SETAC PRESS

Environmental Toxicology and Chemistry, Vol. 22, No. 10, pp. 2416–2431, 2003

Printed in the USA
0730-7268/03 $12.00 + .00

EXAMINATION OF REPRODUCTIVE ENDPOINTS IN GOLDFISH (*CARASSIUS AURATUS*) EXPOSED IN SITU TO MUNICIPAL SEWAGE TREATMENT PLANT EFFLUENT DISCHARGES IN MICHIGAN, USA

JOHN P. GIESY,*† ERIN M. SNYDER,† KRISTA M. NICHOLS,‡ SHANE A. SNYDER,† SERGIO A. VILLALOBOS,† PAUL D. JONES,† and SCOTT D. FITZGERALD§

†Department of Zoology, National Food Safety and Toxicology Center, Institute of Environmental Toxicology,
‡Department of Fisheries and Wildlife and Institute of Environmental Toxicology,
§Department of Pathobiology and Diagnostic Investigation, Michigan State University, East Lansing, Michigan 48824-1222, USA

(*Received* 17 *July* 2002; *Accepted* 20 *February* 2003)

Abstract—Effects of representative mid-Michigan (USA) wastewater treatment plant (WWTP) effluents on the reproductive physiology of common goldfish (*Carassius auratus*) were assessed in situ by measuring plasma concentrations of vitellogenin (VTG), 17β-estradiol (E2), and testosterone (T), and evaluating gonad histology. Caged adult male and female goldfish were exposed for six weeks to WWTP effluents. One riverine site and one lacustrine site were included as references for comparison to WWTP sites. There was differential growth and gonadal development among locations, which confounded the interpretation of biomarker responses. A linear contrast model was developed by analysis of covariance, and adjusted values were developed for concentrations of VTG, E2, and T in the plasma of both male and female fish. In general, VTG concentrations were significantly less in male than in female goldfish. Most concentrations of VTG in male goldfish were less than the method detection limit. There were no significant differences in plasma VTG of either male or female goldfish among locations or between sites at WWTPs and reference sites. Concentrations of E2 in the plasma of female goldfish were similar among WWTP sites, all of which were less than in females at a pond reference location. Concentrations of E2 in the plasma of male goldfish were similar at all WWTP locations, except for one, where they were greater. No consistent trends in hormone concentrations or gonadal histology could be attributed to putative endocrine disrupter exposure in WWTP effluents. The results indicate that the risk for estrogen agonist exposure below these mid-Michigan WWTPs is small.

Keywords—Vitellogenin Hormones Gonads Endocrine disruption Sex steroids

INTRODUCTION

Effluents of wastewater treatment plants (WWTP) have been reported to have adverse effects on the reproductive condition of fish [1,2]. Some of these effects have been attributed to estrogen agonists in the effluents [3–6]. The condition of intersexing, or occurrence of ovo-testis, has been reported. Specifically, oocytes were found in the testes of wild male roach (*Rutilus rutilus*) collected downstream from WWTP effluent discharges in the United Kingdom [7] and exposed in cages [8]. Wastewater effluents and their associated estrogen agonists have been reported to cause induction of the egg-yolk protein precursor vitellogenin (VTG) in male fish [4,5]. Increases in concentrations of VTG in the plasma of male fish, an indicator of exposure to estrogen agonists, have been reported in fish captured downstream of or caged below WWTP effluents [9,10]. The observed effects are consistent with exposure to estrogen agonists, including the relatively weak estrogen agonist degradation products of alkyl phenol ethoxylates [9,10], the more potent natural endogenous estrogens 17β-estradiol (E2) and estrone (E1), as well as ethinylestradiol (EE2) [3].

The observed effects in male fish in the United Kingdom have led to the initiation of similar studies in North America [11]. In the United States, circulating concentrations of VTG; the steroid hormones E2, testosterone (T), and 11-ketotestosterone (11-KT); ratios of circulating estrogens to androgens; and gonad lesions have been evaluated to assess the functional responses of feral male and female carp to environmental endocrine-disrupting chemicals [12,13]. Results of studies on male common carp exposed to WWTP effluents in the United States have indicated elevated concentrations of serum VTG concomitant with significantly decreased concentrations of serum androgens [12,13]. However, a survey of carp in several U.S. streams did not detect significant increases in concentrations of plasma hormones or lesions of the gonads in male carp [14]. The objective of the current investigation was to determine whether WWTP effluents in mid-Michigan could alter the reproductive physiology of caged adult male and female goldfish (*Carassius auratus*).

MATERIALS AND METHODS

Study sites

Rivers of interest in the study were located in lower mid-Michigan (Fig. 1). One reference site (RS) was selected on the Looking Glass River near Eagle, Michigan. There are no WWTP outfalls upstream of the location, and the Looking Glass River is known to be relatively free of contamination but otherwise has characteristics similar to those of streams receiving effluent from WWTPs. A second reference site was the limnology pond site (LP) on the Michigan State University Campus, East Lansing, Michigan. This site is also relatively free of contamination, and the LP also was used as a reference for comparison to cage confinement and exposure conditions for riverine sites.

* To whom correspondence may be addressed (jgiesy@aol.com).

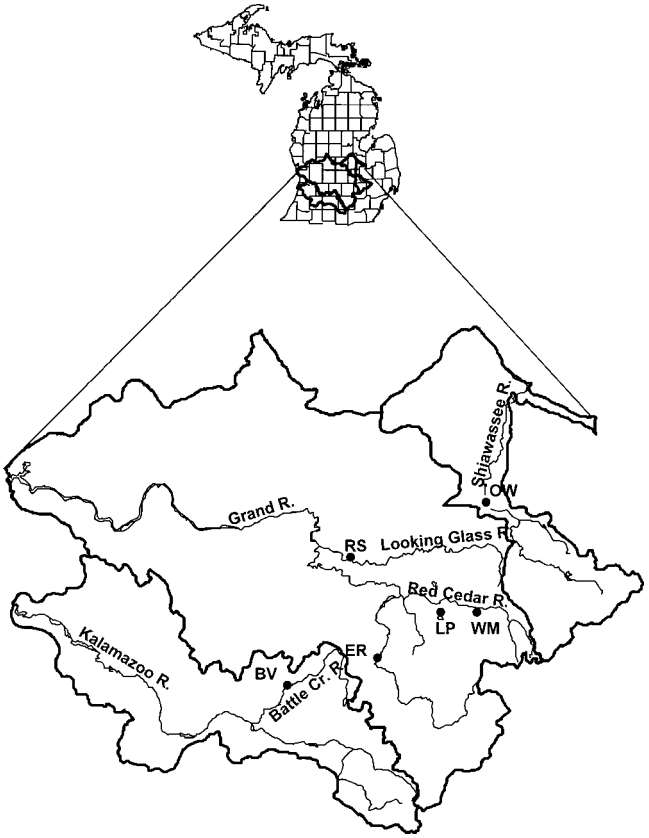

Fig. 1. Map of central, lower Michigan (USA) showing locations of study rivers and sites. Waste water treatment plants indicated are Bellevue (BV), Eaton Rapids (ER), Owosso (OW), and Williamston (WM). Reference sites are the Looking Glass River (RS) and the Michigan State University limnology pond (LP).

Four municipal WWTPs in three different watersheds were chosen based upon type and extent of treatment, volume treated per day, and types of wastewater influents received by the facilities (Fig. 1). These facilities included the Bellevue (BV), Eaton Rapids (ER), Owosso (OW), and Williamston (WM) municipal WWTPs. Average effluent discharges ranged from 174 to 15,330 m^3/d (Table 1). All WWTPs were using at least secondary treatment technologies, and one (OW) employed tertiary treatment such as sand or charcoal filters to remove additional biological oxygen demand (BOD) and phosphorus.

Fish exposure

Common goldfish were obtained from Osage Catfisheries (Osage Beach, MO, USA). Upon receipt, goldfish were placed directly into cages in a holding pond (LP). The fish were not individually weighed at the beginning of the study when they were placed into the cages. It was decided that the individual fish could not be marked, so a paired before and after analysis could not be conducted: it would be stressful and the weights could not be accurately measured on live fish in the field. To estimate the size of the fish at the start of the study, a subsample of 20 fish was collected at random before they were allocated to the study sites. Individuals measuring 10 to 12.5 cm in length had an average weight of 17.33 g, standard deviation (SD) = 1.995, and a range of 13.15 to 19.89 g.

At each site, goldfish were placed in a cage in the river at the point where the river received effluent discharged from the WWTP. Twenty goldfish (10 males, 10 females) undergoing gonadal recrudescence were placed in a single cage at each site, and replicate cages were deployed at the LP site. Fish were selected one at a time and assigned to one of the cages then a second fish was selected and added to the next cage. That is, all of the fish were not selected for one treatment then all of the fish for the next treatment. Fish were selected such that those near the average size were selected first, followed by successively larger and smaller fish. This was done in a way to minimize the bias of sizes of fish among locations. Sex was determined by examining the dorsal edge of the posterior surface of the pectoral fin for the presence of a row of nuptial tubercles, a feature present in males. Cages were assigned randomly to the various locations. The cages and fish were deployed from June 26 until July 1 and remained in place 42 to 43 d (six weeks) until August 8 to August 15, 1996. This period corresponds to the end of the spawning season or the postspawning period for goldfish in temperate regions.

Cages, 0.6·0.6·0.45 m, were constructed of plywood painted with black epoxy paint and black polyethylene mesh and were weathered by exposure to water before use. Cages were secured to metal stakes driven into the riverbed so that cages could not sway or turn in the flow of the river. They were fitted with polyethylene flow deflectors to protect the fish from strong currents. The deflectors also slowed passing food items for the fish to eat. Sites were checked weekly for water stage and water quality, including dissolved oxygen, temperature, and hardness. Effluent water quality data were collected from discharge monitoring reports required by the National Pollutant Discharge Elimination System (http://cfpub.epa.gov/npdes/), and permits were obtained from the Department of Environmental Quality in Michigan. Once per week fish were fed a 1:1 (v:v) mixture of commercial trout pellets (Murray Elevators, UT, USA) and TetraMin flake food (Tetra Sales, Blacksburg, VA, USA).

Fish dissection and sample collection

Following the six-week exposure period, fish were removed from the cages and euthanized with tricaine methanesulfonate (MS-222; Argent Chemical Laboratories, Redmond, VA,

Table 1. Characteristics of waste water treatment plant (WWTP) effluents at four study sites in mid-Michigan (USA) for evaluation of effluent effects on reproductive physiology of goldfish

		Daily flow (m^3)		
WWTP	River	Mean	Maximum	Treatment type
Bellevue	Battle Creek	174	458	Trickling filter
Eaton Rapids	Grand	3,110	8,020	Activated sludge
Owosso	Shiawassee	15,330	49,210	Trickling filter[a]
Williamston	Red Cedar	1,787	8,330	Activated sludge

[a] Indicates treatment beyond traditional secondary technology to further remove biological oxygen demand and phosphorus.

USA). Body weight and standard length were recorded for each fish. Blood was collected immediately from the caudal vasculature with a heparinized 1 cc syringe fitted with a 25 G × 5/8-inch needle. If blood could not be obtained with a syringe, the caudal peduncle was severed with a razor blade and blood was collected from the caudal vasculature with a heparinized hematocrit tube. Blood samples were placed on ice immediately after collection. Fish carcasses were placed into individual plastic sample bags and transported on ice to the laboratory.

The sex of each fish was confirmed by observation of gross gonad appearance upon visceral dissection. Gonads were removed and weighed to the nearest 0.0001 g. Four fish (two males, two females) per site were frozen immediately at −20°C for possible chemical analysis. From each fish, one gonad, one outermost gill arch, and a section of hepatopancreas were preserved in 10% neutral buffered formalin for histological examination.

