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METFORMIN EXPOSURE AT ENVIRONMENTALLY RELEVANT CONCENTRATIONS CAUSES POTENTIAL ENDOCRINE DISRUPTION IN ADULT MALE FISH

Nicholas J. Niemuth,† Renee Jordan,† Jordan Crago,† Chad Blanksma,‡ Rodney Johnson,§ and Rebecca D. Klaper*†

†School of Freshwater Sciences, University of Wisconsin-Milwaukee, Milwaukee, Wisconsin, USA
‡Oak Ridge Institute for Science and Education Research Participation Program, Mid-Continent Ecology Division, National Health and Environmental Effects Research, Laboratory, Office of Research and Development, US Environmental Protection Agency, Duluth, Minnesota, USA
§Mid-Continent Ecology Division, National Health and Environmental Effects Research, Laboratory, Office of Research and Development, US Environmental Protection Agency, Duluth, Minnesota, USA

Abstract: Pharmaceuticals and personal care products (PPCPs) are emerging contaminants that have been found ubiquitously in wastewater and surface waters around the world. A major source of these compounds is incomplete metabolism in humans and subsequent excretion in human waste, resulting in discharge into surface waters by wastewater treatment plant (WWTP) effluent. One pharmaceutical found in particularly high abundance in recent WWTP effluent and surface water studies is metformin, one of the world's most widely prescribed antidiabetic drugs. Interactions between insulin signaling and steroidogenesis suggest potential endocrine-disrupting effects of metformin found in the aquatic environment. Adult fathead minnows (*Pimephales promelas*) were chronically exposed to metformin for 4 wk, at 40 μg/L, a level similar to the average found in WWTP effluent in Milwaukee, Wisconsin, USA. Genetic endpoints related to metabolism and endocrine function as well as reproduction-related endpoints were examined. Metformin treatment induced significant up-regulation of messenger ribonucleic acid (mRNA) encoding the egg-protein vitellogenin in male fish, an indication of endocrine disruption. The present study, the first to study the effects of environmentally relevant metformin exposure in fathead minnows, demonstrates the need for further study of the endocrine-disrupting effects of metformin in aquatic organisms. *Environ Toxicol Chem* 2015;34:291–296. © 2014 The Authors. Published by Wiley Periodicals, Inc. on behalf of SETAC. This is an open access article under the terms of the Creative Commons Attribution-NonCommercial License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited and is not used for commercial purposes.

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INTRODUCTION

Pharmaceuticals and personal care products (PPCPs) are emerging contaminants that have been found ubiquitously in wastewater and surface waters around the world [1–4]. Wastewater treatment plants (WWTPs) are one of the major contributors of PPCPs in the environment. An individual taking a medication excretes a large portion of the medication either unaltered or slightly modified and this eventually enters WWTPs. The PPCPs processed in WWTPs are removed with differing degrees of efficiency [5,6]. Although these compounds generally leave WWTPs at low levels, risk analyses and exposure studies have raised concerns about potential negative impacts of PPCPs even at current environmental levels [4,7–9].

Recent work has revealed the biguanide metformin to be among the most abundant pharmaceuticals being introduced into the environment; it is found in WWTP effluent at concentrations of 1 μg/L to 47 μg/L and in surface waters at concentrations from 0.06 μg/L to 3 μg/L [1,3,7,10,11]. Metformin is one of the most widely prescribed antidiabetic drugs in the world and is also indicated as a potential treatment in various cancers [12] as well as in polycystic ovary syndrome, an endocrine disorder affecting 5% to 15% of reproductive-age women [13].

Metformin is proposed to act primarily by inhibiting complex I of the electron transport chain, impacting cellular energy balance. Complex I inhibition results in an increase in the cellular adenosine monophosphate:adenosine triphosphate (AMP:ATP) ratio, activating the regulatory kinase (K) AMPK. Acting as a cellular energy sensor, AMPK phosphorylates metabolic enzymes and transcription factors, causing inhibition of gluconeogenesis and activation of glycolysis (e.g., inhibiting fructose-1,6-bisphosphatase [FBPase] and up-regulating glucokinase [GK]), and promoting fatty acid oxidation (e.g., down-regulating fatty acid synthase [FASN]) [14].

