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Emerging wastewater contaminant metformin causes intersex and reduced fecundity in fish

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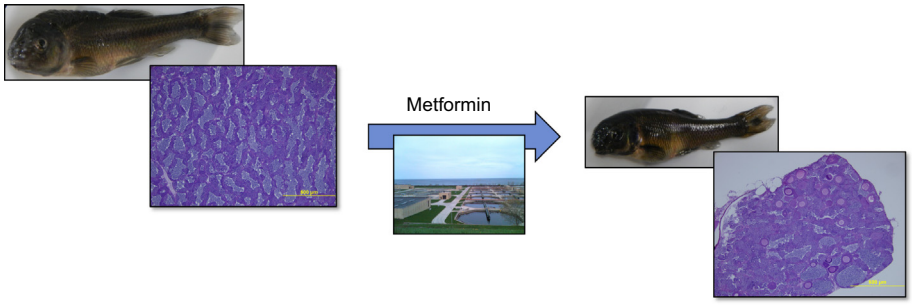
HIGHLIGHTS

- 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



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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

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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.