarly, *in vitro* human models demonstrated decreased testosterone production at therapeutic doses of metformin (50 μM) by almost 45%, with concurrent decreases in mRNA steroid precursor and increases in lactate production. A dose of 500 μM metformin was required to significantly decrease testosterone based on *in vitro* mouse studies (Tartarin et al., 2012). Evidence also exists that metformin impacts behaviors related to reproduction; a study of adult male Siamese fighting fish (*Betta splendens*) found decreases in intermale aggression after multiple weeks of exposure to 40 ng/L metformin, (MacLaren et al., 2018).

With evidence for metformin-induced health consequences resulting from environmentally-relevant exposures across multiple species as mentioned above, further investigation of these outcomes and their underlying mechanisms is warranted (Niemuth et al, 2015; Niemuth and Klaper, 2015; MacLaren et al., 2018). Therefore, the purpose of this study is to evaluate the phenotypic and transcriptomic endpoints of metformin exposure during embryogenesis or larval development at environmentally-relevant levels using zebrafish (*Danio rerio*). Zebrafish are a tractable developmental and toxicological model that is approved by NIH as a human translational model, owing to 70% homology between the zebrafish and human genome (Howe et al., 2013). The results of this study will provide insights into risk assessment for pharmaceuticals in aquatic environments and environmentally-influenced disease.

2. Materials and Methods

2.1 Fish husbandry

Adult zebrafish (wild-type AB strain) were maintained on a 14:10 h light/dark cycle in reverse-osmosis (RO) water buffered with salts (Instant Ocean© Spectrum Brands, Wisconsin, US) at a water temperature of 27–30^{O}C in a recirculating system (Aquaneering, California, US). Adult fish were fed flakes (Aquatox Fish Diet, Zeigler Bros Inc., Pennsylvania, US) twice daily, supplemented with brine shrimp. Adult zebrafish were bred with a sex ratio of 1 male to 2 females in spawning tanks. A total of 180 embryos were collected 4 hours post fertilization (hpf) at approximately the sphere stage of embryonic development. Embryos were cleaned with 58 ppm bleach (Clorox Company, California, US) for 5 minutes, then rinsed with egg water (600mg/L salt in RO water), sorted into groups for their respective exposures, and incubated at 28^{o}C. Zebrafish protocols were approved by the Institutional Animal Care and Use Committee at Wayne State University, according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.2 Chemical exposure

Metformin hydrochloride (HCl; CAS #111–5-70–4, Sigma Aldrich, US) was prepared as a stock solution in a concentration of 10,000 nM in RO water. Embryos were dosed with 1, 10, 100, 1000 or 10,000 nM metformin HCl in fish water (60 mg/L salt in RO water), with fish water as the control condition. Aliquots of the stock solution were used

to form daily preparations of the chemical solutions for exposure. Collected embryos were exposed at 2 different periods: 4 hpf to 5 days post-fertilization (dpf), forming the extended duration exposure group; and 24 hours at 4–5 dpf, forming the short-term exposure group. Exposures were performed in 6 well trays, and each well was plated with 7 mL water and at most 30 embryos per exposure concentration. ~90% of the exposure chemical solution was removed from each well daily and replenished with freshly prepared chemical solution. After the exposure period, larval fish were rinsed 3 times with egg water to end the chemical exposure.

2.3 Morphological screening

Zebrafish embryos were visualized and screened at 24, 48, 72, 96, and 120 hpf for mortality and morphological abnormalities by stereomicroscope (M165C, Leica Microsystem, Germany). The endpoints were percent of hatched embryos (hatch rate), skeletal deformities, improperly inflated swim bladder, yolk sac edema, heart edema, and total abnormalities. Embryos were screened using 6.7X magnification with detailed evaluation occurring at a magnification of 50X. Results were analyzed using a Chi-Square test with significance set at $p < 0.05$, with pairwise comparison with Bonferroni corrections $p < 0.05$ between the control and concentrations.

