

EFFECTS OF PULP AND PAPER MILL EFFLUENTS ON REPRODUCTIVE SUCCESS OF LARGEMOUTH BASS

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(Received 6 February 2002; Accepted 10 July 2002)

Abstract—This study evaluated the effects of bleached and unbleached kraft mill effluent on reproductive success of largemouth bass (*Micropterus salmoides*). Bass were exposed to effluent concentrations (0, 10, 20, 40, or 80%) for 28 and 56 d. Parameters measured included hepatosomatic index (HSI) and gonadosomatic index (GSI) and plasma concentrations of 17 β -estradiol (E₂), 11-ketotestosterone (11-KT), and vitellogenin (VTG). At the end of the 56-d period, bass were moved to hatchery ponds to evaluate spawning success. Spawning mats with eggs either were brought indoors for evaluation of fecundities, hatchabilities, and egg and fry size (measured at age 3 d), or were left in ponds and fry number and size recorded (average age of 14 d). Effluent exposure was verified by measuring resin acids (isopimaric, abietic, and dehydroabietic acids) in bile. Compared to controls, exposed bass had greater concentrations of resin acids in bile. In general, exposed females had lower concentrations of E₂ and VTG ($\geq 20\%$ effluent), whereas males had lower concentrations of 11-KT ($\geq 20\%$ effluent) and increased E₂ ($\geq 20\%$ effluent). The HSI values increased in females ($\geq 10\%$ effluent), and GSI values decreased in both sexes ($\geq 40\%$ effluent). Fecundity, egg size, and hatchability did not differ across treatments, but an increase in the frequency of fry abnormalities and a decrease in fry weights was observed at effluent exposures of 40% and higher. However, results from the pond study, revealed a significant reduction in fry growth and survival ($\geq 10\%$). This decline may have been caused by an increased frequency of deformities, in conjunction with alterations of growth. These changes could have resulted from alterations in egg quality because of failure of parental reproductive systems, from acute embryo toxicity after translocation of contaminants from the mother to the developing embryo, or from both.

Keywords—Reproduction Spawning Mill effluents Largemouth bass

INTRODUCTION

Preliminary results from field and laboratory studies have shown altered reproductive biomarkers for largemouth bass (*Micropterus salmoides*) exposed to pulp and paper mill effluents in Florida, USA [1–3]. In general, these findings are in agreement with several Canadian and Scandinavian studies that have reported alterations in reproductive indicators and biomarkers, including reductions in gonad size, delayed sexual maturation, and reduced production of sex steroids in fish sampled downstream from pulp and paper mills [4–9].

Despite the extensive knowledge on the effects of paper mill effluents on fish reproduction, whether these changes may cause developmental alterations or adversely affect reproductive success in populations of free-ranging fish is not well understood. This lack of knowledge is surprising, given that developing fish embryos and larvae often are considered the most sensitive stages in the life cycle of teleost fish [10]. In addition, what chemical(s) could be held responsible for such changes is still debated. In this respect, in the last few years it has become apparent that chlorinated compounds might not be the primary players, because reproductive alterations are still observed when fish are exposed to unbleached kraft mill effluents, black liquor, and mechanical pulping effluents [11,12]. Nonpersistent compounds capable of altering the en-

docrine system of fish include natural wood components such as fatty acids [13], resin acids, and plant sterols [14–18]. Several reports have implicated β -sitosterol, a plant sterol, as a possible significant factor contributing to the reproductive effects observed in fish exposed to paper mill effluents. In goldfish (*Carassius auratus*), injection of β -sitosterol causes reductions in plasma circulating levels of sex steroids and decreases in gonadal testosterone and pregnenolone production under in vitro conditions [1–3]. This compound can also induce estrogenic effects in fish. It can bind to the estrogen receptor of rainbow trout (*Oncorhynchus mykiss*) and promote expression of the vitellogenin gene in vitro and in vivo [16,19]. In brown trout (*Salmo trutta lacustris*), phytosterols also have been linked to developmental toxicity (dose-dependent egg mortality, smaller egg sizes, and lower weight of yolk-sac larvae) [18]. Other compounds present in paper mill effluents that have been reported to cause reproductive dysfunction in fish include phenol and sulfide. Both of these chemicals inhibited the uptake of radiolabeled cholesterol into carp (*Cyprinus carpio*) ovary from the peripheral circulation and its ovarian conversion to progesterone and pregnenolone [20].

The objective of this study was to assess the potential effects of bleached and unbleached kraft mill effluent (B/UKME) exposure on subsequent reproductive success of largemouth bass. In this study, controlled exposure of bass to different concentrations of B/UKME for up to 56 d was followed by spawning trials that measured effects on fecundity, egg size,

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egg viability, hatchability, and fry growth and survival. In addition, gonad weights and histology and plasma concentrations of sex steroids and vitellogenin (VTG) were measured in adult bass before spawning. Because similar studies are planned after implementation of major process changes, an additional objective of the present investigation was to provide background information for future assessments of the biological responses to process changes.

