2011. Proceedings of the Indiana Academy of Science 119(2):130–143

EVALUATION OF MORPHOLOGY AND SECONDARY SEXUAL CHARACTERISTICS OF THE BLUNTNOSE MINNOW: EVIDENCE OF ESTROGENIC COMPOUND EXPOSURE?

Jason C. Doll: Bureau of Water Quality, Muncie Sanitary District, 5150 W. Kilgore Ave., Muncie, Indiana 47304-4710

ABSTRACT. Estrogenic compounds are known to cause physiological and behavioral changes in fish. Point sources of estrogenic compounds include wastewater treatment plants, combined sewer overflows, and agricultural operations. The West Fork of White River in Delaware County, Indiana is impacted by all three potential point sources of estrogenic compounds. The objective of this study was to evaluate the presence of estrogenic compounds in the West Fork of White River using morphological measurements of bluntnose minnow (*Pimephales notatus*). Fish were sampled using a tote-barge electrofishing unit at five sites on White River and one site on Cabin Creek. After collection, fish were evaluated for sex, total length, total weight, widest head width, interocular distance, gonadosomatic index, gonad weight, egg count, tubercle count, and tubercle score. A total of 346 bluntnose minnows was collected between 25 May and 6 June, 2010. Minimal differences were found between White River and Cabin Creek sites. When evaluating sites only on White River, males were found to have a 31.3% lower tubercle count and 37.5% lower tubercle score at the most downstream site when compared to the most upstream site. Our data suggest that the West Fork of White River is receiving estrogenic compounds from the combined sewer overflows and the Muncie Water Pollution Control Facility, an activated sludge wastewater treatment plant.

Keywords: Rivers, combined sewer overflows, trace organic compounds, wastewater treatment facility

Trace organic compounds are emerging contaminants that have drawn the attention of international agencies (U.S. EPA 1998; OECD 2000). Point sources of ECs include wastewater treatment plants (Purdom et al. 1994), combined sewer overflows (CSOs) (Wilkison et al. 2002), and agricultural operations (Orlando et al. 2004). One of the major concerns of ECs in surface waters is they can alter physiological functions in wildlife (Tyler et al. 1998).

Estrogenic compounds at very low concentrations have been known to disrupt the endocrine system of many animals (e.g., nanogram/L). Environmental effects of ECs on fish include physiological and behavioral changes. For example, estrogen-exposed male fathead minnows (*Pimephales promelas*) exhibit a reduced competitive advantage over non-exposed fish during spawning (Martinovic et al. 2007). Additionally, exposure to ECs has been shown to alter agonistic behavior in mature male fish (Ros et al. 2004). Differences in morphological characteristics and suppressed development of secondary sexual characteristics have also been linked to exposure to ECs (Jobling et al. 1996; Miles-Richardson et al. 1999; Angus et al. 2002; Hassanin et al. 2002; Brian et al. 2007); with the most well known outcome of exposure to ECs being intersex fish (Jobling et al. 1998; Blazer et al. 2007).

Estrogenic compounds remain unregulated in the surface waters of the U.S. Regardless, the extent of environmental ECs must be identified to more accurately describe the effects on all forms of life. The West Fork of White River (hereafter called White River) in Delaware County, Indiana provides an ideal situation to study the potential effects of ECs. This area is located near the headwaters of White River and includes a 64 km segment upstream of the city of Muncie that is primarily influenced by extensive agriculture, including combined animal feeding operations (CAFOs) with only minor urbanization. Within Muncie city limits the river is exposed to urbanization pressures such as CSOs and the Muncie Water Pollution Control Facility (MWPCF) effluent. The MWPCF is a conventional activated sludge

Jason C. Doll, Bureau of Water Quality, Muncie Sanitary District, 5150 W. Kilgore Ave., Muncie, Indiana 47304-4710, tel: 765-747-4896, fax: 765-213-6444 e-mail: jdoll@msdeng.com.

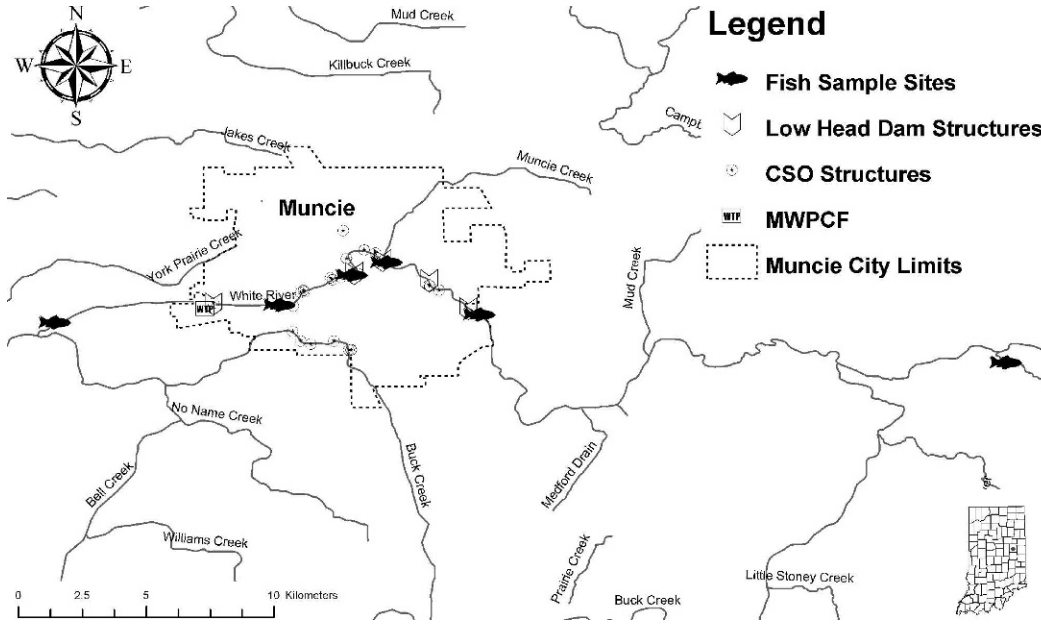

Figure 1.—White River and Cabin Creek sample sites (CSO = combined sewer overflow, MWPCF = Muncie Water Pollution Control Facility).

treatment plant rated at 24 MGD and discharges into White River at river kilometer 501.5. On average the MWPCF discharges 12 MGD of effluent into White River.