2.4 Behavioral analysis

Control and exposed larval fish with an inflated swim bladder and without any observed morphological abnormalities underwent behavioral analysis. At 5 dpf, 24 larval zebrafish from each exposure group were tested with 1 larva per well (each with 2 mL of fish water) in a 24 well plate. Fish were allowed to acclimate for at least 1 hour at 28°C before being placed into the DanioVision Observation Chamber (Noldus Information Technology, Wageningen, Netherlands) with a constant temperature at 28.5 ± 0.5°C. All behavioral tests were performed between 14:00 and 22:00 and consisted of an assay of 3-minute light and dark alternating periods with a total of four light-dark cycles (24 minutes in total). This timeframe was chosen to ensure a stable baseline level of activity, as larval zebrafish are most active in the morning, with stable levels of activity reached between 13:00 – 15:30 (MacPhail et al., 2009). Distance traveled was integrated over 30 second intervals.

The raw data was exported from EthoVisionXT14 into a spreadsheet for quality control. The behavioral data were then analyzed using ANOVA and Tukey's Honest Significant Difference tests. Significance was considered at $p < 0.05$. Quality control and statistics were conducted using R (http://www.r-project.org).

2.5 RNA isolation

A total of 5 larval fish per concentration of metformin HCl were euthanized with tricaine methanesulfonate solution (150 mL fish water, 0.06 g tricaine methanesulfonate, and 0.1 g sodium bicarbonate) for 10 minutes and then pooled in RNALater™ (Thermo Fisher, Massachusetts, US) at 4°C for 24 hours. The RNALater™ solution was removed after 24 hours, and the larval fish pool was stored in −80°C until RNA isolation. RNeasy Lipid Tissue Mini Kit (QIAGEN, Hilden, Germany) was used to isolate RNA according to the

manufacturer's specifications. RNA purity was measured with Qubit® 3.0 Fluorometer (Invitrogen, California, US).

2.6 Transcriptome analysis

3' mRNA-seq libraries were prepared from isolated RNA using QuantSeq 3' mRNA-Seq Library Prep Kit FWD for Illumina (Lexogen, Vienna, Austria). Samples were normalized to 40 ng/μL (total input of 200 ng in 5 μL) and amplified at 17 cycles. Libraries were quantified using a Qubit® 2.0 Fluorometer and Qubit® dsDNA Broad Range Assay Kit (Invitrogen, Carlsbad, CA), and run on an Agilent TapeStation 2200 (Agilent Technologies, Santa Clara, CA) for quality control. The samples were sequenced on a HiSeq 2500 (Illumina, San Diego, CA) in rapid mode (single-end 50 bp reads). Reads were aligned to D. rerio (Build danRer10) using the BlueBee Genomics Platform (BlueBee, Rijswijk, The Netherlands). Quality control of sequencing is shown in Supplementary data (Table S1). Differential gene expression between control and exposure at 3 separate concentrations, low (1 nM), medium (100 nM), and high (10000 nM), was evaluated using DEseq2 (available through GenePattern; Broad Institute, Cambridge, Massachusetts). Genes with significant changes in expression, as defined by absolute log2 fold change value ≥ 0.75 and adjusted p-value < 0.1, were uploaded into Ingenuity Pathway Analysis software (IPA; QIAGEN Bioinformatics, Redwood City, CA) for analysis using RefSeq IDs as identifiers.

3. Results

3.1 Mortality and morphological abnormalities

Metformin exposure, regardless of concentration, did not affect overall mortality rate in the 24-hour exposure. However, the extended duration exposure had a significantly decreased survival rate ($p < 0.05$), specifically when comparing the 1 nM concentration to the control condition ($p < 0.01$). Additionally, the percentage of unhatched eggs in the extended duration exposure groups at 10 and 100 nM, as well as 10,000 nM was decreased compared to control, although it was only significant for the 100 nM concentration ($p < 0.001$; Figure 1). The percentage of unhatched eggs in the 100 nM concentration was also significantly decreased compared to all other concentrations in the extended duration exposure group ($p < 0.001$). Extended-duration exposure to metformin also led to the following overall significant abnormalities when analyzed using a Global Chi-square test: uninflated swim bladders, total abnormalities ($p < 0.001$), and cardiac edema ($p < 0.01$). However, cardiac edema and total abnormalities had no significant difference at any concentration when compared to the control group, and yolk sac edema was only significant at the 1000 nM and 10,000 nM concentrations compared to the control group ($p = 0.015$ and $p = 0.007$, respectively; Figure 1). Additionally, the 1000 nM concentration in the extended duration exposure groups had the highest percentage of fish with abnormalities compared to all other exposure paradigms for skeletal abnormalities (19%), uninflated swim bladder (80%), and cardiac edema (21%), while the percentage of fish with yolk sac edema was highest at the 10,000 nM concentration (26%). The 100 nM concentration had significantly less fish with abnormalities overall (41%) compared to 1 nM (83%), 10 nM (76%), 1000 nM (81%) and 10,000 nM (77%) in the extended duration exposure group ($p < 0.005$). Larval fish exposed