Metformin has also been shown to improve glucose uptake. By acting on metabolic pathways to promote catabolism and glucose uptake, metformin sensitizes cells to insulin, thereby affecting pathways regulated by insulin signaling. Pathways affected by insulin signaling include steroidogenic pathways. Metformin has been shown to affect expression of steroidogenic enzymes such as cytochrome p450 (CYP) 11A and 3β–hydroxysteroid dehydrogenase (3β-HSD) as well as the cholesterol transporter steroid acute regulatory protein, and CYP17 activity in mammals at concentrations from 10 mg/kg/d to 250 mg/kg/d [14]. Metformin administered at a therapeutic dose of 1500 mg/d has also been shown to affect levels of anti-Müllerian hormone in polycystic ovary syndrome patients [15].

Ovaries in women with polycystic ovary syndrome produce high levels of androgens, which cause oligoovulation or anovulation. In many polycystic ovary syndrome patients, insulin resistance and the resulting hyperinsulinemia are thought to contribute to the disease by creating excessive insulin signaling in the ovary, thereby inducing androgenesis [14]. By improving insulin sensitivity, metformin reduces circulating insulin levels and thus attenuates insulin-induced androgenesis in the ovaries of polycystic ovary syndrome patients [14].

The link between insulin signaling and steroidogenesis indicates the potential for antidiabetic drugs including metformin to act as endocrine disruptors. It has been suggested that the prevalence of intersex fish in certain watersheds may be the result of WWTP effluent, which contains a cocktail of potential and known endocrine disruptors [16,17]. Although metformin is one of the most prevalent pharmaceuticals in WWTP effluent [1,3,7], the impact of metformin on aquatic life has only been explored for its metabolic effects as they pertain to aquaculture [18–21] or drug screening [22], and the putative endocrine-disrupting effects of metformin have not been studied at all in aquatic organisms.

The aim of the present study was to determine whether a chronic exposure of adult *Pimephales promelas*, the North American fathead minnow, to levels of metformin found in WWTP effluent [7] would cause detectable endocrine disruption. Fathead minnows are an aquatic species commonly used by the US Environmental Protection Agency (USEPA) to evaluate endocrine disruption in fish [23,24]. We evaluated a host of reproductive endpoints including egg production, plasma hormone levels, and expression of genes associated with metabolism and reproduction in adult fathead minnows mated in pairs during a 28-d chronic metformin exposure.

MATERIALS AND METHODS

Chemicals

Metformin (1,1-dimethylbiguanidine hydrochloride; CAS # 1115–70-4) and ethanol (200 proof; CAS # 64–17-5) were purchased from Sigma-Adrich. A 100-mg/L stock solution of metformin was prepared by adding 50 mg of metformin to 10 mL of ethanol, stirring until dissolved, and then adding this to 490 mL of Milli-Q ultrapure water (EMD Millipore). A final metformin concentration of 40 µg/L, the average concentration found by our previous study in WWTP effluent [7], was achieved by adding 8 mL of this stock solution to 20 L of dechlorinated water in each treatment tank. A stock solution for control tanks was prepared by adding 10 mL of ethanol to 490 mL of ultrapure water; then 8 mL of this vehicle stock was added to 20 L of dechlorinated water in each control tank.

Exposures

Adult fathead minnows (*P. promelas*) were obtained from the laboratory culture maintained within the Klaper laboratory at the University of Wisconsin-Milwaukee School of Freshwater Sciences (Milwaukee, WI, USA). Twelve 40-L tanks were divided into 2 compartments using a clear porous divider, and a male–female pair was placed in each compartment. Two pairs were placed in each tank separated with a clear porous divider, as fathead minnows are group-spawners. Six tanks (12 pairs) were dosed with 40 µg/L metformin, and the remaining 6 tanks were dosed with ethanol vehicle only. Fish were kept on a 16:8-h light: dark cycle and fed Tetramin tropical fish food twice daily (Tetra). Exposures were static, and a 100% water exchange occurred every 4th day and 7th day. Metformin concentrations in water samples from control and treatment tanks were determined by liquid chromatography–tandem mass spectrometry at the Wisconsin State Laboratory of Hygiene (Madison, Wisconsin, USA). Metformin was below the limit of detection in control samples and was found at 41 ± 8 µg/L in treatment samples.

