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IMPACTS OF SEWAGE WASTE WATER ON FEMINIZATION AND VITELLOGENIN EXPRESSION IN MALE FATHEAD MINNOWS

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ABSTRACT. Estrogenic compounds are commonly found in wastewater effluents. Exposure of male fish to these chemicals can lead to 'feminization', including decrease in secondary sex characteristics and production of female-specific proteins such as vitellogenin (VTG). We hypothesized that upon exposure to wastewater from the Muncie Water Pollution Control Facility, Indiana, adult male fathead minnows (*Pimephales promelas*) would respond with a decrease in secondary sex characteristics and increased expression of *vtg* if the effluents contained sufficient estrogens. Adult males were caged at two sites in the West Fork White River: the downstream group was placed directly below the outflow and the upstream group was placed 0.25 km upstream. A third group was housed indoors in aquaria and served as a control. After 21 d, body and organ measurements, secondary sex characteristics, and liver *vtg* gene expression were assessed. While no significant differences were observed in secondary sex characteristics between study groups, 'downstream' males had larger liver somatic index values and showed an up-regulation of liver *vtg* relative to the other two groups. Although our results agree with a previous study in this same area that found 'feminization' of native populations of bluntnose minnows (*P. notatus*), the estrogenic compounds that elicited this response remain unknown.

Keywords: Feminization, fish, estrogens, wastewater, White River, Indiana

INTRODUCTION

An expanding amount of research suggests that effluents from domestic wastewater treatment plants (WWTP) can contain natural (estrone, E1; 17β-estradiol, E2; and estrone, E3) and synthetic (17α-ethinylestradiol, EE2) estrogens (see Limpiyakorn et al. 2011 for a review). The latter form of estrogen is the main ingredient of oral contraceptives and is considered to be the most potent environmental estrogen (Clouzot et al. 2008). Exposure of male fish to estrogens can result in a range of effects from complete sex reversal in the most severe cases to different degrees of 'feminization', including intersex (i.e. testes with oocytes) and decreased expression of secondary sex characteristics (Lange et al. 2012). In more extensive studies with fathead minnows (*Pimephales promelas*) exposed to municipal wastewater, greatly reduced reproductive capacities were observed (Rickwood et al. 2008, Thorpe et al. 2009). Further, female-biased sex ratios have been observed in feral populations of other teleost species such as white suckers (*Catostomus commersoni*) exposed to WWTP effluents (Vajda et al. 2008). The estrogenic ecological effects of these types of effluents are not well understood and are a cause for concern.

A commonly used biomarker of exposure to estrogens in fish is vitellogenin (VTG). Vitellogenin is a phospholipoprotein synthesized in the liver of egg-laying females after estrogen stimulus and is essential in the production of egg yolk proteins and thus embryo survival (Specker and Sullivan 1993). While VTG receptors are present in male fish, the gene is silent unless triggered in the presence of sufficient estrogen concentrations (Maitre et al. 1985; Copeland et al. 1986; LeGuellec et al. 1988). Thus VTG can serve as an efficient marker of estrogenic contamination in aquatic systems (Sumpter and Jobling 1995). Furthermore, induction of VTG in male fish has been