Certain methods exist to effectively identify the presence of ECs (e.g., male blood plasma vitellogenin or a suite of chemical analysis); however, it is more economical to conduct a preliminary analysis of less costly parameters such as secondary sexual characteristics and morphological features that are known to be affected by exposure to ECs.

The objective of this study was to determine the potential presence of ECs in White River by evaluating differences in morphological measurements and secondary sexual characteristics of the bluntnose minnow (*Pimephales notatus*).

METHODS

Sample sites.—Bluntnose minnows were collected from five locations on White River in Delaware County and one location on Cabin Creek in Randolph County (Fig. 1). Cabin Creek (CAB-0.8) in Randolph County represents a control site and is assumed to be minimally influenced by ECs. Of the White River sites, one site (WR-513.1) is located upstream of Muncie and represented the site on White River least impacted by urbanization. This site provides a reference of condition before city limits (Table 1). However, this site is impacted by agricultural operations and potentially exposed to ECs. Three White River sites were located within city limits (WR-509.0, WR-506.6, & WR-504.4) (Table 1). Each of these sites was selected for their proximity to the three most active CSOs in Muncie. Combined sewer overflow activity was measured with ISCO flow meters from 2007 through 2008. Flow at each site is recorded every 15 minutes. The fifth White River site (WR-496.5) was located downstream of the city of Muncie and reflected the cumulative impact of urbanization (CSOs & MWPCF effluent) and agricultural activity (Table 1). Site CAB-0.8 is not impacted by any known CAFOs although row crop agriculture could be contributing estrogen mimics (McDaniel et al. 2008). Therefore, CAB-0.8 is assumed to be minimally impacted by environmental ECs. Movement of fish between sample sites was not measured; however, movement is assumed to be minimal or non-existent due to the multiple low-head dams located throughout the city limits (Fig. 1).

Fish collection.—Fish were collected with a Smith-Root pulsed DC electrofishing totebarge (using 2–3 amps and 30 pulses per second). Sampling at each site was conducted

Table 1.—Bluntnose minnow sample site descriptions.

Site	Latitude	Longitude	Description
WR-496.5	40.178772	−85.495095	Site located directly downstream of the CR 575W bridge.
WR-504.4	40.184836	−85.415999	Site located 0.34 km downstream of Nichols Avenue bridge.
WR-506.6	40.203526	−85.391207	Site located 0.15 km downstream of High Street dam.
WR-509.0	40.199883	−85.378433	Site located 0.2 km downstream of Broadway Avenue bridge.
WR-513.1	40.181462	−85.345779	Site located directly downstream of the 12th Street bridge.
CAB-0.8	40.164628	−85.160482	Site located directly upstream of Windsor Pike.

until a representative sample size was obtained. All bluntnose minnows ≥ 40 mm in total length (TL) were collected and immediately placed on ice and transported to the laboratory for analysis. Only one site was sampled in a given day. Fish were separated by sex (as determined by dissection and visual examination of gonads) and measured for total length (TL, measured to the nearest 1 mm), total weight (TW, 0.01 grams), widest head width (HW, 1 mm), interocular distance (ID, 1 mm), and gonad weight (GW, 0.001 grams). Additional measurements for males included tubercle count (TC) and tubercle score (TS). The TS is a qualitative measurement of tubercle size (U.S. EPA 2002). Each tubercle is mapped as a matrix and given a value of 0 to 3 to reflect developed stage of the tubercle; 0 being absent and 3 being fully developed. All values are then summed to give one total TS for each individual. Fecundity was determined by the number of eggs for each female (EC). Ovaries were removed and preserved in modified Gilson's fluid for later analysis. Eggs were counted individually within two weeks of removal from each ovary. Finally the gonadosomatic index (GSI) (Strange 1996) was calculated for males and females. All measurements followed methods developed for and used with the closely related fathead minnow for determination of exposure to endocrine disrupting chemicals (U.S. EPA 2002; Orlando et al. 2004).

Data analysis.—Analyses were conducted with the dataset separated by sex. All variables were modeled using a generalized linear model framework with multiple error distributions. Different error distributions were considered to ensure a more accurate model to describe the dependent variables. Total weight, HW, ID, GW and GSI were modeled with an error distribution of normal with identity link, log-normal with the identity link, and gamma with inverse link. Count variables (TC, TS, and EC) were modeled with an error distribution of normal with identity link, log-normal with identity link, Poisson with log link, and negative binomial with log link. The final model and error distribution used for data interpretation of each independent variable was chosen based on Akaike's information criterion (AIC) (Lindsey & Jones 1998; Dick 2004). The model with the lowest AIC value is considered the best model. The change in AIC is reported, therefore, values of 0 indicate the lowest AIC. All independent variables are highly correlated to TL therefore TL was included as a fixed effect to account for the variability due to length. To test for differences among site a categorical variable was included as a fixed effect in each model to designate sample site. All models evaluated the site effect in reference to CAB-0.8, the control site, and WR-513.1 on White River without Cabin Creek to establish a linear gradient of antrthopogenic influences on White River only.

Models were fit in the R statistical package version 2.10.1 (R Development Core Team 2009). The glm procedure used for fitting the generalized linear models is available in the stats package. Significance was set at $P < 0.05$ for all analyses.

RESULTS

A total of 346 bluntnose minnow, 169 males and 177 females, was collected between 25 May and 6 June, 2010. Males had significantly longer TL ($t = -5.59$, df= 344, $P < 0.001$),

Table 2.—Descriptive statistics of male bluntnose minnow. TL = total length, TW = total weight, HW = widest head width, ID = interocular distance, TC = tubercle count, TS = tubercle score, GW = gonad weight, and GSI = gonadosomatic index.