Steroid extraction and analysis

Blood samples were stored overnight at 4°C to allow clotting, and then samples were centrifuged at 3,000 *g* for 10 min at 4°C to isolate plasma. Plasma was removed and frozen at −80°C until analysis. Both E2 and T were assayed in plasma by enzyme-linked immunosorbent assay (ELISA) after extraction into diethylether. Up to 50 μl of plasma remaining after VTG determination was transferred, diluted to 1.0 ml with nanopure water, and extracted twice with 5 ml of diethylether [15]. The combined extracts were concentrated to dryness under a stream of nitrogen and were reconstituted in 300 to 500 μl enzyme immunoassay (EIA) buffer (0.1 M phosphate-buffered saline, 0.1% Tween-20). Extraction efficiencies for E2 and T were 78% and 100%, respectively. Plasma samples were analyzed in triplicate for E2 and T using methods specified by the manufacturer of the EIA kits (Cayman Chemical, Ann Arbor, MI, USA). Both E2 (7.8–1,000 pg E2/ml) and T (3.9–500 pg T/ml) standard curves were assayed in duplicate on each plate. Dilution curves of extracted plasma samples were parallel to the standard curves as determined by an *F* test on mean squares [E2, $F(4, 34) = 1.75$, $p = 0.17$; T, $F(5, 33) = 1.15$, $p = 0.3634$). The interassay coefficient of variation for each immunoassay was calculated on the optical density readings at the approximate midpoint of the standard curves (62.5 pg T/ml or 62.5 pg E2/ml) on each plate. Interassay coefficients of variation (%) were 14.46 ($n = 5$) for E2 in plasma, 14.87 ($n = 7$) for plasma T, and 15.44 ($n = 10$) for T in gonadal incubation medium.

Plasma vitellogenin analysis

Vitellogenin was determined by use of a VTG-specific ELISA [15]. Polyclonal antibodies against purified goldfish VTG were raised in New Zealand white rabbits [15]. Concentrations of plasma VTG were determined by a competitive ELISA developed for goldfish and previously described [15]. Purified goldfish VTG standards (0.14–69.4 ng VTG/well) and samples were assayed in duplicate or triplicate on each plate. Intra- and interassay coefficients of variation were 10.3% ($n = 5$) and 25.6% ($n = 11$), respectively. The effective method detection limit (MDL) for the assay was 0.28 ng/ml. For statistical analysis, all values less than this detection limit were set to one-half MDL.

Gross morphology and histology

Tissues for histopathology were fixed in formalin solution for a minimum of 48 h, rinsed by agitating for 30 s in 30 ml of 70% ethanol, and were stored in 70% ethanol until tissue trimming. Samples were trimmed, processed, and embedded in paraffin (SurgiPath, Richmond, IL, USA). Tissues were sectioned at 5 μm with a microtome and stained with hematoxylin and eosin for examination using a light microscope.

Hepatocellular vacuolation was graded on a scale as follows: 0 = no vacuolation; 1 = mild vacuolation with small vacuoles spread throughout the cytoplasm; 2 = moderate vacuolation with larger coalescing vacuoles appearing as large clear zones in many hepatocytes; 3 = severe vacuolation where all or most of the cytoplasm has lost its normal pink coloration due to confluent, large, clear vacuoles.

Testicular lesions were evaluated according to the severity of relative or absolute Sertoli cell proliferation and the percentage of seminiferous tubules affected. Scores ranged from 0 to 4 as follows: 0 = no Sertoli cell proliferation; 1 = <25% Sertoli cell proliferation; 2 = 25 to 50% Sertoli cell proliferation; 3 = 50 to 75% Sertoli cell proliferation; and 4 = >75% Sertoli cell proliferation. Degenerative changes including germ cell syncytia, mineralization of spermatozoa, or necrotic spermatozoa were recorded.

In each female, a single ovary was evaluated by selecting a representative portion, counting 50 follicles, and calculating the percentage of each stage of follicular development observed within those 50 follicles. Follicles were staged according to the following criteria: (1) primary—large nucleus, abundant basophilic cytoplasm, and no yolk vesicles; (2) secondary—numerous eosinophilic yolk vesicles; (3) graafian—large follicle bordered by an amphiphilic egg membrane with a central core of eosinophilic yolk protein; and (4) atretic—degenerative follicle with shrunken, irregular border.

Data analyses

Responses of caged goldfish at different locations were investigated by both parametric and nonparametric statistical methods, depending on whether assumptions for parametric tests were met. Statistical differences in weight among locations at the end of the study were investigated by a one-way analysis of variance (ANOVA). Average weights at each location were compared with the initial estimate of the average weight of the fish used in the study by the general linear model procedure (PROC GLM; SAS Institute, Cary, NC, USA) followed by Dunnett's multiple range test. For parameters that did not meet the assumptions of the ANOVA, location effects were examined by the Kruskal–Wallis, nonparametric one-way ANOVA (SAS; PROC GLM conducted on the ranks of the responses). Condition factor (*K*) was calculated by use of the following relationship: $K = (W/L^3) \cdot 10{,}000$, where W = weight (g) and L = standard length (mm). The gonadosomatic index (GSI) was calculated as the weight of the gonads divided by the whole body weight and multiplied by 100 to express it as a percentage. Relationships between variables were investigated by use of Pearson product-moment correlation coefficients and Spearman rank correlation coefficients. Because there were intercorrelations between a number of parameters, especially with weight and length, relationships between variables were further investigated by the use of partial correlations. Specifically, correlations were calculated by controlling for the effects of length and weight and/or GSI. Differences in responses of males and females were investigated by use

Table 2. Ambient river and gross effluent water quality data from waste water treatment plant field sites June to August 1996 (mid-Michigan, USA). Effluent water data represent numbers from discharge monitoring reports required by National Pollutant Discharge Elimination System permits. All data are averages unless otherwise indicated[a]

	Ambient exposure water[b]					Effluent water[c]					
										BOD[g]	
Site	Depth (m)	Temp (°C)	DO (mg/L)	pH	Hardness (mg/L)	DO[d] (mg/L)	pH[e]	Amm[f] (mg/L)	Cl_2 (mg/L)	mg/ml	% Removal
BV	0.7	20.4	7.20	7.43	280.44	6.83	7.8	0.016	0[h]	8.3	92.7
ER	0.7	20.7	8.53	7.18	251.27	5.43	7.8		0.003	5	95.3
OW	0.4	20.6	8.17	7.57	235.78	8.23	8.38	0.4	0[h]	6.8	
WM	0.8	20.0	8.63	6.82	336.4	6.33	7.85	0.165	0[h]	6.8	
RS	0.8	20.7	6.40	6.80							
LP		23.6	7.35	7.88							

[a] DO = dissolved oxygen; Cl_2 = residual chlorine; BOD = biological oxygen demand; BV = Bellevue; ER = Eaton Rapids; OW = Owosso; WM = Williamston; RS = Looking Glass River; LP = limnology pond.
[b] Ambient water quality parameters measured on days of site check-ups (n = 1–4).
[c] Effluent water quality parameters are means of monthly averages derived from daily grab samples of gross treated effluent.
[d] Average of minimum monthly values June through August.
[e] Average of maximum monthly values.
[f] Amm. = total ammonia concentrations in gross effluent.
[g] A measure of 5-d carbonaceous BOD at 20°C.
[h] Chlorine was not detectable below the method detection limit of 0.0001 mg/L.

of Student's t test on untransformed or log-transformed data. Patterns of relative proportions of primary, secondary, and tertiary follicles were compared by profile analysis by use of the general linear model and multivariate analysis of variance (MANOVA) procedures in SAS (significance was determined by use of the Wilk's Λ criterion and the Hotelling–Lawley trace). Pair-wise comparisons of differences in patterns among locations were made by use of a one-way ANOVA (PROC GLM in SAS) on the difference between the proportions of primary and secondary follicles and the difference between proportions of secondary and tertiary follicles with the Tukey's multiple range test. The effects of covariation with parameters that represented growth (weight), condition (K), and gonadal development (GSI) were removed by calculating adjusted values for the parameters of interest by use of an analysis of covariance (ANCOVA; PROC GLM in SAS). The mean concentrations of VTG, T, E2, and E2/T ratio and in vitro T release were corrected for the effects of the size and developmental stage of fish at different locations. The covariates used in the model were weight (as a measure of growth), K (as a measure of sufficiency of food), and GSI (as a measure of gonadal development). Although each of these parameters were found to be correlated with the other two, an initial assessment of covariance demonstrated that each of these covariates significantly adjusted the means of the response parameters, so all three were used in the ANCOVA. Differences in means of adjusted parameters among locations were investigated by use of a one-way ANOVA (PROC GLM in SAS) followed by Tukey's multiple range test. Proportions of responding and nonresponding individuals were investigated by the chi-squared test (χ^2).

RESULTS

Study site water quality characteristics

In general, effluent and stream water quality parameters and temperatures were similar among sites throughout the study period (June–August 1996; Table 2). Effluent water quality values seem different, but in-stream water quality values illustrate that ambient exposure waters abated extreme values in dissolved oxygen and pH, particularly for ER with low values of dissolved oxygen. The total concentration of ammonia (0.4 mg/L) and pH values were relatively great at OW compared with other WWTPs. Water temperature was consistently greater at LP compared with the streams into which the fish were placed. As can be seen by the BOD values for all of the WWTP (Table 2), all WWTPs apparently were efficiently removing BOD, a general measure of wastewater treatment efficiency. For all WWTPs selected and reporting percentage removal, BOD was on average 92.7 to 95.3% removed by the treatment process. Eighty-five percent removal is the minimum accepted by the National Pollutant Discharge Elimination System permit requirements.

Survival and growth of fish

Survival and growth varied among locations. Statistically significant (ANOVA or Kruskal–Wallis; $p < 0.0001$), site-specific changes were observed in fish weight, condition factor (K), and GSI (Figs. 2–4; Appendix 1). The mean weights of fish at the end of the study were significantly greater at some locations, whereas fish at other locations lost weight. Weights were significantly greater (ANOVA followed by Dunnett's test, $p < 0.05$) at three locations (LP = 125%, WM = 65%, ER = 66%) but lost weight at three of the locations (OW = −7%, BV = −23%, RS = −41%). The weight losses at OW and BV were not significantly different from the initial weights (Dunnett's test, $p < 0.05$).

Fish survival per site at the end of the exposure period ranged from 65 to 100%, with an average survival of 87%. The pattern of growth among sites was similar for males and females. Fish growth, as determined by weight increase, was greatest at the ER, LP, and WM sites and was accompanied by increase in standard length and condition factor (Fig. 3; Appendix 1). The greatest growth was observed at the LP site, which is a pond, rather than a flowing water river site. Fish at the WM and ER sites also exhibited considerable growth. At all sites, the site-specific pattern of growth was accompanied by an increase (not statistically significant for females) in the GSI (Fig. 4), which indicates that increases in whole body weights were accompanied by sexual development. The RS reference site appeared to offer the least natural forage,

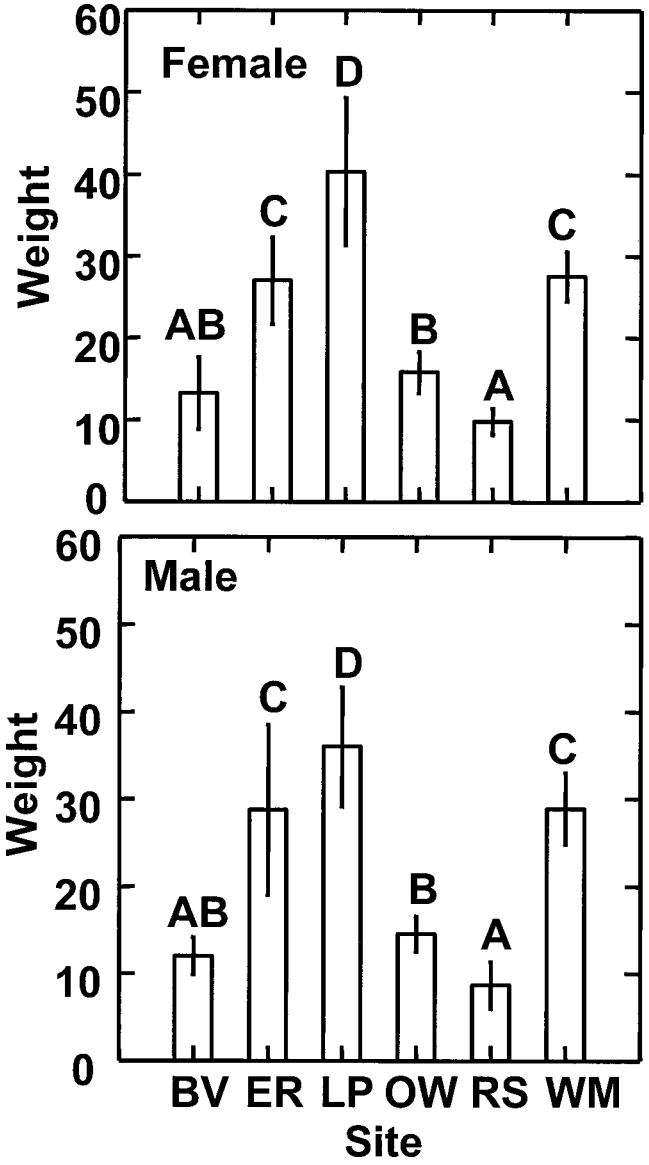

Fig. 2. Average weights for male and female fish at the reference locations and waste water treatment plant sites. The confidence intervals represent ±1.0 standard deviation of the mean. Means labeled with the same letter are not significantly different (Kruskal–Wallis, $p > 0.05$). See Figure 1 for site identifier information.

