Chemosphere 135 (2015) 38–45

Contents lists available at ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

Emerging wastewater contaminant metformin causes intersex and reduced fecundity in fish

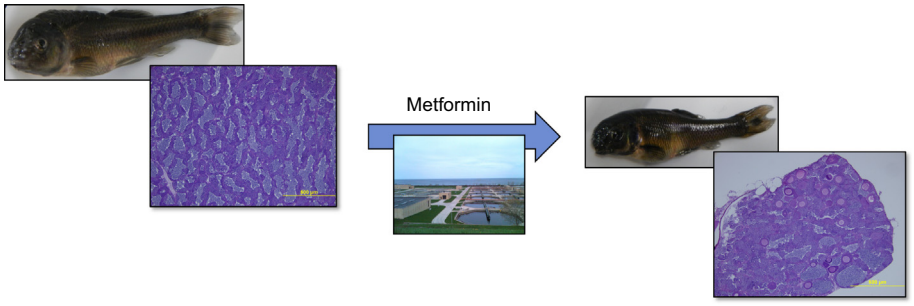
Nicholas J. Niemuth, Rebecca D. Klaper *

School of Freshwater Sciences, University of Wisconsin – Milwaukee, 600 East Greenfield Ave, Milwaukee, WI 53204, USA

HIGHLIGHTS

- Fish were exposed to metformin at concentrations relevant to wastewater effluent.
- Exposure from early life stages to adulthood caused intersex in male fish.
- Exposure caused a reduction in fecundity and in overall size of male fish.
- Results suggest that metformin is a potential endocrine disruptor in the environment.
- Metformin may be another cause of intersex fish seen globally.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 25 February 2015
Received in revised form 26 March 2015
Accepted 30 March 2015
Available online 18 April 2015

Handling Editor: Shane Snyder

Keywords:
Contaminants of emerging concern
Endocrine disrupting compounds
Diabetes
Pharmaceuticals and personal care products

ABSTRACT

The occurrence of intersex fish, where male reproductive tissues show evidence of feminization, have been found in freshwater systems around the world, indicating the potential for significant endocrine disruption across species in the ecosystem. Estrogens from birth control medications in wastewater treatment plant effluent have been cited as the likely cause, but research has shown that endocrine disruption is not solely predictable based on hormone receptor interactions. Many other non-hormone pharmaceuticals are found in effluent at concentrations orders of magnitude higher than estrogens, yet there is little data indicating the impacts of these other medications. The widely prescribed anti-diabetic metformin is among the most abundant of pharmaceuticals found in effluent and is structurally dissimilar from hormones. However, we show here that exposing fathead minnows (*Pimephales promelas*) to a concentration of metformin found in wastewater effluent causes the development of intersex gonads in males, reduced size of treated male fish, and reduction in fecundity for treated pairs. Our results demonstrate that metformin acts as an endocrine disruptor at environmentally relevant concentrations.

1. Introduction

The discovery of intersex male fish, with testis containing oocytes and other features of female gonads, in tributaries of the Potomac river in the United States (Blazer et al., 2012), rivers throughout the United Kingdom (Jobling et al., 2009, 1998), and watersheds downstream of wastewater treatment plants (WWTPs) around the world (Bjerregaard et al., 2006; Tetreault et al., 2011), are an indication of the potential presence of endocrine disrupting compounds (EDCs) in the aquatic environment introduced by human activity. Full-life exposure to WWTP effluent has been shown to cause endocrine disruption in the model fish

* Corresponding author at: 600 E Greenfield Ave, Milwaukee, WI 53204, USA. Tel.: +1 414 382 1713.
E-mail address: rklaper@uwm.edu (R.D. Klaper).

http://dx.doi.org/10.1016/j.chemosphere.2015.03.060
0045-6535/

species roach (*Rutilus rutilus*) (Liney et al., 2006, 2005; Lange et al., 2011) and fathead minnow (*Pimephales promelas*, FHM) (Sowers et al., 2009). Observed effects are often attributed to the presence of estrogens from birth-control medications such as 17α-ethinylestradiol (EE2) in effluent, which have been shown to cause intersex and reproductive decline in FHM at concentrations as low as 1 ng L^{-1} (Parrott and Blunt, 2005). However, some research suggests that EDCs could act through other mechanisms beside classical hormone receptor pathways, such as the neuroendocrine system and the insulin signaling axis (Diamanti-Kandarakis et al., 2009).

Although estrogens can have impacts in the low ng L^{-1} range, many other pharmaceuticals are present in WWTP effluent and surface waters at concentrations orders of magnitude higher (Blair et al., 2013a, 2013b; Chen et al., 2006). Insufficient research has been done to investigate the effects of most of these compounds on organisms in the aquatic environment (Novak et al., 2011). Despite the ubiquitous discharge of medications in WWTP effluent (Heberer, 2002; Fatta-Kassinos et al., 2011), as few as 10% of prescribed pharmaceuticals have been studied for their environmental impacts (Brausch et al., 2012).

One of the most abundant pharmaceuticals found in recent studies of WWTP effluent and surface-waters is the anti-diabetic drug metformin, thought to be the pharmaceutical most deposited into the aquatic environment by mass (Oosterhuis et al., 2013) and detected in effluent at concentrations ranging from 1 to 47 μg L^{-1} (Blair et al., 2013a, 2013b; Ghoshdastidar et al., 2014; Oosterhuis et al., 2013; Scheurer et al., 2012). Initially introduced in the 1950s in the United Kingdom, popularity and prescription of metformin have continued to grow, particularly following its introduction in the United States in 1995, due to its efficacy as an insulin sensitizer, the increasing number of cases of type-2 diabetes around the world (Viollet et al., 2012), and its recent recommendation as a therapy for prevention of type-2 diabetes (American Diabetes Association, 2013). This biguanidine drug is excreted in patients' waste in its active form and, although largely converted to byproducts in WWTPs, is still deposited into the environment in relatively high amounts for a pharmaceutical (Oosterhuis et al., 2013; Blair et al., 2013b), at up to 6 tons per year from individual WWTPs in urban areas (Blair et al., 2013b).

