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An evaluation of biomarkers of reproductive function and potential contaminant effects in Florida largemouth bass (*Micropterus salmoides floridanus*) sampled from the St. Johns River

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Abstract

The objective of this study was to describe and compare several reproductive parameters for Florida largemouth bass (*Micropterus salmoides floridanus*) inhabiting the St. Johns River and exposed to different types and/or degrees of contamination. Welaka was selected as the reference site in this study because of its low urban and agricultural development, Palatka is in close proximity to a paper mill plant, the Green Cove site is influenced by marine shipping activities and Julington Creek site receives discharges of domestic wastewater and storm water runoff from recreational boating marinas. For this study, bass were sampled both prior to (September 1996) and during the spawning season (February 1997). In order to characterize chemical exposure, bass livers were analyzed for up to 90 trace organics and 11 trace metal contaminants. Reproductive parameters measured included gonadosomatic index (GSI), histological evaluation of gonads and plasma concentrations of vitellogenin (VTG), 17β-estradiol (E_2) and 11-ketotestosterone (11-KT). In general, the sum of organic chemicals was highest in livers from Palatka bass and bass from Green Cove and Julington Creek had higher hepatic concentrations of low molecular polycyclic aromatic

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hydrocarbons and polychlorinated biphenyls when compared to fish from Welaka. Metals were more variable across sites, with highest mean concentrations found in bass from either Julington Creek (Ag, As, Cr, Cu, Zn) or Welaka (Cd, Hg, Pb, Se, Tn). Female bass from Palatka and Green Cove had lower concentrations of E_2, VTG and lower GSI in relation to Welaka. Males from Palatka and Green Cove showed comparable declines in 11-KT in relation to males from Julington Creek and GSI were decreased only in Palatka males. These results indicate a geographical trend in reproductive effects, with changes being most pronounced at the site closest to the paper mill (Palatka) and decreasing as the St. Johns River flows downstream. Since reproductive alterations were most evident in bass sampled from the site closest to the paper mill discharge, it is possible that exposure to these effluents might explain at least some of the results reported here. However, the presence of reproductive alterations in fish sampled at a considerable distance from the mill discharge (Green Cove, 40 km) would suggest exposure to chemicals released from sources other than the paper mill plant. It is clear that additional studies are needed to evaluate the potential impact of these reproductive changes in populations of Florida largemouth bass inhabiting the St. Johns River.

Keywords: Endocrine disruption; Reproductive biomarkers; Environmental contaminants; Largemouth bass; *Micropterus salmoides* floridanus; Florida; Fish; Sex hormones; Vitellogenin

1. Introduction

Chemical release of industrial and agricultural contaminants into aquatic environments has prompted scientists to evaluate potential effects of pollutants on both human and ecosystem health. Since fish play a fundamental role in aquatic ecosystems, they have been widely used as monitors of environmental health and quality. These assessments should include an evaluation of reproductive health of wild populations through the use of a battery of reproductive indicators or biomarkers. This is of great importance, because reproductive impairment due to environmental contaminants or to other causes, could lead to gradual declines in population numbers and thus, to an increase in the probability of extinction.

Over the past decade, a great deal of interest has arisen from the potential effects of endocrine active chemicals on wildlife and humans. In fact, endocrine-modulating effects of environmental contaminants have been observed or suspected in almost all taxa, ranging from invertebrates to fish, reptiles, amphibians, birds and mammals. At least 45 chemicals or their metabolites have been suggested as having endocrine-modulating activity that could lead to adverse population-level effects in wildlife (Colborn et al., 1993). Some of these chemicals include chlorinated pesticides (e.g. DDT complex), halogenated aromatic hydrocarbons (e.g. dioxins, furans, and polychlorinated biphenyls or PCBs) and heavy metals. In addition, there is considerable evidence indicating that exposure of fish to complex mixtures such as effluents discharged by sewage and paper mill plants can also lead to endocrine alterations (Matthiessen and Sumpter, 1998; Munkittrick et al., 1998).

The objective of this study was to describe and compare several reproductive parameters for Florida largemouth bass (*Micropterus salmoides floridanus*) inhabiting the St. Johns River and exposed to different types and/or degrees of contamination. For this study, bass were sampled both prior to and during the spawning season. Parameters measured included body weight, length, condition factor, gonadosomatic index (GSI), histological evaluation of gonads, and plasma concentrations of vitellogenin (VTG), 17β-estradiol (E_2) and 11-ketotestosterone (11-KT).