MATERIALS AND METHODS

Description of effluents tested

Georgia-Pacific's Palatka plant (FL, USA) is a kraft mill that produces a 50:50 mix of bleached and unbleached market pulp. During the time our study was conducted, the mill released an estimated 36 million gallons of effluent per day and production averaged 1,452 air-dried metric tons of pulp per day from a furnish that consisted of 80% softwood and 20% hardwoods. Bleaching sequences were $C_{90d10}E_{op}HD_p$ and CEHD for the softwoods and hardwoods, respectively, where C_d = mixture of chlorine (C) and chlorine dioxide (d) in proportions designated by subscripts, E_{op} = extraction with alkali and the addition of elemental oxygen (o) and hydrogen peroxide (p), H = hypochlorite, and D_p = 100% d substitution with the addition of p. At the time of this study, effluents received secondary treatment. This consisted of both anaerobic (500-acre [202.5-ha] basin) and aerobic (500-acre [202.5-ha] basin) biological degradation for a detention period of 40 d. Treated effluents are discharged into Rice Creek, a tributary that runs for about 5 km before its confluence with the St. Johns River (FL, USA). Because Rice Creek is small, effluents can account for a large portion of its total flow (yearly average effluent concentration is estimated to be ~60%, with a range of 50–97%). However, by the time effluents reach the St. Johns River, concentrations have fallen below 10% (S. Holm, Georgia-Pacific, personal communication; also see Quinn et al. [21]). The Palatka paper mill plant has been in operation for more than 50 years. Presently, this mill is implementing a series of important renovations necessary to comply with the U.S. Environmental Protection Agency cluster rule promulgated in 1998. Some of these changes include the use of chlorine dioxide for bleaching instead of elemental chlorine and of oxygen and hydrogen peroxide for bleaching instead of sodium hypochlorite. Improvements in secondary treatment of effluents also are underway.

Animals, holding facility, and exposure conditions

Reproductively active largemouth bass (American Sport Fish, Montgomery, AL, USA) were exposed to one of five effluent concentrations (0 [controls, exposed to well water], 10, 20, 40, or 80%) for either 28 or 56 d, starting the last week in December 1998. Fish were acclimated to the experimental conditions for one week before the experiment started. Concentrations were chosen to represent effluent concentrations likely to be encountered by free-ranging fish inhabiting Rice Creek, the effluent-dominated stream associated with the Palatka mill. In Palatka, fish were held outdoors in 1,500-L round plastic flow-through tanks located approximately 100 m from the effluent discharge. The effluent used for the study was collected directly after being released from the secondary treatment ponds. Water used for the control tanks and to dilute the effluent was obtained from a well located near the tank system. A single high-volume, low-pressure air pump was used to

aerate water in each tank. In-line digital flow meters (Midwest Instruments and Controls, Pleasant Prairie, WI, USA) were used to control well and effluent inputs and maintain the desired effluent concentrations. The average dissolved oxygen, temperature, and pH for the tank system were 6.3 ± 0.14 mg/L, $17 \pm 0.3^\circ\text{C}$, and 7.7 ± 0.04 , respectively. The average flow-through rate was 15 L/min. During the course of the study, fish were fed ad libitum once a week with a commercial pellet (Floating Fish Nuggets, Zeigler, Gardners, PA, USA).

Body measurements, organosomatic indices, and reproductive endpoints

At the end of each exposure period, fish from each tank were weighed with a portable digital scale and total length was recorded. Condition factor was calculated as $\text{weight}/\text{length}^3 \times 100$. Fish were bled from the caudal vein, and blood samples were refrigerated until centrifuged for 10 min at 1,000 g to collect plasma. Plasma was stored at -80°C until analyzed for sex steroids (11-ketotestosterone [11-KT] and 17β -estradiol [E_2]) with a radioimmunoassay technique and VTG (through a direct enzyme-linked immunosorbent assay) as described by Sepúlveda et al. [2]. Fish were euthanized with a blow to the head, then gonads and livers were excised and weighed for the determination of gonadosomatic index (GSI) and hepatosomatic index (HSI) ($100 \times \text{gonad or liver weight}/(\text{body wt} - \text{gonad or liver wt})$). A representative sample of gonad was also saved in 10% buffered formalin for histological examination after standard hematoxylin and eosin staining.