to metformin for the short-term duration had similar abnormality rates as the control fish across all concentrations ($p > 0.05$; Figure 1).

3.2 Behavior

Figure 2 shows the locomotor activity of larval fish at 5 dpf during dark and light cycles. Larval fish traveled longer distances during the dark cycles for all metformin concentrations in both the extended duration and short-term exposures, averaging around 21.6 cm during the dark cycle compared to 3.6 cm during the light cycle across all concentrations in the extended-duration exposure group, and approximately 20 cm versus 6 cm for the short-term exposure group. Distance traveled during the dark cycles decreased significantly after extended duration exposure to 1 nM ($p < 0.01$), 1000 nM ($p < 0.001$) and 10,000 nM ($p < 0.05$) metformin. During the light cycle for the extended duration metformin exposure group, distance decreased significantly when compared to the control group at 1 nM ($p < 0.01$). Distance traveled during the dark cycles for the short-term 10 nM, 100 nM ($p <0.001$), and 10,000 nM ($p < 0.01$) exposure groups increased significantly compared to the control group). During the light cycle for the short-term metformin exposure group, distance traveled for larval fish exposed to 10 nM ($p < 0.01$) and 100 nM ($p < 0.001$) increased significantly compared to control fish.

3.3 Transcriptome

No genes were differentially expressed after short-term exposure. However, a total of 24 genes were differentially expressed after extended duration metformin exposure regardless of concentration, except for *smdt1a* which was not changed at the 1 nM concentration (Table 1). Overall, 19 genes were upregulated and 3 were downregulated across exposure concentrations, and 2 genes were variably dysregulated depending on exposure concentration (Table 1). Metformin at 10,000 nM significantly altered expression of 13 genes, while 1 nM and 100 nM significantly altered expression of 8 and 5 genes, respectively. The significant gene expression profiles were distinct for each exposure concentration, except for *lgmn*, which was significantly upregulated after both 1 and 100 nM exposure, as well as *atp6v0e1*, which was significantly upregulated after both 100 and 10,000 nM exposure. These two genes, along with half of the differentially expressed genes, fell into two categories based on IPA: cardiovascular or neurological development and function. In general, genes implicated in cardiovascular function and development, as well as cardiac abnormality pathways such as dilation, enlargement, and dysfunction, were significantly dysregulated, predominantly at 100 and 10,000 nM. These genes include *abraa*, which was upregulated at 100 nM, as well as *smdt1a, atp2a2a, tnnilc,* and *hsd11b2*, which were significantly upregulated at 10,000 nM. Of the differentially expressed genes implicated in the neurological system, half were significantly dysregulated after 1 nM, including *crygm2d10, nedd9* and *prelid3b*, and the other half were significantly upregulated at 10,000 nm, including *pycr1b, mfsd2ab* and *hjv* (Table 1). Pathways for the genes upregulated at 10,000 nM included proline biosynthesis such as *pycr1b*. No genes associated with the neurological system were significantly dysregulated at the 100 nM concentration. IPA of differentially expressed genes following extended duration exposure at 1 nM revealed pathways involved in metal ion binding and neurological development including *lnx1*. Finally, intracellular process pathways were dysregulated evenly across the

three concentrations, and contained genes encoding for cellular structural components, such as *fam234b*, *hpda*, and *si:ch211–114n24.6,* with 2 genes dysregulated at 1 nM, 2 genes at 100 nM, and 3 genes at 10,000 nM.