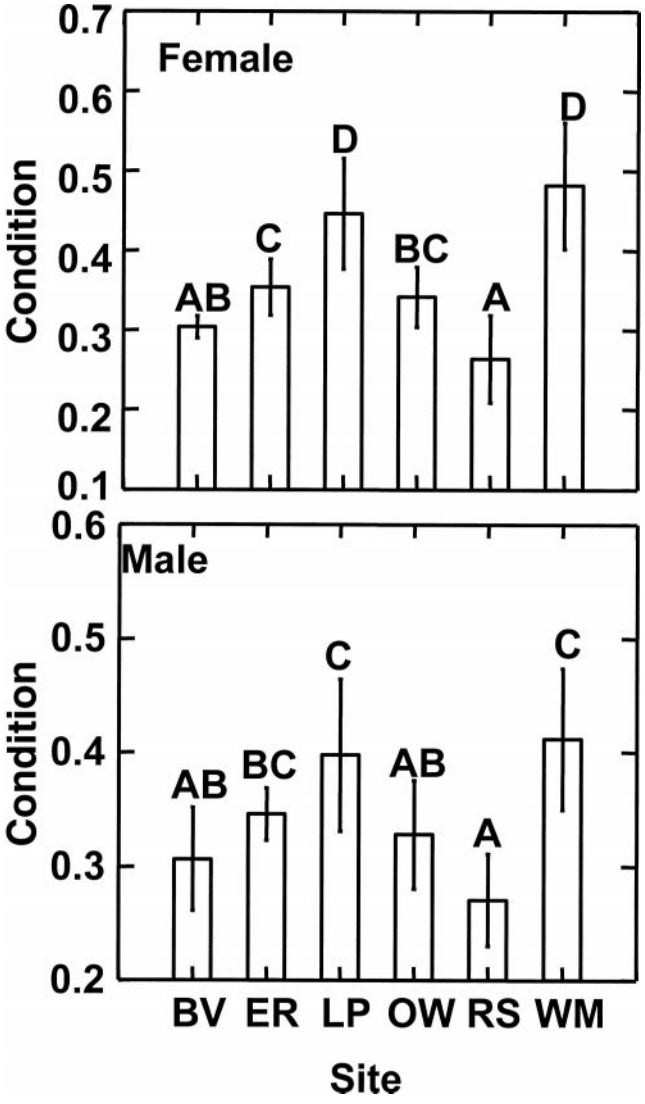

Fig. 3. Average condition factors of male and female fish at the reference locations and waste water treatment plant sites. The confidence intervals represent ±1.0 standard deviation of the mean. Means labeled with the same letter are not significantly different (Kruskal–Wallis, $p > 0.05$). See Figure 1 for site identifier information.

which might explain the lack of growth at that site. However, there was a significant amount of forage at the LP reference location.

Correlations among parameters

The basic morphological parameters of weight, length, and *K* were significantly correlated in both males and females (Table 3). Correlation coefficients were generally greater than 0.5 for these relationships, except for the relationship between standard length and condition factor. This is likely due to the differences in growth observed among sites. The morphological parameters also were correlated to plasma testosterone concentrations in both males and females, but only weight and *K* of females were correlated to plasma E2 concentrations. In both males and females the morphometric parameters also showed significant correlations with the E2/T ratio, but with coefficients of determination generally less than 0.5. In females, plasma E2 concentrations were significantly correlated with plasma T concentrations ($r = 0.719$; $p < 0.001$). However, this association was weaker in males ($r = 0.291$; $p < 0.05$). The ANCOVA significantly reduced the variation in concentrations of VTG, E2, and T among locations (Tables 4 and 5). After means were corrected for the effects of covariates, representing differences in growth and reproductive condition, there were fewer intercorrelations among parameters (Table 6).

Plasma VTG

Plasma VTG concentrations were significantly (ANOVA, $p < 0.001$) greater in female fish (range < MDL − 24.6 mg/ml, $\bar{X} = 3.66$, SD = 5.66 mg/ml) than in males at all sites (range < MDL − 0.963 mg/ml, $\bar{X} = 0.018$, SD = 0.123 mg/ml; Fig. 5; Appendix 1). At BV and ER, VTG was not present at concentrations greater than the MDL of 0.00056 mg/ml in any of the male fish tested. Vitellogenin was detected at relatively low frequencies in males from the LP (4 of 18) and RS (2 of 7) reference sites. At only two of the WTTP exposure

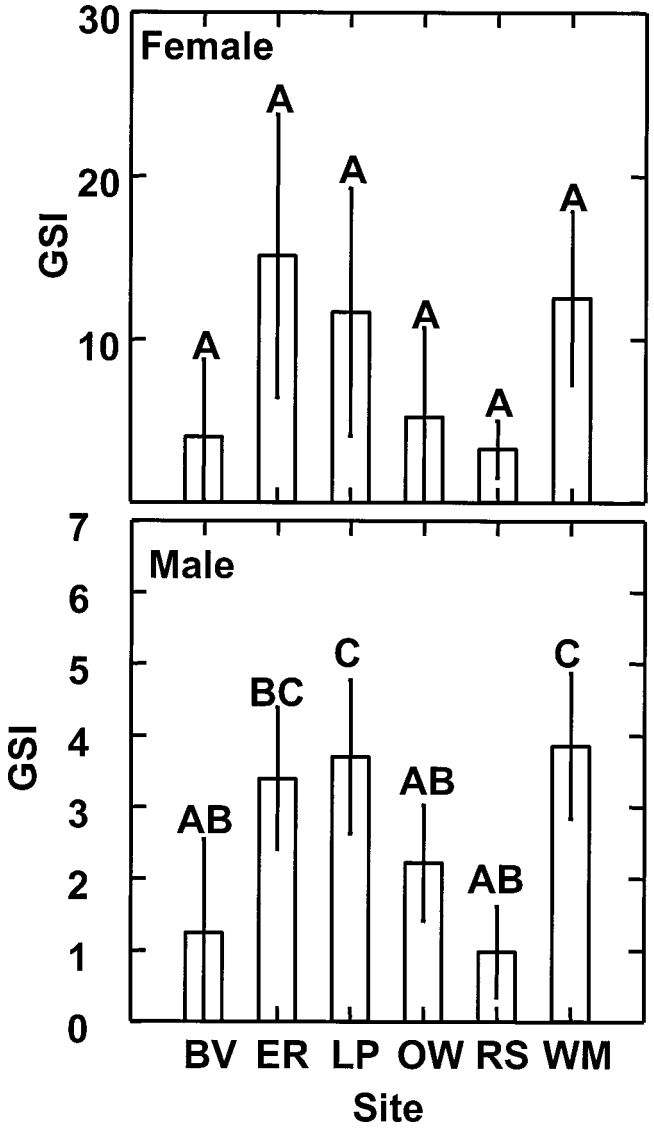

Fig. 4. Average gonadosomatic indices (GSI) of male and female fish at the reference locations and waste water treatment plant sites. The confidence intervals represent ±1.0 standard deviation of the mean. Means labeled with the same letter are not significantly different (Kruskal–Wallis, $p > 0.05$). See Figure 1 for site identifier information.

sites was VTG detected in 50% or more of males; those sites were OW and RS, where 4 of 8 fish and 4 of 7 fish, respectively, contained measurable concentrations of VTG. In contrast, VTG concentrations were greater than the MDL in 50 of 59 female fish caged at the various sites. The greater frequency of VTG detection in females is not surprising given the reproductive significance of VTG. For both sexes, there appeared to be a group of individuals in which VTG was not measurable. This was particularly evident in the female fish where the difference in plasma VTG concentrations between producers and nonproducers of VTG was greater than an order of magnitude. The proportion of females that did not produce VTG was significantly greater (χ^2 test; $p < 0.001$) at the ER and LP sites (3 of 6 and 3 of 22, respectively), with only one nonproducing individual at each of the BV, RS, and WM sites.

Concentrations of VTG in plasma were correlated with *K* and GSI as well as E2 and T concentrations in females. Plasma VTG concentrations were not significantly related to any of the measured parameters in males. The relatively low incidence of VTG production in males at all sites would account for this lack of a relationship. When the mean concentration of VTG was corrected for the effects of the covariates, there were no significant differences in the adjusted concentrations of plasma VTG in either males or females among locations (Fig. 6).

Hormones

Significant differences in mean concentrations of plasma hormones were observed between males and females and among locations (Fig. 7; Appendix 1). Mean E2 concentrations were greater in females than in males at all sites except the RS site; however, the differences between males and females were only significant at the ER and LP sites (Student's *t* test, $p < 0.05$, log-transformed data). Testosterone concentrations were greater in males than in females at all sites; however, the difference was significant only at the OW site (Student's *t* test, $p < 0.05$, log-transformed data).

After the means of the response parameters were adjusted for covariation, there was still a correlation between plasma concentrations of E2 and T in both males and females (Table 6; male $r = 0.627$, $p < 0.001$; female $r = 0.716$, $p < 0.001$). When mean concentrations of plasma E2 were adjusted for the effects of the covariates, there were statistically significant differences among locations for both females ($p < 0.0001$) and males ($p < 0.0001$; Fig. 8). In the case of females, the adjusted mean plasma E2 concentration at WM was greater than those from other locations, but since there was only one corrected value for this location, it could not be determined to be significantly greater. In the case of males, adjusted concentrations of E2 were greater in the plasma of fish from WM and RS than in those from all of the other locations, which were not significantly different from one another.

When plasma concentrations of T were corrected for the effects of size and reproductive condition, there were fewer differences than would have been concluded without correcting for intercorrelations (Fig. 9). In females, adjusted plasma T concentrations were significantly greater only at WM. There were no significant differences in plasma concentrations of T in males among locations.

The ratio of E2 to T (E2/T) was greater in females than in males at all sites except RS; the difference was statistically significant at the ER, LP, and WM sites (Student's *t* test, $p < 0.05$; Fig. 10). There were no statistically significant differences in the E2/T ratio among WWTP locations or between WWTP locations and either of the reference locations ($p > 0.05$).

Hepatopancreas histology

There were few lesions observed in the histological evaluation of the hepatopancreas. The only site-specific effect was vacuolation of the hepatopancreas (Fig. 11). The trends among sites were similar between males and females, but not exactly the same (Fig. 12). The greatest degree of vacuolation was observed in fish from ER, WM, and the reference location (LP).