After 28 d of exposure, male and female fish were euthanized, and the weight and length of each fish were measured. Blood was drawn from the caudal artery or vein using a heparinized microhematocrit capillary tube following tail dissection. Blood was centrifuged for 3 min at 15 000 *g* at 4 °C, and plasma was collected. The liver and gonad of each fish were collected and flash-frozen in liquid nitrogen. A portion of each gonad was collected in a histology cassette and immersed and stored in 10% neutral buffered formalin. Plasma and flash-frozen tissues were stored at –80°C for later analysis.

Gene expression

Ribonucleic acid (RNA) from fathead minnow liver and gonad was extracted using TriZol per the manufacturer's instructions (Invitrogen). The concentration and quality of RNA were measured using a Nanodrop spectrophotometer (Thermo Fisher Scientific). The integrity of the RNA was confirmed using an Experion bioanalyzer (Bio-Rad). The RNA samples were deoxyribonuclease (DNase) treated using RQ1 DNase (Promega), and 500 ng of RNA were used to create complementary (C)DNA using the SuperScript III reverse transcriptase kit (Invitrogen).

We looked at the expression of a number of different genes. The genes selected were those related to metabolism and observed to be affected by metformin exposure in mammals and other fish studies (GK, FBPase, and FASN) [14,15,18–21,25,26]; genes seen to be affected by exposure to chemicals in wastewater in other fathead minnow studies: CYP3A4 and the pregnane X receptor (PXR), both of which are involved in steroid synthesis and the metabolism of xenobiotics; and the steroid synthesis gene CYP19A [27]. We also examined mRNA transcripts encoding for the egg yolk precursor protein vitellogenin (VTG), which is produced in the liver of females in oviparous species including fathead minnows. Expression of VTG mRNA and protein in male fathead minnows has become a metric used by the USEPA as an indicator of endocrine disruption [23,24]. Because of low RNA yields for livers from some fish, only 8 samples per group were used to measure expression of GK, FBPase, FASN, and PXR.

Primer sequences used are shown in Table 1 [27–29]. Primers for GK, FBPase, and anti-Müllerian hormone were designed by searching the *P. promelas* expressed sequence tag database using tBLASTx for the respective mRNA sequence from *Danio rerio*. The highest scoring expressed sequence tag result was then used for primer design using PrimerQuest software (Integrated DNA Technologies) for intercalating dyes.

Gene expression was quantified using the iTaq Universal SYBR Green Supermix 20 µL protocol (Bio-Rad). Reverse transcriptase–polymerase chain reactions were run on the StepOnePlus system (Life Technologies) using the following protocol: 1) 1 cycle at 95 °C for 10 min, and 2) 40 cycles of 95 °C for 15 s and 60 °C for 30 s. The 18 S Universal Primers from Ambion (Life Technologies) were used for normalization across samples. Analyses of reverse transcriptase–polymerase chain reactions results were carried out using the ΔCt method.

Plasma VTG

Plasma VTG protein levels for males were measured using a fathead minnow-specific enzyme-linked immunoabsorbent assay kit for VTG (Cayman Chemical), following the manufacturer's instructions. Absorbance at 450 nm was measured on a Wallac 1420 platereader (PerkinElmer). The VTG protein concentration for samples was calculated by comparison with a standard curve prepared from the Cayman kit. Samples were assayed at 2 dilutions in duplicate. Because of

