Effects of Metformin and its Metabolite Guanylurea on Fathead Minnow (Pimephales promelas) Reproduction

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INTRODUCTION

The occurrence of pharmaceuticals in the environment continues to be a prominent topic of concern for aquatic life. Individual pharmaceuticals are routinely found in surface waters at ng/L to low µg/L concentrations, even in relatively unpopulated areas of the United States (Bradley et al., 2020). Even though these concentrations are generally well below known therapeutic effect levels in humans, there are concerns that they could elicit unintended, adverse effects on exposed aquatic life. As global populations age, pharmaceutical use is only expected to increase, emphasizing the importance of understanding if and how these compounds are causing adverse effects on organisms in the environment. The pharmaceutical metformin, used to treat Type 2 diabetes, is one of the most commonly prescribed pharmaceuticals worldwide. From 2005 to 2015, metformin prescriptions in the United States rose from 2.27 to 235/1000 people (Le & Lee, 2019). This high rate of prescription has made metformin a ubiquitous surface water contaminant, and it is among the highest in concentration and the most frequently detected pharmaceuticals in surface waters. For example, in recent surveys of Great Lakes tributaries (Elliott et al., 2017) and streams from various regions of the
United States (Bradley et al., 2020), metformin was detected in 68% and 71% of samples, respectively, with a maximum concentration of 33.6 µg/L observed in the Cuyahoga River, a tributary of Lake Erie.

Metformin is not metabolized by humans and is rapidly excreted unchanged in urine with a half-life of 4–9 h following oral administration (Scheen, 1996). During wastewater treatment, metformin is incompletely converted into guanylurea (Trautwein & Kummerer, 2011) and may be further mineralized under appropriate conditions (Straub et al., 2019). Consequently, concentrations of guanylurea detected in the environment are often of a similar magnitude or higher than metformin. Guanylurea has only more recently begun to be monitored in the environment, resulting in its detection in United States (Blair et al., 2013; Bradley et al., 2020) and European (Kosma et al., 2015; Oosterhuis et al., 2013; Scheurer et al., 2012) effluents or surface waters at low µg/L concentrations. In surface waters, it is generally detected at higher concentrations than metformin (Caldwell et al., 2015). Median concentrations of metformin reported in surface waters across the United States and Europe were 17 and 235 ng/L, respectively, whereas median guanylurea concentrations in Europe were 2000 ng/L (no United States data were available at the time of review; Caldwell et al., 2019).

In addition to the potential for exposure, both metformin and guanylurea have demonstrated adverse effects in laboratory aquatic toxicity studies. A 2015 study (Niemuth, Jordan, et al., 2015) that exposed reproductively active fathead minnows (Pimephales promelas) to 40 µg metformin/L water for 28 days noted elevated vitellogenin (vtg) gene expression in male livers. A subsequent life cycle study exposing juvenile (30 days post fertilization [dpf]) fathead minnows to 40 µg metformin/L for up to 365 days observed decreased fecundity in spawning pairs and a significant increase in the incidence and severity of intersex in males (Niemuth & Klapar, 2015). Limitations of these initial studies have been noted, including lack of comprehensive chemical characterization and incidence of intersex in controls (Sumpter et al., 2016); numerous other studies have since been conducted to better understand the potential impacts of both metformin and guanylurea on fish, with mixed results. Multiple studies have identified significant effects on early life stage or juvenile fish, but a lack of effects in adults (Craco et al., 2016; Ussery et al., 2018), suggesting a possible developmentally sensitive window to metformin’s effects. However, most recently, a study of fathead minnow embryos exposed to varying concentrations (3.4–269 µg/L) of metformin for 21 days showed no significant impact on hatchability, survival, or growth (Parrott et al., 2022). Likewise, a full life cycle exposure of fathead minnows to metformin (3.0–300 µg/L) demonstrated no impacts on survival, growth, or fecundity (Parrott et al., 2021). Assessment of gonad histology showed no significant impacts on males or females, and no incidence of intersex was observed.

The toxicity of guanylurea to fish species has not been investigated as extensively as metformin, and studies have focused primarily on growth and development, not potential endocrine activity. Ussery and coauthors observed dose-dependent impacts on Japanese medaka (Oryzias latipes) larval growth following a 28-day early life stage exposure to 1–100 ng/L concentrations of guanylurea (Ussery et al., 2019); however, in a full life cycle test, no significant differences were observed in male or female weight, length, or condition factor following 165 days of exposure to 1 or 7.5 µg guanylurea/L, or a mixture of metformin and guanylurea. In that study, transcriptomic, metabolomic, and proteomic analyses of early life stages identified pathways potentially dysregulated by guanylurea, which could lead to the observed growth-related impacts. Other studies with guanylurea in developing brown trout (Salmo trutta f. fario) identified no changes in growth, development, mortality, lipid peroxidation, or stress protein levels (Jacob et al., 2019).

Given the variable effects observed following metformin exposure in small fish species and limited data on the effects of guanylurea, additional studies are needed to provide further weight of evidence for determining the ecological risks of the chemicals. In the present study we sought to better elucidate the potential reproductive toxicity of metformin and guanylurea through a series of experiments with sexually mature fathead minnows. First, an ex vivo exposure was conducted using mature fathead minnow ovary tissue to assess the potential for metformin or guanylurea to directly impact steroidogenesis. Second, a short-term reproduction assay was conducted using fathead minnows to assess the potential endocrine-related reproductive effects of the two compounds. Finally, a 96-h time course exposure using adult male fathead minnows was performed to explore the potential for metformin or guanylurea to affect other biological pathways using whole transcriptome and metabolome analyses.

