A Comparison of the Reproductive Physiology of Largemouth Bass, Micropterus salmoides, Collected from the Escambia and Blackwater Rivers in Florida

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Largemouth bass (LMB), Micropterus salmoides, were taken from the Escambia River (contaminated site) and the Blackwater River (reference site) near Pensacola, Florida. The Escambia River collection occurred downstream of the effluent of two identified point sources of pollution. These point sources included a coal-fired electric power plant and a chemical company. Conversely, the Blackwater River’s headwaters and most of its length flow within a state park. Although there is some development on the lower part of the Blackwater River, fish were collected in the more pristine upper regions. Fish were captured by electroshocking and were maintained in aerated coolers. Physical measurements were obtained, blood was taken, and liver and gonads were removed. LMB plasma was assayed for the concentration of 17β-estradiol (E2) and testosterone using radioimmunoassays. The presence of vitellogenin was determined by gel electrophoresis (SDS-PAGE) and Western blotting using a monoclonal antibody validated for largemouth bass vitellogenin. No differences in plasma concentrations of E2, or testosterone were observed in females from the two sites. Similarly, males exhibited no difference in plasma E2. However, plasma testosterone was lower in the males from the contaminated site, as compared to the reference site. Vitellogenic males occurred only at the contaminated site. Additionally, liver mass was proportionately higher in males from the contaminated site, as compared to males from the reference site. These data suggest that reproductive steroid levels may have been altered by increased hepatic enzyme activity, and the presence of vitellogenic males indicates that an exogenous source of estrogen was present in the Escambia River. Key words: endocrine disrupting chemicals, hepatic detoxification enzymes, hepatosomatic index, steroidogenesis, vitellogenin. Environ Health Perspect 107:199–204 (1999). [Online 29 January 1999] http://ehpnet1.niehs.nih.gov/docs/1999/107p199-204orlando/abstract.html

Certain environmental contaminants can alter the reproductive physiology, growth, and development of vertebrates by disrupting the normal functioning of the endocrine system (1). Such environmental contaminants have been called endocrine disrupting chemicals (EDCs). Examples of EDCs in fish include naturally occurring phytoestrogens or phytooestrogens as well as pesticides from agricultural runoff, heavy metals, halogenated aromatic hydrocarbons, and alkylphenol ethoxylates from industrial effluent (2).

Fish exposed to heavy metals such as aluminum, cadmium, and lead have shown alterations in their hormonal milieu. Exposure to aluminum caused an increase in the plasma concentrations of the thyroid hormones triiodothyronine (T3) and thyroxine (T4), whereas exposure to cadmium and lead caused increased in vitro concentrations of 17β-estradiol (E2) and testosterone (2–4). Some pesticides, such as malathion and endosulfan, decrease plasma E2, testosterone and vitellogenin (Vtg), as well as inhibit germinall vesicle breakdown and spawning in fish (5,6). Other pesticides, for example, α,α′-DDT and α,α′-DDE, were shown to act as estrogen mimics, inducing Vtg production (7,8).

Exposure to halogenated aromatic hydrocarbons (AHs), such as the polychlorinated biphenyl (PCB) mixture Aroclor 1254, decreases steroidogenesis and increases hepatic enzyme activity in fish (9). Similar studies have shown decreases in fertilization and hatching success of fish exposed in the wild to PCBs (10).

Exposure to polychlorinated dibenzo-furan (PCDFs), tetrachlorodibenzodioxin (TCDD), or 3-methylcholanthrene decreased Vtg production, increased hepatic detoxifying enzyme synthesis, and decreased plasma hormone concentrations of T3 and T4 in some fish (5,11,12).

Fish exposed to effluent mixtures from sewage plants and paper mills have exhibited a range of alterations to their reproductive physiology. Exposure to sewage effluent induced Vtg production in male fish, increased liver size, decreased plasma testosterone, and inhibited testicular growth (13–16). Fish exposed to paper mill effluent showed reduced concentrations of steroid hormones, elevated hepatic detoxifying enzyme synthesis, and reduced egg and gonad sizes (17–19).

Many of the aforementioned studies were on nonnative fish collected from the wild or farm-reared fish exposed via cages in situ. Other studies used farm-reared fish and exposed them to contaminants in captivity. These studies evoked the following question: Are there measurable differences in the reproductive physiology of native, wild, adult fish, such as the largemouth bass, Micropterus salmoides, living in a polluted and a relatively pristine river in Florida? This initial study addresses this question by comparing liver size, plasma concentrations of reproductive steroid hormones, and the presence of Vtg in the plasma of largemouth bass from these rivers.