Table 1. Primer sets used

Gene	Primer	Sequence 5'→3'	Tissue tested	Sex tested	Primer design source[a]
VTG	Fwd	TGACAGCTAGTTTGGCTATG	Liver	Male, Female	[28]
VTG	Rev	AATATCATGGATGGGCCTGA	Liver	Male, Female	[28]
GK	Fwd	GAGGATACCGAGTGACTTTGAC	Liver	Male, Female	JGI_ANNO38318.fwd
GK	Rev	CTCTCTTCGCATTCTCCTCATC	Liver	Male, Female	JGI_ANNO38318.fwd
FASN	Fwd	ATTCTGCTGGACGCTTTGTT	Liver	Male, Female	[29]
FASN	Rev	GTCTCCTCTGAACGGACCTG	Liver	Male, Female	[29]
FBPase	Fwd	GACCACATACAGAGCCGAATAG	Liver	Male, Female	JGI_CAAS11466.fwd
FBPase	Rev	TTTGACGTTCAGATCACTCTCTC	Liver	Male, Female	JGI_CAAS11466.fwd
AMH	Fwd	GATCACACACAGGTCGTCATAG	Liver	Male, Female	JGI_CAAX9014.rev
AMH	Rev	CCGTCCTTCTTAACAGCCATATC	Liver	Male, Female	JGI_CAAX9014.rev
CYP3A4	Fwd	TTCCGTTCTTCGGAACGATGCTGA	Liver	Male, Female	[27]
CYP3A4	Rev	TGTGTTTGACACCCACAGGTTCAC	Liver	Male, Female	[27]
CYP11A	Fwd	TGGTGTGCTGGCTAGCCTTCTAAT	Liver	Male, Female	[27]
CYP11A	Rev	AGGTGATACGAGCAGCCGAGATTT	Liver	Male, Female	[27]
PXR	Fwd	GATCATGTCGGATGAGGCCGTC	Liver	Male, Female	[27]
PXR	Rev	CCGCTGCACTCCATCTTCAGG	Liver	Male, Female	[27]
CYP17	Fwd	AGGCTCTGGCAAAGATGGAACTCT	Liver, testes	Male, Female	[27]
CYP17	Rev	AAGAACCACACCAAACTTGCCCTG	Liver, testes	Male, Female	[27]
StAR	Fwd	AAACTGCGTGCTGGCATTTCCTAC	Liver, testes	Male, Female	[27]
StAR	Rev	TGCTCAGCTTCACTGAACGTCTCT	Liver, testes	Male, Female	[27]
CYP19A	Fwd	ATGTTGATCGCGGCTCCAGATACT	Testes	Male	[27]
CYP19A	Rev	TTAACCTGGACAGATGCGAGTGCT	Testes	Male	[27]
3β-HSD	Fwd	GCTGTCAATGTACAAGGGACGGA	Testes	Male	[27]
3β-HSD	Rev	TTGGTCTTTGGGTAGGGCATCTCA	Testes	Male	[27]

VTG = vitellogenin; Fwd = forward; Rev = reverse; GK = glucokinase; FASN = fatty acid synthase; FBPase = fructose-1,6-bisphosphatase; AMH = anti-Müllerian hormone; CYP = cytochrome; PXR = pregnane X receptor; StAR = steroid acute regulatory protein; 3β-HSD = 3β–hydroxysteroid dehydrogenase.
[a]Primer designs beginning JGI are from the National Center for Biotechnology information EST database.

difficulty in obtaining plasma samples, only 5 fish per treatment were used in this assay.

Plasma testosterone

Male plasma testosterone levels were measured using the testosterone enzyme immunoassay kit from Cayman. Plasma samples were ether-extracted and assayed per the manufacturer's instructions. Absorbance at 405 nm was measured on a Wallac 1420 platereader (PerkinElmer). Testosterone concentrations in the samples was calculated by comparison with a standard curve prepared from the Cayman kit. Samples were assayed in duplicate. Because of difficulty in obtaining plasma samples, only 5 fish per treatment were used in this assay.