MATERIALS AND METHODS

Chemicals

Metformin (CASRN 657-24-9; DTXSID2023270) and guanylurea (CASRN 141-83-3; DTXSID3043811) were purchased from Millipore-Sigma as metformin hydrochloride (>99% purity; CASRN 1115-70-4; DTXSID9037246) and guanylurea phosphate (>85% purity, see Supporting Information, Text S1; CASRN 17675-60-4). Mass labeled metformin-d6 and guanylurea-15N4 were purchased from Cayman Chemical and Toronto Research Chemicals, respectively. All solvents were purchased from Fisher Scientific and were of high-performance liquid chromatography grade or better.

Ex vivo chemical exposure

To examine the potential for metformin or guanylurea to directly impact steroidogenesis, production of both 17β-estradiol (E2) and testosterone was assessed for isolated fathead minnow ovary tissues exposed ex vivo as previously described (Villeneuve et al., 2007). Briefly, Medium 199 (Millipore-Sigma) supplemented with 100 µM 2-isobutyl-1-methylxanthene and 2.5 µM 25-hydroxycholesterol was dosed...
with methanol alone (vehicle control; maximum concentration of 0.01% methanol), test chemical in methanol, or prochloraz (5 µM) as a positive control (inhibitor) of steroid synthesis (Villeneuve et al., 2007). Subsamples of ovary tissue weighing 10–20 mg were placed in tubes containing 500 µL of dosed media and incubated at 25 °C for 20 h. Ovary tissue from 10 individual females was used per chemical, and tissue from each fish was divided across treatment groups. Six treatments ranged from 0.001 to 100 µM metformin or guanylurea at log10 intervals. Steroids were measured by radioimmunoassay using a previously described protocol (Jensen et al., 2001).

Fathead minnow chemical exposures

All procedures involving fathead minnows were performed in agreement with Animal Welfare Act and Interagency Research Animal Committee guidelines and under an in-house, approved animal care and use protocol. Adult, sexually mature fish were obtained from an onsite culture facility at the US Environmental Protection Agency (USEPA) laboratory in Duluth, MN. Fish in the test system were held at 25 ± 1 °C under a 16:8 h light:dark photoperiod and fed frozen adult brine shrimp twice daily. Control water (filtered, UV-treated Lake Superior water) or chemical-amended, solvent-free stocks were delivered to individual 20-L glass aquaria containing 10 L of solution at a continuous flow rate of 0.045 L/min. At completion of the experiments, fish were removed from test aquaria and anesthetized with buffered tricaine methane sulfonate (100 mg/L buffered with 200 mg/L NaHCO3). Fish whole-body wet weights were recorded prior to dissection. Glucose was measured in blood from the caudal vein at the time of dissection using a Glucocard Vital (Arkay) blood glucose meter with Glucocard Vital (Arkay) test strips according to the manufacturer’s recommended protocol. Remaining blood was collected into heparinized capillary tubes and centrifuged to obtain plasma. Liver tissue was divided into two separate microcentrifuge tubes and snap-frozen in liquid nitrogen for metabolomics or quantitative real-time–polymerase chain reaction (qRT–PCR) and transcriptomic analyses. Testes were placed in a microcentrifuge tube and snap-frozen for gene expression analysis.

Fish short-term reproduction assay

To examine potential reproductive impacts, a 23-day short-term reproduction assay was performed consistent with standardized protocols for evaluating chemicals as endocrine disruptors (USEPA, 2009). Two spawning pairs of fish were held in each 10-L tank containing test solution, divided by a nylon mesh screen, each with a polyvinyl chloride spawning substrate. Fish were 6–8 months in age and averaged (mean ± SD) 2.70 ± 0.53 and 1.31 ± 0.28 g wet weight for males and females, respectively. Prior to starting chemical exposure, pairs were held in the test system receiving only clean, Lake Superior water to assess fecundity over a 14-day acclimation period. Pairs that spawned successfully were randomly assigned to one of seven exposure treatments: control Lake Superior water, metformin (0.41, 4.1, 41 µg/L), or guanylurea (1.0, 10, 100 µg/L). Concentrations were chosen to encompass environmentally relevant ranges up to those reported in municipal effluents and using similar concentrations previously identified to cause adverse reproductive or growth effects in fish (Niemuth & Klaper, 2015; Ussery et al., 2019). Each treatment was comprised of six tanks (12 pairs) of fish. During the 23 days of exposure, fecundity and fertility of the spawned eggs were assessed daily. Over the course of the 23 days of exposure, one female died in each of the 4.1 µg metformin/L, 41 µg metformin/L, and 1.0 µg guanylurea/L treatments, and three females died in the 10 µg guanylurea/L treatment. Pairs were excluded from further fecundity calculations following the death of a female. One male in the 100 µg/L guanylurea treatment was visibly unhealthy at the end of the experiment (growth on spine, abdominal tumor, multiple damaged fins), so the spawning pair was also removed from fecundity analysis, and the male was not used for subsequent endpoint analyses.

The 96-h time course assay

Initial metabolomic and transcriptomic impacts in response to chemical exposure can be masked by biological compensation over longer term exposures (i.e., the duration of the reproduction assay; Ankley et al., 2012; Ekman et al., 2008b; Schroeder et al., 2017), so a short-term time course exposure was performed to identify potential biological pathways/processes of interest perturbed by metformin or guanylurea exposure. Because previous studies reported endocrine-like impacts in male fathead minnow following exposure to metformin (Niemuth & Klaper, 2015; Niemuth, Jordan, et al., 2015), we focused only on males. Adult, male fathead minnows 7–9 months of age (2.56 ± 0.66 g wet wt [mean ± SD]) were exposed to control water, 41 µg/L metformin, 100 µg/L guanylurea, or a mixture (41 µg/L metformin +100 µg/L guanylurea) of both chemicals for a time course experiment up to 96 h. The mixture was chosen as an environmentally relevant ratio and to replicate a similar ratio previously tested in a full life cycle test in Japanese medaka (Ussery et al., 2019). There were eight replicate tanks/experimental treatment, each with six fish. Two replicate tanks (n = 12 fish) were sampled following exposure durations of 6, 24, 48, and 96 h.

