

Reproductive and biochemical biomarkers in largemouth bass sampled downstream of a pulp and paper mill in Florida

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Abstract

The objective of this study was to evaluate the effects of bleached/unbleached kraft mill effluents (B/UKME) on the reproductive parameters of free-ranging Florida largemouth bass (*Micropterus salmoides floridanus*). The reproductive parameters measured included gonadosomatic index (GSI), histological evaluation of gonads, and plasma concentrations of vitellogenin (VTG), 17 β -estradiol, and 11-ketotestosterone (11-KT). Hepatic ethoxyresorufin-*O*-deethylase (EROD) activity was measured as a marker of exposure to cytochrome P450-inducing agents in these effluents. Endpoints were compared among adult bass sampled from tributary and mainstream effluent-contaminated and reference sites. Females sampled from the site closest to the mill outfall had a significant five-fold increase in EROD activity compared to bass sampled from reference streams. Although sex hormones were significantly reduced in bass from exposed sites, there were no differences in VTG and GSI across sites. The absence of organism-level responses was probably not related to a lack of sensitivity, as previous studies in our laboratory have shown that bass exposed to these effluents exhibit changes in GSI and in other measures associated with reproductive success. In females, inverse relationships were observed between VTG and GSI and EROD activity. These relationships, however, were not consistent within all of the sites studied. Collectively, our findings indicate that hepatic EROD induction is an effective marker of B/UKME exposure in largemouth bass and that it might be associated with antiestrogenic effects in this species.

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1. Introduction

Over the past decade, several Canadian and Scandinavian studies have focused on the effects of bleached kraft pulp mill effluent (BKME) on multiple biochemical and physiological parameters of fish. From these studies, some of the most meaningful responses have been related to altered reproductive function. Specifically, fish exposed to BKME have lower circulating concentrations of reproductive hormones, reduced gonadal size, increased age to sexual maturation, smaller eggs, and reduced expression of secondary sex characteristics compared to fish from reference sites (Andersson et al., 1988; McMaster et al., 1996; Munkittrick et al., 1998).

Effects of paper mill effluents on the reproductive physiology of fish have also been documented in Florida, USA. Female mosquitofish (*Gambusia affinis holbrooki*) inhabiting a stream receiving paper mill effluent were “masculinized” exhibiting elongation of the anal fin, which in male fish forms an intromittent organ used for internal fertilization, or gonopodium (Howell et al., 1980; Parks et al., 2001). More recently, masculinization of female fish has been suggested for both mosquitofish and from two additional species (least killifish, *Heterandria formosa*, and sailfin molly, *Poecilia latipinna*) collected from Rice Creek, the receiving stream for the effluents discharged by the Palatka paper mill plant in Central Florida (Bortone and Cody, 1999). In addition, a survey conducted during 1996 and 1997 with Florida largemouth bass (*Micropterus salmoides floridanus*) reported that alterations in reproductive performance, as measured by decreased sex steroids and gonadosomatic indices (GSI) in both sexes and decreased vitellogenin (VTG) in females, were highly

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evident in fish sampled closest to the Palatka mill outfall (Sepúlveda et al., 2002b).

The objective of this study was to further evaluate the potential impact(s) of environmental exposure to paper mill effluents on the reproductive physiology of free-ranging largemouth bass. This was accomplished by increasing the robustness of the data analysis by increasing the sample size of fish captured and by extending the number of sites sampled in proximity to the mill relative to past studies. We hypothesized that bass sampled in the closest proximity to the mill outfall would respond with decreased reproductive function, reflected in decreased concentrations of sex steroids and VTG and a decreased GSI, relative to fish from reference streams. Because BKME are known to contain cytochrome P4501A (CYP1A)-inducing compounds, hepatic ethoxyresorufin-*O*-deethylase (EROD) activity was used as a biomarker of exposure to these effluents (Bankey et al., 1994; Förlin et al., 1985; Hodson, 1996). Furthermore, because *in vitro* studies with fish hepatocytes have suggested reproductive impairment as a consequence of hepatic CYP1A1 induction (Anderson et al., 1996), a secondary objective of this study was to evaluate the relationship between CYP1A induction and reproductive parameters in bass. We hypothesized that higher hepatic EROD activities would be associated with decreased reproductive function.