Metformin is thought to act as an insulin sensitizer primarily through its impacts on cellular energy balance by inhibiting complex I of the electron transport chain. The resulting decrease in cellular ATP levels activates the regulatory AMP kinase (AMPK), promoting glucose uptake and breakdown as well as fatty acid oxidation, thereby improving insulin sensitivity (Viollet et al., 2012). In addition to its wide prescription as an anti-diabetic, metformin has been indicated as a treatment in cancer as a result of its effects on the AMPK/mTOR energy sensing pathway (Ben Sahra et al., 2010).

Although it does not structurally resemble hormone-like compounds classically identified as EDCs (Blair et al., 2000), steroid synthesis pathways are influenced by insulin signaling, and metformin treatment has been shown to alter expression and activity of enzymes involved in steroid synthesis such as cytochrome p450 CYP17 (Viollet et al., 2012). In fact, metformin has been indicated as a treatment for instances of the endocrine disorder polycystic ovarian syndrome (PCOS) (Tang et al., 2012). Thus, metformin's impacts on steroidogenesis and its use in treating PCOS suggest its potential as a nontraditional EDC in the environment.

Our previous work showed that metformin induces transcription of the mRNA for vitellogenin (VTG) in adult male FHM (Niemuth et al., 2015), an egg yolk protein that is normally expressed only in females and which is used as an indicator for exposure to EDCs both in the laboratory and in wild populations when measured in male fish (Jobling et al., 1998; Ankley et al., 2001; Lattier et al., 2002). We found significantly higher levels of VTG in adult males after four weeks of exposure to metformin at environmentally relevant concentrations. Despite this observation, no intersex tissue changes were observed (Niemuth et al., 2015). However, this was a 28-d adult exposure, which may not have represented the full effects of environmental exposure.

Development is a particularly sensitive period in the life-cycle of an organism, including for sexual differentiation (Johns et al., 2011), and believed to be the most sensitive stage to impacts of toxins in the environment (Luckenbach et al., 2001). Exposure of FHM to EE2 at 10 ng L^{-1} during a window of 10–15 d post-hatch (dph) was sufficient to result in feminization of male gonads observable at sexual maturity 100 dph (van Aerle et al., 2002). However, feminization was not observed in fully mature FHM exposed to 10 ng L^{-1} EE2 for three weeks in adulthood (Pawlowski et al., 2004). Thus, while our previous exposure of adult FHM to metformin resulted only in increased VTG expression (Niemuth et al., 2015), it is plausible that long-term metformin exposure, including exposure during the critical period of male sexual development (up to 90 dph in male FHM(Van Aerle et al., 2004)), could result in more severe endocrine impacts including intersex.

To test this hypothesis we conducted a long-term exposure of FHM to a concentration of metformin found in WWTP effluent discharged into Lake Michigan (Blair et al., 2013a,b), and similar to that found in effluent elsewhere (Scheurer et al., 2012; Ghoshdastidar et al., 2014), beginning in early development. Fish were exposed from fry stage, 30 d post-hatch through adulthood, about 1 year, and gonad histology, secondary sex characteristics, growth, and reproduction from control and treated fish were measured.

2. Materials and methods

2.1. Chemicals and exposure

Metformin (1,1-Dimethylbiguanidine hydrochloride; CAS # 1115-70-4) and ethanol (200 proof; CAS # 64-17-5) were purchased from Sigma–Aldrich (St. Louis, MO). A 100 mg L^{-1} metformin stock was prepared by adding 50 mg of metformin to 500 mL of 2% v/v ethanol in MilliQ ultrapure water (EMD Millipore, Billerica, MA). Treatment tanks were dosed with 400 μL per liter of this stock to yield metformin exposure tanks at 40 μg L^{-1}, similar to concentrations we found in WWTP effluent in Milwaukee, WI (47 μg L^{-1}) (Blair et al., 2013b) and to those found in European (26 μg L^{-1}) (Scheurer et al., 2012) and Canadian (10.6 μg L^{-1}) (Ghoshdastidar et al., 2014) effluents. Control tanks were dosed with 2% v/v ethanol in MilliQ ultrapure water at 400 μL per liter. Tank water used for this study was filtered and dechlorinated at the UW-Milwaukee School of Freshwater Sciences (Milwaukee, WI). Standard metrics (e.g. ammonia, pH, nitrate) were collected weekly and were within normal parameters.

Water samples from tanks were analyzed for metformin by LC–MS/MS at the Wisconsin State Lab of Hygiene (Madison, WI). Metformin has been shown to be stable in aqueous solution at temperatures from 30 to 70 °C, degrading only an estimated 10% over a period of more than 8 d, even at elevated temperatures (Sharma et al., 2010). Metformin is also highly water soluble, with a logP octanol/water of −1.43 (Chou, 2000). Thus one would not expect to see a significant reduction in metformin concentrations due degradation or adsorption over the 3–4 d between water changes, and indeed measured metformin concentrations in our tanks did not decline significantly over the period between water changes, being 38 ± 4 μg L^{-1} (N = 4) on Day 0 and 40 ± 6 μg L^{-1} (N = 3) on Day 3.

Fathead minnow fry were obtained from the culture maintained in the Klaper lab at the UW-Milwaukee School of Freshwater Sciences (Milwaukee, WI). Thirty days post-hatch, fry were divided into 6 tanks of 40 individuals per tank. Three tanks were dosed with metformin at 40 $\mu g\ L^{-1}$ and three were dosed with vehicle alone (giving 3 replicates of 40 fish each for both control and metformin treatment). Fish were kept on a 16:8 light: dark cycle and exposed continuously throughout the experiment by static renewal every fourth and seventh day. Fry were fed freshly hatched Artemia salina (Ocean Star International, Snowville, UT) in the morning and ground Tetramin tropical fish food (Tetra, Blacksburg, VA) in the afternoon for 30 d. After these first 30 d, fish were fed Tetramin tropical fish food twice daily for 290 d, at which point clear males and females were distinguishable.

Upon completion of this initial 320-d group exposure, 36 pairs of fish (18 control: 18 metformin-treated) were used for reproductive assays. As FHM are group spawners, 6 adult male–female pairs were separated out from each exposure tank to identify individual-pair fecundity. Individual pairs were each placed into a compartment of a new spawning tank, with 3 pairs in each spawning tank separated from each other by clear porous dividers. The exposure regimen for control and metformin treatment was continued in spawning tanks.