Site		TL	TW	HW	ID	TC	TS	GW	GSI
RKM-496.5	Mean	67.12	3.12	7.29	5.56	1.35	3.47	0.022	0.004
	SE	1.72	0.30	0.26	0.26	0.76	1.96	0.008	0.001
	Min	50.00	1.06	5	3	0	0	0.001	0.001
	Max	93.00	8.14	11	9	16	43	0.118	0.017
	N	34	34	34	34	34	34	21	21
RKM-504.4	Mean	74.00	4.77	8.z56	6.67	5.78	11.72	0.053	0.008
	SE	2.84	0.55	0.44	0.43	1.68	4.15	0.010	0.001
	Min	59.00	1.95	6	4	0	0	0.001	0.000
	Max	92.00	8.49	11	9	16	44	0.109	0.014
	N	18	18	18	18	18	18	12	12
RKM-506.6	Mean	83.70	6.43	8.77	6.83	11.13	26.57	0.075	0.010
	SE	1.68	0.34	0.21	0.20	1.32	3.65	0.008	0.001
	Min	65.00	2.20	6	5	0	0	0.007	0.002
	Max	95.00	9.25	10	9	18	47	0.142	0.016
	N	30	30	30	30	30	30	26	26
RKM-509.0	Mean	62.03	2.84	6.89	5.14	1.27	2.57	0.020	0.004
	SE	1.76	0.27	0.22	0.22	0.66	1.46	0.007	0.001
	Min	45.00	1.02	5	3	0	0	0.001	0.000
	Max	86.00	7.41	10	8	17	46	0.099	0.015
	N	37	37	37	37	37	37	20	20
RKM-513.1	Mean	56.73	2.03	5.93	4.67	1.53	2.57	0.034	0.006
	SE	2.34	0.30	0.30	0.28	0.86	1.57	0.012	0.002
	Min	41.00	0.57	4	3	0	0	0.006	0.002
	Max	85.00	6.50	10	8	17	40	0.075	0.012
	N	30	30	30	30	30	30	6	6
CAB-8.1	Mean	80.10	6.16	9.55	7.70	8.15	21.15	0.071	0.009
	SE	2.27	0.53	0.39	0.39	1.88	4.97	0.011	0.001
	Min	57.00	1.60	6	4	0	0	0.001	0.000
	Max	93.00	9.09	12	10	18	50	0.132	0.016
	N	20	20	20	20	20	20	16	16
All Sites	Mean	69.37	3.99	7.63	5.91	4.38	10.18	0.047	0.007
	SE	1.11	0.19	0.15	0.14	0.54	1.35	0.004	0.001
	Min	41.00	0.57	4	3	0	0	0.001	0.000
	Max	95.00	9.25	12	10	18	50	0.142	0.017
	N	169	169	169	169	169	169	101	101

greater TW ($t = -6.64$, df= 344, $P < 0.001$), wider HW ($t = -7.22$, df= 344, $P < 0.001$) and wider ID ($t = -7.45$, df= 344, $P < 0.001$) than females (Table 2 & Table 3).

Sites Compared to CAB-0.8

Male.—Five out of the six candidate models describing male morphological measurements and secondary sexual characteristics obtained a better fit with the log-normal error distribution for all sample sites (Table 4). The change in AIC values indicated the log-normal error model for TW, HW, and ID provided a much better fit than the normal and gamma error distributions. Similarly, the change in AIC values indicate the log-normal error distribution is preferred over the normal, Poisson, and negative binomial error distributions when describing male TC and TS. Change in AIC values also indicated the gamma error distribution was the preferred model to describe GW while the normal error distribution was the preferred model to describe GSI.

The intercept and TL were a significant effect in all models describing male morphological measurements and secondary sexual characteristics (Table 5). Compared to CAB-0.8, bluntnose minnows exhibited significantly lower TW

Table 3.—Descriptive statistics of female bluntnose minnow. TL = total length, TW = total weight, HW = widest head width, ID = interocular distance, TC = tubercle count, TS = tubercle score, GW = gonad weight, and GSI = gonadosomatic index.

Site		TL	TW	HW	ID	EC	GW	GSI
WR-496.5	Mean	65.09	2.78	7.05	5.00	943.81	0.356	0.118
	SE	1.36	0.18	0.20	0.20	62.16	0.043	0.011
	Min	52.00	1.26	6	4	441	0.015	0.012
	Max	76.00	4.49	9	7	1536	0.809	0.214
	N	22	22	22	22	21	22	22
WR-504.4	Mean	64.28	2.76	6.72	4.92	975.61	0.292	0.100
	SE	1.16	0.14	0.16	0.14	44.23	0.024	0.006
	Min	49.00	0.98	5	4	628	0.003	0.003
	Max	76.00	4.18	8	6	1794	0.616	0.195
	N	39	39	39	39	38	39	39
WR-506.6	Mean	63.58	2.93	6.18	4.82	1243.70	0.315	0.110
	SE	0.84	0.15	0.13	0.08	216.67	0.024	0.008
	Min	55.00	1.57	5	4	493	0.085	0.036
	Max	73.00	5.31	7	6	7780	0.598	0.226
	N	33	33	33	33	33	33	33
WR-509.0	Mean	57.66	2.19	6.10	4.59	717.22	0.232	0.096
	SE	1.33	0.14	0.15	0.13	58.06	0.026	0.009
	Min	45.00	1.10	5	3	108	0.018	0.016
	Max	70.00	4.27	8	6	1429	0.535	0.174
	N	29	29	29	29	27	27	27
WR-513.1	Mean	59.74	2.23	5.87	4.42	760.42	0.242	0.094
	SE	1.64	0.17	0.22	0.18	46.63	0.033	0.010
	Min	43.00	0.68	4	3	323	0.005	0.007
	Max	80.00	4.65	9	7	1293	0.644	0.202
	N	31	31	31	31	26	31	31
CAB-8.1	Mean	65.39	3.10	6.96	5.17	1238.14	0.471	0.152
	SE	1.27	0.19	0.23	0.20	109.73	0.041	0.013
	Min	54.00	1.40	5	4	496	0.068	0.049
	Max	79.00	5.26	9	7	2297	0.807	0.305
	N	23	23	23	23	22	22	22
All Sites	Mean	62.51	2.65	6.44	4.80	983.89	0.309	0.109
	SE	0.56	0.07	0.08	0.06	50.32	0.014	0.004
	Min	43.00	0.68	4	3	108	0.003	0.003
	Max	80.00	5.31	9	7	7780	0.809	0.305
	N	177	177	177	177	167	174	174

Table 4.—Change in Akaike's Information Criteria (AIC) comparing the generalized linear model; Y = Total Length + Site, for male bluntnose minnows at all sites with different assumed error distributions.