Gonad histology

There were few histologic effects on the ovaries or testes. No ovo-testes were observed in males. There was no significant Sertoli cell proliferation in any testes. No lesions were observed in the ovaries of females (Fig. 13). There was a significant (profile analysis, Wilk's Λ, $p < 0.0002$) difference in

Table 3. Pearson correlation matrix (*r*) for males (top) and females (bottom). Only *r* values where $p < 0.05$ are shown[a]

	Length	Weight	*K*	Gonad	GSI	E2/T	Ln (T)	Ln (E2)
Female								
Length								
Weight	0.929***							
K	0.354**	0.632***						
Gonad	0.577***	0.678***	0.535***					
GSI	0.416**	0.499***	0.507***	0.919***				
E2/T	−0.396**	−0.385*		−0.327*				
Ln (T)	0.517***	0.619***	0.531***	0.536***	0.583***			
Ln (E2)		0.363**	0.415**				0.719***	
VTG			0.572***		0.530***		0.438***	0.399**
Male								
Length								
Weight	0.940***							
K	0.377**	0.620***						
Gonad	0.816***	0.871***	0.557***					
GSI	0.689***	0.689***	0.441***	0.917***				
E2/T	−0.500***	−0.43***	0.323***	−0.488***	−0.563***			
Ln (T)	0.602***	0.652***	0.536***	0.685***	0.659***	−0.428***		
Ln (E2)						0.522***	0.291*	
VTG								

[a] *K* = condition; GSI = gonadosomatic index; E2 = 17β-estradiol; T = testosterone; VTG = vitellogenin.
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

the relative proportions of ovarian follicles in different stages of development among locations. The fewest tertiary follicles were observed in the fish from the RS on the Looking Glass River (Fig. 14). The pattern of follicular development in fish from LP was significantly different from that of RS (Kruskal–Wallis, with Tukey's multiple range test, $p < 0.05$). The pattern at RS was also different from ER and WM ($p < 0.05$). Reference site was the location where fish exhibited the least growth and gonadal development. The greatest proportion of tertiary follicles was observed in females from the LP reference location, where growth and GSI were the greatest.

DISCUSSION

Exposure of fish to complex effluents can be useful in testing for the presence of environmental endocrine-disrupting chemicals [9,10,15]. Exposure to an estrogenic chemical might cause alterations in plasma sex steroid levels in fish, although not necessarily through an estrogen receptor–mediated mechanism [16]. However, confounding responses may result in artifacts that make it difficult to attribute the responses of specific biomarkers to chemicals in effluents. In this study, site-specific differences in growth rate and sexual maturation resulted in alterations in development that confounded the re-

Table 4. Significant Pearson correlation *r* values ($p < 0.05$) for partial correlations with both weight and gonadosomatic index removed as partial variables[a]

	VTG	Length	*K*	Gonad weight	E2/T	Ln (T)
Female						
VTG						
Length	−0.363*					
K	0.395*	−0.875***				
Gonad weight						
E2/T						
Ln (T)				−0.365*		
Ln (E2)					0.512***	0.716***
Male						
VTG						
Length						
K		−0.904***				
Gonad weight						
E2/T				0.349*		
Ln (T)		−0.333*	0.308*			
Ln (E2)					0.566***	0.627***

[a] *K* = condition; E2 = 17β-estradiol; T = testosterone; VTG = vitellogenin.
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

Table 5. Probabilities of type 1 error in rejecting the null hypothesis that there are no differences in the adjusted means of parameters among locations after removing the covariance due to weight, condition (*K*), and gonadosomatic index by analysis of covariance[a]

	Probability (*p*)	
Parameter	Females	Males
VTG	0.1	0.188
E2	<0.0001	<0.0001
T	0.009	0.001
E2/T ratio	0.24	0.479

[a] VTG = vitellogenin; E2 = 17β-estradiol; T = testosterone.

sponses of biomarkers of exposure to endocrine-disrupting chemicals. Although statistical approaches can be used to deconvolute these responses, it still is not possible to state categorically whether the observed responses are primarily due to direct exposure to chemicals or a result of other developmental changes related to differential growth and/or maturation. The results of this study demonstrate the importance of considering the confounding effects of covariates, such as growth, *K*, and gonadal development, on measures of endocrine disruption often applied to fish. The variation observed in these growth parameters was most likely due to differences in availability of food at the various field locations.

Vitellogenin

As has been noted in a variety of other studies in North America, increases in concentrations of VTG in plasma of male fish exposed to WWTP effluents are neither as prevalent, nor as dramatic, as that observed in studies of fish in European rivers [17]. However, in one study conducted in Minnesota (USA), concentrations of plasma VTG as great as 10 mg/ml have been observed in wild male carp caught in a sewage effluent canal [12]. In the current study, the greatest site-specific prevalence of measurable VTG in male fish was 57%, and there were no statistically significant differences in the incidence of measurable VTG of either males or females among locations. When fathead minnows (*Pimephales promelas*) were exposed for three weeks at the same locations, during the same season as the current study, the maximum prevalence of VTG induction in males was 62% at the LP study site [15] compared with 22% of males at LP in this study.

Table 6. Statistical significance (*p*) for full and partial general linear models used to calculate adjusted variables by removal of covariates[a]

	Females	Males
E2 overall model	0.009	0.078
Weight	0.026	0.043
K	0.008	0.30
GSI	0.85	0.18
T overall model	0.0002	<0.0001
Weight	<0.0001	<0.0001
K	0.0256	0.0061
GSI	0.282	0.0267
VTG overall model	<0.0001	0.534
Weight	0.0004	0.51
K	0.0005	0.65
GSI	0.036	0.22

[a] E2 = 17β-estradiol; *K* = condition; GSI = gonadosomatic index; T = testosterone.

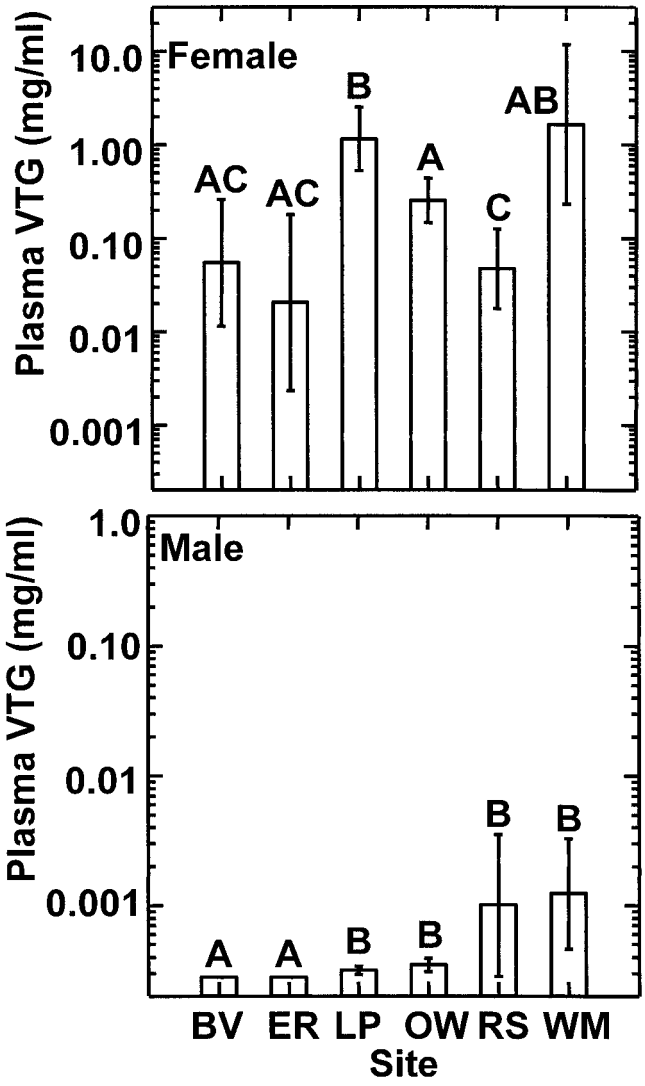

Fig. 5. Mean concentrations of vitellogenin (VTG) in male or female common goldfish. The confidence intervals represent ±1.0 standard deviation of the mean. Means labeled with the same letter are not significantly different (Kruskal–Wallis, $p > 0.05$). See Figure 1 for site identifier information.

The concentrations of estrogenic compounds measured at the various locations were less than the threshold for response to estrogenic compounds. The threshold for induction of VTG varies among species and compounds (Table 7). Concentrations of estrogenic chemicals were measured in 1997 at BV and ER. At site ER, nonylphenol (NP) was detected (0.171–0.806 μg NP/L), but octylphenol (OP) was not detected at the MDL of 2 ng/L. 17β-estradiol was found to be present at a concentration of 477 ng/L, but EE2 was not detected at the MDL of 53 pg/L. At site BV, NP, OP, and EE2 were all detected (22.8–37.0 μg NP/L, 249–673 ng OP/L, 3.23–3.66 ng E2/L, and 0.242–0.759 ng EE2/L) [18]. The total concentrations of estrogen equivalents (measured with an in vitro cell bioassay) contributed by NP, OP, E2, and EE2 in the effluents ranged from 1.9 ng/ml at ER to 16.8 ng/ml at BV [19]. Both E2 and EE2 contributed more than 99.9% of the E2-equivalents measured in these samples. It is not surprising that no significant effects on induction of VTG or gonadal histology were observed in the study reported here. However, E2 and EE2 con-

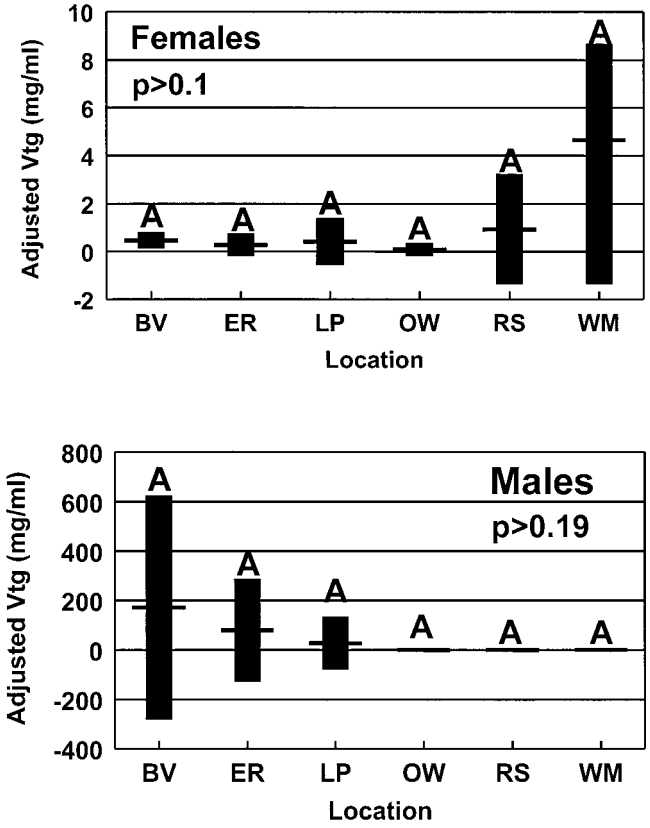

Fig. 6. Plot of adjusted mean plasma vitellogenin (VTG) concentrations in female and male common goldfish caged at reference locations and in rivers receiving waste water treatment plant effluents. Lengths of bars represent ±1.0 standard deviation of the mean. Means are represented by horizontal lines. Means not significantly different from one another are denoted by the same letter. The overall significance of the one-way Kruskal–Wallis test is given in the figure. Predictive equations derived were for females ($p < 0.0001$), VTG = −7.628 + 0.011·weight in grams + 24.55·K + 0.227·GSI, where K is the condition factor, and GSI is the gonadosomatic index. For males, the regression was not significant. See Figure 1 for site identifier information.

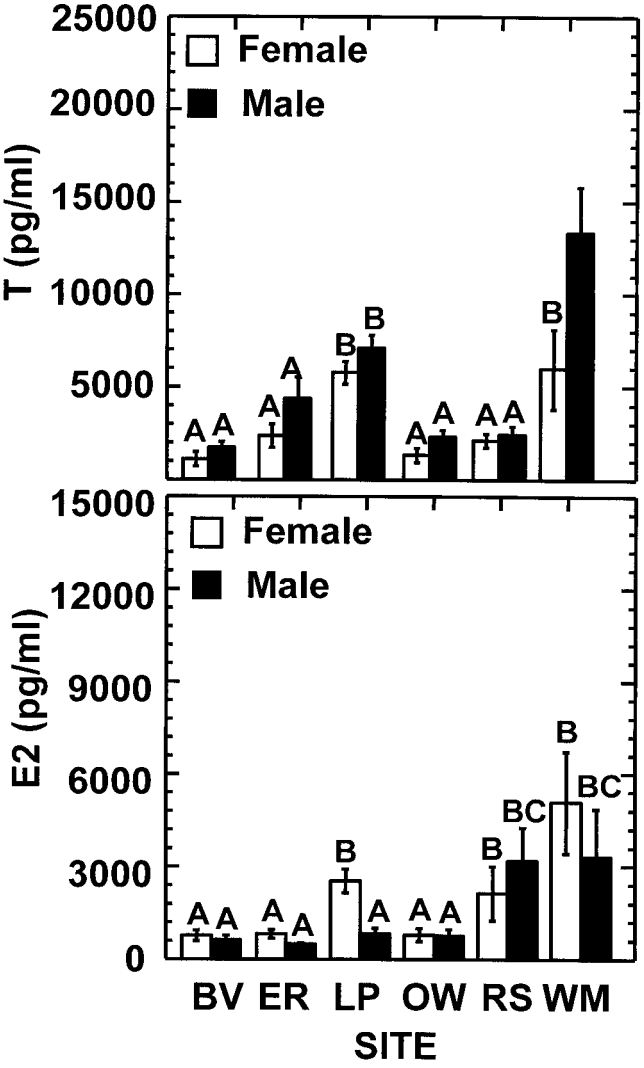

Fig. 7. Mean concentrations of testosterone (T) and estradiol (E2) in male and female common goldfish. Open bars represent females, whereas closed bars represent males. The confidence intervals represent ±1.0 standard deviation of the mean. Means labeled with the same letter are not significantly different (Kruskal–Wallis, $p > 0.05$). See Figure 1 for site identifier information.

centrations in the BV effluent might have been sufficient to induce VTG synthesis in a more sensitive species like rainbow trout. Also, NP concentrations at BV exceeded the threshold for VTG induction in fathead minnows and rainbow trout (Fig. 7).