Materials and Methods

Fish. Seventy adult largemouth bass (LMB), Micropterus salmoides, were captured from the Escambia and Blackwater rivers near Pensacola, Florida (Fig. 1). In the Escambia River, we collected fish immediately downstream of the Crist Electric Generating Plant (a coal-fired electric utility) and the Monsanto Company (a nylon fiber and chemical intermediates manufacturing plant). Of the total, 26 females and 15 males came from the Escambia River and 13 females and 16 males were collected from the Blackwater River. The LMB were collected between 28 February and 7 March 1996. Fish were taken by electroshocking, using a boat-mounted generator and inverter (20). The electrical output parameters of the inverter were approximately 200 volts, 5 amps, and 55% pulse width using alternating or pulsed direct current, based on the conductivity of the water.

After electroshocking, LMB were removed from the water with hand dip-nets and transferred to aerated and insulated holding tanks. Fish were transported to the Environmental Protection Agency Laboratory, Gulf Breeze, Florida, where somatic measurements were taken, blood was collected, and tissues excised.

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Standard length and total mass of each fish were recorded, with means ± standard errors (SE) as follows: Escambia River females, 234 ± 11 mm and 312 ± 41 g; males, 222 ± 14 mm and 303 ± 42 g; Blackwater River females, 291 ± 22 mm and 779 ± 166 g; and males, 275 ± 8 mm and 544 ± 49 g. Fish were anesthetized using MS-222 (50 ppm; Sigma Chemical Company, St. Louis, MO) and blood samples were taken. Fish were euthanized by decapitation and then gonads and liver were removed. Immediately after the mass of these tissues were obtained, a small slice of liver was snap frozen in liquid nitrogen and the rest of the liver and gonads were placed in Bouin’s fixative (21).

All lab work was conducted in full compliance with the guidelines of the University of Florida Institutional Animal Care and Use Committee. Fieldwork was conducted under permit from the Florida Game and Freshwater Fish Commission.

Radioimmunoassays. Plasma concentrations of E₂ and testosterone were measured using a radioimmunoassay (RIA) validated for LMB. The RIA protocol followed here is a modification of the protocol described elsewhere (22). Antibodies to E₂ (stock # E26-47) and testosterone (stock # TS-125) were obtained from Endocrine Sciences, Calabasas, California. Cross-reactivity of the E₂ antibody was as follows: estrone, 1.3%; estradiol, 0.6%; 16-keto-estradiol, 0.2%; all other ligands tested, <0.2%. For the testosterone antibody, the cross-reactivity was as follows: dihydrotestosterone, 44%; Δ1-dihydrotestosterone, 41%; Δ1-androsterone, 18%; 5α-androstan-3β,17β-diol, 3%; 4-androsten-3β,17β-diol, 1.5%; E₁, 0.5%; all other ligands tested, <0.2%. ³H-E₂ and ³H-testosterone were purchased from Amersham Life Sciences, Inc., Arlington Heights, Illinois. E₂ and testosterone standards were bought from Sigma Chemical Company. All other assay constituents were ordered from Becton Dickinson, Franklin Lakes, New Jersey.

From each LMB, blood was drawn from the caudal vein and placed into heparinized Vacutainer tubes (Becton Dickinson) into which aprotinin (2.52 IU/ml blood; Sigma Chemical Company) was added to prevent proteolysis of the vitelligenin. Blood samples were placed on ice (for less than 6 hr) before centrifugation for 20 min at 2,000g. Plasma was removed, snap frozen in liquid nitrogen, and stored at -80°C until assayed for steroid hormones.

Plasma samples (100 μl for E₂ and 75 μl for testosterone) were extracted twice using 5 ml ethyl ether. Ether was vaporized under a stream of filtered, low-humidity air. The samples were resuspended with 100 μl 0.5 M borate buffer (pH 8.0); then 200 μl antibody, 100 μl bovine serum albumin borate buffer, and 100 μl ³H-hormone were added. Antibody final dilutions were 1:55,000 for E₂ and 1:36,000 for testosterone. Final sample volume was 500 μl, and all assay tubes were run in duplicate. E₂ and testosterone concentrations of 1.5625, 3.125, 6.25, 12.5, 25, 50, 100, 200, 400, and 800 pg/tube constituted the standard curves.

Samples were incubated overnight at 4°C; 500 μl 5% charcoal/0.5% dextran/0.5M phosphate-buffered saline (PBS) mixture was then added to separate the bound from free hormone. The tubes were vortexed and centrifuged at 2000g for 30 min. Following centrifugation, the supernatant containing the bound hormone was decanted. Fisher ScintiVerse BD scintillation cocktail (5 ml; Fisher Chemical Company) was added and the samples were counted on a Beckman scintillation counter, model LS 5801 (Beckman Instruments, Irvine, CA).