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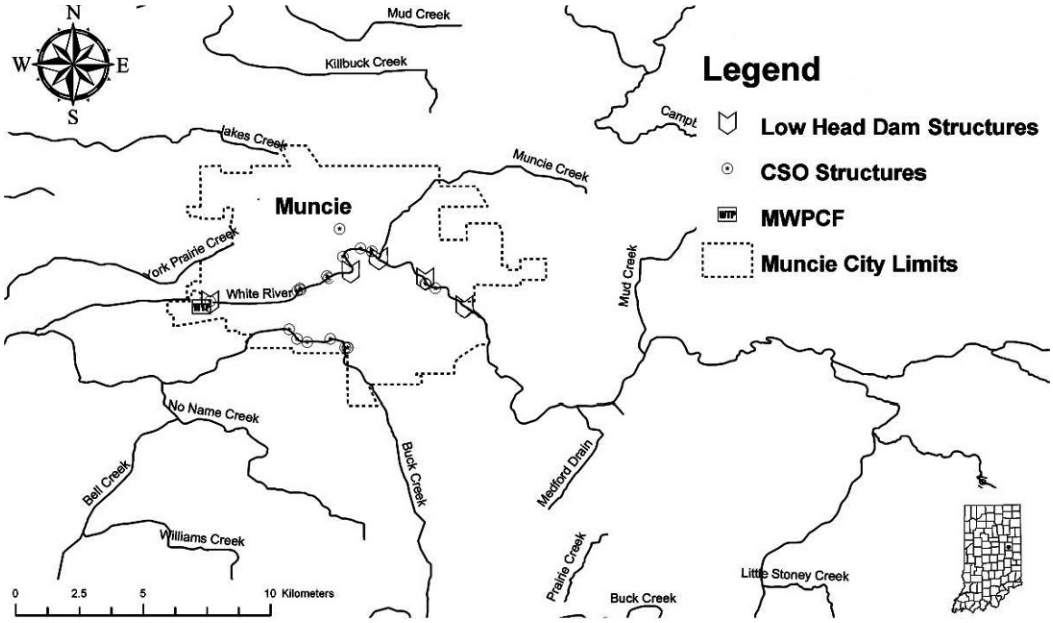

Figure 1.—Map of Muncie, Indiana (dotted lines), showing the location of the combined sewer overflow (CSO) and Muncie Water Pollution Control Facility (MWPCF) along the White River. Fish in this study were caged 0.25 km upstream and 5 m downstream of MWPCF. Adapted from Doll (2011).

associated with decreased sperm quality and decreased fertility, potentially resulting in poor reproductive success (Kidd et al. 2007).

The only study evaluating the potential effects of effluents from a WWTP in Indiana was published by Doll (2011). In this study, free-ranging adult bluntnose minnows (*Pimephales notatus*) were sampled from the West Fork White River (hereafter White River) at different distances up- and downstream from the Muncie Water Pollution Control Facility (MWPCF), Delaware County, Indiana. This facility is a conventional activated sludge treatment plant that discharges an average of 18 MGD (million gallons per day) into the White River. The MWPCF is located at river kilometer 501.5 where the drainage area is $\sim$ 635 km^2 (Hoggatt 1975). The MWPCF serves a population of 67,430 and includes one hospital and Ball State University. In addition, 13 combined sewer overflows are located on the White River within Muncie City limits. Secondary sex characteristics of male minnows collected downstream of the MWPCF had an average of 31.3% fewer tubercles and a 37.5% lower tubercle score compared to those sampled upstream of Muncie (Doll 2011). These results suggest that minnows were being exposed to estrogen-like compounds likely being released from the MWPCF and combined sewage overflows.

The main goal of our study was to further determine the potential estrogenicity of the MWPCF effluent. To do this we conducted an *in situ* exposure using fathead minnows, a commonly used species in ecotoxicology studies. We hypothesized that if estrogens are present at sufficient concentrations in this effluent, male fish caged downstream from the effluent discharge would respond with a decrease in expression of secondary sex characteristics and an induction of VTG.

METHODS

Experimental design.—On June 1, 2011 adult fathead minnow males were exposed for 21 d to three different conditions: 1) caged (n = 4) located approximately 5 m below the MWPCF effluent; 2) caged (n = 3) 0.25 km upstream of the MWPCF (Fig. 1); or 3) kept indoors in the Bureau of Water Quality Laboratory ($\sim$ 38-L glass aquaria, n =4) containing reconstituted reverse osmosis water (APHA 2005) which was changed three times per week. Minnows in cages were allowed to eat naturally occurring food in the river whereas those in the tanks were fed 1 g of frozen brine shrimp every two

days to keep stress at a minimum. These three conditions are referred herein to as downstream, upstream, and control, respectively. This exposure length and species were chosen because they are commonly used in ecotoxicology for assessing impacts of endocrine disrupting compounds (US EPA 2002). Cages were galvanized round minnow traps 419 mm long, 222 mm wide at center, 178 mm wide at ends, and 6 mm wire bar mesh. Openings of each trap were closed off with 3 mm bar mesh fiberglass screen. Minnow cages and tanks contained three to five male adult males each. Dissolved oxygen (DO), temperature, and conductivity (river samples only) were measured with a portable YSI® meter (model 556MPS); turbidity (river samples only) was measured with an Oakton® turbidity meter (model T-100); ammonia, nitrite, and nitrate (indoor tanks only) was measured using Hach® water quality test strips (Hach, Loeveland, CO, USA) daily Monday through Friday. Meters were calibrated and water quality was measured daily. At the conclusion of the exposure, fish were euthanized with MS-222 (300 mg/L) and processed for data collection.