2. Materials and methods

2.1. Description of pulp and paper mill

Georgia-Pacific's Palatka plant is a kraft mill that produces a 50/50 mix of bleached/unbleached market pulp. During the time our study was conducted, the mill released an estimated 36 million gallons of effluent/day and production averaged 1452 air-dried metric tons pulp/day from a furnish that consisted of 80% softwood and 20% hardwoods. Bleaching sequences were C₉₀d₁₀EopHDp and CEHD for the softwoods and hardwoods, respectively, with Cd being a mixture of chlorine (C) and chlorine dioxide (d) in proportions designated by subscripts; Eop being an extraction with alkali and the addition of elemental oxygen (o) and hydrogen peroxide (p); H being hypochlorite; and Dp being 100% d substitution with the addition of p. At the time of this study, effluents received secondary treatment. This consisted of both anaerobic (200-ha basin) and aerobic (200-ha basin) biological degradation for a retention period of 40 days. Treated effluents are discharged into Rice Creek, a tributary that runs for about 5 km before its confluence with the St. Johns River. Because Rice Creek is small, effluents can account for a large portion of its total flow (the yearly average effluent concentration is estimated to be around

60%, with a range of 50–97%). The Palatka paper mill plant has been in operation for over 50 years. Presently, this mill is implementing a series of important renovations necessary to comply with the U.S. Environmental Protection Agency (EPA) Cluster Rule promulgated in 1998. Some of these changes include the use of chlorine dioxide bleaching instead of elemental chlorine and of oxygen and hydrogen peroxide bleaching instead of sodium hypochlorite. Improvements in secondary treatment of effluents are also underway. The reader should refer to Sepúlveda et al. (2001) for a chemical characterization of these effluents.

2.2. Field study: sampling sites and fish collections

During March 1998, approximately 10 largemouth bass of each sex (total of 61 females and 53 males) were collected by electroshocking from six sites within the St. Johns River (mainstream) and tributaries (small creeks) (Fig. 1). Areas sampled included two tributary reference sites, Cedar Creek (located approximately 30 km from the mill) and Etonia Creek (the primary water source for the mill, located 100–200 m upstream of the effluent discharge), and one effluent-dominated tributary site (Rice Creek, a small tributary stream receiving direct discharge from the mill). Fish were also sampled from three mainstream sites: Welaka and Dunn's Creek (reference sites located approximately 40 and 20 km upstream of the effluent discharge, respectively) and Palatka (a contaminated mainstream site receiving discharge from the Rice Creek tributary). The average estimated paper mill effluent concentrations in the Rice Creek and Palatka sites are 60% and less than 10% of discharge, respectively [Georgia-Pacific, personal

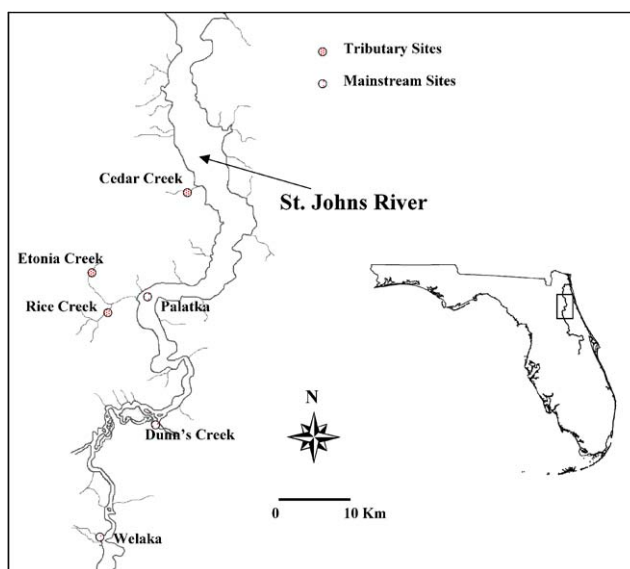


Fig. 1. Map of the St. Johns River (mainstream) and tributaries in North-central Florida from which largemouth bass were sampled during March 1998. The direction of river flow is North.

communication, and see Quinn et al. (2003)]. However, water flow in Rice Creek is tidally influenced; during periods of low flow, mill effluents can account for up to 90% of the total flow (Schell et al., 1993). Reference sites were matched to contaminated sites in most physico-chemical characteristics, with the exception of the presence of effluent. In order to minimize the variation in the parameters measured in relation to the timing of the reproductive season, all fish within each site were collected within an average of 4 h, and all sites were sampled within a 1-week period. Rice Creek was the only exception to this sampling protocol, as there it was necessary to collect largemouth bass on three different occasions over a 2-week period to achieve adequate numbers.