	Distribution				
Y	Normal	Log-Normal	Gamma	Poisson	Negative binomial
TW	811.48	0.00	902.84		
HW	964.98	0.00	989.54		
ID	867.01	0.00	896.38		
GW	13.89	551.26	0.00		
GSI	0.00	933.21	8.67		
TC	866.52	0.00		503.73	363.61
TS	1119.30	0.00		866.82	363.40

Table 5.—Coeficients of the generalized linear model; Y = Total Length + Site, for male bluntnose minnows at all sites with the error distribution determined from Akaike's Information Criteria. Standard error is in parenthesis and significant coefficients are indicated with an asterisk.

			Independent variables					
Y	Distribution	Intercept	TL	WR-496.5	WR-504.4	WR-506.6	WR-509.0	WR-513.1
TW	log-normal	−0.885*	0.020*	−0.470*	−0.001	−0.358*	−0.015	−0.073*
		(0.036)	(4.22E-04)	(0.017)	(0.019)	(0.017)	(0.018)	(0.019)
HW	log-normal	0.373*	0.007*	−0.021*	−0.005	−0.061*	−0.007	−0.038*
		(0.019)	(2.22E-04)	(0.009)	(0.010)	(0.009)	(0.009)	(0.010)
ID	log-normal	0.116*	0.009*	−0.022	−0.010	−0.079*	−0.006	−0.004
		(0.027)	(3.14E-04)	(0.013)	(0.014)	(0.013)	(0.013)	(0.015)
GW	normal	−0.252*	0.004*	−0.005	−0.008	−0.005	−0.009	−0.010
		(0.025)	(2.85E-04)	(0.008)	(0.008)	(0.007)	(0.008)	(0.010)
GW	gamma	235.464*	−2.505*	7.824	0.019	4.429*	−0.557	−6.641
		(23.926)	(0.260)	(5.818)	(3.242)	(2.024)	(7.489)	(8.482)
GSI	normal	−2.81E-02*	4.31E-04*	1.39E-04	1.96E-05	6.42E-05	1.57E-03	2.44E-04
		(3.42E-03)	(3.97E-05)	(1.09E-03)	(1.46E-03)	(9.54E-04)	(1.18E-03)	(1.46E-03)
GSI	gamma	1145.303*	−11.918*	23.143	−2.353	16.046	−7.431	−39.294
		(116.270)	(1.293)	(28.043)	(22.126)	(16.840)	(35.298)	(39.547)
TC	log-normal	−1.835*	0.031*	−0.114	0.069	0.151	0.050	0.218*
		(0.197)	(0.002)	(0.094)	(0.104)	(0.092)	(0.098)	(0.106)
TS	log-normal	−2.306*	0.039*	−0.168	0.014	0.160	0.031	0.234
		(0.252)	(0.003)	(0.120)	(0.133)	(0.118)	(0.124)	(0.136)

Table 6.—Change in Akaike's Information Criteria (AIC) comparing the generalized linear model; Y = Total Length + Site, for female bluntnose minnows at all sites with different assumed error distributions.

	Distribution				
Y	Normal	Log-Normal	Gamma	Poisson	Negative binomial
TW	681.03	0.00	711.09		
HW	956.97	0.00	949.88		
ID	848.27	0.00	839.20		
GW	0.00	263.53	80.59		
GSI	0.00	579.67	40.12		
EC	2740.36	0.00		36370.68	2569.52

and smaller HW at WR-496.5, WR-506.6, and WR-513.1. The coefficient for the other White River sites were negative but were not significant. Fish from WR-506.6 also exhibited significantly smaller ID and GW when modeling with a log-normal and gamma error distribution while no differences were found in GW with the normal error distribution. There were also no significant differences in GSI with the normal or gamma error distribution. Finally, TC was significantly higher at WR-513.1.

Female.—Four out of the six candidate models describing female morphological measurements obtained a better fit with the log-normal error distribution for all sample sites (Table 6). Similar to males the change in AIC values indicated the log-normal error model for TW, HW, and ID provided a better fit than the normal and gamma error distributions. Similarly, the change in AIC values indicate the log-normal error distribution is preferred over the normal, Poisson, and negative binomial error distributions when describing female EC. Change in AIC values also indicated the normal error distribution was the preferred model to describe female GW and GSI.

Total length was a significant effect in all models while the intercept was significant in all models except GSI (Table 7). Female bluntnose minnows weighed significantly less at WR-496.5, WR-504.4, and WR-513.1 when compared to CAB-0.8. Similarly, HW was significantly less at WR-506.6 and WR-513.1. Interocular distance was not significantly different between the White River sites and CAB-0.8. Gonad weight and GSI was significantly less at all White River sites. Finally, EC was significantly less at WR-496.5, WR 509.0, and WR-513.1 when compared to Cabin Creek.

Sites Compared to WR-513.1

Male.—When modeling only White River sites, five out of the seven candidate models describing male morphological measurements and secondary sexual characteristics obtained a better fit with the log-normal error distribution (Table 8). The change in AIC values indicated the log-normal error model for TW, HW, and ID provided a much better fit than the normal and gamma error distributions. Similarly, the change in AIC values indicate the log-normal error distribution is preferred over the normal, Poisson, and negative binomial error distributions when describing male TC and TS. Change in AIC values also indicated the gamma error distribution was the preferred model to describe GW and GSI.

The intercept and TL were a significant effect in all models describing male morphological measurements and secondary sexual characteristics in White River (Table 9). Compared to WR-513.1 bluntnose minnows exhibited significantly higher TW at WR-504.4 and WR-509.0. Male bluntnose minnows also had a longer HW at WR-496.5, WR-504.4, and WR-509.0 while the HW was significantly shorter at WR-506.6. The ID of male bluntnose minnow was also significantly shorter at WR-506.6. Tubercle count and TS was significantly smaller at the most downstream site, WR-496.5, and WR-509.0. For any given length the average tubercle count at WR-496.5 was 31.3% (model coefficient * 100) less than WR-513.1. Similarly, the average TS for any given length was 37.5% less at WR-496.5 compared to WR-513.1.