Data collection.—All fish were measured (total length, mm), weighed (g), and gonads and livers dissected and weighed (± 0.01 g) for determination of gonadosomatic index (GSI) and hepatosomatic index (HSI). These indices were calculated by dividing the weight of the organ by the weight of the fish multiplied by 100. A small section of each liver was placed in RNA*later* (Qiagen, Valencia, CA, USA) and stored at −80°C for analysis of *vtg* expression as described below. Secondary sex characters were measured from each male fish as already described (US EPA 2002; US EPA 2007; Doll 2011), which included tubercle counts and tubercle and fatpad scoring. Tubercle counts were the total number of tubercles present. The size of each tubercle was qualitatively ranked as 1 = present, 2 = enlarged, and 3 = pronounced. The tubercle score was the sum of all individual tubercle ranks per individual fish. The fatpad score is a qualitative ranking and was assigned a 1 = no fatpad visible, 2 = small fatpad evident, 3 = fatpad is clearly visible and is just above body surface, 4 = fatpad is prominent and is clearly above the body surface but not overhanging, and 5 = fatpad is very prominent and overhangs the body surface.

Vitellogenin analysis.—The expression of the gene *vtg*, which codes for the VTG protein, was measured as a marker of estrogen exposure. RNA was extracted (TriSure, Bioline, Taunton, MA, USA), quantified (Nanodrop 1000, Thermo Fisher Scientific, Waltham, MA, USA), DNase-treated (Fermentas Inc., Glen Burnie, MD, USA) and reverse transcribed to cDNA (Applied Biosystems, Foster City, CA, USA). The 260/280 ratio was used as an indicator of RNA quality, and only samples with a ratio of 1.8 or higher were used in gene analysis (33 samples of the 41 total). Elongation factor 1 (*ef1*) was used as the housekeeping gene. Primers for *vtg* and *ef1* were selected from primary literature (Biales et al. 2007; Mager et al. 2008) and purchased through Integrated DNA Technologies (Coralville, IA, USA). Polymerase chain reaction (PCR) products were sequenced on an ABI 3700 (Applied Biosystems, Foster City, CA, USA) at Purdue University's Genomics Core to validate specificity of gene amplification. Gene expression analysis was conducted by quantitative PCR (qPCR) on a Bio-Rad iQ5 (Bio-Rad Laboratories, Hercules, CA, USA) using a DyNAmo™ SYBR® green qPCR kit (Ratastie 2, 01620 Vantaa, Finland). Reactions were comprised of 6.0 µL 2X master mix, 360 nM primer, cDNA template synthesized from 45 ng of DNAse-treated total RNA, and molecular-grade water (12 µL total). Conditions used to amplify samples were: 94°C for 15 sec, 60°C for 30 sec, and 72°C for 30 sec for 40 amplification cycles. No template controls, negative reverse transcriptase controls, and a melt curve analysis were performed for each primer to determine whether nonspecific products were being amplified. Samples were rerun if standard deviation of the Ct values between duplicates was > 0.5. Expression of target genes was normalized relative to the expression of *elf1* ($\Delta Ct = Ct_{vtg\ gene} - Ct_{efl1}$). The relative expression of the target gene in experimental groups compared to the control group was quantified by the $2^{-\Delta\Delta Ct}$ method (Pfaffl 2001).

Data analysis.—Water-quality parameters, *vtg* hepatic expression, and all morphological measurements with exception of tubercle and fatpad scores, were compared across sites using analysis of variance (ANOVA) followed by Tukey's multiple comparison test. Tubercle and fatpad scores were compared across groups using chi-square. All tests were conducted using

Table 1.—Water quality taken from the White River in Muncie, Indiana, during the time of fathead minnow cage deployment (June 2 – June 22, 2011). Different small letters denote significant differences between the means (t-test, $p = < 0.05$). *Not measured.