4. Discussion

Our findings show that metformin induces wide-ranging effects following embryonic exposure in zebrafish, specifically morphological and neurobehavioral abnormalities, as well as changes in transcriptome, notably in cardiovascular and neurological development and function pathways. While neurobehavioral outcomes were only significant for the short-term exposure group, phenotypic and transcriptomic outcomes primarily occurred following the extended duration metformin exposure, specifically significant mortality, percentage of unhatched eggs, cardiac edema, and differential gene expression (Figure 1; Table 1). This suggests that embryonic (< 4 dpf), but not larval exposure is a critical window for metformin sensitivity in zebrafish. Numerous studies investigating various substances, including solvents and EDCs, in zebrafish have shown that earlier embryonic exposures, such as 0–4 hpf, results in greater sensitivity to toxicants than exposures occurring even at 24 hpf (Maes et al., 2012; Massei et al 2015; McGee et al, 2012; Chatterjee et al., 2021). To our knowledge, critical windows for metformin exposure have not previously been studied in zebrafish, but have been examined in other species. For example, a study in Japanese medaka (*Oryzias latipes*) showed significantly higher uptake of an environmentally relevant metformin concentration (60.5 E^{-10} nM) in fish exposed before their chorion hardened (< 6 hpf) compared to fish exposed after 24 hpf (Ussery et al., 2019). The extended duration exposure in the current study started at 4 hpf, encompassing early embryonic development, likely explaining the greater number and significance of endpoints observed compared to the short term exposure group, which started at 4 dpf.

Alternatively, the difference in outcomes between the extended duration and short-term exposure groups may be due to cumulative dose rather than a critical window of sensitivity. This is less likely however, because the amount of metformin present in exposed larval medaka returned to background levels within 24 hours after being placed in clean water (Ussery et al., 2019), suggesting a high rate of clearance that can overcome metformin bioaccumulation at lower concentrations, and potentially over short term exposures as well. Additionally, a previous study examining metformin accumulation in zebrafish similarly showed minimal tissue concentration following a 30-day exposure and 30-day recovery period, at doses up to 1000 μg/L metformin (approximately 128 nM) (Lin et al., 2021).

The primary morphological abnormality seen in this study was cardiac edema, which occurred in the extended duration metformin exposure, specifically at the higher doses of 1000 and 10,000 nM. It is not surprising that fish only in the extended duration were affected due to timing of cardiovascular system development in zebrafish, which starts with cardiac progenitor cells at approximately 5 hpf, differentiation at 15 hpf, and pacemaker formation by 48 hpf (Bakkers, 2011), again supporting our hypothesis that early embryonic development is a critical window for metformin exposure. Nonetheless, this finding is unexpected because metformin was previously suggested as cardioprotective in certain disease states, such as Type 2 diabetes with congestive heart failure (Eurich

et al., 2013), although more recent studies suggest that metformin use does not decrease risk of exacerbation in patients with congestive heart failure(Weir et al., 2018). No studies have investigated metformin-related cardiac endpoints after early developmental exposure or in healthy organisms, and thus may explain why our results are contradictory to previous studies. Based on the role of *lgmn* in axon and tissue regeneration in the cardiovascular system, upregulation of this gene at 1 and 100 nM metformin may have been cardioprotective at these lower exposure concentrations for which no cardiac phenotype was observed. Conversely, our transcriptomic findings at the higher metformin concentrations are supportive of cardiotoxicity.

Notably, mutations of the gene *atp6v0e1*, which was upregulated at 100 and 10,000 nM in the extended duration metformin group, increase incidence of pericardial edema in larval zebrafish (Daly et al., 2017). The genes *atp2a2a, hsd11b2*, and *tnni1c*, were all significantly upregulated at 10,000 nM. These genes are involved in calcium ion homeostasis in the intracellular space (Sancak et al., 2013), sarcoplasmic reticulum, and developing heart (Frank et al., 2018) and myocardium respectively (Genge et al., 2016), with *tnni1c* also helping to regulate myocardial contraction. Further, *tnni1c* is a zebrafish homologue of a gene associated with cardiomyopathy in humans, TNN13 (Shih et al., 2015), while *hsd11b2* is upregulated after stressor exposure, and the human ortholog has been implicated in the development of hypertension (Tokarz et al., 2013; Kamide et al., 2006). Another gene, *abraa,* which was upregulated after 100 nM extended duration metformin exposure, is stress-inducible and upregulated during hypertrophic growth of the heart (Triodl et al., 2009; Arai, Spencer an Olson 2002), and involved in blood circulation and heart development (Troidl et al., 2009; Chong et al., 2012). Although in-utero metformin exposure does not appear to increase risk of congenital abnormalities in humans (Given et al., 2018; Gilbert, Valois and Koren, 2006), metformin-induced cardiac edema has potential implications for the fitness of wild fish, especially since metformin is known to adversely impact other organ systems, such as reproduction in fathead minnows (*Pimephales promelas*) (Niemuth et al., 2015; Niemuth and Klaper, 2015).