Reproduction and tissue histology endpoints

Reproduction was assessed as total eggs laid per pair over the 28-d exposure period, counted daily. Routine histological embedding of formalin-fixed gonad tissues, sectioning, hematoxylin and eosin staining, and slide preparation were performed by Histology Tech Services. Histopathological evaluation of male gonad sections was conducted at the USEPA (Duluth, Minnesota, USA).

Statistics

Data were tested for normality using the Shapiro–Wilk test and for equality of variances using Levene's test. Gene expression of treated and untreated males and females in the liver was analyzed using a 2-way analysis of variance (ANOVA). When treatment, sex, or treatment × sex were found to be significantly different, groups were compared using independent-samples t tests. Analyses of gene expression for male gonad, plasma VTG, plasma testosterone, and egg number were performed using an independent-samples t test. Significance was considered to be $p < 0.05$. All analyses were performed and outliers were removed using SPSS Ver 22 for MAC.

RESULTS

Metabolic gene expression (GK, FBPase, FASN)

No differences in expression were found in the liver between treatment or sex for the metabolic genes GK (2-way ANOVA: sex $p = 0.922$, $df = 1$, $F = 0.010$; treatment $p = 0.963$, $df = 1$, $F = 0.002$) or FBPase (2-way ANOVA: sex $p = 0.977$, $df = 1$, $F = 0.001$; treatment $p = 0.070$, $df = 1$, $F = 3.581$). No treatment effect was observed for FASN (2-way ANOVA: treatment $p = 0.974$, $df = 1$, $F = 0.974$). However, FASN was significantly higher in males compared with females (2-way ANOVA: sex $p = 0.001$, $df = 1$, $F = 13.178$; Figure 1).

Detoxification gene expression (CYP3A4, CYP11A, CYP17, PXR)

A significantly higher expression in the liver of males compared with females was found for PXR (2-way ANOVA: sex $p < 0.001$, $df = 1$, $F = 35.073$), but no difference between treatments (2-way ANOVA: treatment $p = 0.334$, $df = 1$, $F = 0.969$; Figure 2). No differences in liver expression were found between sex or treatment for CYP3A4, CYP11A, or CYP17 (2-way ANOVA: CYP3A4, sex $p = 0.653$, $df = 1$, $F = 0.025$, treatment $p = 0.994$, $df = 1$, $F < 0.001$; CYP11A, sex $p = 0.148$, $df = 1$, $F = 2.235$, treatment $p = 0.218$, $df = 1$, $F = 1.597$; CYP17, sex $p = 0.861$, $df = 1$, $F = 0.031$, treatment $p = 0.180$, $df = 1$, $F = 1.864$).

Endocrine-related gene expression (steroid acute regulatory protein, anti-Müllerian hormone, VTG, CYP17, CYP19A, 3β-HSD)

As seen in Figure 3, VTG mRNA was expressed at a significantly higher level in metformin-treated males compared with control males (2-way ANOVA: sex $p < 0.001$, treatment $p = 0.010$, sex × treatment $p = 0.004$; t test: control males vs metformin-treated males: $p = 0.002$, $df = 16.885$, $t = 3.769$). As expected, females expressed VTG mRNA at levels significantly higher than males (t test: control male vs control
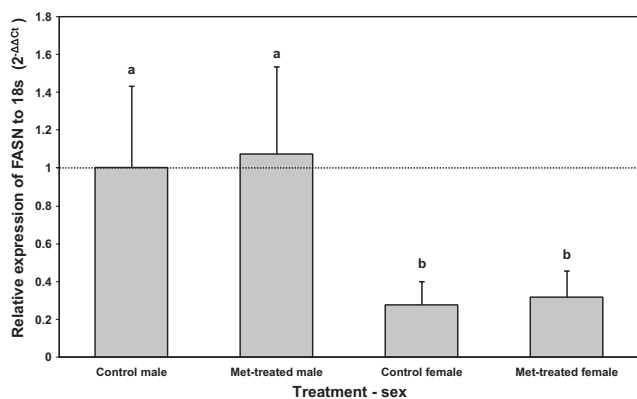
Figure 1. Relative messenger ribonucleic acid (mRNA) transcript expression of fatty acid synthase (FASN) mRNA transcripts in liver of male and female fathead minnow exposed to control and 40 μg/L metformin (Met) for 28 d. Error bars represent standard error of the mean (SEM). $n = 8$ for all groups. Two-way analysis of variance: sex $p = 0.001$, $df = 1$, $F = 13.178$; treatment $p = 0.974$, $df = 1$, $F = 0.001$; sex × treatment $p = 0.946$, $df = 1$, $F = 0.005$. t test: control males versus metformin-treated males: $p = 0.970$, $df = 14$, $t = -0.038$; control male versus control female: $p = 0.016$, $df = 14$, $t = -2.751$; control male versus metformin-treated female: $p = 0.023$, $df = 14$, $t = -2.544$; metformin-treated male versus control female: $p = 0.021$, $df = 14$, $t = -2.590$; metformin-treated male versus metformin-treated female: $p = 0.031$, $df = 14$, $t = -2.406$; control female versus metformin-treated female: $p = 0.955$, $df = 14$, $t = 0.057$.