After segregation of pairs for fecundity studies, all fish were fed frozen Artemia salina (San Francisco Bay Brand, Newark, CA) in the morning and Tetramin tropical fish food in the afternoon for the remainder of the experiment. Mating pairs were exposed for an additional 40 d after splitting. Eggs from each FHM pair were counted daily, beginning the day subsequent to splitting. Remaining fish were exposed for an additional 45 d post-split. In all, mating pairs were exposed for a total of 360 d (final N: control pairs = 17, metformin-treated pairs = 17); remaining fish were exposed for a total of 365 d (final N: control males = 8, control females = 54, metformin-treated males = 15, metformin-treated females = 45).

At the end of each respective exposure period, fish were euthanized in dechlorinated water containing MS-222 (Ethyl m-Aminobenzoate Methanesulfonate; CAS# 886-86-2; MP Biomedicals, Santa Ana, CA) buffered with sodium bicarbonate (CAS# 144-55-8; Sigma–Aldrich, St. Louis, MO) each at 200 $mg\ L^{-1}$. Each fish was weighed, measured, and photographed for future reference. Condition factor (K) for each fish was calculated using Fulton's condition factor, $K = 100 * (W/L3)$, with weight (W) in g and length (L) in cm. Secondary sex characteristics of male fathead minnows were scored on a 0–9 scale using the method of Dammann et al. (2011), summing scores for tubercles (0–3), dorsal pad (0–3), and banding (0–3) for each fish; 0 being absent and 3 being pronounced. One gonad from each fish was collected in a histology cassette and immersed in fixative as described below.

2.2. Histology

For mating pairs and remaining fish, upon sacrifice gonad tissues from each male and female fish were placed in histology cassettes and immersed in Bouin's fixative (Sigma Aldrich, St Louis, MO) overnight (control male N = 25, metformin-treated male N = 31, control female N = 64, metformin-treated females N = 55). Cassettes were transferred to 50% ethanol for 48 h to destain and then stored in 70% ethanol. Preserved tissues were paraffin-embedded, sectioned, and H&E stained by Histology Tech Services (Gainesville, FL).

Scoring of male tissues for intersexuality was performed on a 0–7 scale using the method of Jobling et al. (1998), illustrated in Fig. 1: (a) normal male testis, score of 0; (b) intersex score of 2, infrequent perinucleolar follicles (PNF) scattered throughout otherwise normal testis; (c) score of 3, frequent PNF scattered throughout testis; (d) score of 4, frequent PNF and possibly cortical alveolar oocytes (CAO) clumping throughout testis; (e) score of 5, less than 50% of testis is composed of PNF, CAO, and possibly also early or late vitellogenic oocytes (EVO, LVO); (f) score of 6, >50% of testis composed of PNF, CAO, EVO, LVO, and possibly mature oocytes; (g) score of 7, 100% conversion of gonad to oocytes of varying stages.

2.3. Statistics

Because of the nested design of our experimental setup, data were analyzed using models to test for effects of treatment as well as any possibly confounding tank effect, in which apparent differences between treatments would be driven by fish in a single tank. No significant tank effect was found for any of the metrics reported in this study ($p > 0.05$). For normally distributed data (weight, length, and condition factor), a two-way nested ANOVA was applied to determine significance of any tank or treatment effects. For non-normal, ordinal data (male histology and sex scores) a generalized linear model (GLM) for ordinal data was employed to determine tank and treatment effects. Cumulative clutch number per pair were analyzed using a general linear model for repeated measures. Clutch size data were analyzed using a GLM for Tweedie (1.5) distributed data. Percent intersex was compared between treatments using a Student's t-test for independent samples, treating each replicate tank as an independent sample. Correlations between data sets were determined using linear regression analysis. Outliers were removed and all analyses performed using SPSS v22 for Mac.

3. Results

3.1. Histology

Metformin-exposed male FHMs had a significant occurrence of intersexuality, with an average score of 0.2 for control males and an average score of 3.8 for treated males (Fig. 2, panel a). There was a significant difference in the percent of intersex fish per treatment (histology score >0), 13% for controls versus 84% for metformin-treated males (Fig. 2, panel b). Fig. 1 panels a through g demonstrate the morphology of normal male testis and differing degrees of intersexuality observed in metformin-exposed male fish. Fig. 1, panel a (score of 0) is representative of normal testis observed in control male fish. Fig. 1, panel d (score of 4) is representative of the average score observed in treated male gonad, in which oocytes including perinucleolar follicles and primary oocytes occur frequently throughout testicular tissue. Sections from exposed male fish ranged in intersexuality from histologically normal testis (score of 0, seen in 5 of 31 fish; Fig. 1a) up to gonad sections that were entirely oocytes (score of 7, seen in 5 of 31 fish; Fig. 1g). No histological differences were observed in female gonad sections.

3.2. Size and condition factor

We also observed significant differences in weight and condition factor for metformin-treated males (two-way nested ANOVAs, weight: $p = 0.011$, $df = 1$, $F = 18.596$; condition factor: $p < 0.001$, $df = 1$, $F = 69.221$), with mean weights of 4.0 ± 0.2 g and 3.1 ± 0.1 g and condition factors of 1.51 ± 0.04 and 1.34 ± 0.04 for control and treated males respectively. No significant differences