Exposure verification

Both metformin and guanylurea concentrations were measured throughout flow-through exposures using liquid chromatography–tandem mass spectrometry (LC–MS/MS). Water from each tank was collected and diluted with acetonitrile containing 10 ng of both metformin-d6 and guanylurea-15N4. The sample was directly injected onto an Agilent 1200 system (Agilent Technologies) coupled to an Agilent 6410 mass spectrometer. Analytes were quantified by an internal standard quantification method using MassHunter Quantitative Analysis (Ver B.06.00) software. Limits of
quantification (defined as lowest standard within ±20% accuracy) were 0.17 and 0.5 µg/L for metformin and guanylurea, respectively, and the limits of detection (defined as 3 times the value of blank response in internal standard spiked reagent water) were 0.097 and 0.31 µg/L, respectively. Additional details on sample preparation and LC–MS/MS analyses are provided in the Supporting Information, Text S2 and Table S1).

During the 96-h time course assay, metformin and guanylurea were measured in each individual tank containing fish at study initiation (0 h), and at 6, 24, 48, 72, and 96 h. During the 23-day reproduction assay, all tanks were measured daily for the first 2 days. Beginning on day 3, even and odd tanks were alternated, and chemical concentrations in each treatment were measured at least 3 times/week (three tanks measured/treatment each day) until study termination. Thus, chemical concentration in each individual tank was measured on 6 or 7 separate days across the 23-day study. Method performance was monitored by inclusion of method blanks, spiked Lake Superior water (n = 6/concentration; final concentration of 5.0 or 50 µg/L), and duplicate sample analyses. Mean (SD) spike recovery was 110.8 (19.1)% and 95.0 (11.7)% for metformin and guanylurea, respectively. Duplicate tank samples (metformin n = 13; guanylurea n = 12) had a mean relative percent difference of 6.9 (6.4)% and 9.8 (8.5)% for metformin and guanylurea, respectively.

**qRT-PCR measurements**

To quantify the abundance of mRNA transcripts in liver or gonad of male fathead minnows, qRT–PCR was used. Several endocrine function–related genes, previously shown to be impacted in male fathead minnows exposed to metformin (Niemuth & Klaper, 2018), were measured in tests. These included androgen receptor (ar), cytochrome P45019a aromatase (cyp19a), two steroid dehydrogenases (3βhsd and 17βhsd), and a sulfotransferase (sult2a1). In the liver, additional endocrine-responsive genes (estrogen receptor [esr1] and vtg), and genes associated with glucose metabolism (glucokinase [gck] and pyruvate kinase [pkhl]) were measured. Complete methods details including primer and probe sequences are described in the Supporting Information, Text S3 and Table S2.

**Plasma steroid measurements**

Plasma steroids were measured in both male and female fish from the 23-day reproduction assay. Sample preparation and analysis by LC–MS/MS was performed as previously described (Blackwell & Ankley, 2021), with the addition of 11-ketotestosterone-d3 as the internal standard for 11-ketotestosterone (11-KT) quantification. Briefly, 8–10 µL of plasma was spiked with 0.1 ng of mass-labeled internal standards and extracted by solid–liquid extraction using Phenomenex Novum Mini 96-well plates. Plasma was pooled from individuals within a single treatment aquarium, if necessary, to reach the desired sample volume. Final extracts were analyzed by LC–MS/MS using an Agilent 1290 system coupled to an Agilent 6490 mass spectrometer. Steroids were quantified with an internal standard quantification method using MassHunter Quantitative Analysis (Ver B.06.00) software. Method performance was monitored by inclusion of method blanks, a pooled female and male plasma quality control (QC) sample, and spiked pooled plasma (matrix spike; spiked at 10 ng/ml) with each batch. No analytes were detected above the reporting limit in method blanks (n = 4) except for 11-KT in two replicates at concentrations less than 0.53 ng/ml (lowest reported concentration in male plasma in the present study was 8.5 ng/ml). For the three steroids routinely detected in the pooled plasma QC (E2, testosterone, androstenedione [A4]; n = 3), the relative standard deviation (RSD) ranged from 2.8% to 6.6%. For the three steroids routinely detected in the male plasma QC (11-KT, testosterone, and A4; n = 4), the RSD ranged from 6.0% to 8.8%. Matrix spikes (n = 3) ranged from 82% to 84% average recovery for A4, E2, and testosterone, whereas the average 11-KT recovery was slightly lower at 69%.

**RNA sequencing**

For whole transcriptome analysis, total RNA was isolated from male liver samples using Tri-Reagent (Molecular Research Center) following the manufacturer’s protocols and was quantified using a Nanodrop ND-1000 (Thermo Fisher Scientific). The RNA quality was checked using the RNA ScreenTape Analysis on an Agilent 4200 TapeStation (Agilent Technologies). The RNA libraries were prepared using the TruSeq Stranded mRNA Library Prep Kit (Illumina) for 48 samples according to the manufacturer’s protocol with an epMotion 5075 automated liquid handling platform (Eppendorf). Sequencing was performed by the Research Technology Support Facility Genomics Core at Michigan State University (East Lansing, MI, USA). Libraries were run in single-end 50-base format in Rapid Run mode on an Illumina HiSeq. 4000 System. All the RNA sequencing data that we discuss have been deposited in the National Center for Biotechnology Information (NCBI)’s Gene Expression Omnibus (Edgar et al., 2002) and are accessible there (NCBI, 2022).