2.3. Body measurements and reproductive biomarkers

On capture, bass were weighed using a portable digital scale and the total length was recorded. Condition factor was calculated as $K = \text{weight}/\text{length}^3 \times 100$. Fish were bled from the caudal vein, and blood samples were refrigerated until centrifugation for 10 min at 1000g for the collection of plasma. Plasma was stored at -80°C until analyzed for sex steroids [11-ketotestosterone (11-KT) and 17β -estradiol (E_2)] using a radioimmunoassay technique and VTG (through a direct enzyme-linked immunosorbent assay) as described in Sepúlveda et al. (2002b). Fish were euthanized, and gonads and livers were excised and weighed for the determination of gonadosomatic and hepatosomatic (HSI) indices ($100 \times \text{gonad or liver weight}/\text{body weight} - \text{gonad or liver weight}$). A representative sample of gonad was also saved in 10% buffered formalin for histological examination after standard Mayer's hematoxylin and eosin staining. Testes were cut longitudinally and ovaries were cut transversally. Tissue samples were then embedded in paraffin, sectioned at $5\ \mu\text{m}$, mounted on glass slides, air dried, and stained. Ovaries and testes were classified into different stages of sexual maturation as described in Sepúlveda et al. (2002b). In addition, the number of atretic follicles was counted in each histologic section. Fish were decapitated for the removal of sagittal otoliths, which were used for the determination of age as described in Crawford et al. (1989).

2.4. Hepatic EROD activity

Buffers, substrates, and cofactors were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Snap-frozen liver pieces (approximately 250 mg of tissue, w/w) were homogenized with 3 vol (v/w) of homogenizing buffer consisting of 10 mM TRIS (pH 7.4), 250 mM sucrose, 1 mM EDTA, 0.2 mM dithiothreitol, and 0.1 mM phenylmethylsulfonyl fluoride. Samples were homogenized for approximately 10 s and the homo-

genates were further centrifuged at 8000g for 10 min. The resulting supernatant was further centrifuged at 12000g for 20 min to obtain an S-9 fraction containing cytosolic and microsomal fractions and stored at -80°C until analyzed. The S-9 protein concentrations were assayed with a Bio-Rad (Richmond, CA, USA) protein assay kit using bovine serum albumin as a standard. Hepatic EROD activities were measured in triplicate using the S-9 fractions and a Spectromax Fmax 96-well fluorescent microplate reader at an excitation wavelength of 544 nm and emission of 590 nm. For this reaction, 5 μL of enzyme (S-9) was mixed with 195 μL of assay buffer (0.1 M NaPO_4 , pH 7.8) and 5 μL of substrate (100 μM ethoxyresorufin in methanol). The reaction was started by adding 5 μL NADPH and the fluorescence change recorded for 2 min at 30°C . Negative controls were run in the absence of enzyme and positive controls in the presence of rat microsomal fractions. Reaction linearity was demonstrated over the course of the reactions. A resorufin standard curve (0, 1, 2.5, 5, 10, and 20 pmol/well) was generated for each set of samples, and the initial rates were determined by dividing the rate of change in fluorescence per minute by the slope of the resorufin standard curve. Results are expressed as picomoles of resorufin formed/minute/milligram S-9 protein.

2.5. Statistical analyses

Pair-wise 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 between sites. Data sets that did not meet the criteria of normality and homogeneity of variance (PROC UNIVARIATE) were log or arcsin transformed. Since preliminary analyses showed differences in reproductive parameters in fish from different-size streams, type of stream (tributary or mainstream) was used as a cofactor and age was used as the covariate. Since fish parameters from the reference sites were not statistically different within each type of stream, data were pooled. For this study, the exposed sites, Palatka and Rice Creek, were compared to the reference sites, Welaka and Dunn's Creek and Cedar and Etonia Creeks, respectively. In addition, regressions of liver EROD activity and several reproductive parameters were calculated. The frequency distributions of different gonadal developmental stages were compared between sites using a χ^2 test (PROC FREQ). Statistical significance was assessed at $P \leq 0.05$.