Steroids

It has been hypothesized that relative, rather than absolute, concentrations of individual sex steroid hormones are indicative of the reproductive health and fitness of fish and exhibit differences between males and females. In a previous study conducted with common carp, variation in the E2/T ratio in female carp was affected more by its relationship with T than with E2 [14]. Although T is an androgen and an important sex steroid in male fish, it is also important in the ovary [20]. Because T serves as a precursor for E2 in fish ovary [20], it is not surprising that T concentrations might strongly influence the variation in the E2/T ratio in female carp. Previous authors have found that feral female carp evaluated in situ and in reference areas exhibited E2/T ratios greater than 1.0, whereas feral males in rivers had E2/T ratios that were generally less than 1.0 [12,14]. In this study the E2/T ratio was less than 1.0 for both males and females. In all cases, except for goldfish from RS, the E2/T ratio was greater in females than in males. The E2/T ratio was, on average, 2.5-fold (n = 6, SD = 1.3) greater in females than in males.

Stress

The effects of generalized and confinement stress on reproductive physiology, primarily fecundity and gonad histology, plasma E2, T, 11-KT, and VTG concentrations, have been reported previously [21]. Stress is known to cause significant reductions in circulating sex steroid concentrations in some species of fish [22], possibly including carp [23]. These investigations provide some insight into the potential causes for differences between LP and WWTP exposure sites. Cortisol is the primary steroid produced by neuroendocrine stimulation of the interrenal glands upon stimulation by a stressor. Stress and the subsequent induction of cortisol can increase the degree of ovarian atresia and decrease concentrations of plasma E2, T, and VTG and, to a lesser extent, 11-KT [24,25]. Investigators have determined that the effects of cortisol on reproductive endocrinology and recrudescence for several spe-

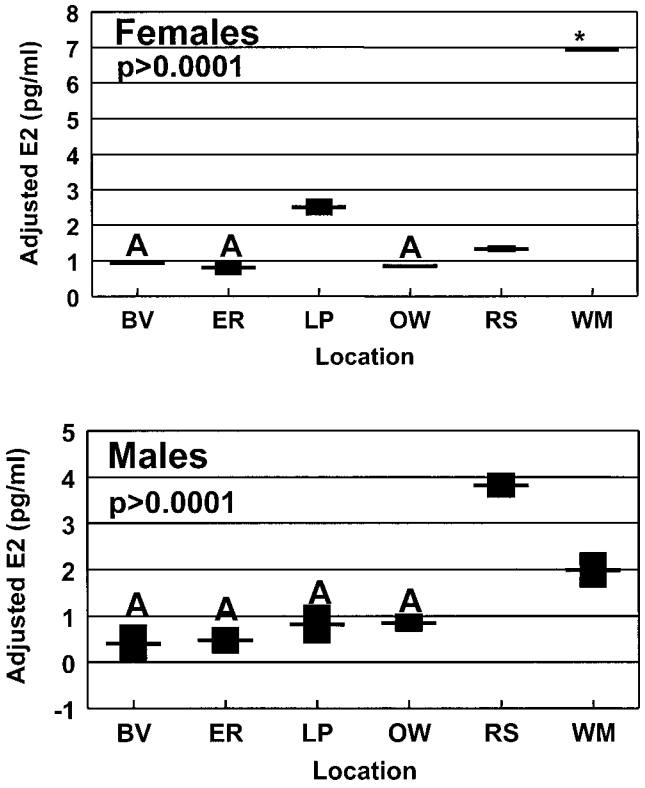

Fig. 8. Plot of adjusted mean plasma estradiol (E2) concentrations in female and male common goldfish caged at reference locations and in rivers receiving wastewater treatment plant effluents. Lengths of bars represent ±1.0 standard deviation of the mean. Means are represented by horizontal lines. Means not significantly different from one another are denoted by the same letter. The overall significance of the one-way Kruskal–Wallis test is given in the figure. Predictive equations derived were for females ($p < 0.009$), E2 = −2,105 − 3.36·weight in grams (WT) + 10,909·K + 10.65·GSI, where K is the condition factor, and GSI is the gonadosomatic index. For males ($p < 0.077$), E2 = 1,287 − 33.9·WT + 4,435·K − 293·GSI. See Figure 1 for site identifier information.

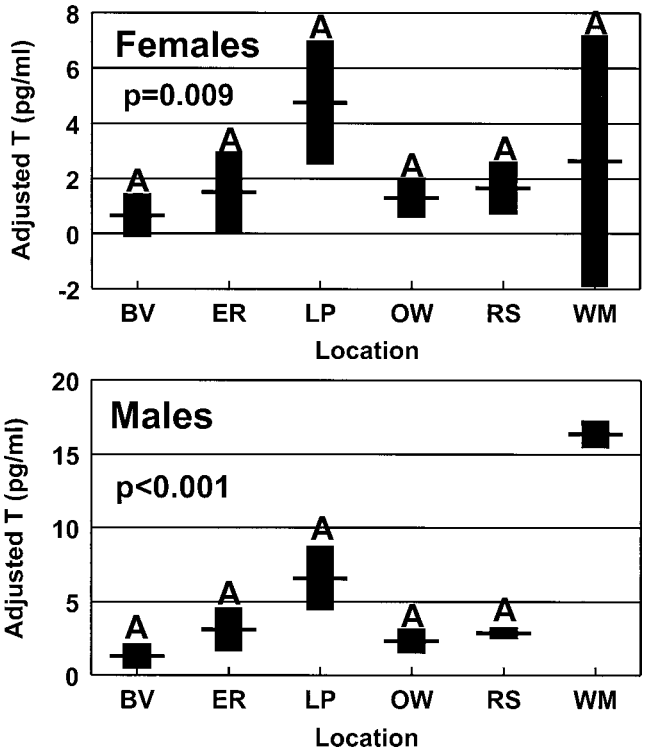

Fig. 9. Plot of adjusted mean plasma testosterone (T) concentrations in female and male common goldfish caged at reference locations and in rivers receiving wastewater treatment plant effluents. Lengths of bars represent ±1.0 standard deviation of the mean. Means are represented by horizontal lines. Means not significantly different from one another are denoted by the same letter. The overall significance of the one-way Kruskal–Wallis test is given in the figure. Predictive equations derived were for females ($p < 0.0002$), T = −2,415 + 51·weight in grams (WT) + 11,011.8·K + 79.5·GSI, where K is the condition factor, and GSI is the gonadosomatic index. For males ($p < 0.0001$), T = −8,208 − 15·WT + 30,351·K + 1,237·GSI. See Figure 1 for site identifier information.

cies of fish are not elicited by direct action on ovarian steroidogenesis. Rather, it may be more likely that the effects observed are mediated at levels other than the gonads in the hypothalamic-pituitary-gonad axis [26]. Furthermore, even with low levels of capture and confinement stress and associated reduction in plasma sex steroid hormone concentrations, site-specific differences among controls and fish exposed to bleached kraft mill effluent were discernible for the white sucker (*Catostomus commersoni*) [27]. Even at low concentrations, NH_3 can cause chronic stress that results in changes in physiology and immune function of fish [28]. The ammonia concentration in the effluent of OW was high and approached values for acute toxicity in more sensitive species.

Gonadal morphology

The observation of no effects on gonadal morphology, either at the gross or histological level of investigation in this study, is similar to the study of fathead minnows conducted simultaneously at the same locations [15]. However, the results are different from those of similar studies in Europe. Histological changes in the gonads of male roach (*R. rutilus*) have been observed downstream from WWTP effluents in the United Kingdom [9,10]. Previous studies have demonstrated decreased GSI in male, female, and intersex fish exposed to sewage effluent [7,10]. In contrast, in the current study the

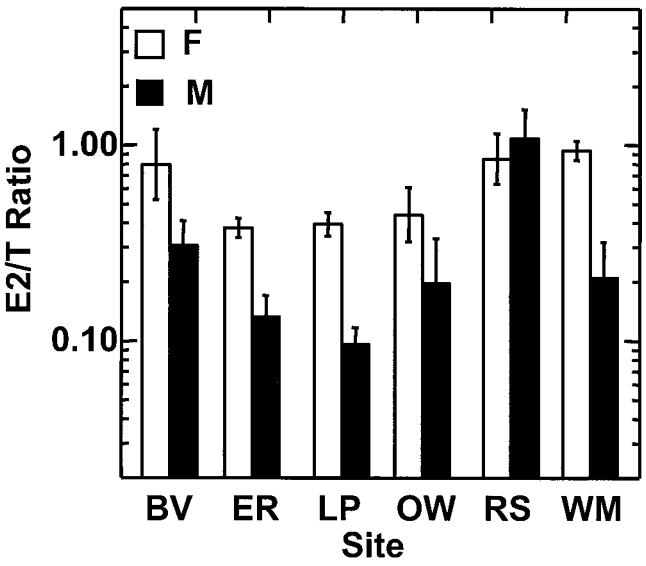

Fig. 10. Ratio of estradiol (E2) to testosterone (T) in male and female goldfish. The confidence intervals represent ±1.0 standard deviation of the mean. Means depicted by the same letter are not significantly different (Kruskal–Wallis, $p > 0.05$). See Figure 1 for site identifier information.

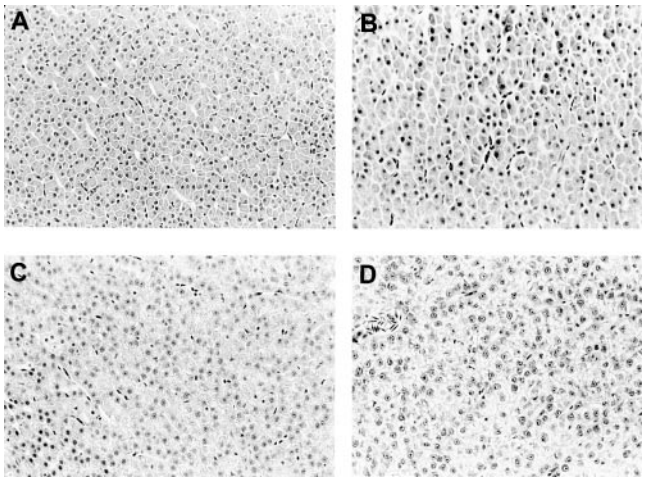

Fig. 11. Photomicrographs of goldfish hepatopancreas illustrating scoring scale for vacuolation. (**A**) score 0, (**B**) score +1, (**C**) score +2, and (**D**) score +3. Magnification ×350.

least GSI was observed in males and females from the reference location RS, where fish were exposed in a river but not to WWTP effluent. The common goldfish is a cyprinid species, as is the roach. Thus although the difference may be due to the species being used in the studies, it seems more likely that the differences are due to the fact that the roach is exposed to greater concentrations of estrogenic substances in some of the United Kingdom rivers studied. Furthermore, the roach were wild fish and might have been exposed their entire lives, which might result in exposures of greater duration and perhaps at critical periods of sexual development.