**Metabolomics**

Metabolite profiling was performed on male livers from the 41 µg/L metformin exposure and the 100 µg/L guanylurea exposure for both the 96-h time point (time course experiment) and the 23-day reproduction assay. Individual livers were extracted using a dual-phase extraction process (Ekman et al., 2012), after which the polar and nonpolar extracts were dried under vacuum. (We report only results from the polar extracts.) The dried polar extracts were reconstituted in 65 µL of 0.1 M sodium phosphate-buffered deuterium oxide (pH 7.4) containing 20 µM sodium 2,2-dimethyl-2-silapentane-5-sulfonate-d3, as a nuclear magnetic resonance (NMR) chemical shift reference, and then transferred to a 1.7-mm NMR tube. The NMR data were acquired at 20 °C using a Bruker Avance 600 MHz NMR spectrometer equipped with a 5-mm cryoprobe.
Data analysis

For steroid production, gene expression, plasma steroid concentrations, and fecundity-related data, metformin and guanylurea treatments were analyzed separately. A Kolmogorov–Smirnov test was used to test whether data were normally distributed, and Bartlett’s test was used to test for equal variance. Data were log-transformed to meet assumptions of normal distribution, when appropriate. For data meeting parametric assumptions, one-way analysis of variance (ANOVA) was used to test for differences across treatment groups. Dunnett’s test was used to determine whether treatments differed significantly from controls. A nonparametric Kruskal–Wallis test was used for data that did not meet parametric assumptions, followed by Dunn’s multiple comparison test to determine whether treatments differed significantly from controls. For all analyses, differences were considered significant at \( p \leq 0.05 \). Statistical analyses were performed using GraphPad Prism (Ver 8.4.3).

For the RNA-seq analysis, Bowtie2 (Ver 2.3.5) using default settings was used to map sequencing reads to the full transcriptome of the fathead minnow genome. The fathead minnow transcriptome was based on the gene annotation of the latest fathead minnow genome reference (GCF_016745375.1; Martinson et al., 2022). The RSEM (Ver 1.3.1) software was employed to obtain estimated read count matrices for all samples. Read counts were then normalized with the weighted trimmed mean of M-values, and differential gene expression analyses were carried out with the edgeR R package (Ver 3.26.8) using the edgeR exact test for identification of differentially expressed genes (DEGs; Robinson et al., 2009). A 5% false discovery rate (FDR) was used as the cutoff of DEGs. The gene ontology (GO)-term enrichment analysis of DEGs was conducted with GOATools (Ver 1.1.6; Klopfenstein et al., 2018). A 5% FDR was used as the cutoff for significantly enriched GO terms.

For the metabolomics analysis, binned \(^1\)H-NMR spectra data were imported into Microsoft® Excel and normalized to unit total intensity. Principal component analysis (PCA) was used for a general assessment of the data (SIMCA-13.0, Umetrics) and to identify outliers using the Hotelling’s \( T^2 \) test at the 95% confidence interval (Jackson, 1991; Wikström et al., 1998). Three fish (one 96-h control fish, one 96-h guanylurea-exposed fish, and one 23-day guanylurea-exposed fish) were identified as outliers and removed from the dataset. Next, the binned and normalized data were used to construct t-test filtered difference spectra to determine endogenous metabolites that were significantly affected (\( p < 0.05 \)) by the exposures. Details on this approach, including procedures for controlling for false positives, have been reported previously (Collette et al., 2010). The identities of these metabolites were assigned using Chenomx NMR Suite 8.6 and the Human Metabolome Database (Wishart et al., 2007). To further evaluate impacts of the exposures, we calculated the total intensity values for the t-test filtered difference spectra by summing the absolute values of the magnitudes for all significantly different bins (Collette et al., 2019). This provided a single metric that integrated both the breadth and intensities of the metabolite changes. Comparison of impacts across exposures (relative to the corresponding controls) provided an assessment of the relative extent of perturbation produced by each exposure. A one-way ANOVA with a post hoc Tukey’s test was used to determine significant differences across the exposures.

RESULTS

Ex vivo steroidogenesis

Neither metformin nor guanylurea caused significant changes in steroid synthesis by fathead minnow ovary exposed ex vivo (Figure 1). Prochloraz (5 µM) significantly reduced E2 production (\( p < 0.05 \)) in experiments for both metformin and guanylurea. Production of testosterone was significantly reduced (\( p < 0.05 \)) by prochloraz in the guanylurea experiment, but testosterone production in the metformin experiment, although reduced relative to controls, was not significant (\( p = 0.060 \)).

Exposure verification

Target nominal exposure concentrations during the short-term reproduction assay were 0.41, 4.1, and 41 µg/L for metformin and 1.0, 10, and 100 µg/L for guanylurea. Mean measured concentrations of metformin averaged 118% of nominal (measured 0.52, 4.75, and 46.2 µg/L), whereas guanylurea averaged 108% of nominal (measured 1.13, 11.1, and 99.1 µg/L) over the duration of the 23-day reproduction assay (Supporting Information, Table S3). No metformin was detected in guanylurea or control treatments, and no guanylurea was detected in metformin or control treatments during our study.

Measured concentrations of metformin over the 96-h time course in the metformin and metformin + guanylurea treatment averaged 119%–128% and 108%–119%, respectively, of the nominal concentration of 41 µg/L (Supporting Information, Table S4). Guanylurea in the guanylurea and metformin + guanylurea treatment averaged 87%–94% and 95%–103% of the nominal concentration of 100 µg/L in the 96-h study (Supporting Information, Table S4). No metformin was detected...
in guanylurea or control treatments, and no guanylurea was detected in metformin or control treatments during our study.