Table 7.—Coeficients of the generalized linear model; Y = Total Length + Site, for female bluntnose minnows at all sites with the error distribution determined from Akaike's Information Criteria. Standard error is in parenthesis and significant coefficients are indicated with an asterisk.

			Independent variables					
Y	Distribution	Intercept	TL	WR-496.5	WR-504.4	WR-506.6	WR-509.0	WR-513.1
TW	log-normal	−0.893*	0.021*	−0.044*	−0.034*	0.012	0.003	−0.053*
		(0.044)	(0.001)	(0.017)	(0.015)	(0.016)	(0.017)	(0.017)
HW	log-normal	0.331*	0.008*	0.001	−0.001	−0.035*	0.005	−0.033*
		(0.029)	(4.29E-04)	(0.012)	(0.010)	(0.011)	(0.011)	(0.011)
ID	log-normal	0.152*	0.008*	−0.013	−0.012	−0.011	0.015	−0.024
		(0.034)	(0.001)	(0.014)	(0.012)	(0.013)	(0.013)	(0.013)
GW	normal	−0.591*	0.016*	−0.109*	−0.159*	−0.125*	−0.125*	−0.135*
		(0.092)	(0.001)	(0.037)	(0.032)	(0.033)	(0.036)	(0.035)
GSI	normal	−0.021	0.003*	−0.032*	−0.049*	−0.037*	−0.037*	−0.043*
		(0.034)	(4.93E-04)	(0.013)	(0.012)	(0.012)	(0.013)	(0.013)
EC	log-normal	2.419*	0.001*	−0.107*	−0.077	−0.028	−0.189*	−0.170*
		(0.144)	(0.002)	(0.052)	(0.046)	(0.047)	(0.051)	(0.050)

Female.—The same error distributions were selected for only White River sites as with all sites compared to CAB-0.8. The log-normal error distribution was the best model to describe TW, HW, ID, and EC while the normal error distribution was the preferred model to describe GW and GSI (Table 10).

The intercept and TL were a significant effect in all models describing female morphological measurements and secondary sexual characteristics (Table 11). Female TW was significantly greater at WR-506.6 and WR-509.0 compared to WR-513.1. Head width was also significantly greater at all sites except WR-506.6 compared to WR-513.1. Interocular distance was greater at WR-509.0 and EC was greater at WR-504.4 and WR-506.6 compared to WR-513.1. Gonad weight and GSI were not significantly different among sample sites.

DISCUSSION

The number of potential ECs and estrogen mimics that have been found in surface waters of the U.S. is quite extensive (Sonnenschein & Soto 1998). It is not only important to evaluate the presence of common ECs (i.e., 17β-estradiol) but the cumulative impact of all ECs and its mimics should also be evaluated. The synergistic effects of ECs have been successfully evaluated using a variety of biological endpoints (Folmar et al. 1996; Denslow et al. 1999; Miles-Richardson 1999; Harries et al. 2000; Jones et al. 2000; Bringolf & Summerfelt 2003). Many of these field and laboratory studies have used ubiquitous species such as common carp (*Cyprinus carpio*), fathead minnow, rainbow trout (*Oncorhynchus mykiss*), Japanese medaka (*Oryzias latipes*), and zebrafish (*Danio rerio*) (Tyler et al. 1996; Nilsen et al. 2004; Hassanin et al. 2002). While these species are ubiquitous they may not be found in enough numbers to facilitate field research projects or local monitoring programs, or may not be present in specific habitats of interest within those ranges. Therefore, an alternative was to choose a species that is closely related to the fathead minnow and locally abundant. Due to the closely linked phylogeny of fathead minnow and bluntnose minnow, it is assumed that many of the physical reactions known to occur with the fathead minnow would also occur in the bluntnose minnow.

This study documented differences in morphological measurements and secondary sexual

Table 8.—Change in Akaike's Information Criteria (AIC) comparing the generalized linear model; Y = Total Length + Site, for male bluntnose minnows at White River sites only with different assumed error distributions.

	Distribution				
Y	Normal	Log-Normal	Gamma	Poisson	Negative binomial
TW	685.78	0.00	770.30		
HW	830.01	0.00	864.14		
ID	746.37	0.00	779.37		
GW	24.20	475.28	0.00		
GSI	2.11	781.90	0.00		
TC	760.33	0.00		394.60	300.13
TS	985.51	0.00		607.39	288.69

characteristics of bluntnose minnow that are known to be affected by exposure to ECs in other species. While this study could not identify the exact cause of the differences found it does suggest a more extensive study to identify the cause is warranted.

This study evaluated a gradient along White River as well as compared the findings of White River to that of a site on Cabin Creek, a stream which is thought to be minimally affected by ECs. Although, since this study did not evaluate water samples for the presence of any known ECs or estrogen mimics it is difficult to be certain that the Cabin Creek site was in fact not impacted by ECs. For the purposes of this study Cabin Creek is used as a reference to compare the findings of White River to a smaller tributary.

The upstream-downstream gradient on White River reflects sites that are impacted by two distinct sources. The most upstream site is impacted by agricultural activities including 25 CAFOs (IDEM 2009). The remaining sites downstream are influenced by progressively increasing urbanization pressures in addition to the upstream pressures. For example, the three sites within City limits are each located downstream of a CSO. Finally the most downstream site is subjected to the combined influences of all upstream sources as well as the effluent of the MWPCF.

The morphological and secondary sexual characteristic differences found in our study were similar to those found by others. For example, studies have reported reduced number and prominence of nuptial tubercles in male fathead minnows following exposure to 17β-estradiol and other natural steroid estrogens (Miles-Richardson et al. 1999; Harries et al. 2000; Brian et al. 2007). Miles-Richardson et al. (1999) found a reduction in the size of nuptial tubercles following exposure to 17β-estradiol for 14 days. Whereas Harries et al. (2000) documented a reduction in the number of nuptial tubercles after exposure to the highest concentrations of 4-nonylphenol (4-NP) during a three week exposure period. The chemical 4-NP has been shown to be weakly active as an endocrine mimic compared with 17β-estradiol (White et al. 1994). The present study found a similar response in the closely related bluntnose minnow. For example, the TC and TS at WH-496.5 was 31.3% and 37.5% smaller than that measured at WH-513.1. These two major findings, with the support of the literature, suggest that the cumulative impact of the MWPCF and CSOs may be leading to a substantial reduction in the secondary sexual characteristics of the bluntnose minnow through EC exposure.