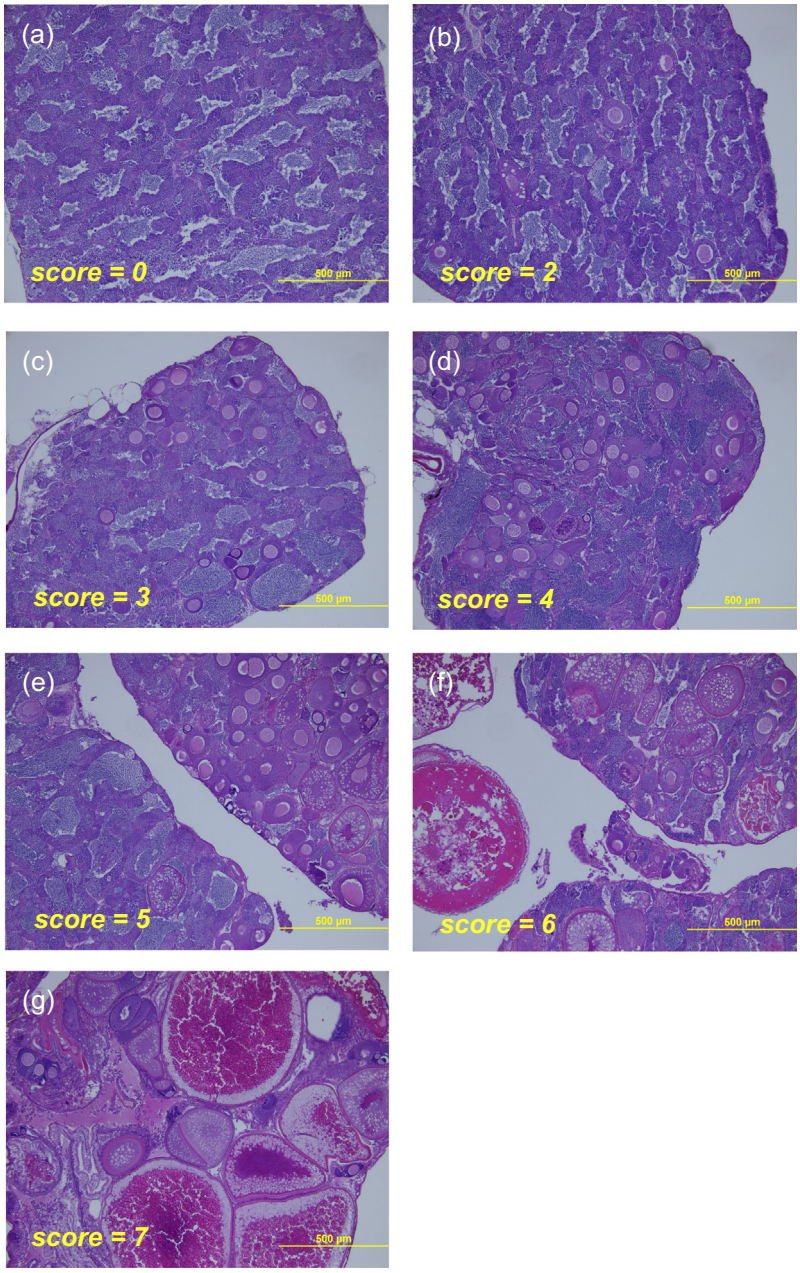


Fig. 1. Scoring of FHM male gonad histology. Representative male gonad histology slides: (a) normal male testis, score of 0, as seen in a control male; (b) intersex score of 2, infrequent perinucleolar follicles (PNF) scattered throughout otherwise normal testis, here in an exposed male fish; (c) score of 3, frequent PNF scattered throughout testis, seen in an exposed male; (d) score of 4, frequent PNF and possibly cortical alveolar oocytes (CAO) clumping throughout testis; (e) score of 5, less than 50% of testis is composed of PNF, CAO, and possibly also early or late vitellogenic oocytes (EVO, LVO); (f) score of 6, >50% of testis composed of PNF, CAO, EVO, LVO, and possibly mature oocytes (MSO); (g) stage 7, 100% conversion of gonad to oocytes of varying stages.

in weight, length, or condition factor were detected between treatments for female minnows (two-way nested ANOVAs: $p > 0.05$).

3.3. Reproduction

Significant differences were also found for cumulative clutches laid per mating pair over time between control and metformin-treated minnows, with significantly fewer clutches for metformin-exposed pairs (Fig. 3a). Mean clutch size per pair also showed significant differences between control and metformin-treated fish, with significantly smaller clutches for treated fish (Fig. 3b). Importantly, treated pairs with males showing intersex in histology still produced eggs, including the 3 treated pairs with males having an intersex gonad histology score of 7 (complete conversion to ova, Fig. 1 panel g) each producing a single clutch with a mean of 140 ± 90 eggs over the course of observation.

3.4. Correlations between endpoints

A small but significant negative correlation was found between male intersex histology score and clutch number for mating pairs (linear regression: $R^2 = 0.199$, $\beta = -0.4$, $p = 0.013$, $df1 = 1$, $df2 = 28$, $F = 6.967$). A similar negative correlation was also found between male intersex score and clutch size ($R^2 = 0.138$, $\beta = -9.250$, $p = 0.044$, $df1 = 1$, $df2 = 28$, $F = 4.465$). No clear correlation was found between size (weight, length, condition factor) and

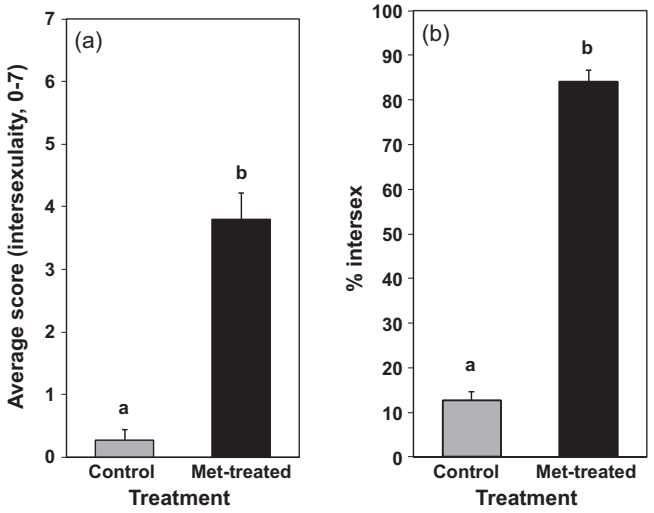


Fig. 2. Intersex scoring for histological sections of gonad from control males and FHM males exposed to 40 μg L^{-1} metformin. (a) Intersex histology scores (0–7) for males from control and metformin-treated groups. Includes scores for males from mating pairs (exposed 360 d) and remaining males (exposed 365 d). Error bars represent SEM. N=: control males, 25; treated males, 31. Metformin-treated males were found to have a significantly higher intersexuality score (generalized linear model for ordinal numbers: $p < 0.001$, $df = 1$, $\chi 2 = 34.265$). (b) Percent of fish showing intersex (intersex histology score > 0) for control and metformin-treated males. Error bars represent SEM. N=: control tanks, 3; metformin-treated tanks, 3. Males from metformin-treated tanks had a significantly higher incidence of intersex (t-test: $p < 0.001$, $df = 4$, $t = -21.578$).

reproductive output (clutch size, clutches per pair) for male or female minnows ($p > 0.05$).