	Temperature(°C)	Conductivity(μS)	D.O.(mg/l)	Turbidity(NTU)
Upstream (n = 13)				
Mean	20.8[a]	0.46[a]	8.20[ac]	63.5
Median	19.9	0.48	8.40	24.8
Range	5.60	0.20	2.50	283
St. Dev.	1.76	0.06	0.85	85.8
Downstream (n = 14)				
Mean	18.9[b]	0.81[b]	9.20[b]	34.0
Median	18.7	0.79	9.10	15.1
Range	2.70	0.63	1.80	149
St. Dev.	0.83	0.18	0.53	43.7
Control (n = 15)				
Mean	22.0[c]	-[*]	8.50[ac]	-
Median	22.3	-	8.50	-
Range	3.41	-	1.52	-
St. Dev.	0.95	-	0.42	-

SAS 9.3, and significance was declared at $\alpha = 0.05$.

RESULTS

Water quality.—There were significant differences in water temperature and DO across the three treatments; however, overall differences were relatively small (ranges of temperature 18–21°C and DO 6.6–8.9 mg/l) (Table 1). Conductivity was almost twice as high in the 'downstream' site compared to the 'upstream' site (0.81 ± 0.18 μS and 0.46 ± 0.06 μS, respectively). Nitrogenous compounds were measured in the control tanks only. Nitrate and nitrites were not detected, and total ammonia averaged 0.44 ± 0.32 mg/L during the experiment.

Morphometric measurements.—There were no mortalities during this experiment. At the time of dissection, four fish from the 'downstream' and two fish from the 'upstream' site were classified as females and were eliminated from further analyses. The 'upstream' fish were larger than the other two groups, but not significantly (Table 2). The only parameter that differed among treatments was LSI, with males from the 'downstream' site having the largest value (upstream 1.38 ± 0.8; downstream 2.04 ± 0.8; control 0.96 ± 0.3).

Secondary sex characteristics.—Secondary sex characteristics of male fathead minnows are summarized in Table 2. No significant differences were observed across treatments. However, the 'control' group had the highest tubercle count, fatpad score and weight of all the groups.

Vitellogenin expression.—While upstream males showed no significant difference in *vtg* expression compared to control fish, downstream males displayed an up-regulation in the expression of this gene (Figure 2).

DISCUSSION

We documented a significant induction of *vtg* in male fathead minnows after a 21-d exposure to the MWPCF effluent. These males also had significantly higher LSI than the upstream and control group. However, secondary sex characteristics did not significantly differ among the three treatments. Although there were differences in water quality parameters between treatment groups, overall differences were relatively small (ranges of temperature 18–21°C and DO 6.6–8.9 mg/l; Table 1). These values fell well within acceptable limits for fish and would not have induced the differences in *vtg* expression and LSI observed. These results suggest presence and exposure of fish to estrogens or estrogen-mimic compounds. To our knowledge, the present study is only the second published evaluating the potential impacts of sewage effluents on fish in Indiana. The first study in Indiana also showed evidence of feminizing contaminants being released by

Table 2.—Means (± SD) of morphological parameters measured in male fathead minnows at the end of the study. Gonadosomatic index (GSI), liver somatic index (LSI), interocular distance (ID), widest head width (HW), tubercle count (TC), fatpad score (FPS), and fatpad weight (FPW). * Indicates significance p = 0.0001. [a] = only one fish measured.

Location	Total weight (g)	Total length (mm)	GSI (%)	LSI (%)	ID (mm)	HW (mm)	TC	FPS	FPW (g)
Upstream	3.99	71.6	0.86	1.38	6.63	8.5	12.1	1.25	0.04[a]
(n = 8)	(0.8)	(4.8)	(0.4)	(0.8)	(0.9)	(0.8)	(8.4)	(0.5)	
Downstream	3.41	66.6	1.34	2.04*	6.00	7.89	15.6	1.87	0.02
(n = 15)	(0.6)	(2.6)	(1.0)	(0.8)	(0.7)	(0.7)	(3.7)	(0.8)	(0.02)
Control	3.33	65.7	0.97	0.96	6.22	8.00	16.1	2.00	0.08
(n = 18)	(1.1)	(6.7)	(0.4)	(0.3)	(0.8)	(1.0)	(5.9)	(1.0)	(0.08)

the MWPCF, as wild bluntnose minnows exposed to effluent at the downstream site were observed to have reduced expression of secondary sex characteristics (Doll 2011).