While some of our findings agree with the published literature, we did document some discrepancy. Orlando et al. (2004) reported a smaller ID in both females and males from a site contaminated with ECs. This study only documented a significant difference between WR-506.6 and CAB-0.8, although it was a small difference. The remaining sites had a negative coefficient but not significant. Additionally, Orlando et al. (2004) reported reduced GW in male fathead minnow but no difference in female GW from a site contaminated with feedlot effluent when compared to a control site. Reduced male GSI and GW have also been

Table 9.—Coeficients of the generalized linear model; Y = Total Length + Site, for male bluntnose minnows at White River sites only with the error distribution determined from Akaike's Information Criteria. Standard error is in parenthesis and significant coefficients are indicated with an asterisk.

			Independent variables				
Y	Distribution	Intercept	TL	WR-496.5	WR-504.4	WR-506.6	WR-509.0
TW	log-normal	−0.955*	0.020*	0.026	0.072*	0.038	0.087*
		(0.028)	(4.62E-04)	(0.016)	(0.020)	(0.020)	(0.015)
HW	log-normal	0.335*	0.007*	0.018*	0.034*	−0.022*	0.032*
		(0.014)	(2.33E-04)	(0.008)	(0.010)	(0.010)	(0.008)
ID	log-normal	0.118*	0.009*	−0.016	−0.004	−0.073*	−0.001
		(0.021)	(3.42E-04)	(0.012)	(0.015)	(0.015)	(0.011)
GW	normal	−0.244*	0.004*	0.003	0.003	0.007	0.017
		(0.023)	(2.81E-04)	(0.009)	(0.010)	(0.009)	(0.010)
GW	gamma	247.688*	−2.736*	15.773	8.835	13.892	5.759
		(25.871)	(0.302)	(9.514)	(8.671)	(8.706)	(10.144)
GSI	normal	−2.59E-02*	4.17E-04*	−2.16E-04	−1.09E-04	6.92E-05	1.11E-03
		(3.32E-03)	(4.04E-05)	(1.34E-03)	(1.45E-03)	(1.36E-03)	(1.38E-03)
GSI	gamma	1109.926*	−11.967*	62.647	37.310	55.840	31.753
		(118.754)	(1.397)	(43.204)	(40.911)	(39.476)	(46.251)
TC	log-normal	−1.513*	0.029*	−0.313*	−0.117	−0.018	−0.159*
		(0.138)	(0.002)	(0.077)	(0.096)	(0.097)	(0.073)
TS	log-normal	−1.925*	0.036*	−0.375*	−0.174	−0.003	−0.189*
		(0.174)	(0.003)	(0.097)	(0.121)	(0.122)	(0.092)

Table 10.—Change in Akaike's Information Criteria (AIC) comparing the generalized linear model; Y = Total Length + Site, for female bluntnose minnows at White River sites only with different assumed error distributions.

	Distribution				
Y	Normal	Log-Normal	Gamma	Poisson	Negative binomial
TW	588.70	0.00	603.20		
HW	826.41	0.00	822.15		
ID	731.14	0.00	726.61		
GW	0.00	255.56	67.91		
GSI	0.00	534.32	46.42		
EC	2384.91	0.00		32244.36	2221.04

reported in common carp collected at a site with high concentrations of 4-NP, bisphenol A and 17β-estradiol (Hassanin et al. 2002). Our study did not detect a large difference in male GSI or GW while a significant difference was found between all White River sites and CAB-0.8 for female GSI and GW. Although a reduction in female GW and GSI would suggest increased testosterone rather than increased ECs. It should also be noted that Orlando et al. (2004) analyzed their dataset with only the log-normal error distribution where our analysis suggested the preferred model assumes either a gamma or normal error distribution for GW and GSI. Additionally, this study assumes a similar response between bluntnose minnows and fathead minnows. Furthermore, this study found a reduction of 10–15% in GW and 3–5% in GSI when comparing White River sites to CAB-0.8.

Field studies, such as the present one, are not as common as laboratory studies. It is assumed that this is due to ECs not being regulated by the U.S. EPA. Regardless, the literature on the environmental impact, namely fish morphology, and the findings presented here are providing more evidence of a need to screen for these chemicals. Known outcomes from exposure to ECs range from morphological changes in tubercles to more extreme differences in sexual dimorphism such as presence of oocytes within male testis (Jobling et al. 1996; Andersen et al. 2003; Palace et al. 2006; Barry 2009). Additionally, the near extinction of fathead minnows from an experimental lake in Ontario was documented after exposure to ECs (Kidd et al. 2007).

Table 11.—Coeficients of the generalized linear model; Y = Total Length + Site, for female bluntnose minnows at White River sites only with the error distribution determined from Akaike's Information Criteria. Standard error is in parenthesis and significant coefficients are indicated with an asterisk.

			Independent variables				
Y	Distribution	Intercept	TL	WR-496.5	WR-504.4	WR-506.6	WR-509.0
TW	log-normal	−0.952*	0.021*	0.009	0.019	0.065*	0.057*
		(0.043)	(0.001)	(0.017)	(0.015)	(0.015)	(0.016)
HW	log-normal	0.295*	0.008*	0.043*	0.027*	−0.002	0.038*
		(0.026)	(4.27E-04)	(0.011)	(0.009)	(0.009)	(0.010)
ID	log-normal	0.133*	0.008*	0.012	0.013	0.014	0.039*
		(0.032)	(0.001)	(0.013)	(0.011)	(0.012)	(0.012)
GW	normal	−0.755*	0.017*	0.024	−0.026	0.008	0.011
		(0.080)	(0.001)	(0.032)	(0.027)	(0.028)	(0.029)
GSI	normal	−9.35E-02*	3.14E-03*	7.57E-03	−8.14E-03	3.78E-03	5.84E-03
		(2.90E-02)	(4.71E-04)	(1.15E-02)	(9.88E-03)	(1.02E-02)	(1.06E-02)
EC	log-normal	2.243*	0.001*	0.063	0.093*	0.142*	−0.019
		(0.147)	(0.002)	(0.051)	(0.044)	(0.045)	(0.048)