**Short-term reproduction assay**

**Apical effects.** Over the course of the 23-day exposure, one female died in each of the 4.1 µg metformin/L (day 5), 41 µg metformin/L (day 6), and 1.0 µg guanylurea/L treatments (day 16), and three females died in the 10 µg guanylurea/L treatment (days 8, 15, and 22). Exposure to metformin or guanylurea did not significantly impact fecundity relative to controls as measured by cumulative eggs/female or spawns/pair (Figure 2). Average fecundity ranged from 17 to 25 eggs/female/day, and average cumulative eggs/female ranged from 382 to 565 for the 23-day exposure. Fecundity did trend higher in a dose-dependent manner with exposure to increasing concentrations of metformin, but these differences were not significant. Additional apical endpoints including wet weight and gonadosomatic index showed no significant changes relative to controls, and neither male nor female blood glucose showed significant differences across any treatment relative to controls (Supporting Information, Figure S1).

**Gene expression.** Neither metformin nor guanylurea caused any significant effects relative to controls in expression of endocrine-associated genes in male gonad, including 3βhsd, 17βhsd, ar, cyp19a, and sult2a1, or esr1 and vtg expression in the male liver (Supporting Information, Figure S2). Similarly, no
significant changes were observed in gene expression of two genes related to energy metabolism, gck or pklr, in male liver. One statistical outlier was noted in gck expression from the guanylurea 100μg/L treatment; however, results were not statistically significant compared with controls with or without the outlier, and the outlier was not removed in the analysis or figure.

Plasma steroids. Plasma steroids were measured in both male and female fathead minnows from the 23-day assay. Four steroids (11-KT, A4, E2, and testosterone) were routinely detected in either male (11-KT, A4, and testosterone) or female (E2, A4, and testosterone) plasma (Supporting Information, Figure S3). Relative to controls, increased variance was observed in male A4 and testosterone and in female testosterone; however, there were no significant differences among the mean plasma steroid concentrations in any treatment relative to controls.

RNA sequencing. Whole transcriptome analysis was performed on male livers at 6, 24, 48, and 96 h of the time course experiment. Sequence read mapping rates to the fathead minnow transcriptome were consistent within and across groups, with a grand mean (SD) read mapping rate of 82.7 (0.6)%. Metformin exposure at 41 μg/L induced no discernable effects on the hepatic transcriptome of males exposed for 6–96 h, because only one DEG was identified in the 6-h metformin treatment (Figure 3). Guanylurea induced changes after 24 h of exposure, with 63 DEGs identified (35 up-regulated and 28 down-regulated; Figure 3 and Supporting Information, Table S5). Of the 35 up-regulated DEGs, 5 were between 2.5- and 9.9-fold higher, 4 were between 10- and 99.9-fold higher, and 27 were above 100-fold; for down-regulated DEGs, 4 were 2.5- to 9.9-fold lower, 7 were between 10- and 99.9-fold lower, and 17 were 100-fold lower in expression than controls. At 48 h, only two DEGs were detected for the guanylurea treatment. This time point showed increased transcriptomic variability, which may explain the lower number of DEGs. The greatest number of DEGs was observed after 96 h of exposure to guanylurea, at which point 472 DEGs were identified, 146 up-regulated and 326 down-regulated (Supporting Information, Table S5). Of the 146 up-regulated DEGs, 44 were between 2.5- and 9.9-fold higher, 52 were between 10- and 99.9-fold higher, and 50 were above 100-fold; for down-regulated DEGs, 52 were 2.5- to 9.9-fold lower, 118 between 10- and 99.9-fold lower, and 156 were 100-fold lower in expression than controls. The mixture of both metformin (41 μg/L) and guanylurea (100μg/L) showed less obvious effects than guanylurea alone, and again the greatest number of DEGs (46) was observed at 96 h (Figure 3 and Supporting Information, Table S5). Seven DEGs were in common from the guanylurea 24 h, guanylurea 96 h, and guanylurea + metformin 96 h treatment groups: solute carrier family 5 (sodium/glucose cotransporter), member 1 (slc5a1); mucin-like proteocadherin (mucpcdh); peptide transporter PEPT1 (solute carrier family 15 [oligopeptide transporter] member 1; slc15a1); alkaline phosphatase (alp3); MAM and LDL-receptor class A domain-containing protein 1 (madr1); apolipoprotein Bb, tandem duplicate 1 (apoobb.1); and lactase-phlorizin hydrolase (lactase-glycosylceramidase; lct). Interestingly, in the guanylurea 6-h treatment, all seven of these genes were up-regulated, whereas in the 96 h treatment with guanylurea or metformin + guanylurea, all were down-regulated.

Using a gene set enrichment analysis approach, 47 pathways were significantly impacted in the guanylurea 96-h treatment (Supporting Information, Table S6). These pathways were categorized as cellular component, biological process, and molecular function. The top impacted cellular component term (extracellular space, GO:0005615) and biological process terms (peptidase activity, GO:0008233; catalytic activity acting on a protein, GO:0140096) were each enriched relative to controls. Under molecular functions, multiple metabolic processes (heterocycle metabolic process, GO:0046483; organic cyclic compound metabolic process, GO:1901360; nucleobase-containing
compound metabolic process, GO:0006139; cellular aromatic compound metabolic process, GO:0006725; nucleic acid metabolic process, GO:0090304) were down-regulated relative to controls.

**Metabolomics.** A PCA of the male fathead minnow hepatic metabolome did not identify significant differences (p > 0.05) following 96-h exposure to 41 µg/L metformin or 100 µg/L guanylurea based on PCA (Supporting Information, Figure S4). After 23 days of exposure, significant effects relative to controls were observed in the 100 µg/L guanylurea treatment (p = 0.013), but not metformin treatment (p = 0.896; Supporting Information, Figure S4). Individual t-test filtered difference spectra identified several resonances from glucose that were altered relative to controls following exposure to 100 µg/L guanylurea at both 96 h and 23 days. Interestingly, several of these glucose resonances were increased relative to controls at 96 h, whereas by 23 days, many of the same resonances were decreased relative to controls (Supporting Information, Figure S5); however, no significant effects on blood glucose were detected across any treatment of guanylurea at 23 days (Supporting Information, Figure S1). It is also noteworthy that this decrease in liver glucose at 23 days was accompanied by a concomitant decrease in glycogen. Conversely, multiple branched chain amino acids (valine, leucine, and isoleucine) were not significantly impacted by the 96-h exposure but were increased after 23 days of exposure.