Another notable effect of extended duration metformin exposure was significantly increased mortality, occurring only at the 1 nM concentration, thus exhibiting a non-monotonic response. This finding conflicts with studies examining metformin and its metabolites in other aquatic species, such as brown trout (*Salmo trutta*) and Japanese medaka (Ussery et al., 2019; Jacob et al., 2018), in which metformin exposure did not affect mortality. However, the study with medaka involved exposure after hatching (at approximately 1 nM) (Ussery et al., 2019), while the study with brown trout exposed embryos starting at 48 dpf (Jacob et al., 2019), both missing our hypothesized early embryonic critical window of sensitivity to metformin exposure. Non-monotonic responses are common in endocrine disrupting chemicals (EDCs) such as metformin, particularly at low doses (Vandenburg 2014; Hill, Myers and Vandenburg 2018). In fact, exposure to environmentally-relevant metformin concentrations during bovine oocyte development led to an increase in the number of 2-cell embryos at the highest concentration (10,000 nM), but led to arrested growth at lower concentrations, which decreased the percentage of embryos past the 8-cell stage (Pikiou et al., 2015). Similar to mortality, percentage of unhatched eggs was also affected in the extended duration metformin exposure group in a non-monotonic pattern, with a

significant decrease only at a mid-range concentration (100 nM). Although few studies have examined metformin-induced changes in hatching rate, one study examining metformin exposure of fathead minnow egg and larval stages revealed a similar outcome to the current study with no significant effect on percentage of unhatched eggs at concentrations < 100 nM (Parrott et al., 2021). Zebrafish hatching rate is plastic in response to adverse environmental factors, such as salinity (Ord, 2019), and other EDCs, such as tributylin (Liang et al., 2017). However, unlike the non-monotonic pattern seen with metformin exposure, tributylin exposure increased percentage of unhatched eggs in a monotonic fashion once concentrations exceeded 1 ng/L (Zhang et al., 2011).

The neurobehavioral changes in the short-term metformin exposure group also exhibited a non-monotonic response, with significantly more distance traveled during dark periods at 10, 100, and 1000 nM compared to the control group.. During the light periods, zebrafish in the short-term metformin exposure group exhibited hyperactivity at 10 and 100 nM. Zebrafish are generally more active during dark periods than light periods (Ogungbemi et al., 2019), potentially due to a search for better lit environments that maximize their ability to feed (Burgess and Granato, 2007). Although no significant transcriptomic changes occurred in the short-term metformin exposure groups, proline biosynthesis was implicated following the extended duration exposure. Studies have shown that proline exposure leads to hyperactivity in zebrafish (Savio et al., 2012). Hypoactivity was the main neurobehavioral outcome seen after extended duration metformin exposure, with significantly less distance traveled during the dark cycles after extended duration exposure to 1, 1000, and 10,000 nM metformin. During the light cycle for the extended duration metformin exposure group, distance decreased significantly when compared to the control group at 1 nM. A previous study found that approximately 28% of 91 different chemicals cause hypoactivity in developing zebrafish, including pharmaceuticals, flame retardants, PAHs, and pesticides (Dach et al., 2019). A gene implicated in retinal morphogenesis, *atp6v0e1*, was upregulated at 100 and 10,000 nM in the extended duration exposure. A previous study examining a *atp6v0e1* mutation in larval zebrafish showed aberrant retinal morphology and impaired photoreception that resulted in altered responses to changing light conditions, notably hypoactivity during dark periods (Daly et al., 2017).