Definitive conclusions on the cause of observed differences in this study cannot be determined. Nor is it certain that the patterns observed here are linked to exposure to ECs. A cause and effect conclusion and confirmatory analysis can be determined by blood analysis for vitellogenin (VTG) from male fish with simultaneous evaluation of surface water concentrations of common ECs. Since the mid-1990s VTG production has been a commonly used biomarker of ECs (Sumpter & Jobling 1995; Tyler et al. 1996; Panter et al. 2002; Maltais & Roy 2007). Vitellogenin is part of the hypothalamic/pituitary/gonadal axis and serves as a precursor to egg yolk proteins of oviparous vertebrates (Wallace 1985). Under normal conditions males do not produce VTG; however, they do possess the gene capable of producing VTG (Chen 1983). Laboratory and field studies have documented VTG production in males by a variety of fish species when exposed to environmentally relevant concentrations of ECs (Folmar et al. 1996; Angus et al. 2002; Seki et al. 2006; Maltais & Roy 2007).

In conclusion, I documented evidence that suggests White River is contaminated with ECs. Next, to determine the source and to confirm these findings, a more thorough study should be conducted to include the analysis of male bluntnose minnow blood plasma for VTG expression, gonad histology, and chemical analysis of common ECs found in surface waters.

ACKNOWLEDGMENTS

I thank the Bureau of Water Quality for providing funds for this research. I also thank Drew Holloway, Luke Etchison, and Cami Miller with their assistance in data collection. I am grateful to Rick Conrad and two anonymous reviewers for assistance in the preparation of this manuscript.

LITERATURE CITED

Andersen, L., H. Holbech, A. Gessbo, L. Norrgren & G.I. Petersen. 2003. Effects of exposure to 17α-ethinylestradiol during early development on sexual differentiation and induction of vitellogenin in zebrafish (*Danio rerio*). Comparative Biochemistry and Physiology Part C: Toxicology and Pharmacology 134(3):365–374.

Angus, R.A., S.A. Weaver, J.M. Grizzle & R.D. Watson. 2002. Reproductive characteristics of a male mosquitofish (*Gambusia affinis*) inhabiting a small southeastern U.S. river receiving treated domestic sewage effluent. Environmental Toxicology and Chemistry 21(7):1404–1409.

Barry, C. 2009. A reconnaissance of the intersex condition of *Micropterus dolomieu* of the upper Ohio River basin as an indicator of anthropogenic endocrine disruptors. Master's thesis. Marshall University, Huntington, West Virginia.

Blazer, V.S., L.R. Iwanowicz, D.D. Iwanowicz, D.R. Smith, J.A. Young, J.D. Hedrick, S.W. Foster & S.J. Reeser. 2007. Intersex (testicular oocytes) in smallmouth bass from the Potomac River and selected nearby drainages. Journal of Aquatic Animal Health 19:242–253.

Brian, J.V., C.A. Harris, M. Scholze, A. Kortenkamp, P. Booy, M. Lamoree, G. Pojana, N. Jonkers, A. Marcomini & J.P. Sumpter. 2007. Evidence of estrogenic mixture effects on the reproductive performance of fish. Environmental Science and Technology 41:337–344.

Bringolf, R.B. & R.C. Summerfelt. 2003. Reduction of estrogenic activity of municipal wastewater by aerated lagoon treatment facilities. Environmental Toxicology and Chemistry 22(1):77–83.

Chen, T.T. 1983. Identification and characterization of estrogen responsive gene products in the liver of the rainbow trout. Canadian Journal of Biochemical Cell Biology 61:802–810.

Denslow, N.D., M.C. Chow, K.J. Kroll & L. Green. 1999. Vitellogenin as a biomarker of exposure for estrogen or estrogen mimics. Ecotoxicology 8:385–398.

Dick, E.J. 2004. Beyond 'lognormal versus gamma': discrimination among error distributions for generalized linear models. Fisheries Research 70:351–366.

Folmar, L.C., N.D. Denslow, V. Rao, M. Chow, D.A. Crain, J. Enblom, J. Marcino & L.J. Guillette, Jr. 1996. Vitellogenin induction and reduced serum testosterone concentrations in feral male carp (*Cyprinus carpio*) captured near a major metropolitan sewage treatment plant. Environmental Health Perspectives 104(10):1096–1101.

Harries, J.E., T. Runnalls, E. Hill, C.A. Harris, S. Maddix, J.P. Sumpter & C.R. Tyler. 2000. Development of a reproductive performance test for endocrine disrupting chemicals using pair-breeding fathead minnows (*Pimephales promelas*). Environmental Science and Technology 34:3003–3011.

Hassanin, A., S. Kuwahara, Nurhidayat, Y. Tsukamoto, K. Ogawa, K. Hiramatsu & F. Sasaki. 2002. Gonadosomatic index and testis morphology of common carp (*Cyprinus carpio*) in rivers contaminated with estrogenic chemicals. Journal of Veterinary Medical Science 64(10):921–926.

IDEM (Indiana Department of Environmental Management). 2009. CONFINED_FEEDING_OPERATIONS_IDEM_IN: Confined Feeding Operations in Indiana (Indiana Department of Environmental Management, Point Shapefile). Available from: http://inmap.indiana.edu/dload_page/environment.html.

Jobling, S., M. Nolan, C.R. Tyler, G. Brighty & J.P. Sumpter. 1998. Widespread sexual disruption in wild fish. Environmental Science and Technology 32:2498–2506.

Jobling, S., D. Sheahan, J.A. Osborne, P. Matthiessen & J.P. Sumpter. 1996. Inhibition of testicular growth in rainbow trout (*Oncorhynchus mykiss*) exposed to estrogenic alkylphenolic chemicals. Environmental Toxicology and Chemistry 15(2):194–202.

Jones, P.D., W.M. De Coen, L. Tremblay & J.P. Giesy. 2000. Vitellogenin as a biomarker for environmental estrogens. Water Science and Technology 42:1–14.

Kidd, K.A., P.J. Blanchfield, K.H. Mills, V.P. Palace, R.E. Evans, J.M. Lazorchak & R.W. Flick. 2007. Collapse of a fish population after exposure to a synthetic estrogen. Proceedings of the National Academy of Sciences of the United States 104(21):8897–8901.