**DISCUSSION**

The primary goals of the experiments we performed were to: (1) examine whether metformin and its metabolite guanylurea directly impact steroidogenesis in fathead minnow ovary tissue, (2) assess whether the two chemicals could impact reproduction in adult fathead minnows, and (3) identify whether there was evidence for modulation of other biological pathways using ‘omics techniques in a short-term (6–96 h) time course exposure.

**Steroidogenesis**

Metformin is known to alter steroid production in humans and is used in treatment of some disorders related to steroid hormone dysregulation. Human polycystic ovary syndrome is often treated with metformin; in this condition, metformin has demonstrated the ability to reduce circulating androgen concentrations, perhaps through impacts on steroidogenesis (Lashen, 2010). The direct impacts of metformin on steroidogenesis have been investigated in multiple human cell lines. In forskolin-induced human thecal-like androgen-producing tumor cells, metformin directly inhibited A4 production at 50 and 200 µM, and reduced testosterone and 17α-hydroxyprogesterone production at 200 µM (Attia et al., 2001). In human adrenal NIC-H295R cells, androstenedione production and 3β-hsd gene expression were reduced following 48 h of treatment with 10 mM metformin (Hirsch et al., 2012). Because guanylurea is a bacterial biotransformation product and human exposure is of little concern, its effects in humans, including those on steroidogenesis, have not been considered.

In the present study, fathead minnow ovary explants were exposed to up to 100 µM of metformin or guanylurea with no observable impacts on testosterone or E2 production (Figure 1). If metformin is inducing the effects observed in human cell lines through inhibition of mitochondrial respiratory chain complex I (see Viollet et al., 2012 for review), it was hypothesized this would also occur in fish given the high conservation of mitochondrial proteins across species. The significant response to the prochloraz-positive control indicates that the overall assay design and power was sufficient to detect effects.

The three lowest treatments (0.001–1.0 µM) encompass the range of environmentally relevant concentrations of both metformin and guanylurea. The full range of tested concentrations exceeded expected environmental concentrations by over 100-fold and exceeded average therapeutic doses of metformin in human serum (Hess et al., 2018; Owen et al., 2000). Even at these elevated doses, neither metformin nor guanylurea elicited significant changes to in vitro steroid production. In the aforementioned human in vitro studies (Attia et al., 2001; Hirsch et al., 2012), only androstenedione production was impacted in the concentration range we tested. Other effects of metformin were noted at 200 µM and 10 mM. Even if concentrations of metformin or guanylurea above the 100 µM we tested (12 900 µg/L and 10 200 µg/L, respectively) are capable of inducing changes in teleost steroidogenesis, effects would not be expected in wild fish because these concentrations are far above environmentally relevant levels.

**Reproduction**

The desire to explore the reproductive toxicity of metformin and guanylurea was primarily motivated by a previous full life cycle study of fathead minnows exposed in static conditions to 40 µg metformin/L for up to a full year (Niemuth & Klaper, 2015). The authors noted a high frequency of intersex male fish and a decrease in fecundity of spawning pairs. Subsequent analysis of gene expression in testes identified a significant increase in transcript abundance of 3β-hsd, 17β-hsd, ar, cyp19a, and sult2a1 (Niemuth & Klaper, 2018). Some of the primary criticisms of that study included a lack of comprehensive exposure verification and elevated incidence of intersex in control male (Sumpter et al., 2016). An additional study exposing spawning pairs of fathead minnows to 41 µg metformin/L for 28 days under static conditions (Niemuth, Jordan, et al., 2015) identified no impacts on cyp19a and 3β-hsd gene expression in testes or fecundity of spawning pairs and no impacts on plasma testosterone concentration in males. These authors did, however, observe a small but significant increase in male hepatic vtg expression, potentially indicating that the effects observed in the full life cycle study could be mediated in adult stages. Given the lack of consensus among these and other studies, our aim was to provide additional evidence on the ability (or lack thereof) of
metformin and/or guanylurea to directly impact biological pathways critical for reproduction in adult fish.

We assessed the impacts of metformin and guanylurea on reproduction by exposing spawning pairs of fathead minnows to varying concentrations of metformin (measured at 0.52, 4.75, and 46.2 µg/L; Supporting Information, Table S3) or guanylurea (measured at 1.13, 11.1, and 99.1 µg/L; Supporting Information, Table S3) for 23 days. The concentration ranges chosen encompassed those observed in surface waters up to those encountered in treated municipal wastewater. Both metformin and guanylurea failed to induce significant effects on reproduction-related endpoints in fathead minnows in the present study—from the gene transcript levels to the apical response of reproduction. Five endocrine-related gene transcripts (3βhsd, 17βhsd, ar, cyp19a, and sult2a1) measured in male testes and two (vtg and esr1) in male liver were not significantly impacted by either chemical (Supporting Information, Figure S2). Plasma sex steroids, another indicator of potential endocrine effects, were not significantly impacted by either chemical in either male or female fish (Supporting Information, Figure S3). Lastly, overall egg production and spawning frequency were not significantly different across any metformin or guanylurea treatment (Figure 2).