To our knowledge, no studies have examined the effects of metformin on neurological development during embryogenesis. Brain morphogenesis in zebrafish occurs in two phases with the first phase occurring between 17 – 24 hpf, during which the main structures are formed, and the second phase occurring between 24 – 36 hpf, during which the amount of brain tissue and volume of the ventricle volumes increase substantially (Lowery et al., 2009). The extended duration metformin exposure occurred during these critical periods, while the short term exposure occurred when zebrafish had a fully developed brain. Environmental contaminants can induce different effects depending on window of exposure. For example bifenthrin exposure in zebrafish showed transcriptional responses that first occurred during exposure, while behavioral responses did not occur until after a 14 day recovery period, and then only at concentrations <10 ng/L (Frank et al., 2018). However, in adult male Wistar rats the opposite effect was seen, in that behavioral effects during bifenthrin exposure were quickly extinguished during the recovery period (Syed et al., 2018). For bifenthrin, this suggests that different endpoints stem from exposure in organisms with mature versus

developing brains. Thus it is not surprising that metformin induced different endpoints under different exposure paradigms, but the mechanism of these differences should be explored further in future studies.

In conclusion, the findings of the present study contribute to the limited studies of developmental metformin toxicity in embryogenesis and larval zebrafish. Our findings indicate that early embryonic (< 4 dpf) exposure is a critical window for metformin sensitivity in zebrafish, and can lead to non-monotonic responses in mortality, percentage of unhatched eggs, and neurobehavior. Cardiac edema, along with transcriptomic changes associated with the cardiovascular system, was the most prevalent abnormality resulting from metformin exposure, specifically at the higher doses of 1000 and 10,000 nM. Furthermore, our data indicates several future areas of exploration, such as the potential impacts of differential gene expression on metformin-induced outcomes, particularly differing neurobehavioral and transcriptomic endpoints stemming from exposures during different stages of neurological development and maturity. Although metformin exposure has been studied in obese and diabetic animals with therapeutic results, our results indicate that metformin exposure can potentially lead to adverse outcomes in healthy individuals and should also be explored further. Overall, our results indicate the potential for adverse phenotypic, behavioral, and transcriptomic changes caused by the endocrine disruptor, metformin. As environmental concentrations of this prescription medication are likely to increase, continued investigation of the potential effects of chronic, low level exposures is needed not only for aquatic organisms, but also for humans, particularly related to early life exposures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Extended duration metformin exposure leads to decreased survival in zebrafish
- Significant abnormalities were seen with extended duration metformin exposure
- Extended and short term exposure had a significant effect on behavioral activity
- Metformin exposure dysregulates several development pathways and genes