Extraction efficiencies were 95.13% for E₂ and 99.89% for testosterone. Assays were validated by running an internal standard curve and a plasma dilution curve on pooled LMB plasma and comparing them to the assay standard curve. For the internal standard curve, 1.0625, 3.125, 6.25, 12.5, 25, 50, 100, 200, 400, and 800 pg of hormone standard was combined with 100 μl stripped plasma. Different volumes, 100, 80, 60, 40, 20, and 0 μl, of stripped plasma were combined with 0, 20, 40, 60, 80, and 100 μl, respectively, of pooled plasma to make the plasma dilution curve. Both internal standards and plasma dilution curves were assayed as described above. Parallelism between the internal standards, plasma dilutions, and the assay standard curves was tested using homogeneity of slopes (SuperANOVA, Abacus Concepts, Berkeley, CA).

**Gel electrophoresis and Western blotting.** The yolk protein precursor Vtg was detected in the plasma of LMB using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. For both techniques, unless stated otherwise, all assay chemicals were purchased from Fisher Chemical Company. The Vtg monoclonal antibody (mAb NL 1080 (1CB-3C11), validated for LMB Vtg (23)) was characterized in the laboratory of Nancy Denslow (Protein Chemistry Core Laboratory, Interdisciplinary Center for Biotechnology Research, University of Florida).

LMB plasma proteins were separated using an 8% Tris-glycine SDS-PAGE following a modified protocol (24, 25). Molecular weight markers and LMB plasma samples were diluted with Laemmli sample buffer (1:1 and 1:9 ratios, respectively). Rainbow Colored Protein Molecular Weight Markers (7 μl; Amersham) and 10 μl of LMB plasma samples were loaded into the gels in duplicate. Gels were run at 50 volts DC for 25 min and then at 120 volts for approximately 90 min at room temperature. One gel was stained with Coomassie Brilliant Blue (R250) for 45 min and destained overnight at room temperature.
The other gel was used for identification of the Vtg protein band by Western blotting.

For Western blotting, the protein bands were transblotted to a polyvinylidenedifluoride (PVDF) membrane (Immobilon-P, Millipore Corp., Bedford, MA). Protein transfer was performed at 20 volts overnight at 4°C. The membrane was blocked to prevent nonspecific binding with 5% Bovine serum albumin (25 g dry milk, 500 ml Tris-buffered Tween-20 (TTBS), 0.1g NaNO₃) for 1 hr at room temperature. The membrane was then incubated overnight at 4°C with the primary antibody, mcAb HL 1080 (1C8-3C11), diluted (1:1) in 5% Bovine serum albumin. After washing the membrane in TTBS, it was probed with secondary antibody (goat IgG, antimouse linked to alkaline phosphatase; Bio-Rad Laboratories, Richmond, CA) diluted in 5% Bovine serum albumin (1:3000) for 2 hr at room temperature. The membrane was again washed in TTBS. Plasma Vtg was visualized by adding the chromogens, bromochloroindolyl phosphate, and nitro-blue tetrazolium chloride to the membrane (50 mg/ml each). LMB Vtg was detected by observation of a dense band at approximately 200 kDa.

**Statistics.** The gonadosomatic index (GSI, gonad mass/total body mass x 100) and the hepatosomatic index (HSI, liver mass/total body mass x 100) data were analyzed using a two-sample t-test. Robustness of the t-test was invoked for the assumptions of normal distribution and homogeneity of variance (26).

To control for differences in steroidogenic capability of fish of different sizes, plasma concentration of E₂ and testosterone were analyzed using a one-way analysis of covariance (ANCOVA), with total body mass as the covariant. Heterogeneity of variance was corrected by log transforming the data (26).

The Fisher's exact test was chosen to analyze the data for the presence of circulating Vtg. A chi-square test could be used to analyze this type of nominal value, but since less than 80% of the expected values had n <5, the Fisher's exact test was the appropriate choice (26,27).

All data are reported as the mean ± one SE, and significance was determined at p<0.05. All reported values are nontransformed data.

**Results**

There was no difference in the GSI for females (F = 0.055, degrees of freedom (df) = 1.33; p=0.82) or males (F = 0.01; df = 1.24; p=0.94) between sites. The GSIs for females from the contaminated and reference sites were 1.69 ± 0.35 and 1.56 ± 0.39, respectively, whereas the GSIs were 0.28 ± 0.05 for contaminated males and 0.29 ± 0.03 for reference males.