2. Materials and methods

2.1. Study area and sampling protocol

Four bass sampling sites were selected from a downstream segment of the St. Johns River (approx. 80 km in length), from its confluence with the Oklawaha River and downstream to the city of Jacksonville (Fig. 1). Sites from south to north

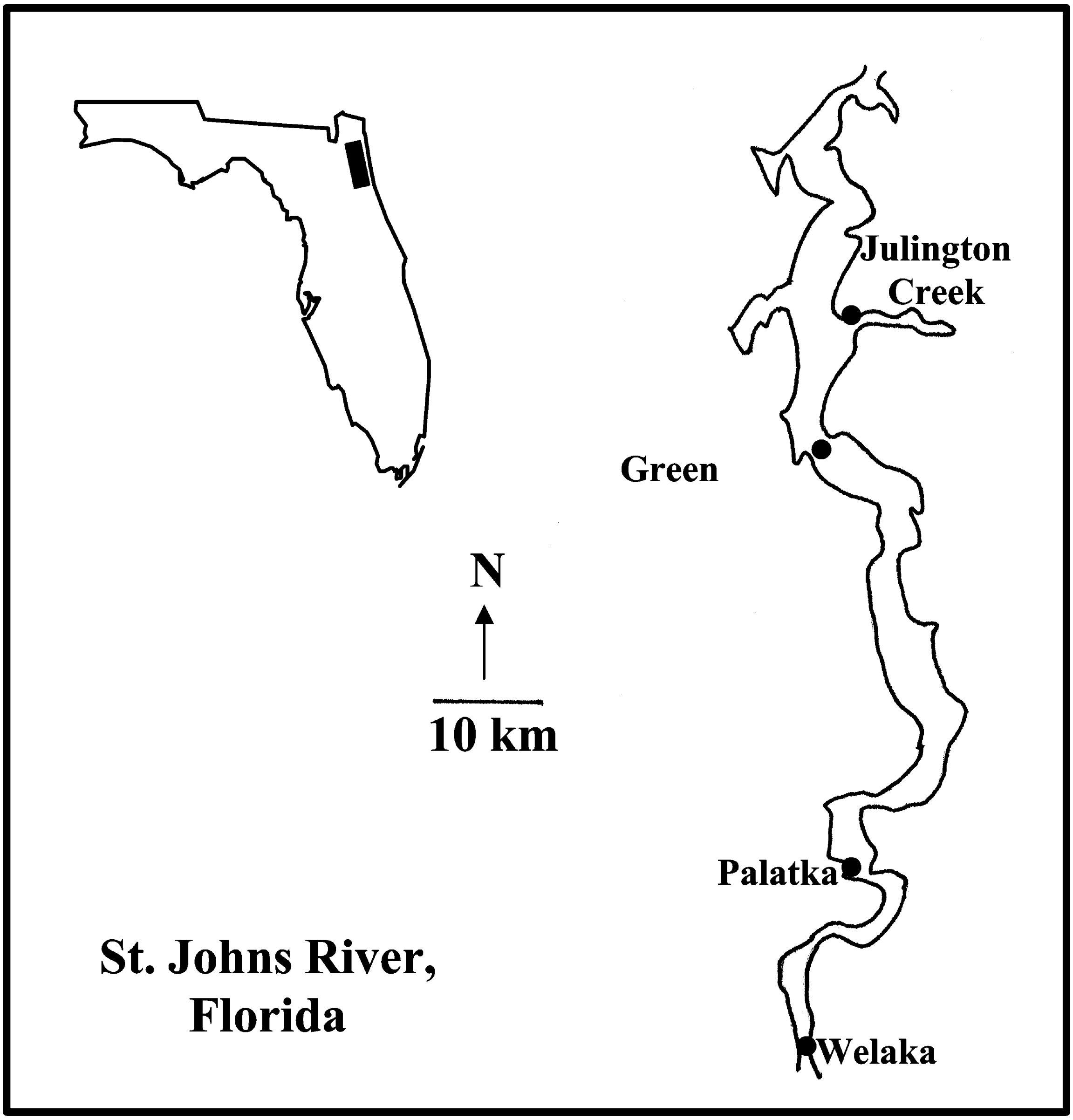

Fig. 1. Map of the St. Johns River in North-Central Florida showing the study sites. The direction of River flow is north.

were as follows: Welaka, near the confluence of the Oklawaha and the St. Johns River; Palatka (21 km north of Welaka) at the Rice Creek confluence; Green Cove (40 km north of Palatka) upstream of the Black Creek confluence; and Julington Creek (10 km north of Green Cove), located at the confluence of Julington Creek with the St. Johns River (Fig. 1). It was anticipated that these four sites would reflect differing contaminant exposure and body burdens. Welaka (the most upstream site) was selected as the reference or control site in this study because of its low urban and agricultural development. Activity of this rural community centers on sports fishery industry. The Palatka and Green Cove sites are representative of urban, industrial and agricultural development. The Palatka site is in close proximity (< 5 km) to a paper mill plant, whereas Green Cove site is influenced by marine shipping activities. Finally, the Julington Creek site receives discharges of domestic wastewater, and storm water runoff from recreational boating marinas.