3. Results

A summary of several physiological and reproductive parameters measured from largemouth bass sampled

Table 1
Age, length, weight, and condition factor (*k*) of largemouth bass sampled along the St. Johns River during the spawning season (March) of 1998

Site ^a	Total no. fish sampled	Age (years)	Length (cm)	Weight (g)	Condition factor (<i>k</i>)
Tributaries, females					
Cedar (10) and Etonia (7) Creeks (reference)	17	4.5±0.2	40.6±1.7	1062±159	1.41±0.06
Rice Creek (exposed)	13	3.6±0.3*	41.4±1.6	1012±121	1.35±0.06
Tributaries, males					
Cedar (8) and Etonia (10) Creeks (reference)	18	5.0±0.5	34.4±1.1	678±72	1.56±0.04
Rice Creek (exposed)	6	4.6±1.4	35.8±2.5	622±91	1.34±0.08
Mainstream, females					
Welaka (10) and Dunn's (10) Creek (reference)	20	3.5±0.2	37.0±1.0	698±68	1.33±0.04
Palatka (exposed)	11	3.4±0.3	37.9±1.3	809±87	1.43±0.04
Mainstream, males					
Welaka (10) and Dunn's (10) Creek (reference)	20	4.0±0.3	35.0±0.7	606±37	1.39±0.02
Palatka (exposed)	9	5.0±0.5	37.1±1.0	705±57	1.36±0.05

Fish were collected from tributaries or mainstream sites. Values reported are means±SEM (sample sizes are in parentheses). Asterisks indicate differences in relation to reference sites (Cedar and Etonia Creeks for tributaries or Welaka and Dunn's Creek for mainstream sites).

^aLocations of sites can be found in Fig. 1.

Table 2
Reproductive endpoints of largemouth bass sampled along the St. Johns River during the spawning season (March) of 1998

Site ^a	Total no. fish sampled	GSI (%)	HSI (%)	E ₂ (pg/mL)	11-KT (pg/mL)	VTG (mg/mL)
Tributaries, females						
Cedar (10) and Etonia (7) Creeks (reference)	17	2.61±0.47	1.3±0.18	1322±145	491±76	0.83±0.22
Rice Creek (exposed)	13	2.08±0.54	0.99±0.11	482±130*	274±49*	0.27±0.09
Tributaries, males						
Cedar (8) and Etonia (10) Creeks (reference)	18	0.38±0.04	1.48±0.21	521±81	1562±173	0.06 and 1.51 ^b
Rice Creek (exposed)	6	0.25±0.07	1.23±0.32	312±45	237±118*	<0.001 ^c
Mainstream, females						
Welaka (10) and Dunn's Creek (10) (reference)	20	2.97±0.39	1.07±0.08	1703±200	491±58	0.87±0.18
Palatka (exposed)	11	4.30±0.7	1.12±0.12	612±73*	300±48*	0.50±0.11
Mainstream, males						
Welaka (10) and Dunn's Creek (10) (reference)	20	0.48±0.02	0.94±0.07	593±102	1537±171	2.58 ^b
Palatka (exposed)	9	0.44±0.04	0.85±0.11	387±47	775±119*	<0.001 ^c

GSI, gonadosomatic index; HSI, hepatosomatic index; E₂, 17β-estradiol; 11-KT, 11-ketotestosterone; VTG, vitellogenin.

Fish were collected from tributaries or mainstream sites. Values reported are means±SEM (sample sizes are in parentheses). Asterisks indicate differences in relation to reference sites (Cedar and Etonia Creeks for tributaries or Welaka and Dunn's Creek for mainstream sites).

^aLocations of sites can be found in Fig. 1.

^bVTG concentrations were above detection limits in two males from Cedar Creek and in one male from Welaka.

^cAll samples fell below the detection limit.

along the St. Johns River during March 1998 are presented in Tables 1 and 2. Except that females from the Rice Creek tributary were younger compared to those from the reference tributary sites, fish size and condition were similar across sites (Table 1).