female: $p < 0.001$, $df = 18$, $t = 19.076$; control male vs metformin-treated female: $p < 0.001$, $df = 20$, $t = 19.898$; metformin-treated male vs control female: $p < 0.001$, $df = 20$, $t = 9.663$; metformin-treated male vs metformin-treated female: $p < 0.001$, $df = 22$, $t = 10.242$), but there was no significant difference between control and treated females (t test: $p = 0.765$, $df = 20$, $t = -0.303$). No differences in expression were found between sex or treatment for steroid acute regulatory protein or anti-Müllerian hormone in the liver (2-way ANOVA:
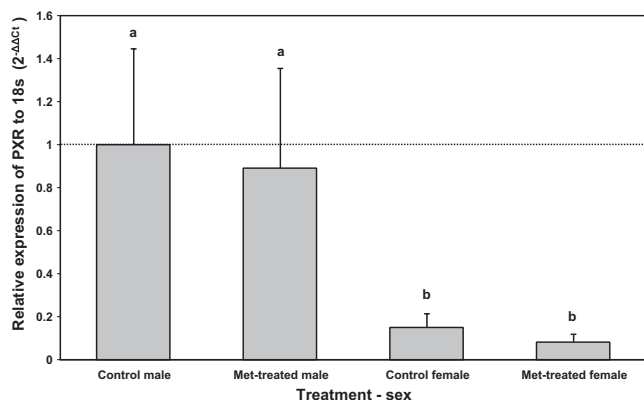

Figure 2. Relative messenger ribonucleic acid (mRNA) transcript expression of pregnane X receptor (PXR) mRNA transcripts in liver of male and female fathead minnow exposed to control and 40 μg/L metformin (Met) for 28 d. Error bars represent standard error of the mean (SEM). Control males $n = 8$; metformin-treated males $n = 7$; control females $n = 7$; metformin-treated females $n = 8$. Two-way analysis of variance: sex $p < 0.001$, $df = 1$, $F = 35.073$; treatment $p = 0.334$, $df = 1$, $F = 0.969$; sex × treatment $p = 0.735$, $df = 1$, $F = 0.117$. t test: control males versus metformin-treated males: $p = 0.700$, $df = 13$, $t = -0.394$; control male versus control female: $p = 0.002$, $df = 13$, $t = -3.780$; control male versus metformin-treated female: $p < 0.001$, $df = 14$, $t = -5.521$; metformin-treated male versus control female: $p = 0.009$, $df = 12$, $t = -3.101$; metformin-treated male versus metformin-treated female: $p < 0.001$, $df = 13$, $t = -4.642$; control female versus metformin-treated female: $p = 0.274$, $df = 13$, $t = -1.142$.
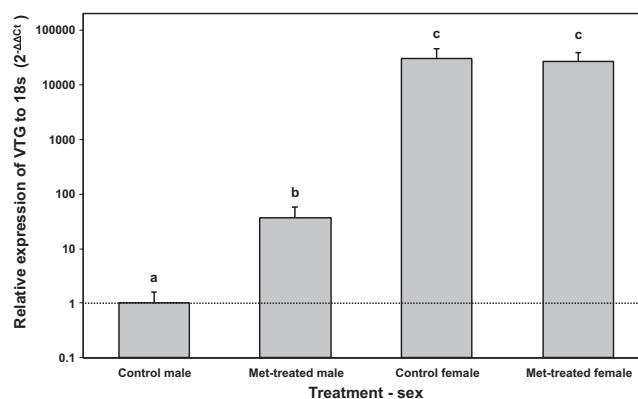