Largemouth bass were collected during September 1996 (pre-spawning season; 46 females and 30 males) and February 1997 (spawning season; 36 females and 48 males) by electroshocking from the four sites. In order to minimize possible seasonal variation in reproductive parameters, all fish in this study were collected within a 1-week period. Fish were weighed using a portable digital scale to the nearest 0.1 g and body length measured (total length, from the tip of the mouth to the tip of the tail) to the nearest millimeter. Condition factor was calculated as $K = 100 \times$ weight/length^3. Immediately after fish were caught, they were bled from the caudal vein using 3-ml syringes and 1.5 inch, 20-G needles. Blood samples were transferred to 5-ml heparinized vacutainers and kept on ice until centrifugation for 10 min at $1000 \times g$ for the collection of plasma. Plasma was pipetted into 2-ml cryotubes and stored at -80°C until analyzed. Fish were euthanized with a blow to the head and gonads excised weighed for the determination of GSI ($100 \times$ gonad weight/(body weight $-$ gonad weight), and a section preserved in 10% buffered formalin for assessment of reproductive status through histological evaluation. Gonadal information was obtained only during the spawning season collections.

2.2. Reproductive biomarkers

Plasma samples from largemouth bass were analyzed for E_2 and 11-KT using radioimmunoassay (RIA) procedures. All plasma samples were assayed in duplicate and values are reported as pg/ml of plasma. Plasma samples (50 μl) were extracted twice with 5 ml diethyl ether prior to RIA analysis (Scintillation Counter Pachard Tricarb, Model 1600). Plasma samples were corrected for extraction efficiencies of 87 ± 3.5% and 83 ± 2.8% for E_2 and 11-KT, respectively. Standard curves (1, 5, 10, 25, 50, 100, 250, 500 and 1000 pg) were prepared in buffer with known amounts of radioinert E_2 (ICN Biomedicals, Costa Mesa, CA, USA) and 11-KT (Sigma Chemical, St. Louis, MO, USA). The minimum concentration distinguishable from zero was 5.1 pg/ml for E_2 and 9.3 pg/ml for 11-KT. Specific antibodies against sex steroids were purchased from either ICN Biomedicals (E_2) or Helix Biotech, Richmond, BC, Canada (11-KT). Cross-reactivities of the E_2 antiserum with other steroids were: 11.2% for estrone; 1.7% for estriol; < 1.0% for 17α-estradiol and androstenedione; and < 0.1% for all other steroids examined. Cross-reactivities of the 11-KT antiserum with other steroids were: 9.65% for testosterone; 3.7% for α-dihydrotestosterone, < 1.0% for androstenedione and < 0.1% for all other steroids examined. Pooled samples (approximately 310 pg/ml of E_2 and 275 pg/ml of 11-KT) were assayed serially in 10, 20, 30, 40, and 50-μl volumes (final volume of 50 μl with charcoal-stripped plasma). The resulting inhibition curves were parallel to the respective standard curves, with the tests for homogeneity of regression indicating that the curves did not differ. Further characterization of the assays involved measurement of known amounts of hormones (1, 2, 5, 10, 25, 50, 100, 250 and 500 pg) in 50 μl charcoal-stripped plasma [(for E_2: $Y = -9.63 + 1.12X$, $R^2 = 0.9081$; for 11-KT: $Y = 13.8 + 0.93X$, $R^2 = 0.8767$; Y = amount of hormone (pg); X = amount of hormone added (pg)]. Interassay and intra-assay coefficients of variation were 5.8 and 7.9%, respectively, for E_2, and 6.4 and 8.4% for 11-KT.