3.5. Secondary sex characteristics

In addition, metformin-treated males had a decreased secondary sex score when compared to control males, however this was not statistically significant (Fig. 4, panel a). Total score for each fish was based on the sum of three scores: tubercles (0–3), dorsal pad (0–3), and banding (0–3). Importantly, treated males with an intersex gonad histology score as high as 6 (>50% testis-ova; Fig. 1, panel f) had a secondary sex score of 9 (Fig. 4, panel c), having pronounced tubercles, dorsal pad, and banding while having significant testis-ova (see Fig. 4, panel b for comparison to control).

4. Discussion

Our results demonstrate that metformin, which has recently been found to be a major emerging contaminant in wastewater and surface waters, has a significant impact on the reproductive system of FHMs and can cause intersex in male fish at concentrations emerging from WWTPs. Full life-cycle metformin exposure resulted in significant induction of testis-ova in male gonad (Fig. 2), a condition seen in fish in freshwater systems around the globe. The high concentration of this compound found in WWTP effluent and watersheds worldwide warrants further investigation of the impacts that this emerging contaminant may have on fish populations.

This is the first study to look at the endocrine-disrupting effects of full life-cycle exposure to metformin in fish. Our previous study, exposing fully-grown FHM in a 28-d chronic exposure to levels of metformin found in WWTP effluent, found a significant upregulation of VTG mRNA in livers of metformin-treated adult males, indicating possible endocrine disruption, but no histological or reproductive effects were seen (Niemuth et al., 2015). The current study reveals impacts on reproduction and gonad histology when FHM are exposed for a full life-cycle beginning in early development. This work further demonstrates the need to consider more than chronic adult assays for the study of impacts on reproduction. Moreover, our results demonstrate the need to investigate what portion of the life-cycle is responsible for these impacts of metformin exposure in vertebrates.

Fish are known to be significantly more sensitive to EDCs in early development, particularly during sexual differentiation (Johns et al., 2011), which is not completed in FHM males until 90 d post-hatch (Van Aerle et al., 2004). Although metformin may not be the only compound in effluent that can cause endocrine disruption, histological changes found in this study (Fig. 1) resemble those seen in studies with FHM (Sowers et al., 2009) and roach (*Rutilus rutilus*) (Lange et al., 2011) exposed for a full life-cycle to WWTP effluent, as well as those found in fish downstream of WWTPs (Jobling et al., 2009, 1998; Blazer et al., 2012).

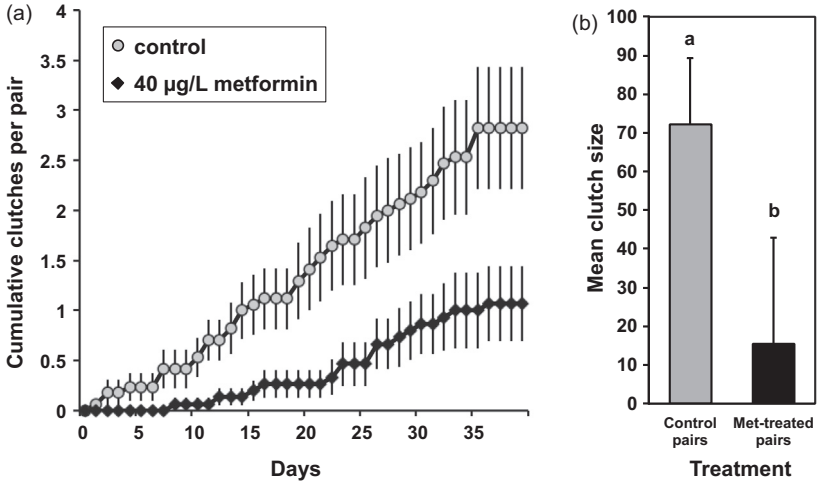


Fig. 3. Reproductive output for control and 40 μg L^{-1} metformin-treated FHM pairs. (a) Cumulative clutches laid per pair over 40 d for control and metformin-treated pairs. Error bars represent SEM. N=: controls pairs, 17; treated pairs, 14. Metformin-treated pairs laid significantly fewer clutches when compared to control pairs (general linear model for repeated measures: $p = 0.031$, $df = 1$, $F = 5.167$). (b) Mean clutch size per pair over the 40 d observation period for control and metformin-treated pairs. Error bars represent SEM. N=: control pairs, 17; treated pairs, 14. Mean clutch size was significantly smaller for metformin-treated pairs (generalized linear model for Tweedie 1.5 distributed data: $p = 0.011$, $df = 1$, $\chi 2 = 6.419$).

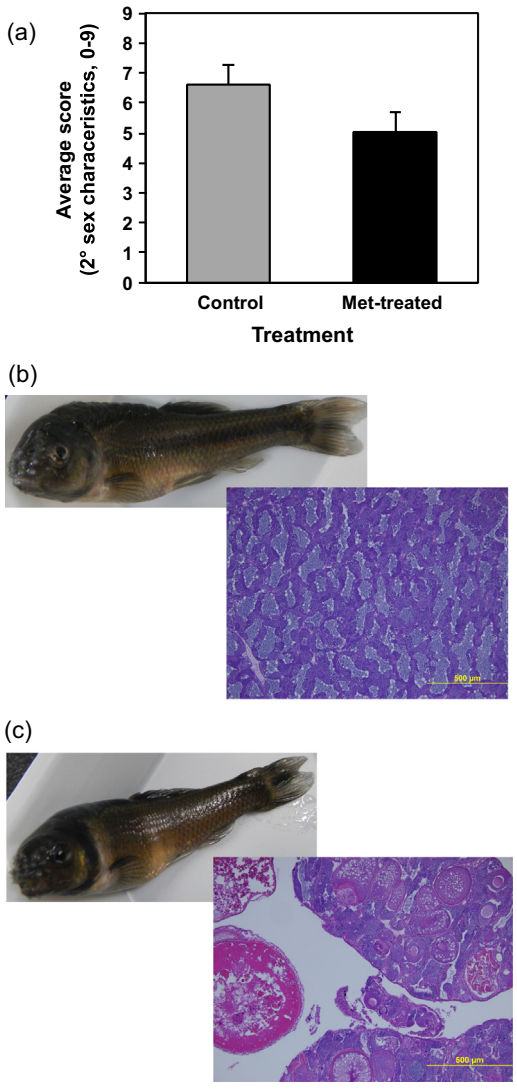


Fig. 4. Scoring of secondary sex characteristics for control males and FHM males exposed to 40 μg L^{-1} metformin. (a) Sex scores (0–9) for males from control and metformin treatment groups. Combines scores for males from mating pairs (exposed 360 d) and remaining males (exposed 365 d). Error bars represent SEM. N=: control males, 17; treated males, 30. Metformin-treated males had a lower score, but not significantly (generalized linear model for ordinal numbers: $p = 0.135$, $df = 1$, $\chi 2 = 2.233$). (b) A control male with a secondary sex score of 9 and a gonad histology score of 0. (c) A metformin-treated male with a secondary sex score of 9 and a gonad histology score of 6.