There was no evidence to indicate a difference in the HSI between sites for females (F = 0.02; df = 1.36; p<0.88). However, the males from the contaminated site had a larger HSI when compared to the reference site males (F = 11.22; df = 1.29; p<0.002) (Fig. 2).

Plasma concentrations of the steroid hormones E₂ (F = 0.21; df = 1.31; p>0.65) and testosterone (F = 0.04; df = 1.22; p>0.85) did not differ for females between sites (Fig. 3). However, it is interesting to note the relatively small sample size and large variance in the female plasma testosterone concentrations (Fig. 4).

There was no statistical difference in plasma E₂ concentrations in males (F = 0.68; df = 1.15; p>0.68) between sites (Fig. 3). There was a difference, however, in the concentration of plasma testosterone in males (F = 14.34; df = 1.22; p<0.001). Males from the contaminated site had significantly lower circulating levels of testosterone compared to reference males (Fig. 4).

In the measurement of the reproductive steroid hormones E₂ and testosterone, the total number of samples analyzed is different from the number of fish collected from each site. This is due to an insufficient volume of plasma collected.

For the presence of Vtg in the blood, no statistical difference was observed in female (p>0.30) or male LMB (p=0.23) between sites. Twelve of 27 contaminated females compared to 8 of 12 reference females had detectable concentrations of circulating Vtg. Even though there was no statistical difference in the presence of Vtg in males between sites, it is important to note that 3 of 17 males from the contaminated site had detectable concentrations of plasma Vtg. Conversely, no males out of a sample size of 14 from the reference site were measurably vitellogenic (Figs. 5 and 6).

**Discussion**

The results of this study show an increase in liver size, decrease in plasma testosterone concentration, and the presence of Vtg in the plasma of some of the males from the Escambia River. There was no difference in liver size, plasma testosterone concentration, and the presence of Vtg in females. Also, there was no difference in plasma E₂ in either females or males between sites.

When comparing the reproductive physiology of adult fish from two sites, it is important to know that they are at the same reproductive stage. Every end point measured in this study, including liver size, reproductive steroid hormones, and vitellogenin, varies with the stage of reproduction (28–30). The design of this study accounted for this concern, as all fish were collected within a 10-day period. To further test the assumption that all fish exhibited a similar reproductive stage, we examined their GSI. The GSI has been shown to be a reasonably good indicator of reproductive maturity (31,32). The GSI of both female and male largemouth bass collected from the Blackwater (reference) and Escambia (contaminated) Rivers. Values are means ± standard error; n is shown within each bar.

*Significant difference (p<0.05).
LMB were not significantly different between sites, suggesting they were not at different stages of reproductive maturity. Limited histological examination of the gonads supported this conclusion. Therefore, the reproductive physiology of LMB from the two sites could be compared with confidence.

HSI has been shown to be a good indicator of liver size in fish (33). Liver size of female LMB did not differ between sites. In contrast, liver size of males from the Escambia River (contaminated site) were significantly larger than the liver size of males collected from the Blackwater River (reference site). What could cause this increase in liver size? We suggest two hypotheses to explain these data.

First, Vtg production occurs in the liver of oviparous and viviparous female fish upon estrogen stimulation (34). Males, however, can also produce Vtg when exposed to natural estrogens such as E2, to a synthetic estrogen in a lab setting, or by exposure in their environment (35). This production of Vtg causes an increase in the size of the liver (33). In this study, Vtg was expressed only in the males from the contaminated site. Even though there was no difference in the mean HSI of vitellogenic males (0.800) compared to nonvitellogenic males (0.830), perhaps the nonvitellogenic males were induced, but producing Vtg at concentrations below the assay sensitivity (5 μg/ml). Another possible hypothesis is that the increase in liver size of the contaminated-site males was due in part to Vtg production, but also to zona radiata protein (ZRP) synthesis. ZRPs are inducible by natural and environmental estrogens such as certain alkyl phenol ethoxylates. Because both Vtg production (33) and hepatic detoxification enzyme synthesis are known to increase liver size (17,37), it seems reasonable to expect that ZRP production would also increase liver size.

There was no significant difference in female liver size between sites. Because female LMB from the contaminated site were exposed to the same contaminants as the males, one would expect a similar increase in liver size due to stimulation of Vtg and/or ZRP synthesis. This expectation is not reflected in the data because vitellogenic female livers are greatly increased in size due to stimulation from endogenous E2 (33). We hypothesize that further stimulation by an environmental contaminant would produce a relatively smaller change in size that would be difficult to discern in the already stimulated liver.