VTG concentrations in plasma of largemouth bass were quantified by Direct Enzyme-Linked Immunosorbent Assay (ELISA). VTG was first purified by anion exchange chromatography (LMB VTG 102396B) and its protein concentration determined by the Bradford method (Bradford, 1976) for use as a standard. Standard curves were constructed by adding serial dilutions of purified largemouth bass VTG (0 mg/ml to 0.001 mg/ml) to male control plasma and processed the same way as samples. Male control plasma was made from a pool of plasma from fish collected at an uncontaminated site, which was shown by Direct ELISA and Western Blot analysis to have non-detectable VTG. The monoclonal antibody, Mab 3G2 Ascites 109 AB (produced by the Hybrydoma Core, University of Florida) was used in the ELISA assay. This antibody reacts with high specificity and sensitivity to largemouth bass VTG, with little or no cross-reaction with other plasma proteins. Plasma samples were diluted 1:200 (male samples) or 1:10 000 (female samples) in phosphate buffer saline azide (PBSZ 0.15 M NaCl, 10 nM phoshapte, 0.02% NaN_3, pH 7.2) with aprotinin (10 K IU/ml), 50 μl was added in triplicate to microtitre plate wells and incubated overnight at 4°C in a humidified chamber. Plates were washed with PBSZ plus tween (PBSTZ, 0.05% Tween-20), blocked with 360 μl/well of blocking buffer [1% bovine serum albumin (BSA) and 10 mM Tris BSTZ] for 2 h at room temperature and washed again with PBSTZ. Purified monoclonal antibody was diluted with blocking buffer with aprotinin to 3 μg/ml for male runs and to 0.1 μg/ml for female runs, coated onto 96-well microtitre plates (50 μl/well) and stored overnight at 4°C in a humified chamber. The next day plates were washed with Tris BSTZ, incubated with 50 μl/well polyclonal biotinylated goat mouse anti-VTG IgG antibody (H + L) (Pierce, Rockford, IL, USA), diluted to 1:1000 with blocking buffer, and incubated for 1 h at room temperature. Plates were washed with Tris BSTZ, and incubated with 50 μl/well of strep-avidin-alkaline phosphatase, diluted to 1:1000 with blocking buffer, for 1 h at room temperature. After a final wash with Tris BSTZ, 100 μl/well of *p*-nitro phenyl phosphate in carbonate buffer (pH 9.6) was added to each well and incubated at room temperature in the dark for 30 min. The intensity of yellow color that developed was quantified at 405 nm with an automated ELISA reader (Spectra Max 250, Molecular Devices, Sunnyvale, CA, USA). Standard curves fitted by quadratic regression were used to calculate VTG concentrations, with R^2 values usually between 0.95 and 0.99. In order to test for interassay and intra-assay variation, each assay was run with a positive control that had a known VTG concentration. Samples were rerun if the coefficient of variation between triplicates exceeded 10%. The minimum concentration detectable in this assay was 0.001 mg/ml. VTG values are reported as mg/ml of plasma.

Ovaries were classified into four stages of sexual maturation: previtellogenic (stage 1, primary and secondary oocytes, no vitellogenic oocytes) and early, mid, or late vitellogenic (stages 2, 3, or 4 ranging from the presence of few and small vitellogenic oocytes to large oocytes containing numerous vitelline granules). Similarly, testes were classified into three stages of sexual maturation: low; moderate; or high spermatogenic activity (stages 1, 2, or 3 ranging from a thin germinal epithelium and scattered spermatogenic activity, to a thick germinal epithelium with high proliferation and maturation of sperm).

2.3. Exposure assessment

Bass livers were collected and analyzed for up to 90 trace organics and 11 trace metal contaminants to characterize potential chemical exposure. Livers from 9 to 11 females were collected from each of the sites during September 1996 and February 1997. Livers from the 1997 collection were pooled for each of the four sites and analyzed as composite samples. Sample collections, laboratory analysis and quality control procedures were carried out as previously described (St. Johns Water Management District, 1998). In brief, for determination of organics, samples were serially extracted using dichloromethane, filtered, concentrated and purified using a high performance liquid chromatographic gel permeation chromatography (HPLC-GPC) cleanup procedure. The purified extract was then analyzed through gas chromatography-mass spectrometry (GC-MS) for determination of polycyclic aromatic hydrocarbons (PAHs) and phthalates, or through GC/electron capture detection for analysis of PCBs, hexachlorocyclohexanes (BHCs), pesticides (e.g. dichlorodiphenyltrichloroethane and derivatives, DDTs) and other chlorinated compounds. For metals, samples were freeze dried, homogenized and then digested with a mixture of hot nitric acid and hydrogen peroxide. Metal concentrations were measured either by cold-vapor atomic absorption spectroscopy for mercury, or inductively coupled plasma/mass spectrometry (ICP-MS) for the ten other metals. Contamination data are reported on a dry weight basis and were not corrected for lipid content, nor percent recoveries. In this study, livers had a mean lipid and moisture content of 9.3 ± 1.1 and $79.3 \pm 0.3\%$, respectively.

2.4. Statistical analyses

Pairwise comparisons were conducted using a two-way analysis of covariance (ANCOVA) (PROC GLM, SAS Institute, 1988) within sexes to test for differences in the dependent variables among sites. Data sets that did not meet the criteria of normality and homogeneity of variance (PROC UNIVARIATE) were log or arcsin transformed. For these analyses, season (spawning or non-spawning) was used as the second cofactor and body weight was used as the covariate. If the ANCOVA showed a significant site effect, a Dunnett's multiple comparison test was used to examine if Palatka, Green Cove, or Julington Creek differed from Welaka. The frequency distributions of different gonadal developmental stages were compared among sites using a χ^2-test (PROC FREQ). Statistical significance was assessed at $P < 0.05$.