Lindsey, J.K. & B. Jones. 1998. Choosing among generalized linear models applied to medical data. Statistics in Medicine 17:59–68.

Maltais, D. & R.L. Roy. 2007. A lateral flow immunoassay for rapid evaluation of vitellogenin levels in plasma and surface mucus of the copper redhorse (*Moxostoma hubbsi*). Environmental Toxicology and Chemistry 26(8):1672–1676.

Martinovic, D., W.T. Hogarth, R.E. Jones & P.W. Sorensen. 2007. Environmental estrogens suppress hormones, behavior, and reproductive fitness in male fathead minnows. Environmental Toxicology and Chemistry 26(2):274–278.

McDaniel, T.V., P.A. Martin, J. Struger, J. Sherry, C.H. Marvin, M.E. McMaster & G. Tetreault. 2008. Potential endocrine disruption of sexual development in free ranging male northern leopard frogs (*Rana pipiens*) and green frogs (*Rana clamitans*) from areas of intensive row crop agriculture. Aquatic Toxicology 88(4):230–242.

Miles-Richardson, S.R., V.J. Kramer, S.D. Fitzgerald, J.A. Yamini, S.J. Barbee & J.P. Giesy. 1999. Effects of waterborne 17β-estradiol on secondary sex characteristics and gonads of fathead minnows (*Pimephales promelas*). Aquatic Toxicology 47:129–145.

Nilsen, B., K. Berg, J. Eidem, S.I. Kristiansen, F. Brion, J.M. Porcher & A. Goksoyr. 2004. Development of quantitative vitellogenin-ELISAs for fish test species used in endocrine disruptor screening. Analytical and Bioanalytical Chemistry 378(3):621–633.

OECD (Organization for Economic Co-Operation and Development). 2000. Report of the OECD expert consultation on testing in fish – EDF2. Tokyo, March 2000. OECD, rue Andre Pascal, Paris.

Orlando, E.F., A.S. Kolok, G.A. Binzcik, J.L. Gates, M.K. Horton, C.S. Lambright, L. Earl Gray, Jr., A.M. Soto & L.J. Guillette, Jr. 2004. Endocrine-disrupting effects of cattle feedlot effluent on an aquatic sentinel species, the fathead minnow. Environmental Health Perspectives 112(3):353–358.

Palace, V.P., K.G. Wauthier, R.E. Evans, P.J. Blanchfield, K.H. Mills, S.M. Chalanchuk, D. Godard, M.E. McMaster, G.R. Tetreault, L.E. Peters, L. Vandenbyllaardt & K.A. Kidd. 2006. Biochemical and histopathological effects in pearl dace (*Margariscus margarita*) chronically exposed to a synthetic estrogen in a whole lake experiment. Environmental Toxicology and Chemistry 25(4): 1114–1125.

Panter, G.H., T.H. Hutchinson, R. Lange, C.M. Lye, J.P. Sumpter, M. Zerulla & C.R. Tyler. 2002. Utility of a juvenile fathead minnow screening assay for detecting (anti-) estrogenic substances. Environmental Toxicology and Chemistry 21(2):319–326.

Purdom, C.E., P.A. Hardiman, V.V.J. Bye, N.C. Eno, C.R. Tyler & J.P. Sumpter. 1994. Oestrogenic effects of effluents from sewage treatment works. Chemistry and Ecology 8(4):275–285.

R Development Core Team. 2009. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna Austria. ISBN 3-900051-07-0, http://www.R-project.org.

Ros, A.F., R. Bruintjes & R.S. Santos. 2004. The role of androgens in the trade-off between territorial and parental behavior in the Azorean rock-pool blenny, *Parablennius parvicornis*. Hormones and Behavior 46:491–497.

Seki, M., S. Fujishima, T. Nozaka, M. Maeda & K. Kobayashi. 2006. Comparison of response to 17β-Estradiol and 17β-Trenbolone among three small fish species. Environmental Toxicology and Chemistry 25(10):2742–2752.

Sonnenschein, C. & A.M. Soto. 1998. An updated review of environmental estrogen and androgen mimics and antagonists. Journal of Steroid Biochemistry and Molecular Biology 65(1):1–6.

Strange, R.J. 1996. Field examination of fishes. Pp. 433–446. *In* Fisheries Techniques (B.R. Murphy & D.W. Willis, eds.). American Fisheries Society, Bethesda, Maryland.

Sumpter, J.P. & S. Jobling. 1995. Vitellogenesis as a biomarker for oestrogenic contamination of the aquatic environment. Environmental Health Perspective 103:173–178.

Tyler, C.R., B.V. Eerden, S. Jobling, G. Panter & J.P. Sumpter. 1996. Measurement of vitellogenin, a biomarker for exposure to oestrogenic chemicals, in a wide variety of cyprinid fish. Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology 166(7):418–426.

Tyler, C.R., S. Jobling & J.P. Sumpter. 1998. Endocrine disruption in wildlife: a critical review of the evidence. Critical Reviews in Toxicology 25:319–361.

U.S. EPA (Environmental Protection Agency). 1998. Research plan for endocrine disruptors. EPA/600/

R-98/042. Office of Research and Development, Washington, D.C.

U.S. EPA (Environmental Protection Agency). 2002. A short-term method for assessing the reproductive toxicity of endocrine-disrupting chemicals using the fathead minnow (*Pimephales promelas*). EPA/600/R-01/067. Duluth, Minnesota: Mid-Continent Ecology Division.

Wallace, R.A. 1985. Vitellogenesis and oocyte growth in nonmammalian vertebrates. Developmental Biology 1:127–177.

White, R., S. Jobling, S.A. Hoare, J.P. Sumpter & M.G. Parker. 1994. Environmentally persistent alkylphenolic compounds are estrogenic. Endocrinology 135:175–182.

Wilkison, D.H., D.J. Armstrong & D.W. Blevins. 2002. Effects of wastewater and combined sewer overflows on water quality in the Blue River Basin, Kansas City, Missouri and Kansas, July 1998–October 2000. U.S. Geological Survey Water-Resources Investigations Report 02–4107, 162 pp.

Manuscript received 4 October 2010, revised 10 January 2011.