Importantly, metformin is now being found at relatively high concentrations in watersheds (in the ng to μg L^{-1} range when it has been measured), and often at orders of magnitude higher concentrations than known EDCs such as EE2 (Scheurer et al., 2012; Oosterhuis et al., 2013; Blair et al., 2013a; Ghoshdastidar et al., 2014). EE2 has endocrine disrupting effects at concentrations in the single ng L^{-1} range and is found in effluent at 1–62 ng L^{-1} and in surface waters at up to 5 ng L^{-1} (Pawlowski et al., 2004). Metformin is found in effluent at 1–47 μg L^{-1} and in surface waters at 12 ng L^{-1} to 3 μg L^{-1} throughout North America and Europe (Blair et al., 2013a,b; Ghoshdastidar et al., 2014; Oosterhuis et al., 2013; Scheurer et al., 2012). The data presented here and in our previous work (Niemuth et al., 2015), exposing FHM to a concentration of metformin found in effluent (40 μg L^{-1}), demonstrate the potential endocrine disrupting effects of metformin. While the estrogenic potency of metformin likely differs from known EDCs such as EE2 (and remains to be determined), the widespread detection of this compound in WWTP effluent and freshwater systems highlights the need to determine the potential impacts of this compound in wild fish populations at environmental concentrations.

Effects of metformin on gonadal development have also been documented in at least one study in mammals, with altered testicular development in progeny of mice administered metformin during pregnancy (Tartarin et al., 2012). Studies of the endocrine disrupting effects of metformin are important because it currently is being considered as a treatment for gestational diabetes (Feig and Moses, 2011). Importantly, metformin is known to readily cross the placenta, and has been detected in cord plasma at upwards of 800 μg L^{-1}, 67.5% of the concentration measured in maternal plasma and 20 times the concentration used in the present study (Charles et al., 2006). These findings, taken together with the significant development of intersex in male fish seen here, recommend further research into the potential impacts of metformin on vertebrate development.

Changes in gonad morphology have obvious implications for reproduction, but changes in size may also have reproductive impacts in fish. Metformin treated males were significantly smaller than their control counterparts, and metformin-treated pairs produced significantly fewer clutches than controls (Fig. 3). As in this study, impacts on size and fecundity were also observed in FHM exposed for a full life-cycle to EE2, a synthetic estrogen used in oral contraceptives (Länge et al., 2001). Female length has been shown to be correlated with fecundity in certain fish species (Koops et al., 2004), and, in FHM, mating pairs with males of larger size relative to females have been shown to be more reproductively successful (Pollock et al., 2008). Although treated males were smaller in this study, no significant correlation was found between fecundity and size. We found a small but significant negative correlation between male gonad intersex histology score and fecundity, measured as clutch size and clutch number. However, the small size of this correlation indicates that gonad histology may not be a good predictor of reproductive output. The implications of these results are that, while size-based health metrics such as condition factor may be a good indicator of fecundity in healthy wild fish populations, in environmental conditions where fish are exposed to EDCs (and metrics such as size may be confounded), exposure effects such as changes in gonad histology may not be ideal predictors of reproductive output.

Interestingly, metformin-exposed males with high intersex gonad histology scores still displayed high secondary sex characteristics (as illustrated in Fig. 4). In addition, mating pairs with these males were still able to produce eggs, even in cases where histology showed completely intersex testis. These results suggest that disruption is not the result of upstream changes to steroidogenesis, a conclusion that accords with the results of our previous 28-d study (Niemuth et al., 2015). We have suggested that metformin may impact endocrine pathways through its impacts on insulin signaling (Niemuth et al., 2015). However, the exact mechanism by which metformin may act as an EDC remains to be elucidated.

The U.S. Environmental Protection Agency currently uses a combination of high throughput in vitro and short-term in vivo assays to determine the endocrine disrupting effects of chemicals (U.S. Environmental Protection Agency, 2012). However, budgetary and ethical considerations have resulted in a push towards increased and even exclusive use of in vitro and in silico assays to screen for potential EDCs (U.S. Environmental Protection Agency, 2011). These assays rely on binding of chemicals to hormone receptors and similarity of chemicals to known EDCs, in particular estrogen mimics (Rotroff et al., 2013; Zang et al., 2013). Metformin, because biguanidines do not structurally resemble

known hormone mimics, likely would not be detected as an EDC in in vitro or in silico assays. While hormones such as EE2 are the classical EDCs and the focus of much research, the work presented here indicates a need to broaden our investigations of potential EDCs and predictive models of what are important pathways beyond traditional hormone receptor and response assays being developed for high throughput screening.

Overall the research presented here demonstrates that metformin should be considered a compound of concern. Given its environmental persistence and presence worldwide (Scheurer et al., 2012; Blair et al., 2013a), this compound merits further research on its potential environmental impacts as well as its impacts on vertebrate development more generally and should be added to the list of potential EDCs.

Acknowledgements

This study was conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee of the University of Wisconsin – Milwaukee. This work was supported by the UW-Milwaukee School of Freshwater Sciences. Special thanks to members of the Klaper lab for their assistance in the sacrifice of animals for this study.

References

Ankley, G.T., Jensen, K.M., Kahl, M.D., Korte, J.J., Makynen, E.A., 2001. Description and evaluation of a short-term reproduction test with the fathead minnow (*Pimephales promelas*). Environ. Toxicol. Chem. 20, 1276–1290.