Second, when fish are exposed to toxic substances, these substances must be removed from circulation to reduce cell damage (38). The liver is the main site of detoxification, and the presence of certain contaminants in the blood stimulates the synthesis of hepatic detoxification enzymes. In hepatocytes, enzymes are produced that conjugate the toxin with a glucuronide or a sulfate group. These groups make compounds such as organochlorines more hydrophilic and, consequently, more readily cleared by the kidneys (39). Another route for removal of contaminants is via biliary excretion through the digestive tract. Livers that are stimulated and actively producing these enzymes are known to increase in size (17,37).

Although the difference in male liver size could be a function of the increase in the production of hepatic detoxification enzymes, how can there be no significant difference in female liver size between sites? Again, we suggest the relative increase in the female liver size, due to an increase in enzyme production, is small compared to the size increase associated with active vitellogenesis in the female.

Is there a relationship between the increase in liver size (possibly due to stimulation of hepatic detoxification enzyme synthesis and vitellogenesis) in the contaminated-site males and the decrease in plasma concentration of testosterone? Some hepatic detoxification enzymes belong to the P450 superfamily. Included in this family are some of the conversion enzymes of the steroidogenic pathway (for example, the P450 side-chain cleavage enzyme, which converts cholesterol to pregnenolone). Some studies have shown an increase in detoxifying enzyme production with a concomitant decrease in specific steroid hormones (9,17,18).

Our steroid hormone data demonstrated that males from the contaminated site had lower plasma testosterone concentrations. Plasma testosterone concentrations in females were not significantly different between sites. Although we cannot state definitively, the trend in the data suggests that with a larger sample size, the relationship in plasma testosterone concentrations in females between the two sites might be similar to that observed in the male LMB.

Alteration in the normal hormonal profile of fish can be caused by contaminants interacting with the endocrine system at one or more places in the hypothalamic–pituitary–gonadal (H-P-G) axis (40). For example, a contaminant could decrease plasma testosterone by providing negative feedback to the pituitary, thereby decreasing the release of gonadotropins. Similar negative
feedback could occur at the level of the gonad (4f). Phytoestrogens, such as β-sitossterol found in paper mill effluent, can decrease the plasma concentrations of testosterone by reducing the availability of cholesterol or its conversion to pregnenolone (42). Compounds such as HAls found in paper mill effluent, polynuclear aromatic hydrocarbons (PAHs) produced by the burning of fossil fuels, or alkyl phenol ethoxylates, commonly associated with many manufacturing processes, have been shown to disrupt the endocrine system. These contaminants can induce vitellogenesis, provide negative feedback to reduce steroidogenesis, or cause an increase in hepatic enzymes known to degrade steroids and detoxify contaminants (43-45).

There was no significant difference between sites in the proportion of females or males producing Vtg. However, such large amounts of plasma vitellogenin is an abnormal condition in males (35,46). Some studies have shown very low concentrations of Vtg in male fish (47). However, the results of these studies are suspect, because in each of these studies, the fish were kept in water of questionable quality (river water, which at the time of these studies, was not known to contain endocrine-disrupting chemicals). It is important to recognize that the presence of Vtg in three males occurred only in LMB from the Escambia River. These males had Vtg bands that were similar in density and thickness to the vitellogenic females (Fig. 6). We found no vitellogenic males from the Blackwater River. Although the exact source could not be determined here, this research suggests the presence of a xenoestrogen in the Escambia River.

There is a growing concern with the realization that environmental contamin-ants of human origin can alter the reproductive physiology of fish and other vertebrates, as well as invertebrates, in ways previously undocumented. This alteration is caused by relatively low concentrations of pollutants disrupting normal endocrine system function. Previous endocrine disruption research in fish has shown a range of effects such as decreased egg size and sperm motility, reduced embryo/fry viability, modified hormone profiles, and altered reproductive behavior (2).

In this study, we asked whether we could measure a difference in the reproductive physiology of fish living in a polluted river and a reference river. To our knowledge, this is the first study to demonstrate endocrine disruption in a wild, native gamefish exposed to industrial effluent in the state of Florida. This research is important in that it shows a measurable alteration in several parameters in the reproductive physiology of the largemouth bass collected from the Escambia River.

Future studies should investigate the source of the xenoestrogen(s) and probe the mechanism(s) effecting the decrease in circulating testosterone. It would be interesting to examine both steroid hormone metabolism and detoxification enzyme activity (e.g., ethoxyresorufin-o-deethylase; EROD) in the livers of LMB from both sites.

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