In relation to the reproductive parameters in females, organosomatic indices were similar between the exposed and reference sites (Table 2). Concentrations of sex hormones were significantly reduced in females from the

exposed tributary and mainstream sites (overall reductions of 65% and 42% for E₂ and 11-KT, respectively). Although VTG concentrations appeared decreased in females sampled from exposed streams, these differences were not statistically significant. For both types of stream, there were no differences in the frequency distribution of ovarian stages in females sampled from reference streams in relation to exposed fish (Fig. 2A). Approximately 74% of the females were in moderate to

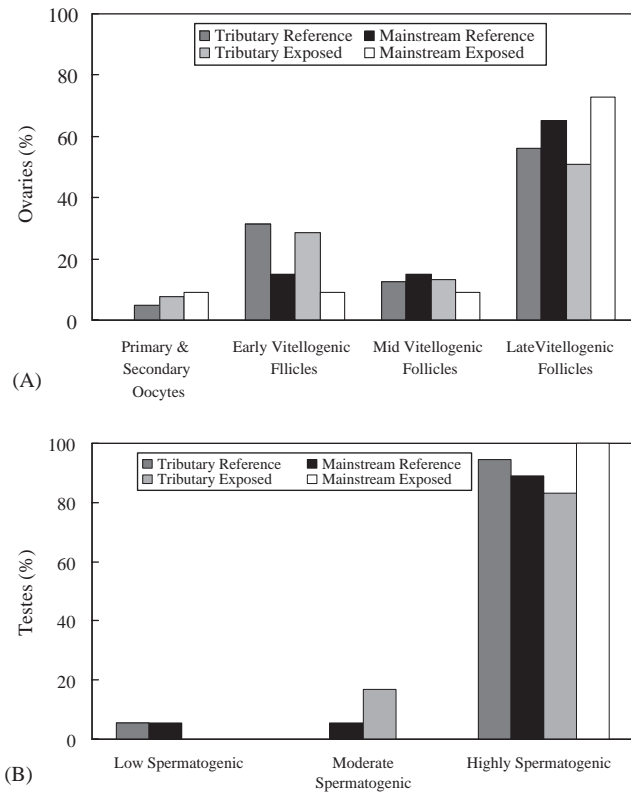


Fig. 2. Differences in the frequency distribution of ovarian (A) and testicular (B) reproductive stages in largemouth bass sampled along the St. Johns River during the spawning season (March) of 1998. There were no differences in reproductive condition between exposed and reference tributary and mainstream sites ($\chi^2 = 0.2-1.1$, $P > 0.05$ and $\chi^2 = 0.2-0.3$, $P > 0.05$ for females and males, respectively).

advanced vitellogenesis, indicating that the populations sampled were at or near spawning. In addition, there were no differences in the number of atretic follicles/histological sections between females from the reference and exposed sites (5.4 ± 1.1 and 7.4 ± 2.9 , for reference and exposed streams, respectively).

Similarly to those in females, organosomatic indices in males did not differ across sites (Table 2). In males, E_2 did not change among the exposed and reference sites, but 11-KT decreased in tributaries and mainstream exposed sites in relation to controls (overall decline of 67%). Of the 53 males examined, 6% (3 individuals) had measurable circulating levels of VTG. These fish included 2 males from Cedar Creek with concentrations of 0.06 and 1.51 mg/mL and 1 male from Welaka with a concentration of 2.58 mg/mL. Although these VTG concentrations fell within ranges normally found in females, E_2 concentrations in these males were not increased (overall average of 411 pg/mL). There were no differences in the reproductive condition of male bass across sites (Fig. 2B).

Largemouth bass collected from mainstream sites appeared to be at a more advanced reproductive state

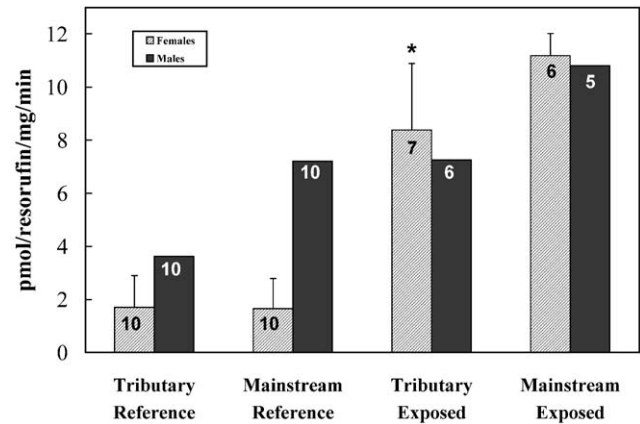


Fig. 3. Mean \pm SEM liver EROD concentrations in largemouth bass sampled along the St. Johns River during the spawning season (March) of 1998. Fish were collected from tributaries and mainstream sites. Numbers inside histograms indicate sample sizes (n). Females from the tributary Rice Creek had significantly higher EROD activity compared to females from reference streams (Cedar and Etonia Creeks) (ANCOVA, Dunnett's multiple comparison test; $\alpha = 0.05$).

compared to bass sampled from tributary streams. For instance, both females and males from the tributary streams had lower sex steroid concentrations compared to fish sampled from mainstream sites (mean of 958 vs. 1315 pg/mL of E_2 in females and 1229 vs. 1300 pg/mL of 11-KT in males, respectively). These lower hormone concentrations were translated as smaller GSI in largemouth bass from the tributary sites (mean of 2.38% vs. 3.45%, for females and 0.34% vs. 0.47% for males, respectively).