Gonadal morphology—intersex

Estrogenic contaminants associated with WWTP effluents have been implicated as a potential cause of an unusual incidence of an intersex condition observed in wild fish in rivers in the United Kingdom [7]. Ovo-testis also can be induced in female carp by administration of androgenic steroids such as methyltestosterone applied to feed [29]. It is possible for estrogenic steroids to induce ovo-testis in adult fish. Exposure of sexually mature male common carp for two or three months to E2 in water at 1 pg E2/L caused formation of ovo-testes in previously regressed testes of some exposed fish [30]. The lowest observed effect concentrations for histological effects of E2 in male and female fathead minnows were found to be 0.1 and 0.5 nM (27.2 and 136 ng E2/L), respectively [31]. The concentration to produce a 10% effect (EC10) for reduced fecundity in fathead minnows has been reported to be 23 pM (6,218 pg E2/L) [32]. The concentrations of E2 were 477 and 3,230 to 3,660 pg/L in the effluents from ER and BV, respectively [18]. Concentrations of EE2 were <53 pg/L at ER, but 242 to 759 pg/L at BV. Thus the concentration of E2 and the total concentration of E2 and EE2 would be expected to be sufficient to induce lesions in the gonads of both male and female common carp and fathead minnows, but were less than the concentration required to cause decreased reproductive output in fathead minnows. However, exposure to these concentrations in WWTP effluents for six weeks did not cause observable gonadal lesions in common goldfish in this study. This finding could have been due to the duration of exposure, to variation in concentration of estrogenic chemicals in WWTP effluents, or to the form of the xenoestrogens, which may not

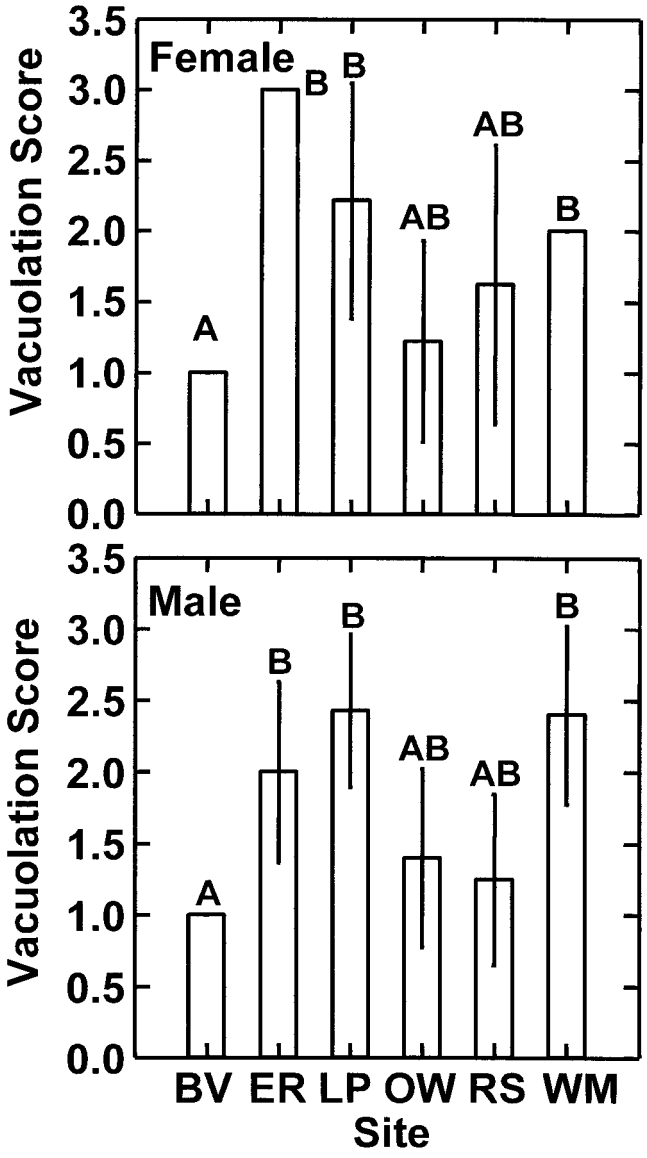

Fig. 12. Hepatopancreas vacuolation scores for male and female goldfish at two reference locations and four sites receiving wastewater treatment plant effluents. The confidence intervals represent ±1.0 standard deviation of the mean. Means labeled with the same letter are not significantly different (Kruskal–Wallis, $p > 0.05$). See Figure 1 for site identifier information.

have been biologically available to the fish in the cages. It is unknown whether the greatest exposure of fish to these compounds is through the diet or water. Also, in the study conducted by Gimeno et al. discussed previously [30], regression of the testes appeared to be a prerequisite for formation of ovo-testis. Testicular regression was not observed in our study. The fact that no intersexing or occurrence of ovo-testes was observed in male common goldfish exposed to WWTP effluents in this study suggests that the concentrations of estrogenic compounds in the effluents were not sufficient to cause these effects during a six-week exposure.

Exposure to certain alkylphenolic compounds via water can induce the formation of ovo-testis in fish, such as in Japanese medaka [34] exposed to these chemicals during critical periods of sexual differentiation. The lowest observed effect concentration for NP to induce intersexing in Japanese medaka was reported to be 50 μg/L [34]. A lowest observed effect level

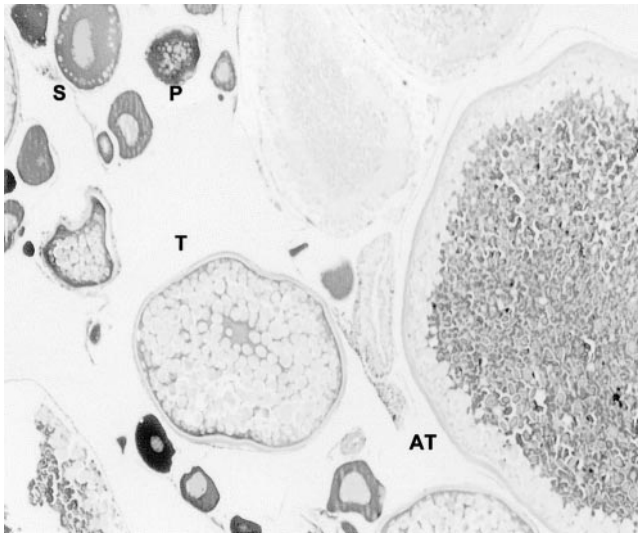

Fig. 13. Photomicrograph of oocytes in goldfish. Examples of primary (P), secondary (S), tertiary (T), and atretic (AT) follicles are indicated. Magnification ×350.

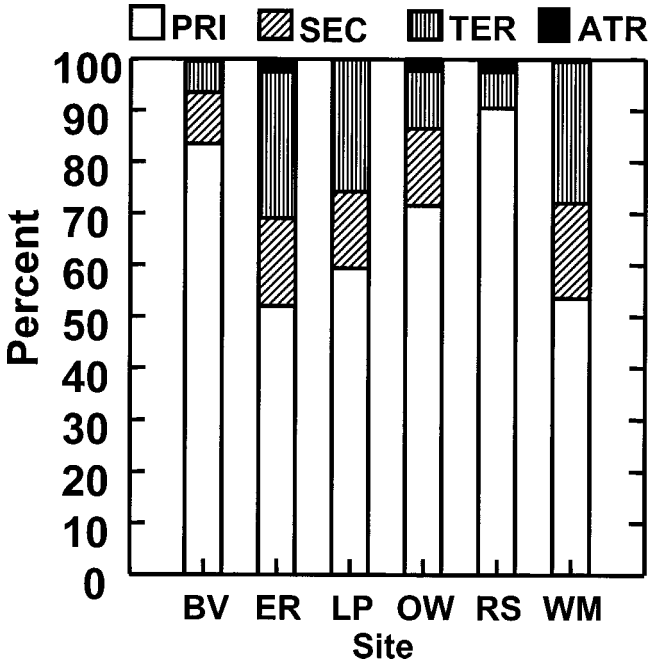

Fig. 14. Histogram of the pattern of oocyte development. Values are percentages of total oocytes classified as either primary (PRI), secondary (SEC), tertiary (TER), or atretic (ATR). See Figure 1 for site identifier information.

based on gonadal histology for NP of between 0.4 and 3.4 μg/L has been reported [35]. This is similar to the lowest observed effect level based on decreases in egg production and increases in concentrations of E2 in plasma of fathead minnows [36]. Concentrations of nonylphenol and octylphenol were 0.171 to 0.806 and 37 μg/L at ER and BV, respectively, whereas the concentrations were 22.8 and 0.25 μg/L, respectively, at BV. Based on results with other species, no effects on gonadal histology would have been expected to occur at ER. However, the concentrations of NP observed at BV were in the range where they could have caused effects on gonadal histology if adult goldfish exposed for six weeks were as sensitive as adults of other model species and if the chemicals were all biologically available.

No histological effects on testes, including Sertoli cell proliferation, were observed in the current study. Exposure of male fathead minnows (*Pimephales promelas*) to 2 nM (5.4 ng/L) E2 for 14 d (or 19 d) or to 1.6 to 3.4 μg/L NP for 42 d caused severe or moderate Sertoli cell proliferation in the testes, respectively. In this study, the greatest concentrations of E2, which were observed at ER and BV, were 0.48 and 3.23 to 3.66 ng E2/L, whereas the concentrations of NP were 0.171 to 0.806 and 22.8 to 37.0 μg NP/L, respectively [18]. Using the minimum lowest observed effect level and the maximum measured exposure concentration (MMEC), a risk ratio (RR) was calculated as the ratio of these two values (RR = MMEC / no observed effect level [NOEL]). The RR for NP was found to be approximately 10, whereas that for E2 was found to be 0.6. Thus, based on studies with other species, the male com-

Table 7. Reported threshold concentrations for induction of vitellogenin in male fishes

Species	Compound	Threshold	Reference
Fathead minnow (*Pimephales promelas*)	Estradiol	27 ng/L	[32]
Fathead minnow (*Pimephales promelas*)	Estradiol	100 ng/L	[41]
Fathead minnow (*Pimephales promelas*)	Estradiol	30 ng/L	[42]
Fathead minnow (*Pimephales promelas*)	Estradiol	50 ng/L	[43]
Sunshine bass (*Morone saxatalis* × *M. chrysops*)	Estradiol	10,000 ng/L	[44]
Japanese medaka (*Oryzias latipes*)	Estradiol	1,000 ng/L	[44]
Channel catfish (*Ictalurus punctatus*)	Estradiol	1,000 ng/L	[44]
Rainbow trout (*Oncorhynchus mykiss*)	Estradiol	1–10 ng/L	[3]
Rainbow trout (*Oncorhynchus mykiss*)	Estradiol	0.1–10 ng/L	[40]
Sheepshead minnow (*Cyprinodon variegatus*)	Estradiol	100 ng/L	[45]
Sheepshead minnow (*Cyprinodon variegatus*)	Estradiol	200 ng/L	[45]
Fathead minnow	Estrone	31.8 ng/L	[41]
Rainbow trout	Estrone	25 to 50 ng/L	[46]
Zebrafish (*Danio rerio*)	Ethinyl estradiol	9.05 ng/L	[47]
Zebrafish (*Danio rerio*)	Ethinyl estradiol	5 to 10 ng/L	[47]
Fathead minnows	Nonylphenol	0.65 to 8.1 μg/L	[48]
Rainbow trout	Nonylphenol	20.3 μg/L	[49]
Rainbow trout	Octylphenol	4.8 μg/L	[49]
Rainbow trout	Octylphenol	1–10 μg/L	[3]
Roach	Octylphenol	100 μg/L	[3]
Sheepshead minnow	Octylphenol	5.4 μg/L	[50]
Japanese medaka	Octylphenol	20–230 μg/L	[50]

mon goldfish did not express a response in Sertoli cell proliferation that would be consistent with exposure to estrogen agonists. The RR values were near the threshold for response. There were other strong and weak estrogen agonists present, such as OP and EE2, respectively, but the concentrations of both of these compounds, although measurable, would not add significantly to the estimated RR.