The results of our experiment are largely in agreement with others reported in the literature. As just noted, a previous study (Niemuth, Jordan, et al., 2015) similarly identified no impacts on cyp19a and 3βhsd in adult males. These authors did report elevated hepatic gene expression of vtg in male fathead minnows, which was not replicated in our study. Elsewhere, juvenile fathead minnows (75–85 dpf) exposed to 1.0, 10, or 100 µg metformin/L for 7 days had elevated whole-body expression of four genes associated with reproduction and development (vtg, esr1, gonadotropin-releasing hormone 3 [gnrh3], and cyp3A), although similar changes were not observed in adult male fish following a 7-day exposure at the same concentrations (Crago et al., 2016). Most recently, a study exposing fathead minnows to 3.0, 31, or 322 µg/L metformin over a full life cycle identified no adverse effect on fecundity and zero incidence of oocytes in testes (Parrott et al., 2021). Gene expression in testes was not investigated, but a lack of effect on gonad histology and spawning suggests that treated individuals would not differ significantly from controls. It is presumed the gene expression profile of intersex male gonad would be markedly different from that of a normal gonad, and thus the change in gene expression reported by Niemuth and coauthors (Niemuth & Klapel, 2015) may be inherently associated with intersex tissue. Because the occurrence of intersex in male fathead minnows is uncommon and has thus far not been replicated in other exposures to metformin, the weight of evidence does not indicate metformin as a primary cause of intersex gonad development. Based on our data as well as that of Parrott et al. (2021), metformin does not appear to be a potent reproductive toxicant in fish.

To our knowledge, ours is the first study to evaluate the reproductive toxicity of guanylurea in a fish species. Because guanylurea is the primary transformation product of metformin and is commonly found at higher concentrations than metformin, it is important to understand whether it can adversely impact fish reproduction. Most previous studies have not monitored guanylurea during exposure to metformin (Crago et al., 2016; Niemuth & Klapel, 2015; Niemuth, Jordan, et al., 2015; Ussery et al., 2018), and if guanylurea was formed as a transformation product during exposure, any observed effects could possibly have been caused by guanylurea. However, as with metformin, the present study identified no adverse reproductive impacts of guanylurea, suggesting it is not a reproductive toxicant in fish at environmentally relevant concentrations.

Additional biological effects

Further biological impacts of metformin, guanylurea, or a combination of both were investigated through a time course exposure. Because the greatest impacts reported in previous studies were in males (Niemuth & Klapel, 2015, 2018), only male fish were used for the time course. Metformin had surprisingly little impact across the 96-h exposure, with only a single DEG identified after 6 h of exposure to 41 µg/L, which is likely not a biologically relevant impact to the transcriptome because no other DEGs were identified across the other time points during the 96-h metformin exposure. Changes to the hepatic metabolome were also negligible after 96 h of exposure to 41 µg metformin/L. Additional analysis of male hepatic metabolome from the 23-day reproduction study likewise identified a negligible impact on hepatic metabolite profiles, providing no indication that a longer time scale would have identified transcriptome-related impacts from metformin.

The overall result of metformin inducing no significant effects across the entire array of biological endpoints is somewhat surprising. Metformin has been demonstrated to inhibit mitochondrial respiratory chain complex I, which leads to impacts on adenosine monophosphate (AMP)/adenosine triphosphate ratios, and activation of AMP-activated protein kinase, leading to a cascade of effects resulting in the desired clinical impact of decreased blood sugar, among other changes (see Viollet et al., 2012 for review). Given the conservation of mitochondrial respiration proteins across eukaryotes, a similar action and subsequent impacts would reasonably be expected in fathead minnows. This lack of observable impacts may simply be a product of dose, because the study was designed around environmentally relevant concentrations up to a maximum of 41 µg/L or 0.32 µM. Although plasma concentrations were not directly measured in the present study, a low octanol/water partition coefficient (log K<sub>OW</sub> ~1.4) and high dissociation constant (pK<sub>a</sub>; 11.5) indicate that metformin would not be expected to bioaccumulate to any significant degree. In addition, prior research has shown metformin uptake in zebrafish embryos and larvae (Ussery et al., 2018) and in brown trout larvae with a maximal reported bioconcentration factor of 0.2 L/kg in whole larvae (Knoll et al., 2020). Thus, the exposure concentration used for the 96-h time course would produce plasma concentrations considerably lower than human therapeutic plasma concentrations.
which average 10–20 µM (Hess et al., 2018; Owen et al., 2000). These data could be viewed as a no-observed-transcriptional-effect level (Ankley et al., 2006; Lobenhofer et al., 2004) given the lack of observable biological perturbation at the highest tested concentration of 41 µg/L.

Metformin has previously been shown to induce growth-related effects in early life stage Japanese medaka, which had significantly decreased wet weight and length following 28 days of exposure to 3.2 µg/L or more of metformin (Ussery et al., 2018). Several fatty acids and amino acids were noted as significantly changed relative to controls, providing a possible basis for the changes in growth. However, a subsequent full life cycle study of Japanese medaka exposed to 3.2 µg metformin/L from embryos to 165 days post hatch found no significant differences in wet weights (Ussery et al., 2018). A separate early life stage exposure of fathead minnows to metformin for 21 days identified no significant effects on growth at concentrations up to 269 µg/L (Parrott et al., 2022). This may indicate increased susceptibility to metformin at early stages of development, although the impacts were not consistent across two different fish species and do not appear to translate to adverse growth effects in adults.

Unlike metformin, guanylurea did show a moderate impact on hepatic gene expression and metabolite profiles over the 96-h time course experiment. The greatest number of DEGs were identified following 96 hpf exposure to 100 µg/L guanylurea. Effects of a mixture of metformin and guanylurea induced fewer DEGs than guanylurea alone, but did show a significant impact, indicating the effects were likely driven by guanylurea. Far fewer DEGs were identified in the mixture treatment, which may be due to higher variability across individuals of this treatment. Metformin and guanylurea could also be competing at the same molecular targets; however, with the current dataset and experimental design, the reason for this discrepancy between guanylurea alone or in mixture with metformin can only be speculated on.