Female largemouth bass sampled from the effluent-exposed site of Rice Creek had a five-fold increase in EROD activity compared to bass sampled from reference streams (Fig. 3). Initial rate EROD activities in male bass were approximately two-fold higher than those observed in females (means of 6.7 vs. 3.0 pmol resorufin/mg/min for males and females, respectively); however, no significant differences were observed in the EROD activities in males captured from different sites. Regression analyses between reproductive parameters and liver EROD activities in all female largemouth bass sampled revealed that VTG and GSI were inversely related to liver EROD activity ($P < 0.007$, $R^2 = 0.3$). However, when this analysis was conducted within each site, this significant relationship was maintained only in females from Rice Creek ($P = 0.02$, $R^2 = 0.7$ for VTG and GSI).

4. Discussion

An objective of the present study was to compare reproductive endpoints in bass sampled from B/UKME-exposed streams with those observed in fish collected

from reference streams. Our approach was to conduct a thorough examination of the potential effects of these effluents in largemouth in multiple sites, as a previous study (conducted in 1996/1997) had indicated that the adverse reproductive changes in bass sampled along the St. Johns River were most evident in the site closest to the Palatka pulp and paper mill outfall (Sepúlveda et al., 2002b). The latter study, however, was not designed solely to look for effluent-related changes and therefore included the sampling of several other sites impacted with other sources of contamination. Accordingly, an additional objective was to compare the results of the present study with those obtained in the 1996/1997 survey.

For both years of study, female and male bass collected from reference sites had higher concentrations of E_2 and 11-KT, respectively, than fish from exposed sites. Although there was a decline in the plasma concentration of VTG in females from exposed sites compared to those from reference streams during both years of study (94% and 56% declines for 1996/1997 and 1998, respectively), this decline was significant only during 1996/1997. Higher declines in VTG in females sampled during that year could explain the concomitant decrease in GSI and the lack of such a decline in 1998. In fact, during 1998, GSI were either slightly decreased in males (19%, although not statistically significant) or not affected at all in females. These results suggest that variations in gonad weight are probably more related to variations in local environmental conditions than to contaminant exposure and/or that higher declines in sex hormones and VTG are necessary before declines in GSI are observed. In this regard, several studies have reported declines in GSI in fish exposed to BKME (Gagnon et al., 1994b; Gibbons et al., 1998; Larsson et al., 1988; Munkittrick et al., 1991, 1992, 1994). However, other studies have reported that decreases in gonadal size may not always occur after exposures of fish to BKME (McMaster et al., 1996), which would suggest differences in reproductive responsiveness to contaminant exposure across species.

Gonads were evaluated histologically to ensure that observed differences in concentrations of sex steroids and VTG between fish from exposed and reference sites were not caused by different stages of sexual maturity. Within each type of stream, the results showed similar stages of ovarian and testicular development among bass collected from all three sites. In addition, the similarity in the number of atretic follicles in ovaries from females from clean and contaminated streams and the absence of any noticeable lesions in the testes examined would suggest that alterations in sex steroid concentrations in bass from exposed sites (declines in E_2 and 11-KT in females and males, respectively) were probably not enough to cause damage to gonadal tissue.

Many studies of the effects of paper mill effluents report a concomitant increase in HSI in fish with high EROD activities (Bankey et al., 1994; Huuskonen and Lindström-Seppä, 1995; Larsson et al., 1988). Although we did observe an increase in EROD activities in some of the fish analyzed, this induction was not associated with increases in liver size (hepatomegaly). Typically, increases in liver weight following exposure to Ah receptor agonists is due to a proliferation of the smooth endoplasmic reticulum as a result of increased synthesis of CYP1A-related proteins (Larsson et al., 1988). Although not investigated, it is likely that the modest EROD induction observed among some of the fish in the present study was not of the magnitude to increase proliferation of the endoplasmic reticulum. Alternatively, the absence of an increase in HSI in the present study could have been related to the timing of fish sampling. Bass were collected during the reproductive season (March), and it is well known that the weight of the liver in this species undergoes seasonal variations, with highest values in winter and spring (December–April) and lowest values in the summer months (Sepúlveda et al., 2002a). Thus, any increases in liver weight due to an enhanced activity of xenobiotic biotransformation enzymes would have been masked by the physiological increases that are associated with reproductive status (this is particularly true in the case of females due to increases in the synthesis of VTG).