Fish undergoing active gonadal growth and development provide the most useful information regarding the endpoints examined in this study [22]. A number of factors, including timing and duration of exposure, could affect the outcome. Studies of the reproductive tract of common carp have demonstrated that there are windows during development during which the developing fish gonad is particularly susceptible to the effects of xenoestrogens [37]. In male carp, the period between days 24 and 110 of development is the window of greatest sensitivity to the effects of 4-*tert*-pentylphenol, which, like NP, is a weak xenoestrogen, based on effects on gonadal development [38]. The exact age of the goldfish used in this study is unknown, but they would have been sexually mature with completely differentiated gonads at the beginning of the six-week exposure. Although it appears that there were no significant estrogenic effects on fish, the lack of response of the gonads may have been due to the fact that they were not in the most sensitive life stage. However, using younger, smaller fish was not practical in the riverine setting in which this study was conducted. Smaller fish would have been subject to greater physical stress, and it would not have been possible to obtain sufficient blood and tissue samples from smaller fish for measurement of all of the selected biomarkers of exposure to estrogen agonists.

Exposure of female fish to presumed chemical stressors, in both the field and laboratory, has resulted in increased ovarian atresia, increased proportions of perinucleolar or primary follicles in the ovary, and alterations in concentrations of VTG and sex steroid hormones [35,39]. In the current study the incidence of ovarian atresia was small. Ovarian follicle atresia has been associated with stress in fish. Atresia of ovarian follicles also has been associated with exposure of fish to E2 [31]. Those authors reported increased atresia of ovarian follicles in fathead minnows exposed to waterborne E2 at concentrations of 10 or 2 nM (27.24 and 5.40 ng/L) for 19 d. The mechanism involved in producing this effect is unknown and might not be mediated through the estrogen receptor. Ovarian development varied among locations, but there was no trend that could not be explained by differential growth among the locations. The fact that there was a greater incidence of primary follicles in female goldfish from RS than LP (both reference sites) suggests that differences observed among reference and exposure sites were primarily due to differential growth and associated gonadal development. The least ovarian development was observed in females from RS, where the least growth was observed and the GSI was also the least.

Given the argument that the primary concern with WWTP effluent exposure is constituent estrogen mimics, it would seem that male gonad histology (rather than female) would be most affected. The lack of lesions in the testes was consistent among RS and exposure sites and does not suggest endocrine disruption by estrogen agonist exposure. This observation, along with the fact that there were no consistent trends in the responses of biomarkers, indicates that the WWTP effluents investigated in this study are not causing overt endocrine disruption or impacts on reproductive function of fish.

The risk of exposure to endocrine-modulating substances in these representative Midwestern U.S. municipal WWTP and in random surface waters of the United States [14] appears to be small relative to the reproductive abnormalities observed in the United Kingdom. In the United Kingdom, WWTP effluents can comprise up to 36% of the river flow regimes [10]. The WWTP discharge permits required by the Clean Water Act (http://www.epa.gov/r5water/cwa.htm) in the United States are dictated first by technology (secondary treatment is required for most publicly owned WWTP), and then by the water quality and quantity of the effluent receiving stream. The greater the contribution of effluents to flows in U.S. streams, the more stringent the effluent water quality standards. Furthermore, with the advent of the Clean Water Act and the allocated funding provided to municipalities in the 1970s, the most economically feasible and best treatment technologies were employed to remove pollutants from wastewater. In Michigan, as in the rest of North America, trickling filter treatment plants still operate, particularly in small communities, but the preferred technology is activated sludge. Some environmental contaminants are lipophilic and are adsorbed to sediments in the aquatic environment. For lipophilic contaminants with these properties, such as alkylphenols, the activated sludge process has been observed to be a more efficient sink. In the United Kingdom, the prevalence of trickling filter technologies, the relatively great volumes of flow from most WWTP, and the lesser dilution of the effluents by receiving streams are possible explanations for the differences observed in fish endocrine disruption when compared with the United States.

Differences in the responses among species of fish may offer insight into the varied outcomes between WWTP and industrial effluent effects on the reproductive physiology of fish. One of the species used in caging studies in the United Kingdom has been the rainbow trout [9,10], whereas most studies conducted in North America have employed cyprinid fish [12–14]. Trout are capable of synthesizing VTG at low temperatures that appear to inhibit the ability of cyprinid fish to do the same [40], and therefore temperature differences among studies and sites might also be a factor.

Acknowledgement—We thank the collaborative efforts within the Aquatic Toxicology Laboratory at Michigan State University. Sylvia Heaton of the Michigan Department of Environmental Quality provided information regarding Michigan WWTPs. Glen Van Der Kraak and Z.X. Yao provided technical assistance and training in the development and application of the VTG ELISA. Municipal WWTP owners and operators were extremely helpful, and their cooperation and interest in the study are appreciated. This research was funded, in part, by the Chlorine Chemistry Council of the Chemical Manufacturer's Association; the National Institute for Environmental Health Sciences Project NIEHS-ES-04911, and the U.S. Environmental Protection Agency Office of Water Project CR-822983-01-0.

REFERENCES

1. McMaster ME. 2001. A review of the evidence for endocrine disruption in Canadian aquatic ecosystems. *Water Qual Res J Canada* 36:215–231.
2. Schmidt H, Bernet D, Wahli T, Meier W, Burkhardt-Holm P. 1999. Active biomonitoring with brown trout and rainbow trout in diluted sewage plant effluents. *J Fish Biol* 54:585–596.
3. Routledge EJ, Sheahan D, Desbrow C, Brighty GC, Waldock M, Sumpter JP. 1998. Identification of estrogenic chemicals in STW effluent. 2. In vivo responses in trout and roach. *Environ Sci Technol* 31:1559–1565.
4. Sheahan DA, Brighty GC, Kirby SJ, Hurst MR, Kennedy J, Morris S, Routledge EJ, Sumpter JP, Waldock MJ. 2002. Estrogenic ac-

tivity measured in a sewage treatment works which treats industrial inputs containing high concentrations of alkylphenolic compounds—A case study. *Environ Toxicol Chem* 21:507–514.
5. Sole M, Lopez De Alda MJ, Castillo M, Porte C, Ladegaard-Pedersen K, Barcelo D. 2000. Estrogenicity determination in sewage treatment plants and surface waters from the Catalonian area (NE Spain). *Environ Sci Technol* 34:5076–5083.
6. Jobling S, Sumpter JP. 1993. Detergent components in sewage effluent are weakly oestrogenic to fish: An in vitro study using rainbow trout (*Oncorhynchus mykiss*) hepatocytes. *Aquat Toxicol* 27:361–372.
7. Jobling S, Nolan M, Tyler CR, Brighty GC, Sumpter JP. 1998. Widespread sexual disruption in wild fish. *Environ Sci Technol* 32:2498–2506.
8. Rodgers-Gray TP, Jobling S, Kelly C, Morris S, Brighty G, Waldock MJ, Sumpter JP, Tyler CR. 2001. Exposure of juvenile roach (*Rutilus rutilus*) to treated sewage effluent induces dose-dependent and persistent disruption in gonadal duct development. *Environ Sci Technol* 35:462–470.
9. Harries JE, Sheahan DA, Jobling S, Matthiessen P, Neall P, Routledge EJ, Rycroft R, Sumpter JP, Tylor T. 1996. A survey of estrogenic activity in United Kingdom inland waters. *Environ Toxicol Chem* 15:1993–2002.
10. Harries JE, Sheahan DA, Jobling S, Matthiessen P, Neall P, Sumpter JP, Tylor T, Zaman N. 1997. Estrogenic activity in five United Kingdom rivers detected by measurement of vitellogenesis in caged male trout. *Environ Toxicol Chem* 16:534–542.
11. Hewitt M, Servos M. 2001. An overview of substances present in Canadian aquatic environments associated with endocrine disruption. *Water Qual Res J Canada* 36:191–213.
12. Folmar LC, Denslow ND, Rao V, Chow M, Crain DA, Enblom J, Marcino J, Guillette LJ Jr. 1996. Vitellogenin induction and reduced serum testosterone concentrations in feral male carp (*Cyprinus carpio*) captured near a major metropolitan sewage treatment plant. *Environ Health Perspect* 104:1096–1101.
13. Bevans HE, Goodbred SL, Miesner JF, Watkins SA, Gross TS, Denslow ND, Schoeb T. 1996. Synthetic organic compounds and carp endocrinology and histology in Las Vegas Wash and Las Vegas and Callville Bays of Lake Mead, Nevada, 1992 and 1995. Water Resources Investigations 96-4266. U.S. Geological Survey, Carson City, NV.
14. Goodbred S, Gilliom RJ, Gross TS, Denslow NP, Bryant, WL, Schoeb TR. 1997. Reconnaissance of 17β-estradiol, 11-ketotestosterone, vitellogenin, and gonad histopathology in common carp of United States streams: Potential for contaminant-induced endocrine disruption. Open-File Report 96-627. U.S. Geological Survey, Sacramento, CA.
15. Nichols K, Snyder E, Miles-Richardson S, Pierens S, Snyder S, Giesy JP. 1999. Effects of exposure to municipal wastewater in situ on the reproductive physiology of the fathead minnow (*Pimephales promelas*). *Environ Toxicol Chem* 18:2001–2012.
16. Tremblay L, Van Der Kraak GJ. 2002. Use of a series of homologous in vitro and in vivo assays to evaluate the endocrine modulating actions of β-sitosterol in rainbow trout. *Aquat Toxicol* 43:149–162.
17. Jones PD, DeCoen WM, Tremblay L, Giesy JP. 2000. Vitellogenin as a biomarker for environmental estrogens. *Water Sci Technol* 42:1–14.
18. Snyder SA, Keith TL, Verbrugge DA, Snyder EM, Gross TS, Kannan K, Giesy JP. 1999. Analytical methods for detection of selected estrogenic compounds in aqueous mixtures. *Environ Sci Technol* 33:2814–2820.
19. Snyder SA, Villeneuve DL, Snyder EM, Giesy JP. 2001. Identification and quantification of estrogen receptor agonists in wastewater effluents. *Environ Sci Technol* 35:3620–3625.
20. McMaster ME, Munkittrick KR, Jardine JJ, Robinson RD, Van der Kraak GJ. 1995. Protocol for measuring in vitro steroid production by fish gonadal tissue. *Can Tech Rep Fish Aquat Sci 1992.* 1–29.
21. Donaldson EM. 1990. Reproductive indices as measures of the effects of environmental stressors in fish. *Am Fish Soc Symp* 8: 109–122.
22. McMaster ME, Munkittrick KR, Van der Kraak GJ. 1992. Protocol for measuring circulating levels of gonadal sex steroids in fish. *Can Tech Rep Fish Aquat Sci* 1836:1–29.
23. Santos AJG, Furukawa K, Kobayashi M, Bando K, Aida K, Hanyu I. 1986. Plasma gonadotropin and steroid hormone profiles during ovulation in the carp *Cyprinus carpio*. *Bull Jap Soc Sci Fish* 52: 1159–1166.
24. Carragher JF, Sumpter JP, Pottinger TG, Pickering AD. 1989. The deleterious effects of cortisol implantation on reproductive function in two species of trout, *Salmo trutta* and *Salmo gairdneri* Richardson. *Gen Comp Endocrinol* 76:321.
25. Pickering AD, Pottinger TG, Carragher JF, Sumpter JP. 1987. The effects of acute and chronic stress on the levels of reproductive hormones in the plasma of mature brown trout, *Salmo trutta* L. *Gen Comp Endocrinol* 68:249–259.
26. Pankhurst NW, Van der Kraak GJ, Peter RE. 1995. Evidence that the inhibitory effects of stress on reproduction in teleost fish are not mediated by the action of cortisol on ovarian steroidogenesis. *Gen Comp Endocrinol* 99:249–257.
27. Jardine JJ, Van Der Kraak GJ, Munkittrick KR. 1996. Capture and confinement stress in white sucker exposed to bleached kraft pulp mill effluent. *Ecotoxicol Environ Saf* 33:287–298.
28. Russo RC. 1985. Ammonia, nitrate, and nitrite. In Rand GM, Petrocelli SR, eds, *Fundamentals of Aquatic Toxicology*. Taylor & Francis, Bristol, PA, USA, pp 455–471.
29. Nagy A, Bercsenyl M, Csanyi V. 1981. Sex reversal in carp (*Cyprinus carpio*) by oral administration of methyltestosterone. *Can J Fish Aquat Sci* 38:725–728.
30. Gimeno S, Komen H, Jobling S, Sumpter J, Bowmer T. 1998. Demasculinization of sexually mature male common carp, *Cyprinus carpio*, exposed to 4-*tert*-pentylphenol during spermatogenesis. *Aquat Toxicol* 43:93–109.
31. Miles-Richardson SR, Kramer VJ, Fitzgerald SD, Barbee SJ, Giesy JP. 1999. Effects of waterborne exposure of 17β-estradiol on secondary sex characteristics and gonads of fathead minnows (*Pimephales promelas*). *Aquat Toxicol* 47:129–145.
32. Kramer VJ, Miles-Richardson S, Pierens SL, Giesy JP. 1998. Reproductive impairment and induction of alkaline-labile phosphate, a biomarker of estrogen exposure, in fathead minnows (*Pimephales promelas*) exposed to waterborne 17β-estradiol. *Aquat Toxicol* 40:335–360.
33. Atz JW. 1964. Intersexuality in fishes. In Armstrong CN, Marshall AJ, eds, *Intersexuality in Vertebrates Including Man.* Academic, New York, NY, USA, pp 145–232.
34. Gray M, Metcalf CD. 1997. Induction of testis-ova in Japanese medaka (*Oryzias latipes*) exposed to p-nonylphenol. *Environ Toxicol Chem* 16:1082–1086.
35. Miles-Richardson SR, Pierens SL, Nichols KM, Kramer VJ, Snyder EM, Snyder SA, Render JA, Fitzgerald SD, Giesy JP. 1999. Effects of waterborne exposure to 4-nonylphenol and nonylphenol ethoxylate on secondary sex characteristics and gonads of fathead minnows (*Pimephales promelas*). *Environ Res* 80:S122–S137.
36. Giesy JP, Pierens SL, Snyder EM, Miles-Richardson S, Kramer VJ, Snyder SA, Nichols, KM, Villeneuve DA. 2000. Effects of 4-nonylphenol on fecundity and biomarkers of estrogenicity in fathead minnows (*Pimephales promelas*). *Environ Toxicol Chem* 19:1368–1377.
37. Gimeno S, Gerritsen A, Bowmer T, Komen H. 1996. Feminization of male carp. *Nature* 384:221–222.
38. Gimeno S, Komen H, Venderbosch PWM, Bowmer T. 1997. Disruption of sexual differentiation in genetic male common carp (*Cyprinus carpio*) exposed to an alkylphenol during different life stages. *Environ Sci Technol* 31:2884–2890.
39. Johnson LJ, Casillas E, Collier TK, McCain BB, Varanasi U. 1988. Contaminant effects on ovarian development in English sole (*Parophrys vetulus*) from Puget Sound, Washington. *Can J Fish Aquat Sci* 45:2133–2146.
40. Hernández I, Poblete A, Amthauer R, Pessot R, Krauskopf M. 1992. Effect of seasonal acclimatization on estrogen-induced vitellogenesis and on the hepatic estrogen receptors in the male carp. *Biochem Int* 28:559–567.
41. Panter GH, Thompson RS, Sumpter JP. 1998. Adverse reproductive effects in male fathead minnows (*Pimephales promelas*) exposed to environmentally relevant concentrations of the natural oestrogens, oestradiol and oestrone. *Aquat Toxicol* 42:243–253.
42. Panter GH, Thompson RS, Sumpter JP. 2000. Intermittent exposure of fish to estradiol. *Environ Sci Technol* 34:2756–2760.
43. Tyler CR, van Aerle R, Hutchinson TH, Maddix S, Trip H. 1999. An in vivo testing system for endocrine disruptors in fish early life stages using induction of vitellogenin. *Environ Toxicol Chem* 18:337–347.
44. Thompson S, Tilton F, Schlenk, D, Benson WH. 2000. Compar-