3. Results

Fish chemical data is summarized in Table 1. Unfortunately, this data set was not powerful enough to detect clear trends across sites, probably because of the small number of samples analyzed, which resulted in a high degree of variation. Nevertheless, with the exception of BHCs, the sum of organics appeared highest in livers from bass collected from Palatka when compared to fish from Welaka (increases ranged from 1.5 to 7-fold). There was also a trend for a decline in organic chemicals in fish from Green Cove and Julington Creek in relation to fish from Palatka, with several groups being lower (low molecular PAHs and BHCs) or comparable (chlorinated benzenes and other chlorinated pesticides) to values found in Welaka fish (Table 1). Metals were more variable across sites, with highest mean concentrations found in bass from either Julington Creek (Ag, As, Cr, Cu, Zn) or Welaka (Cd, Hg, Pb, Se, Tn). Liver metal concentrations found in this study, however, are below those known to induce sublethal effects in other species of freshwater fish (Jarvinen and Ankley, 1999).

The average ± S.E.M. weight, length and condition factor for female fish in this study were 1069 ± 86 g, 401 ± 10 mm and 1.49 ± 0.1, respectively. The corresponding values for males were 739 ± 46 g, 364 ± 6 mm and 1.43 ± 0.04, respectively. Pre-spawning females from Green Cove and Julington Creek were smaller and lighter (358 ± 17 mm and 699 ± 151 g for both sites combined) when compared to pre-spawning females from Welaka (500 ± 3 mm and 1776 ± 242 g), whereas male body measurements did not differ across sites. For both sexes, body weights, lengths and condition factor did not differ across seasons.

Reproductive parameters/biomarkers for bass are presented in Table 2. For both sexes, although changes in sex steroids and VTG among sites were observed as early as 5 months prior to spawning (September), differences were most evident in spawning fish (February).

Females from Palatka and Green Cove had GSI that were approximately half of those seen in Welaka fish (Table 2). For both sampling periods, sex hormones (11-KT and E_2) were decreased in females from Green Cove and Palatka when compared to Welaka bass. Whereas E_2 showed a seasonal increase in fish from all sites (increased from an average of 325 pg/ml in pre-spawning bass to 927 pg/ml in spawning fish), 11-KT decreased only in Palatka and Welaka females (from 406 to 206 pg/ml). VTG concentrations were approximately twelve times lower in spawning females from Palatka and Green Cove in relation to bass from Welaka (means of 0.42 and 4.9 mg/ml, respectively). Although Julington Creek females had approximately half the concentration of this protein when compared to bass from Welaka, this difference was not significant. Seasonal changes in VTG concentrations were observed in females from all sites, except Green Cove (from

Table 1
Organic (μg/kg dry wt.) and metal (mg/kg dry wt.) contaminant data from livers of largemouth bass sampled along the St. Johns River during September 1996 and March 1997 ($n = 9$, 11, 10 and 10 females for sites Welaka, Palatka, Green Cove and Julington Creek, respectively). Liver samples from 1997 were pooled and run as one sample

Chemical Group	Welaka[a]	Palatka	Green Cove	Julington Creek
Organics (Σ of)[b]				
1) Low molecular weight PAHs	30.0	52.2	24.0	17.1
2) High molecular weight PAHs	3.70	27.1	10.6	15.3
3) DDTs	15.5	26.4	19.3	17.7
4) PCBs	40.0	158	99.2	98.0
5) Chlordanes	8.20	11.8	8.90	12.1
6) BHCs	2.80	1.10	0.88	0.25
7) Chlorinated Benzenes	79.7	117	60.0	71.1
8) Cyclodiene Pesticides	1.10	3.10	1.60	2.60
9) Other Chlorinated Pesticides	2.80	3.80	2.00	1.80
Metals (Means)				
Silver (Ag)	0.05	0.04	0.02	0.36
Arsenic (As)	0.35	0.41	0.46	0.62
Cadmium (Cd)	0.11	0.09	0.05	0.07
Chromium (Cr)	0.17	0.31	0.26	0.36
Copper (Cu)	4.80	10.3	5.00	15.7
Mercury (Hg)	1.30	0.97	0.51	0.50
Nickel (Ni)	0.06	0.10	0.09	0.08
Lead (Pb)	0.10	0.01	0.02	0.04
Selenium (Se)	2.00	1.70	1.70	1.80
Tin (Tn)	0.16	0.12	–[c]	0.10
Zinc (Zn)	32.0	38.9	31.7	40.0

[a]Location of sites can be found in Fig. 1.