A common finding in studies that have focused on the reproductive effects of BKME on fish is the decline in the concentration of circulating sex steroids (Adams et al., 1992; Gagnon et al., 1994a; Hodson et al., 1992; McMaster et al., 1995, 1996; Munkittrick et al., 1998). The consequences of this endocrine alteration have varied greatly between species and have ranged from no organism responses to decreased gonadal sizes, secondary sexual characteristics, and egg sizes (McMaster et al., 1996). These observations provide evidence for species differences in the susceptibility to BKME, but also show the inherent difficulty when trying to compare biological responses in fish populations inhabiting highly different environments and exposed to complex mixtures likely to vary in chemical composition. In this respect, except for a decline in GSI during 1996/1997, the results from our field efforts suggest that decreased hormone concentrations in response to paper mill effluent exposure may not always be associated with obvious reproductive impairment, such as a reduction in gonad weights. The absence of organism-level responses is probably not related to a lack of sensitivity, since laboratory *in vivo* experiments on the impacts of BKME on the reproductive performance of largemouth have shown effects on gonad weights and other measures of reproductive success (Sepúlveda et al., 2001, 2003). However, in these laboratory studies, the lowest observed effect level (LOEL) for declines in sex steroids and VTG has

generally been lower (20% effluent) than the LOEL associated with declines in GSI (40%). These observations would then suggest an insufficient exposure to BKME in the populations of largemouth bass sampled closest to the mill outfall (Rice Creek and Palatka). Although effluent concentrations are high in Rice Creek, the scarcity of bass in this stream would indicate the absence of adequate prey and/or nesting substrate, thus making this area unsuitable for long-term residency. Fish from mainstream Palatka, on the other hand, are being exposed to a highly diluted effluent (less than 10% v/v) because of the high water flow present in the St. Johns River.

Measurements of EROD activity have been widely used as a biomarker for the exposure of fish to several groups of CYP1A-inducing compounds, including polychlorinated dibenzo-*p*-dioxins and dibenzofurans, polychlorinated biphenyls (PCBs), and polycyclic aromatic hydrocarbons (PAHs). Although effluents from modern mills are generally free of the majority of these chlorinated persistent compounds, researchers have found that they are still capable of inducing EROD in fish. For instance, Lindesjoo et al. (2002) reported increased EROD activity in rainbow trout (*Oncorhynchus mykiss*) exposed to effluents from a total chlorine-free mill. The compound(s) responsible for such an induction is not of the highly hydrophobic chlorinated type, but rather of the moderately hydrophobic planar PAH-type form present as natural components of wood (e.g., resin acids) and readily metabolized by fish (Hodson, 1996; Pacheco and Santos, 2002).

Since BKME are known to contain CYP1A-inducing agents, the modulation of EROD activities has played a major role in the study of the fate and biological effects of paper mill effluent discharges. In general, low constitutive hepatic CYP1A expression results in low initial EROD activities in fish from unpolluted sites, with significant increases in activities in fish sampled close to pulp mill outfalls (Bankey et al., 1994; Courtenay et al., 1993; Förlin et al., 1985; Lindström-Seppa and Oikari, 1989; Soimasuo et al., 1995).

In the present study, EROD activities were five-fold higher in females from the site closest to the mill outfall (Rice Creek) compared to those in females from the reference site. There was also a trend toward a gradient of increased EROD activities from Rice Creek to its confluence with the St. Johns River (a distance of only 3 km), with females from this latter site having EROD activities that were comparable to values from the reference areas (1.2 pmol/min/mg). This rapid fall in EROD activities would suggest that compounds capable of causing enzyme induction are present in high enough concentrations only in water and/or sediments from Rice Creek and that by the time they reach the St. Johns

River they are diluted enough for EROD activities to fall to background levels. In males, EROD activities were more variable across sites and were somewhat higher in bass sampled from Rice Creek and Palatka (average of 9.1 pmol/min/mg) compared to those in males from reference sites (average of 5.4 pmol/min/mg), although these differences were not statistically significant.