Ben Sahra, I., Le Marchand-Brustel, Y., Tanti, J.-F., Bost, F., 2010. Metformin in cancer therapy: a new perspective for an old antidiabetic drug? Mol. Cancer Ther. 9, 1092–1099.

Bjerregaard, L.B., Korsgaard, B., Bjerregaard, P., 2006. Intersex in wild roach (Rutilus rutilus) from Danish sewage effluent-receiving streams. Ecotoxicol. Environ. Saf. 64, 321–328.

Blair, R.M., Fang, H., Branham, W.S., Hass, B.S., Dial, S.L., Moland, C.L., Tong, W., Shi, L., Perkins, R., Sheehan, D.M., 2000. The estrogen receptor relative binding affinities of 188 natural and xenochemicals: structural diversity of ligands. Toxicol. Sci. 54, 138–153.

Blair, B.D., Crago, J.P., Hedman, C.J., Klaper, R.D., 2013a. Pharmaceuticals and personal care products found in the Great Lakes above concentrations of environmental concern. Chemosphere 93, 2116–2123.

Blair, B.D., Crago, J.P., Hedman, C.J., Treguer, R.J.F., Magruder, C., Royer, L.S., Klaper, R.D., 2013b. Evaluation of a model for the removal of pharmaceuticals, personal care products, and hormones from wastewater. Sci. Total Environ. 444, 515–521.

Blazer, V.S., Iwanowicz, L.R., Henderson, H., Mazik, P.M., Jenkins, J.A., Alvarez, D.A., Young, J.A., 2012. Reproductive endocrine disruption in smallmouth bass (Micropterus dolomieu) in the Potomac River basin: spatial and temporal comparisons of biological effects. Environ. Monit. Assess. 184, 4309–4334.

Brausch, J.M., Connors, K.A., Brooks, B.W., Rand, G.M., 2012. Human pharmaceuticals in the aquatic environment: a review of recent toxicological studies and considerations for toxicity testing. Rev. Environ. Contam. Toxicol. 218, 1–99.

Charles, B., Norris, R., Xiao, X., Hague, W., 2006. Population pharmacokinetics of metformin in late pregnancy. Ther. Drug Monit. 28, 67–72.

Chen, M., Ohman, K., Metcalfe, C., 2006. Pharmaceuticals and endocrine disruptors in wastewater treatment effluents and in the water supply system of Calgary, Alberta, Canada. Water Qual. Res. J. Can. 41, 351–364.

Chou, C.H., 2000. Uptake and dispersion of metformin in the isolated perfused rat liver. J. Pharm. Pharmacol. 52, 1011–1016.

Dammann, A.A., Shappell, N.W., Bartell, S.E., Schoenfuss, H.L., 2011. Comparing biological effects and potencies of estrone and 17β-estradiol in mature fathead minnows, *Pimephales promelas*. Aquat. Toxicol. 105, 559–568.

Diamanti-Kandarakis, E., Bourguignon, J.-P., Giudice, L.C., Hauser, R., Prins, G.S., Soto, A.M., Zoeller, R.T., Gore, A.C., 2009. Endocrine-disrupting chemicals: an Endocrine Society scientific statement. Endocr. Rev. 30, 293–342.

Fatta-Kassinos, D., Meric, S., Nikolaou, A., 2011. Pharmaceutical residues in environmental waters and wastewater: current state of knowledge and future research. Anal. Bioanal. Chem. 399, 251–275.

Feig, D.S., Moses, R.G., 2011. Metformin therapy during pregnancy: good for the goose and good for the gosling too? Diabetes Care 34, 2329–2330.

Ghoshdastidar, A.J., Fox, S., Tong, A.Z., 2014. The presence of the top prescribed pharmaceuticals in treated sewage effluents and receiving waters in Southwest Nova Scotia, Canada. Environ. Sci. Pollut. Res. Int. 22, 689–700.

Heberer, T., 2002. Occurrence, fate, and removal of pharmaceutical residues in the aquatic environment: a review of recent research data. Toxicol. Lett. 131, 5–17.

Jobling, S., Nolan, M., Tyler, C.R., Brighty, G., Sumpter, J.P., 1998. Widespread sexual disruption in wild fish. Environ. Sci. Technol. 32, 2498–2506.

Jobling, S., Burn, R.W., Thorpe, K., Williams, R., Tyler, C., 2009. Statistical modeling suggests that antiandrogens in effluents from wastewater treatment works contribute to widespread sexual disruption in fish living in English rivers. Environ. Health Perspect 117, 797–802.

Johns, S.M., Denslow, N.D., Kane, M.D., Watanabe, K.H., Orlando, E.F., Sepúlveda, M.S., 2011. Effects of estrogens and antiestrogens on gene expression of fathead minnow (*Pimephales promelas*) early life stages. Environ. Toxicol. 26, 195–206.

Koops, M.A., Hutchings, J.A., McIntyre, T.M., 2004. Testing hypotheses about fecundity, body size and maternal condition in fishes. Fish Fish. 5, 120–130.

Länge, R., Hutchinson, T.H., Croudace, C.P., Siegmund, F., Schweinfurth, H., Hampe, P., Panter, G.H., Sumpter, J.P., 2001. Effects of the synthetic estrogen 17α-ethinylestradiol on the life-cycle of the fathead minnow (*Pimephales promelas*). Environ. Toxicol. Chem. 20, 1216–1227.

Lange, A., Paull, G.C., Hamilton, P.B., Iguchi, T., Tyler, C.R., 2011. Implications of persistent exposure to treated wastewater effluent for breeding in wild roach (Rutilus rutilus) populations. Environ. Sci. Technol. 45, 1673–1679.

Lattier, D.L., Reddy, T.V., Gordon, D.A., Lazorchak, J.M., Smith, M.E., Williams, D.E., Wiechman, B., Flick, R.W., Miracle, A.L., Toth, G.P., 2002. 17alpha-ethynylestradiol-induced vitellogenin gene transcription quantified in livers of adult males, larvae, and gills of fathead minnows (*Pimephales promelas*). Environ. Toxicol. Chem. 21, 2385–2393.

Liney, K.E., Jobling, S., Shears, J.A., Simpson, P., Tyler, C.R., 2005. Assessing the sensitivity of different life stages for sexual disruption in roach (Rutilus rutilus) exposed to effluents from wastewater treatment works. Environ. Health Perspect 113, 1299–1307.