ative vitellogenic responses in three teleost species: Extrapolation to in situ field studies. *Mar Environ Res* 51:185–189.
45. Folmar LC, Hemmer M, Hemmer R, Bowman C, Kroll K, Denslow ND. 2000. Comparative estrogenicity of estradiol, ethynyl estradiol and diethylstilbestrol in an in vivo, male sheepshead minnow (*Cyprinodon variegatus*), vitellogenin bioassay. *Aquat Toxicol* 49:77–88.
46. Purdom CE, Hardiman PA, Bye VJ, Eno NC, Tyler CR, Sumpter JP. 1994. Estrogenic effects of effluents from sewage treatment works. *Chemical Ecology* 8:275–285.
47. Van den Belt K, Verheyen R, Witters H. 2001. Reproductive effects of ethynylestradiol and 4*t*-octylphenol on the zebrafish (*Danio rerio*). *Arch Environ Contam Toxicol* 41:458–467.
48. Harries JE, Runnalls T, Hill E, Harris CA, Maddix S, Sumpter JP, Tyler CR. 2000. Development of a reproductive performance test for endocrine disrupting chemicals using pair-breeding fathead minnows (*Pimephales promelas*). *Environ Sci Technol* 34: 3003–3011.
49. Jobling S, Sheahan D, Osborne JA, Matthiessen P, Sumpter JP. 1996. Inhibition of testicular growth in rainbow trout (*Oncorhynchus mykiss*) exposed to estrogenic alkylphenolic chemicals. *Environ Toxicol Chem* 15:194–202.
50. Gronen S, Denslow N, Manning S, Barnes S, Barnes D, Brouwer M. 1999. Serum vitellogenin levels and reproductive impairment of male Japanese medaka (*Oryzias latipes*) exposed to 4-*tert*-octylphenol. *Environ Health Perspect* 107:385–390.

Appendix

Statistics for biological parameters measured in goldfish exposed to wastewater treatment plant effluents (mid-Michigan, USA)[a]

		VTG (ng/ml)	Length (cm)	Weight (g)	Condition (*K*)	Gonad (g)	GSI	Plasma T (pg/ml)	Plasma E2 (pg/ml)	In vitro T production
Males										
BV	Range (*n*)	0–0 (13)	6.5–8.3 (13)	9–15.7 (13)	0.23–0.39 (13)	0.01–0.41 (7)	0.075–3.45 (7)	363–4,123 (13)	78.1–1,669 (13)	1.71–7.43 (10)
	Mean (SD)	0 (0)	7.33 (0.53)	12.0 (2.05)	0.31 (0.04)	0.15 (0.142)	1.24 (1.194)	1,723 (1,084)	616 (538)	4.30 (1.75)
LP	Range (*n*)	0–0.098 (18)	8.8–10.8 (18)	27.7–52.9 (18)	0.31–0.57 (18)	0.74–2.35 (15)	2.09–6.33 (15)	2,411–12,614 (17)	239–2,705 (17)	1.84–34.3 (15)
	Mean (SD)	0.006 (0.023)	9.68 (0.64)	36.0 (6.7)	0.40 (0.07)	1.27 (0.42)	3.70 (1.035)	7,077 (2,829)	817 (728)	12.03 (10.1)
OW	Range (*n*)	0–0.001 (8)	6.6–8.3 (8)	11.7–16.7 (8)	0.28–0.41 (8)	0.19–0.54 (6)	1.37–3.42 (6)	894–3,577 (8)	78.1–1,430 (8)	2.62–9.66 (6)
	Mean (SD)	0 (0)	7.65 (0.54)	14.6 (1.9)	0.33 (0.04)	0.34 (0.13)	2.22 (0.73)	2,305 (974)	745 (592)	4.98 (2.86)
ER	Range (*n*)	0–0 (9)	7.7–11 (9)	17–47 (9)	0.31–0.37 (9)	0.56–1.47 (7)	1.79–4.45 (7)	1,677–12,140 (9)	345–643 (9)	4.41–403 (7)
	Mean (SD)	0 (0)	9.32 (1.02)	28.8 (9.1)	0.35 (0.02)	0.97 (0.32)	3.40 (0.911)	4,363 (3,222)	490 (101)	63.4 (150)
RS	Range (*n*)	0–0.963 (7)	6–7.5 (8)	4.5–11.9 (8)	0.21–0.32 (8)	0.04–0.219 (6)	0.54–2.08 (6)	471–4,444 (8)	558–7,824 (8)	3.64–11.8 (6)
	Mean (SD)	0.138 (0.364)	6.80 (0.67)	8.69 (2.50)	0.27 (0.04)	0.092 (0.07)	0.979 (0.571)	2,408 (1,250)	3,177 (2,833)	6.94 (3.00)
WM	Range (*n*)	0–0.152 (7)	8.3–10.3 (7)	23.3–34.2 (7)	0.31–0.49 (7)	0.843–1.45 (5)	2.73–5.08 (5)	2,643–19,542 (7)	1,278–11,778 (7)	7.87–14.6 (5)
	Mean (SD)	0.023 (0.057)	8.91 (0.71)	28.9 (3.73)	0.41 (0.06)	1.12 (0.25)	3.86 (0.889)	13,336 (6,059)	3,307 (3,769)	10.0 (2.69)
Female										
BV	Range (*n*)	0–3.67 (6)	7–8.9 (6)	10.2–20.5 (6)	0.29–0.32 (6)	0.09–1.36 (4)	0.74–9.06 (4)	424–2,536 (6)	410–1,394 (6)	0.516–16.8 (4)
	Mean (SD)	0.889 (1.50)	7.53 (0.75)	13.3 (3.96)	0.30 (0.01)	0.52 (0.60)	4.01 (3.98)	1,099 (851.627)	767 (397)	8.53 (9.17)
LP	Range (*n*)	0.135–22.9 (22)	8.3–11.6 (22)	30.5–57.8 (22)	0.37–0.62 (22)	0.34–14.4 (19)	1.07–25.2 (19)	1,772–11,960 (19)	563–6,296 (19)	3.02–119 (19)
	Mean (SD)	6.595 (5.784)	9.68 (0.05)	40.3 (8.8)	0.45 (0.07)	4.58 (3.46)	11.7 (7.37)	5,763 (2,544)	2,528 (1,610)	66.4 (39.7)
OW	Range (*n*)	0.027–5.1 (11)	7.2–8.3 (11)	12–20.1 (11)	0.28–0.40 (11)	0.11–2.15 (9)	0.87–14.2 (9)	367–4,404 (11)	78.1–1,593 (11)	1.14–165 (9)
	Mean (SD)	0.897 (1.53)	7.73 (0.36)	15.8 (2.4)	0.34 (0.04)	0.83 (0.83)	5.22 (5.15)	1,323 (1,220)	782 (659)	34.7 (52.7)
ER	Range (*n*)	0–6.98 (6)	8.5–9.8 (6)	21–35 (6)	0.31–0.40 (6)	1.14–6.58 (4)	4.75–21.1 (4)	1,011–4,078 (6)	412–1,176 (6)	24.45–80.8 (4)
	Mean (SD)	3.022 (2.742)	9.12 (0.44)	27 (4.8)	0.35 (0.03)	4.5 (2.35)	15.1 (7.25)	2,356 (1,389)	815 (302)	48.6 (24.2)
RS	Range (*n*)	0–0.866 (8)	6.2–8.7 (10)	7.4–12.3 (10)	0.13–0.32 (10)	0.10–0.579 (8)	1.41–6.89 (8)	765–3,389 (7)	799–6,842 (7)	0.91–1.95 (8)
	Mean (SD)	0.235 (0.331)	7.25 (0.65)	9.81 (1.5)	0.26 (0.05)	0.33 (0.14)	3.26 (1.624)	2,108 (889)	2,128 (2,116)	1.28 (0.41)
WM	Range (*n*)	1.93–24.6 (6)	7.9–8.8 (6)	23.6–30.9 (6)	0.42–0.61 (6)	2.30–4.85 (4)	8.12–16.5 (4)	875–13,459 (6)	1,285–10,560 (6)	12.8–53.6 (4)
	Mean (SD)	10.413 (8.495)	8.32 (0.31)	27.5 (2.7)	0.48 (0.07)	3.59 (1.2)	12.5 (4.43)	5,960 (4,729)	5,077 (3,644)	37.4 (17.5)

[a] VTG = vitellogenin; GSI = gonadosomatic index; E2 = 17β-estradiol; T = testosterone; BV = Bellevue; LP = limnology pond; OW = Owosso; ER = Eaton Rapids; RS = Looking Glass River; WM = Williamston.