It is important to mention, however, that within each “exposed” and “reference” site there was variation in the amount of EROD induction. For instance, bass sampled from reference streams sometimes had higher EROD activities than fish from exposed streams, and vice versa. Part of this variation could be due to the degree of mobility of largemouth bass. In this respect, Snyder et al. (1986) reported that 38% of the bass marked and released in the lower St. Johns River, FL, were recaptured in the same area as tagged, and of the remaining 62%, 44% had moved a distance of less than 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).

The degree of EROD activities among female and male largemouth bass is indicative of a suppression of biotransformation activity in female fish that can occur during reproduction (Elskus et al., 1989; Larsen et al., 1992; Stegeman and Hahn, 1994). Although the exact mechanism of this regulation remains unclear, the suppression is likely related to higher increases in E_2 concentrations in spawning females in relation to males. In this regard, Navas and Segner (2001) have demonstrated that E_2 downregulation of CYP1A expression and EROD activities is E_2 -receptor dependent and does not overcome CYP1A-induction responses. If a similar pattern occurs in spawning largemouth bass, it is likely that basal EROD activities may be higher in nonreproductive bass. In this respect, constitutive EROD activities of adult bass in this study are lower than the values reported in immature largemouth bass exposed to up to 8% BKME for 263 days (Bankey et al., 1994), although the induction levels are similar (up to six-fold). Accordingly, the comparative differences in initial-rate EROD activities are probably related to having worked with adult spawning bass as opposed to juvenile fish. On the other hand, the relative induction levels in females from Rice Creek are comparable to values observed by Haasch et al. (1993) in largemouth bass exposed in the laboratory to β -naphthoflavone for up to 4 days and in caged bass exposed to waters from a PAH- and PCB-contaminated river for up to 7 days (overall inductions of 4.4 and 6, respectively).

Of considerable interest in aquatic toxicology is whether alterations in hepatic EROD activities are related to changes in reproductive or other physiological functions. Interestingly, studies on the effects of paper mill effluents on fish have provided most of the

information available on this subject. In general, there does not appear to be a clear relationship among titers of steroid hormones and other reproductive alterations and hepatic EROD activities in fish from sites impacted by BKME (Gagnon et al., 1994a,b; Munkittrick et al., 1994; Soimasuo et al., 1998). Our results would suggest an inverse relationship between female reproductive endpoints (VTG and GSI) and EROD activity, but only when all sites are included in the analyses. Within sites, however, these negative relationships hold significant for only a single site (Rice Creek). These findings are suggestive of an association between EROD induction and antiestrogenic effects in largemouth bass. English sole (*Parophrys vetulus*) exposed naturally to Puget Sound sediments contaminated with PCBs and PAHs also showed significant correlations between chemical exposure, MFO induction, and reduced concentrations of plasma E_2 (Johnson et al., 1988), and in vitro studies have shown a positive relationship between a compound's antiestrogenicity and its ability to induce CYP1A proteins in fish (Anderson et al., 1996). In addition, these authors reported a depression of estrogen receptor binding capacity in relation to increased EROD activity in juvenile rainbow trout injected with 50 mg/kg of β -naphthoflavone. These findings indicate alterations in the affinity of E_2 for the estrogen receptor, probably due to Ah receptor-mediated changes in the phosphorylation state of the estrogen receptor (Anderson et al., 1996). Since CYP1A in fish does not participate in the catabolism of E_2 , it is unlikely that increases in EROD activities are related to increases in the oxidative metabolism of this hormone (Snowberger and Stegeman, 1987). Clearly additional studies are needed for a better understanding on the involvement of EROD enzymatic activity in endocrine modulation and its potential effects on fish reproduction.

In conclusion, our results showed a significant reduction in sex hormones in bass from effluent-exposed sites, but there were no differences in VTG and GSI across sites. The absence of organism-level responses in feral fish was probably not related to a lack of sensitivity, but rather to insufficient exposure. Bass sampled from the site closest to the mill outfall had a significant five-fold increase in EROD activity compared to bass sampled from reference streams. In females, inverse relationships were observed between VTG and GSI and EROD activity. Collectively, our findings indicate that hepatic EROD induction is an effective marker of B/UKME exposure in largemouth bass and that it might be associated with antiestrogenic effects in this species.

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