Seven DEGs identified in common at 24 h of exposure to guanylurea and 96 h of exposure to guanylurea or guanylurea + metformin demonstrated a time-dependent profile of expression whereby genes were enriched at 24 h but decreased after 96 h. This change may be a compensatory response following the initial exposure to guanylurea, although it is currently unclear whether these genes remain down-regulated over a longer duration because they were not measured in the 23-day study. The hepatic metabolome, however, was compared for both 96-h and 23-day male fish. Multiple glucose resonances were increased at 96 h but decreased at 23 days (Supporting Information, Figure S4), supporting a compensatory response hypothesis (increased glucose availability to fuel cellular adaptation to guanylurea exposure). In contrast, after 23 days of exposure to guanylurea, glucose and glycogen were both decreased relative to controls, suggesting a depletion of cellular energy resources. However, the response was clearly not severe, because the other endpoints that were collected in our study, including blood glucose (Supporting Information, Figure S1), suggest only minimal effects. In an effort to place the magnitude of the metabolomic responses into context, we compared the extents of impact to the guanylurea and metformin exposures with those determined previously for exposures to bisphenol A (Ekman et al., 2012) and ethinylestradiol (Ekman et al., 2008a) that were conducted in a similar fashion in fathead minnows (Figure 3B). Exposure to guanylurea or metformin at both time points produced relatively minor impacts on the hepatic metabolome in comparison with these other environmental contaminants.

Looking more closely at specific genes that were affected by the exposures to guanylurea, we noted that one of the seven genes that was consistently impacted and down-regulated at 96 h, is a sodium/glucose cotransporter (slc5a1), which may be implicated in the decreased glucose-related metabolites observed in male liver after 23 days of exposure. Expression of slc5a1 is often associated with the intestine, where it plays an important role in glucose transport from the gut (Subramaniam et al., 2019). Although not directly monitored in the present study, a similar decrease of slc5a1 in the intestine of fathead minnows could be reasonably expected. Changes in glucose absorption in the gut or within the liver could both impact liver glucose and glycogen, consistent with the metabolomic profile observed after 23 days (Supporting Information, Figure S5).

Focusing more broadly on GO terms, several interesting categories of effect were identified after the 96-h guanylurea exposure. Multiple metabolic processes, which all encompassed the same list of 17–22 impacted genes (Supporting Information, Table S6) were down-regulated compared with controls. A previous study on the effects of guanylurea on Japanese medaka identified growth-related impacts in a 28-day exposure to concentrations as low as 1 ng/L (Ussery et al., 2019). Subsequent proteomic analyses identified multiple proteins associated with metabolic processes, both up- and down-regulated (Ussery et al., 2021). These prior results along with those in the present study—changes in specific glucose transport expression, multiple metabolic processes, and changes in glucose-related metabolite concentrations at 23 days—all indicate guanylurea is capable of altering energetic pathways essential for homeostasis. The biological outcomes may be similar to the desirable therapeutic outcomes of metformin treatment in humans, indicating that similar molecular targets and processes may be involved for both metformin and guanylurea; however, because guanylurea is not formed via human metabolism and human exposure is not expected, it has not been studied in depth as metformin has. Thus there is no direct mechanistic data on the possible mode of action of guanylurea, and potential molecular targets leading to the outcomes we observed can only be inferred. In this regard, the increase in hepatic branched chain amino acids (BCAAs) measured after 23 days of guanylurea exposure is noteworthy. Specifically, Rivera and co-authors recently reported time-dependent suppression of the expression and activity of BCAA catabolic enzymes in response to metformin exposure in vitro (skeletal muscle cells), and suggested that BCAA accumulation would occur as a result (Rivera et al., 2020).

As mentioned previously in the Steroidogenesis section, metformin is known to impact mitochondrial metabolism in part
through inhibition of respiratory chain complex proteins (Viollet et al., 2012). Two recent studies on the impacts of guanylurea on zebrafish indicate that guanylurea may cause adverse effects by increasing reactive oxygen species (ROS) in developing embryos and in the brain of adult zebrafish (Elizalde-Velázquez et al., 2021, 2022). If guanylurea is acting in a similar way as metformin by inhibiting mitochondrial respiratory chain complex proteins, this could lead to an increase in ROS (Wang et al., 2015), as was observed in these studies. Thus we recommend that future studies focus on the potential molecular mechanism of action for guanylurea, particularly inhibition of electron transport chain proteins.

CONCLUSIONS

Our study provides multiple lines of evidence indicating that neither metformin nor guanylurea are directly endocrine active in adult fathead minnows at environmentally relevant concentrations. Neither compound significantly altered steroid production by fathead minnow ovary exposed ex vivo, nor did they impact reproduction in vivo in a 23-day short-term reproduction study. When the data are taken together, the results of the present study along with those of a recent full life cycle study (Parrott et al., 2022) do not support the conclusions of earlier studies indicating that metformin may act as an endocrine-disrupting chemical (Niemuth & Klaper, 2015; Niemuth, Jordan, et al., 2015). Our study is the first to test the potential impacts of guanylurea on fish reproduction, and at the environmentally relevant concentrations tested, it shows no evidence of reproductive toxicity. These conclusions do not preclude the possibility that metformin or guanylurea may be active at more sensitive life stages, as appears to perhaps be the case based on other early life stage exposures of both metformin and guanylurea. Hepatic metabolome and whole hepatic transcriptome analyses in the present study both revealed that guanylurea induced more significant impacts in adult fathead minnows. The results were consistent with potential effects on carbohydrate homeostasis, although we can only speculate as to the potential significance of these to ecological fitness. Because metformin use is expected to increase with an aging global population, it is important to understand the potential ecological impacts of both chemicals on aquatic species.

Supporting Information—The Supporting Information is available on the Wiley Online Library at https://doi.org/10.1002/etc.5450.

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