Liney, K.E., Hagger, J.A., Tyler, C.R., Depledge, M.H., Galloway, T.S., Jobling, S., 2006. Health effects in fish of long-term exposure to effluents from wastewater treatment works. Environ. Health Perspect (Suppl 114), 81–89.

Luckenbach, T., Kilian, M., Triebskorn, R., Oberemm, A., 2001. Fish early life stage tests as a tool to assess embryotoxic potentials in small streams. J. Aquat. Ecosyst. Stress Recover 8, 355–370.

Niemuth, N.J., Jordan, R., Crago, J., Blanksma, C., Johnson, R., Klaper, R.D., 2015. Metformin exposure at environmentally relevant concentrations causes potential endocrine disruption in adult male fish. Environ. Toxicol. Chem. 34, 291–296.

Novak, P.J., Arnold, W.A., Blazer, V.S., Halden, R.U., Klaper, R.D., Kolpin, D.W., Kriebel, D., Love, N.G., Martinović-Weigelt, D., Patisaul, H.B., Snyder, S.A., Vom Saal, F.S., Weisbrod, A.V., Swackhamer, D.L., 2011. On the need for a National (U.S.) research program to elucidate the potential risks to human health and the environment posed by contaminants of emerging concern. Environ. Sci. Technol. 45, 3829–3830.

American Diabetes Association, 2013. Standards of medical care in diabetes – 2013. Diabetes Care. vol. 36, S11–S66.

Oosterhuis, M., Sacher, F., Ter Laak, T.L., 2013. Prediction of concentration levels of metformin and other high consumption pharmaceuticals in wastewater and regional surface water based on sales data. Sci. Total Environ. 442, 380–388.

Parrott, J.L., Blunt, B.R., 2005. Life-cycle exposure of fathead minnows (*Pimephales promelas*) to an ethinylestradiol concentration below 1 ng/L reduces egg fertilization success and demasculinizes males. Environ. Toxicol. 20, 131–141.

Pawlowski, S., van Aerle, R., Tyler, C.R., Braunbeck, T., 2004. Effects of 17alpha-ethinylestradiol in a fathead minnow (*Pimephales promelas*) gonadal recrudescence assay. Ecotoxicol. Environ. Saf. 57, 330–345.

Pollock, M.S., Fisher, S.E., Squires, A.J., Pollock, R.J., Chivers, D.P., Dubé, M.G., 2008. Relative body size influences breeding propensity in fathead minnows: implications for ecotoxicology testing procedure. Water Qual. Res. J. Can. 43, 257–264.

Rotroff, D.M., Dix, D.J., Houck, K.A., Knudsen, T.B., Martin, M.T., McLaurin, K.W., Reif, D.M., Crofton, K.M., Singh, A.V., Xia, M., Huang, R., Judson, R.S., 2013. Using in vitro high throughput screening assays to identify potential endocrine-disrupting chemicals. Environ. Health Perspect 121, 7–14.

Scheurer, M., Michel, A., Brauch, H.-J., Ruck, W., Sacher, F., 2012. Occurrence and fate of the antidiabetic drug metformin and its metabolite guanylurea in the environment and during drinking water treatment. Water Res. 46, 4790–4802.

Sharma, V.K., Nautiyal, V., Goel, K.K., Sharma, A., 2010. Assessment of thermal stability of metformin hydrochloride. Asian J. Chem. 22, 3561–3566.

Sowers, A.D., Gaworecki, K.M., Mills, M.A., Roberts, A.P., Klaine, S.J., 2009. Developmental effects of a municipal wastewater effluent on two generations of the fathead minnow, *Pimephales promelas*. Aquat. Toxicol. 95, 173–181.

Tang, T., Lord, J.M., Norman, R.J., Yasmin, E., Balen, A.H., 2012. Insulin-sensitising drugs (metformin, rosiglitazone, pioglitazone, D-chiro-inositol) for women with polycystic ovary syndrome, oligo amenorrhoea and subfertility. Cochrane Database Syst. Rev. 5, CD003053.

Tartarin, P., Moison, D., Guibert, E., Dupont, J., Habert, R., Rouiller-Fabre, V., Frydman, N., Pozzi, S., Frydman, R., Lecureuil, C., Froment, P., 2012. Metformin exposure affects human and mouse fetal testicular cells. Hum. Reprod. 27, 3304–3314.

Tetreault, G.R., Bennett, C.J., Shires, K., Knight, B., Servos, M.R., McMaster, M.E., 2011. Intersex and reproductive impairment of wild fish exposed to multiple municipal wastewater discharges. Aquat. Toxicol. 104, 278–290.

U.S. Environmental Protection Agency, 2011. Endocrine Disruptor Screening Program for the 21st Century (EDSP21 Work Plan). The Incorporation of In Silico Models and In Vitro High Throughput Assays in the Endocrine Disruptor Screening Program (EDSP) for Prioritization and Screening. [WWW Document].

<http://www.epa.gov/endo/pubs/edsp21_work_plan_summary _overview_ final.pdf>.
U.S. Environmental Protection Agency, 2012. Tier 1 Screening Battery [WWW Document]. <http://www.epa.gov/endo/pubs/assayvalidation/tier1battery.htm>.
Van Aerle, R., Pounds, N., Hutchinson, T.H., Maddix, S., Tyler, C.R., 2002. Window of sensitivity for the estrogenic effects of ethinylestradiol in early life-stages of fathead minnow, *Pimephales promelas*. Ecotoxicology 11, 423–434.
Van Aerle, R., Runnalls, T.J., Tyler, C.R., 2004. Ontogeny of gonadal sex development relative to growth in fathead minnow. J. Fish Biol. 64, 355–369.
Viollet, B., Guigas, B., Sanz Garcia, N., Leclerc, J., Foretz, M., Andreelli, F., 2012. Cellular and molecular mechanisms of metformin: an overview. Clin. Sci. 122, 253–270.
Zang, Q., Rotroff, D.M., Judson, R.S., 2013. Binary classification of a large collection of environmental chemicals from estrogen receptor assays by quantitative structure-activity relationship and machine learning methods. J. Chem. Inf. Model. 53, 3244–3261.