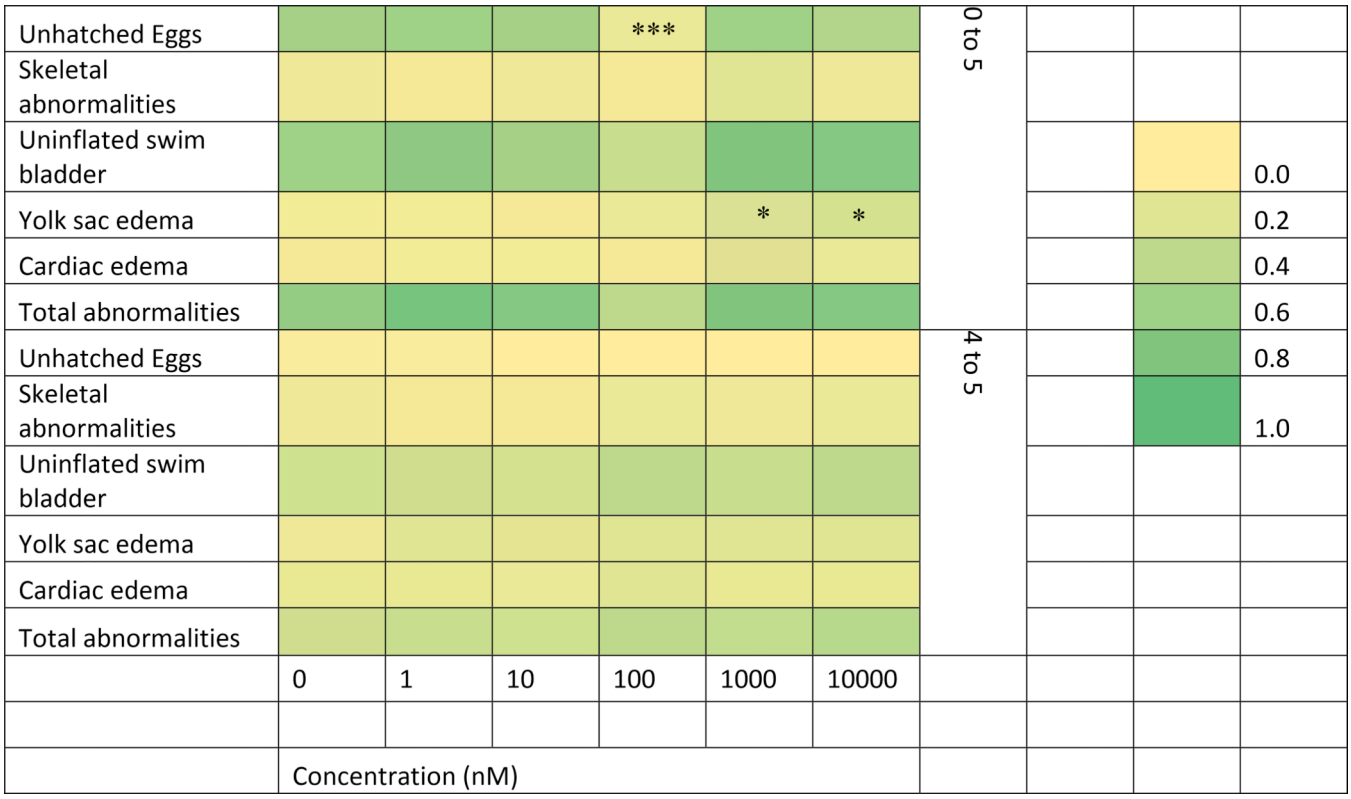

Figure 1:

Abnormality rate of control fish and fish exposed to metformin starting from 4 hours post-fertilization to 5 days post-fertilization (0–5 dpf) or 4 – 5 dpf. * Indicates significant difference from control ($p < 0.05$), *** ($p < 0.001$).