Figure 3. Relative messenger ribonucleic acid (mRNA) transcript expression of vitellogenin (VTG) mRNA transcripts in liver of male and female fathead minnow exposed to control and 40 μg/L metformin for 28 d. Error bars represent standard error of the mean (SEM). Control males $n = 10$; metformin-treated males $n = 12$; control females $n = 10$; metformin-treated females $n = 12$. Two-way analysis of variance: sex $p < 0.001$, $df = 1$, $F = 358.444$; treatment $p = 0.010$, $df = 1$, $F = 7.382$; sex × treatment $p = 0.004$, $df = 1$, $F = 9.427$. t test: control males versus metformin-treated males: $p = 0.002$, $df = 16.885$, $t = 3.769$; control male versus control female: $p < 0.001$, $df = 18$, $t = 19.076$; control male versus metformin-treated female: $p < 0.001$, $df = 20$, $t = 19.898$; metformin-treated male versus control female: $p < 0.001$, $df = 20$, $t = 9.663$; metformin-treated male versus metformin-treated female: $p < 0.001$, $df = 22$, $t = 10.242$; control female versus metformin-treated female: $p = 0.765$, $df = 20$, $t = -0.303$.

steroid acute regulatory protein, sex $p = 0.640$, $df = 1$, $F = 0.223$, treatment $p = 0.508$, $df = 1$, $F = 0.448$; anti-Müllerian hormone, sex $p = 0.799$, $df = 1$, $F = 0.065$, treatment $p = 0.480$, $df = 1$, $F = 0.509$). No difference in expression between treatments was found for steroid acute regulatory protein, CYP17, CYP19A, or 3β-HSD in male gonad (t test: steroid acute regulatory protein, $p = 0.748$, $df = 12.189$, $t = 0.382$; CYP17 $p = 0.571$, $df = 17$, $t = 0.580$; CYP19A $p = 0.325$, $df = 21$, $t = -1.007$; 3β-HSD $p = 0.548$, $df = 22$, $t = 0.610$).

Plasma VTG and testosterone levels

Although plasma VTG protein levels were found to be higher in metformin-treated male fathead minnows (control, 210 ± 50 ng/μL, $n = 5$; metformin-treated, $10\ 000 \pm 5000$ ng/μL, $n = 5$), this difference was not significant (t test: $p = 0.125$, $df = 4.001$, $t = -1.938$). There was no difference found in plasma testosterone levels between control and treated males (t test: $p = 0.996$, $df = 4$, $t = -0.005$).

Reproduction and histology

There was no difference in mean total egg number between control and treated mating pairs (control 160 ± 50 eggs/pair, $n = 12$; metformin-treated, 230 ± 60 eggs/pair, $n = 12$; t test: $p = 0.429$, $df = 22$, $t = -0.805$). No significant difference was observed for spawning frequency or clutch size between treatments. The histopathology of male gonad samples from treated and untreated samples was similar, with normal appearances of gonadal cells in testis.

DISCUSSION

Metformin causes induction of VTG expression in male fathead minnow liver, indicating potential endocrine disruption. Expression of VTG was found to be significantly up-regulated in metformin-treated males, a more than 30-fold overexpression

(Figure 3). Expression of VTG mRNA has been demonstrated as a sensitive marker of endocrine disruption in zebrafish (*D. rerio*) [30] and fathead minnow [23,31]. Although not significant, our VTG enzyme-linked immunoabsorbent assay kit also showed higher VTG protein levels in plasma of metformin-treated males. Although there was no indication of hormone or reproductive changes, this could be because of the length or timing of exposure.