[b](1) Anthracene, fluorene, naphthalene, 2-methylnapthalene, 1-methylnapthalene, 2,6-dimethylnapthalene, 2,3,5-trimethylnapthalene, biphenyl, acenaphthylene, 1-chloronapthalene, 2-chloronapthalene, acenaphthene, phenanthrene, 1-methylphenanthrene; (2) fluoranthene, pyrene, perylene, chrysene, benzo[*a*]anthracene, benzo[*b*]fluoroanthene, benzo[*k*]fluoranthene, benzo[*e*]pyrene, benzo[*a*]pyrene, indenol[*1,2,3-c,d*]pyrene, dibenzo[*a,h*]anthracene, benzo[*g,h,I*]perylene; (3) 2,4′-DDT, 4,4′-DDT, 2,4′-DDE, 4,4′-DDE, 2,4′-DDD, 4,4′-DDD; (4) Σ of 25 isomers; (5) oxychlordane, δ-chlordane, *cis*-chlordane, *cis*-nonachlor, *trans*-nonachlor, heptachlor, heptachlor epoxide; (6) α-BHC, β-BHC, δ-BHC, γ-BHC; (7) 1,2-dichlorobenzene, 1,3-dichlorobenzene, 1,4-dichlorobenzene, 1,2,4,5-tetrachlorobenzene, 1,2,4-trichlorobenzene, hexachlorobenzene, hexachlorobutadiene, hexachlorocyclopentadiene, hexachloroethane;(8) aldrin, dieldrin, endrin, endrin aldehyde, endrin ketone; (9) endosulfan I, endosulfan II, endosulfan sulfate, mirex.

[c]Not determined.

September to February it increased almost 500-fold from 0.005 to 2.6 mg/ml). Sex steroid and VTG differences among sites occurred despite the fact that female bass were in similar reproductive condition ($\chi^2 = 3.2$, $P = 0.78$, with 80% of females being in stage 4 or late vitellogenic).

Males from Palatka and Julington Creek had lower and higher GSI, respectively when compared to males from Welaka (Table 2). Pre-spawning males from Palatka and Julington Creek had lower concentrations of 11-KT when compared to Welaka, but when sampled during the spawning season, Welaka males had concentrations of 11-KT that were over twice of those found in males from other sites. With the exception of spawning males from Julington Creek, E_2 concentrations were increased in bass from all sites in relation to Welaka males. There were seasonal changes in the concentration of 11-KT in males from all sites, except Green Cove (increased from a mean of 294 pg/ml in September to 704 pg/ml in February) and declines in E_2

Table 2
Reproductive parameters of largemouth bass sampled along the St. Johns River during the pre-spawning (September 1996) and spawning (February 1997) seasons

Site[a]	Fish (n)	E_2 (pg/ml)	11-KT (pg/ml)	Vitellogenin (mg/ml)	GSI[b] (%)
Pre-spawning					
Females					
Welaka	12	341 ± 39	213 ± 18	0.006 ± 0.003	–[c]
Palatka	11	231 ± 23*	198 ± 19	0.007 ± 0.002	–
Green Cove	10	434 ± 45	393 ± 42**	Non-detected	–
J. Creek	13	305 ± 41	279 ± 17	0.004 ± 0.002	–
Males					
Welaka	8	208 ± 22	392 ± 11	0.003 ± 0.002[d]	–
Palatka	9	269 ± 18*	210 ± 19**	0.006 ± 0.003	–
Green Cove	7	362 ± 19*	399 ± 43	Non-detected	–
J. Creek	6	329 ± 20*	289 ± 37**	0.002 ± 0.001	–
Spawning					
Females					
Welaka	8	1283 ± 220	305 ± 26	4.9 ± 1.5	4.1 ± 0.6
Palatka	12	631 ± 112*	474 ± 0.41	0.63 ± 0.48*	1.9 ± 0.4*
Green Cove	9	1035 ± 229	596 ± 94*	0.14 ± 0.06*	2.0 ± 0.5*
J. Creek	7	892 ± 88	377 ± 48	3.3 ± 1.8	4.0 ± 0.6
Males					
Welaka	15	242 ± 25	990 ± 154	0.004 ± 0.002	0.3 ± 0.02
Palatka	8	463 ± 102**	425 ± 21**	0.01 ± 0.005	0.2 ± 0.02*
Green Cove	12	452 ± 73**	400 ± 73**	0.006 ± 0.002	0.4 ± 0.06
J. Creek	13	184 ± 30**	546 ± 26**	0.006 ± 0.003	0.4 ± 0.03*

Values reported are means ± S.E.M. Asterisks indicate differences in relation to Welaka (ANCOVA, Dunnett's multiple comparison test; $*P = 0.02–0.001$; $**P < 0.0005$).
[a] Location of sites can be found in Fig. 1.
[b] GSI = 100 × gonad weight/(body weight − gonad weight).
[c] Not determined.
[d] The average VTG concentrations in males are based on values above detection limits (> 0.001 mg/ml). This corresponded to 30, 71, 47 and 47% of the samples analyzed for male bass from Welaka, Palatka, Green Cove and J. Creek, respectively. Only values above detection limits were included for statistical comparisons.

from pre-spawning to spawning condition were observed only in Julington Creek males (declined from 329 pg/ml to 184 pg/ml). Approximately half of the males sampled (47%) had measurable concentrations of VTG (> 0.001 mg/ml). Although male bass had comparable concentrations of VTG across sites, as with females, plasma concentrations of this protein changed seasonally increasing from a mean of 0.003 mg/ml in September to a mean of 0.006 mg/ml in February. Similarly to what was observed with females, there were no differences in the reproductive condition of male bass across sites ($\chi^2 = 1.1$, $P = 0.76$, with 73% of males being in stage 3 or high spermatogenic activity).