Hundreds of studies around the country and the world have quantified natural and synthetic estrogens in effluents from sewage waste water treatment plants (Limpiyakorn et al. 2011). Although in most cases estrogens are found at very low concentrations (low ng/l), feminization and complete reproductive failure of male fathead minnows has been shown after chronic exposure to < 5 ng/l to EE2 (Parrott and Blunt 2005; Filby et al. 2007; Kidd et al. 2007). This reproductive failure in males can result from alterations in testicular development, but it can also be the result of more subtle effects such as VTG production. Indeed, VTG production by males can lead to decreased fertility and even kidney pathology (Zha et al. 2008). Alterations in behavior have also been reported after exposure of male fish to estrogens (Dammann et al. 2011). Fathead minnow males caged downstream from the MWPCF also had enlarged livers. Since VTG is produced by the liver, this hepatomegalia is consistent with these fish being exposed to estrogens (Gunnarsson et al. 2009). VTG induction and LSI have been shown to have a positive relationship in fathead minnows (Barber et al. 2007).

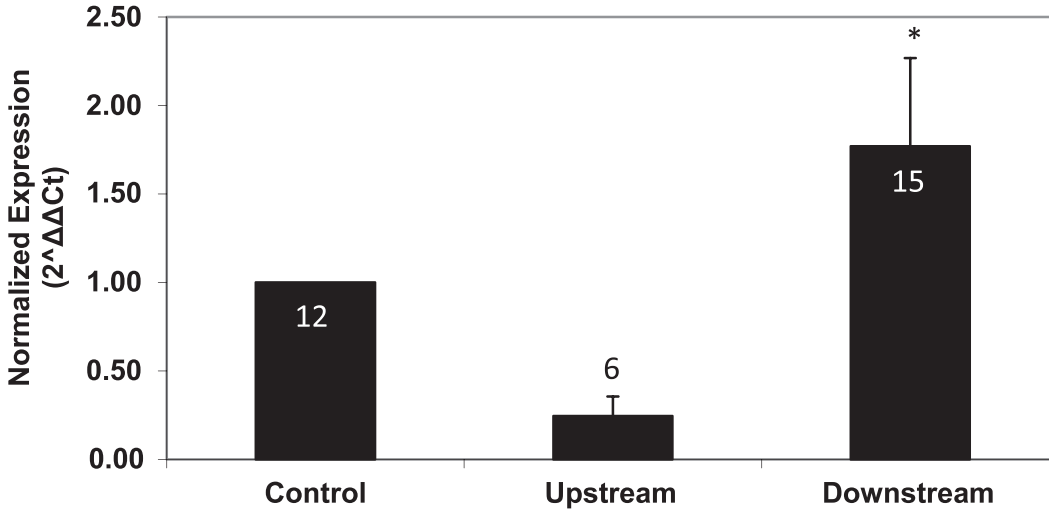

Figure 2.—Mean ± SD of relative *vtg* gene expression from livers of male fathead minnows caged downstream and upstream from the Muncie Water Pollution Control Facility (MWPCF) for 21 d in relation to the controls which were kept indoors in aquaria for the same length of time. Males downstream responded with an up- regulation (denoted with an asterisk; p = 0.05) in *vtg* expression compared back to the control males (assigned a value of 1). Numbers on bars indicate sample sizes.

Results of this study showed indications of feminization downstream from the MWPCF. The lack of feminization in male secondary sex characteristics indicates that the estrogenic contaminants are not potent enough to elicit feminizing effects after a relatively short exposure (i.e. 21 d). However, these contaminants are still of concern for chronic fish exposures, as seen with the decreased expression of secondary sex characteristics in feral fish found at this downstream site (Doll 2011).

In conclusion, effluents from the MWPCF are likely releasing estrogens into the White River. More studies are needed that measure the types(s) and concentrations of estrogens and potentially other types of contaminants from this effluent as well as the potential impacts on natural populations of fish and other aquatic organisms inhabiting this site.

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