Vitellogenin mRNA expression is an early and sensitive indicator of exposure to endocrine-disrupting compounds [23,30,31], being detectably up-regulated in male fathead minnow in as little as 24 h following exposure to the synthetic estrogen 17α-ethinylestradiol (EE$_2$) at concentrations as low as 2 ng/L [23]. Although not causing severe impacts in this short exposure [23], EE$_2$ is capable of producing severe endocrine effects with prolonged exposure [32]. Thus, it follows that the significant up-regulation of VTG seen in the present study may be indicative of the potential for greater endocrine-disrupting impacts from extended metformin exposure, such as may occur in the environment.

Vitellogenin expression is thought to be regulated primarily though estrogen receptors in the liver [33]. However, metformin does not structurally resemble hormone-like compounds classically identified as endocrine disruptors, and its impact on expression of genes like VTG that are associated with endocrine disruption may not be the direct result of the compound's binding to hormone receptors. Instead, these changes may occur as a result of the drug's effects on insulin signaling. Insulin is a peptide hormone and is known to influence many aspects of endocrine signaling by effecting steroid synthesis. It is this impact of insulin signaling on steroidogenesis that makes metformin a useful treatment in certain instances of polycystic ovary syndrome [14]. Although effects seen in the present study may possibly be the result of impacts from altered insulin signaling on steroid synthesis pathways, no differences in the mRNA levels for steroidogenic enzymes or changes in testosterone levels were observed, and the precise mechanism by which metformin induces VTG expression in male fathead minnows remains to be elucidated.

In the mammalian liver, metformin's major effects are generally thought to be on regulation of metabolic gene expression and their products through AMPK [14], including GK [25] and FASN [26]. In trout (*Oncorhynchus mykiss*), intraperitoneal injection or administration of metformin in food at 20 mg/kg/d to 50 mg/kg/d was shown to affect expression of GK, FASN, and FBPase in the liver [18–21]. In the present study with fathead minnow at a low level of exposure, no treatment effect was seen for expression of GK, FBPase, or FASN in liver. Thus, chronic low-level exposure to metformin in water does not appreciably affect expression-level regulation of these metabolic pathways in fathead minnow liver. One reason for these differences may be that the present study was designed specifically to mimic the type of exposure one might find at a WWTP outflow, exposing fish to a low dose of metformin in water, rather than exposing them through food or intraperitoneal injection as in other studies [18–21]. In this case metformin and other emerging contaminants would be expected to be primarily taken up by fish across their gills, and thus the amount of a given compound that crosses into the bloodstream will depend, among other things, on its lipophilicity [34] or transport via organic cation transporters as in mammals [35]. Although similar transport pathways may exist in fish gills as in

mammalian gut and liver [36], it is not known whether an analogous transporter exists in the fathead minnow gill. This may partly explain the very different results observed for different methods of administration.

Although not affected by treatment, the observed sexual dimorphism for PXR expression in the present study (Figure 1) resembles that seen previously in fathead minnow [37] and zebrafish [38]. Differences in PXR expression between males and females may be indicative of a difference in metabolism between the two sexes [37]. FASN also showed significant differences between sexes (Figure 2), and, although its sexual dimorphism has not been previously studied as is the case for PXR, metabolic differences between sexes may also account for observed differences in expression of this fatty acid synthesis gene.

The present study, the first to examine the impacts of metformin exposure to fish at environmentally relevant concentrations, demonstrates the need for further examination of the effects of this compound on aquatic life. Recent research suggests that wild fish such as walleye (*Sander vitreus*) and northern pike (*Esox lucius*) may be more sensitive to endocrine disruptors than fathead minnows [39]. Studies of the effects of metformin at higher doses, over longer periods of exposure, or in other species, may provide a more definitive answer as to the endocrine impacts of metformin exposure and any impacts on reproduction it may have for organisms exposed to this drug in the aquatic environment.

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