4. Discussion

Although dioxins were not measured in livers of largemouth bass in this study, there is some data on 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in sediment and fish from Rice Creek, the tributary receiving the direct discharge of the

Palatka mill. Schell et al. (1993) reported up to 52.8 ppt of TCDD in sediments collected from Rice Creek, with significant declines in the concentration of this chemical at the confluence with the St. Johns River (6.8 ppt). These authors also reported concentrations of TCDD in liver and gonads of largemouth bass (range 1.8–8.8 ppt), bowfin, *Amia calva* (11.2–46.1 ppt) and brown bullhead catfish, *Ictalurus nebulosus* (1.8–2.8 ppt) collected from Rice Creek.

In the present study, GSI were decreased only in females from Palatka and Green Cove. This decline could have been due to a decrease in circulating VTG levels, which in turn could have resulted from an imbalance between E_2 and 11-KT concentrations. GSI in males was lowered only in fish collected from the site closest to the mill discharge (Palatka), despite a similar decline in 11-KT in males from Green Cove and Julington Creek (46% decline overall). Similarly to what was observed with females from Julington Creek, males from this site exhibited normal GSI, which could have been due to a lack of an increase in E_2 in relation to males from Palatka and Green Cove. These results would suggest that examining the ratio of E_2 to 11-KT concentrations might be an important marker to consider when evaluating endocrine disruption in this species. Previous studies with largemouth bass have reported that E_2/11-KT ratios above 2 and below 1.0 are expected in healthy reproductively active female and male bass, respectively (Sepúlveda, 2000). In the present study, spawning female and male bass from Welaka had E_2/11-KT ratios of 4.2 and 0.3, respectively, whereas bass collected from the remaining streams exhibited an imbalance between the concentration of these two sex steroids (overall E_2/11-KT ratios of 1.5 in females and of 1.1 in males). Elevated plasma concentrations of E_2 relative to testosterone (T) have also been reported in male largemouth bass inhabiting other contaminated sites (Escambia River) in Florida (Orlando et al., 1999).

In the present study, VTG was detected in 47% of the males sampled. These concentrations, however, were low and although increased from September to February, its high variability precluded the detection of any differences across sites. Finding detectable concentrations of this protein in plasma of male fish has generally been considered as a sign of potential endocrine disruption associated with exposure to estrogenic compounds (Sumpter and Jobling, 1995; Denslow et al., 1999). Some studies, however, have documented low background concentrations of VTG in different species of male fish, and have regarded such concentrations as physiologically normal (Copeland and Thomas, 1988; Ding et al., 1989). This is not completely unexpected, since male fish (including bass) are known to have circulating levels of E_2, the single known inducer of VTG production (Rosenblum et al., 1987; Guiguen et al., 1993; Zaki et al., 1995). Although at this time we cannot rule out the possibility that males could have been exposed to chemicals with estrogenic effects, the mere finding of low concentrations of this protein complicates the interpretative value of this biomarker as a definitive sign of endocrine disruption in this species. Indeed, results from the present study would suggest that low plasma VTG concentrations (< 0.01 mg/ml) are probably normal in male largemouth bass.

In summary, female bass sampled from Palatka and Green Cove had lower concentrations of E_2, VTG and lower GSI in relation to Welaka. Males from Palatka and Green Cove showed higher increases in E_2 and comparable declines in 11-KT in relation to males from Julington Creek and GSI were decreased only in Palatka males. These results indicate a geographical trend in reproductive effects, with changes being most pronounced at the site closest to the paper mill (Palatka) and decreasing as the St. Johns River flows downstream (north). Several studies have shown reproductive alterations in different species of fish exposed to bleached kraft mill effluents (BKME). One of the most consistent findings from these studies has been a decline in the concentration of sex steroids in plasma of exposed animals. For example, BKME-exposed white suckers (*Catostomus commersoni*) from Jackfish Bay, Canada, had decreased concentrations of several sex steroid hormones (T, 11-KT, E_2 and 17,20β-dihydroxy-4-pregnen-3-one or 17,20β-P, a maturation inducing steroid) (McMaster et al., 1996).