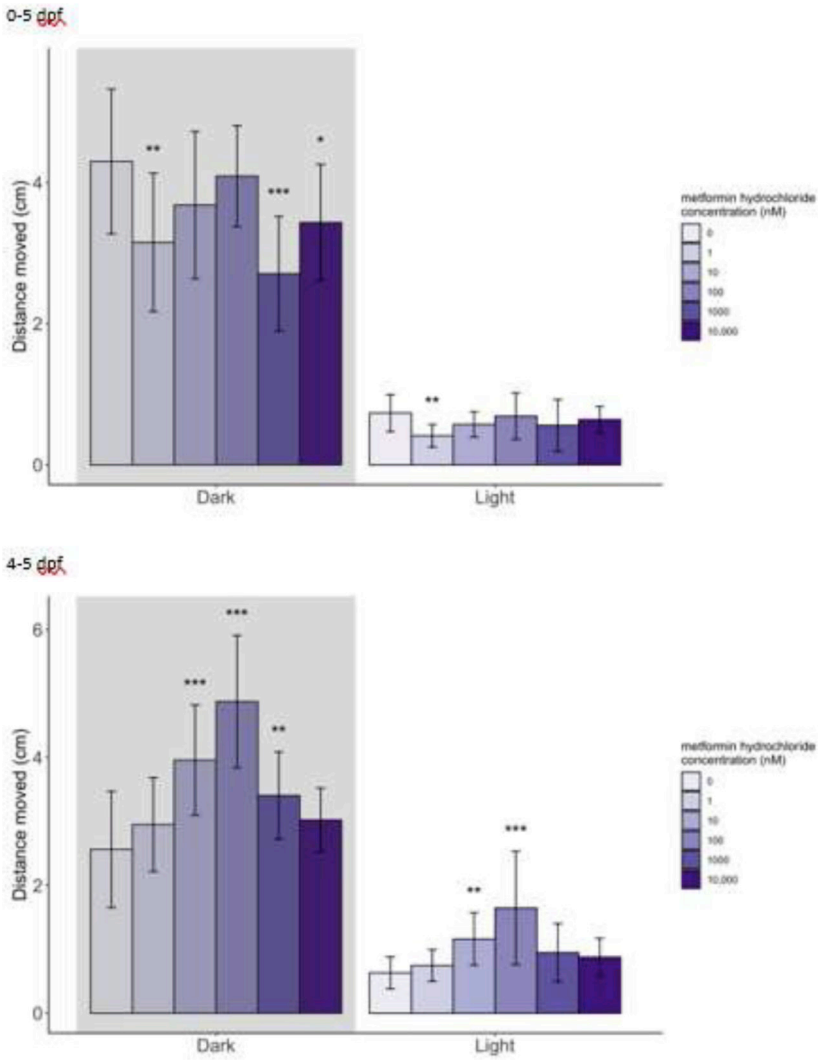

Figure 2:

Average distance moved ± standard deviation by larval zebrafish during light and dark cycles following extended duration metformin exposure starting from 4 hours post-fertilization to 5 days post-fertilization (dpf; 0–5 Day) or short term 4 – 5 dpf (4–5 Day). 24 larval zebrafish were tested for each exposure duration. All behavioral tests were performed between 14:00 and 22:00 and consisted of an assay of 3-minute light and dark alternating periods with a total of four light-dark cycles (24 minutes in total), these periods were integrated into 30 second intervals for data analysis. Significant difference from control at $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (**).

Table 1:

Differential expression for all genes altered in zebrafish following extended duration metformin exposure starting at 4 hours post-fertilization through 5 days post-fertilization. Significant absolute log2 fold changes (value ≥ 0.75 and adjusted p-value < 0.1) in bold. (ND = no difference in expression)

		Exposure concentration		
Gene symbol	**Gene name**	**1 nM**	**100 nM**	**10,000 nM**
Cardiovascular				
lgmn	legumain	**1.1**	**0.8**	0.5
abraa	actin binding Rho activating protein a	−0.1	**0.8**	0.4
smdt1a	single-pass membrane protein with aspartate-rich tail 1a	ND	0.3	**0.8**
atp2a2a	ATPase sarcoplasmic/endoplasmic reticulum Ca2+ transporting 2a	0.6	0.3	**0.8**
tnni1c	troponin I, skeletal, slow c	0.3	0.5	**0.9**
hsd11b2	hydroxysteroid (11-beta) dehydrogenase 2	0.7	0.5	**0.9**
atp6v0e1	ATPase H+ transporting V0 subunit e1	0.7	**0.8**	**0.9**
Neurological				
crygm2d10	crystallin, gamma M2d10	**−0.9**	−0.3	−0.2
nedd9	neural precursor cell expressed, developmentally down-regulated 9	**0.9**	0.2	0.7
prelid3b	PRELI domain containing 3B	**1.2**	0.6	0.6
pycr1b	pyrroline-5-carboxylate reductase 1b	0.6	0.5	**0.8**
mfsd2ab	major facilitator superfamily domain containing 2ab	0.7	0.4	**1.0**
hjv	hemojuvelin BMP co-receptor	0.5	0.5	**1.0**
Metabolic Processes				
ssr1	signal sequence receptor, alpha	**0.8**	0.1	0.6
lnx1	ligand of numb-protein X 1	**0.8**	0.2	0.5
Immune System				
lect2l	leukocyte cell-derived chemotaxin 2 like	0.1	−0.5	**−0.8**
ccl25b	chemokine (C-C motif) ligand 25b	0.8	0.5	**1.0**
Intracellular Processes				
si:ch211–74f19.2	Si:ch211–74f19.2	**−0.9**	−0.2	−0.3
fam234b	family with sequence similarity 234 member B	**0.8**	0.3	0.7
hpda	4-hydroxyphenylpyruvate dioxygenase a	0.4	**0.8**	0.5
si:ch211–114n24.6	Si:ch211–114n24.6	0.3	**0.8**	0.6
ypel5	yippee-like 5	−0.5	−0.3	**−1.1**
si:ch211–181d7.3	Si:ch211–181d7.3	0.4	0.2	**0.9**
stc1l	stanniocalcin 1, like	0.3	0.3	**0.8**