These hormonal changes have generally been associated with decreased gonadal sizes, secondary sexual characteristics and egg sizes and increased age to maturity. Results from studies on white sucker indicate that the pituitary–gonadal-axis is affected after exposure to BKME. Fish from exposed sites had significantly lower plasma levels of gonadotropin (GtH-II) and showed depressed responsiveness of sex steroids and 17,20β-P after injections with gonadotropin releasing hormone (GnRH) (Van Der Kraak et al., 1992). In vitro incubations of ovarian follicles collected from BKME-exposed females also exhibited reduced production of T, E_2 and 17,20β-P 2 under basal and human chorionic gonadotropin stimulated conditions (Van Der Kraak et al., 1992). The similarities between both types of studies would suggest that reductions in plasma steroid levels in BKME-exposed fish are due to alterations in ovarian steroid production. In a review of whole organism responses of fish exposed to different kinds of mill effluents (including unbleached pulps), 80% of the populations examined showed increased age to sexual maturation and reduced gonadal size was reported in 58% of the studies (Sandström, 1996). Results from our laboratory have also shown altered reproductive parameters in largemouth bass exposed to BKME (Sepúlveda, 2001). Indeed, bass exposed to these effluents responded with changes at the biochemical level (decline in sex steroids in both sexes and VTG in females) that were usually translated into tissue/organ-level responses (declines in GSI in both sexes and in ovarian development in females). Although the compounds responsible for these effects have not yet been identified, it has been hypothesized that these changes might be related to exposure to natural components of wood (such as resin acids, sterols and lignins), which have been reported to have weak estrogenic activity (Van Der Kraak et al., 1998).

The presence of reproductive alterations in fish sampled at a considerable distance from the mill discharge (Green Cove, 40 km) would suggest exposure to chemicals released from sources other than the paper mill plant. In this respect, fish from Green Cove had approximately three times the tissue burden concentration of high molecular weight PAHs and over twice the concentrations of PCBs when compared to fish from Welaka. There is considerable evidence from laboratory and field studies showing endocrine alterations in fish due to exposure to these types of compounds. For example, reduced plasma T in males and E_2 and progesterone in females, accompanied by an increase in several sex steroid-metabolizing enzymes, was observed in carp (*Cyprinus carpio*) injected with 250 mg/kg of the commercial PCB, Aroclor 1248 (Yano and Matsuyama, 1986). Atlantic croaker (*Micropogonius undulates*) fed benzo[*a*]pyrene, a common type of PAH, at a rate of 0.4 mg/70 g/day for 30 days during the period of ovarian recrudescence experienced decreased GSI with a concomitant reduction in plasma E_2 and T concentrations (Thomas, 1988). Similarly, several field studies have documented altered reproductive activity in fish residing in PCB and PAH-contaminated waters. For instance, gonadal development was impaired and E_2 concentrations were depressed in English sole (*Pleuronectes vetulus*) from highly contaminated areas of Puget Sound, Washington. The reproductive impairments were statistically correlated with elevated PAH concentrations, as measured by the presence of fluorescent aromatic compounds in the bile of the fish (Johnson et al., 1988). PCB levels in the liver and ovarian tissue of this species of fish have also been associated with the spawning of fewer eggs (Johnson et al., 1997).

Although field studies are important and necessary because they provide ecological relevance, they are subject to many limitations. For example, it is often difficult to derive cause and effect relationships from these types of studies because fish are likely to be exposed to a variety of chemicals as well as non-chemical stressors. In addition, there is a great deal of uncertainty in regards to the extent (doses and lengths) and mode (routes) of chemical exposure of free-ranging fish, which can seriously hinder data interpretation. In this respect, it is important to consider the degree of mobility of the species under study if conclusions regarding point sources of pollution are intended. Information on the range of movement of Florida largemouth bass is limited. Snyder et al. (1986) reported that 38% of the bass marked and released in the lower St. Johns River were recaptured in the same area as tagged and of the remaining 62%, 44% had moved a distance of < 2 km. In another study, 84% of specimens tagged moved less than 8 km, with a maximum distance of 20 km (cited by Hardy, 1978). Due to the mobile and sedentary habitat utilization characteristics of this species, a direct relationship between tissue chemical data and point source of contamination must be assessed with caution. Nevertheless, the present study is the first to evaluate the potential reproductive effects of environmental contaminants in Florida largemouth bass inhabiting the St. Johns River.

In conclusion, lower and potentially sensitive levels of biological organization (biochemical: such as sex hormones and VTG) were altered in largemouth bass from contaminated streams. Overall, these changes were associated with impacts at higher and less sensitive levels of organization (organ: such as gonad weights). Since reproductive alterations were most evident in bass sampled from the site closest to the paper mill discharge, it is possible that exposure to these effluents might explain at least some of the results reported here. It is clear that additional studies are needed to evaluate the potential impact of these reproductive changes in populations of Florida largemouth bass inhabiting the St. Johns River. Future field study designs should incorporate the capability for testing the relationship between chemical exposure and biological responses and should be accompanied by controlled laboratory studies that explore dose-response relationships to better interpret the data generated.

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