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, respectively, ranging from the presence of few and small vitellogenic oocytes to large oocytes containing numerous vitelline granules). Testes were classified into three stages of sexual maturation: low, moderate, or high spermatogenic activity (stages 1, 2, or 3, respectively, ranging from a thin germinal epithelium and scattered spermatogenic activity, to a thick germinal epithelium with high proliferation and maturation of sperm).

Assessment of effluent exposure

Exposure of fish to the pulp and paper mill effluents was evaluated through the analysis of total (free and conjugated) resin acid bile concentrations (isopimaric, dehydroabietic, and abietic acids). For this analysis, bile was collected and pooled by sex from five males and five females per treatment (28- and 56-d exposure groups). Concentrations of resin acids were determined by gas chromatography–mass spectrometry by using the method described by Morales et al. [22]. This technique was modified by adding sulfatase (5 units/ μl) to enhance hydrolysis of sulfate conjugates in bile. In addition, a different derivatizing agent was used (triethylxonium tetrafluoroborate, 1 ml of a 1 M solution, Sigma, St. Louis, MO, USA).

Spawning study

At the end of the 56-d exposure (mid-February), 15 males and 20 females were collected from each of the treatment tanks and put, as a group, into a 0.04-ha spawning pond (total of five ponds, each with 35 bass). Although these ponds were lined, they had a sediment layer on top of the liner (~35 cm), which allowed for limited vegetation growth. The average depth of each pond was approximately 80 cm (45–150 cm). Two weeks before fish were moved, ponds were cleaned of

vegetation, filled with well water, and provided with 20 spawning mats or nests. Spawning mats (Spawntex Spawning Mat, Aquatic Eco-systems, Apopka, FL, USA) measured 60×50 cm. The mats were distributed uniformly within each pond, and fixed to the sediment with four pieces of thin stainless steel wire. Fish were monitored daily for signs of spawning activity. Dissolved oxygen, temperature, and pH were measured daily at a depth of approximately 30 cm, and averaged 8.9 ± 0.15 mg/L, $20 \pm 0.14^\circ\text{C}$, and 8.2 ± 0.04 , respectively, with no differences across ponds. After spawning behavior was detected (males exhibited territorial behavior 10 d after being transferred to ponds), the mats were checked via snorkeling every other day for the presence of eggs. Approximately one half of the mats seen with eggs were collected and moved to the laboratory for controlled hatchability studies (see below). The remaining mats were left in the ponds for future monitoring of fry survival and growth.

Indoor hatchability study

In this study, all eggs collected from a mat were assumed to have been spawned by a single female. Although some potential exists for more than one female to spawn in one mat, our observations suggest this is an unlikely possibility because when examined under the microscope, all eggs collected from a single mat were always at the same developmental stage and were very similar in size. Also, we never observed more than one defined cluster of eggs in each mat. Thus, in this study, total number of eggs collected per mat is defined as a clutch and is considered as a direct measure of fecundity.

In the laboratory, eggs were collected from mats after immersion in a 1.5% sodium sulfite solution (anhydrous 97%; Acros Organics, Suwanee, GA, USA) for 5 min. The eggs then were rinsed with well water (not chlorinated), and dead eggs (white opaque rather than bright yellow) as well as debris were removed. The number of eggs was determined volumetrically to estimate fecundity, by using a graduated glass cylinder (from several trials, we found that 500 eggs were ~ 1 ml). Egg diameter was determined from each clutch after measuring 30 eggs under a dissecting microscope equipped with an ocular metric scale. Viable eggs were left in fish-hatching jars (Midland Fish Hatching Jar, Brookfield, WI, USA) for a total of 3 d. These jars received well water at a flow rate of approximately 3 L/min, with an average dissolved oxygen, temperature, and pH of 7.7 ± 0.19 mg/L, $21 \pm 0.03^\circ\text{C}$, and 7.6 ± 0.05 , respectively. The jars were treated daily with hydrogen peroxide (500 mg/ml of 35% active ingredient, static bath for 30 min; Sigma) to prevent fungal growth. On day three, fry were collected from each jar and counted with an automatic fry counter (Jensorter Fry Counter, Model FC, Bend, OR, USA). The number of fry produced by day 3, expressed as a percentage of viable eggs present on day 0, was used as an estimate of hatchability.

Outdoor hatchability study

Approximately one half of the mats with eggs were left to hatch in the ponds. Fry were first seen schooling above the mats at about 7 d of age, but were not collected until they were at least 9 d old. The fry were collected with fry nets. Because collecting the whole school at once was difficult, nests were visited every other day for up to five visits. As a result of this sampling strategy, the range of fry ages collected was 9 to 19 d. In the laboratory, small fry (<6 mm) were counted with the automatic fry counter, and large fry were counted

manually. In this study, fry production per pond was expressed per spawned female (determined as the number of spawning mats left with eggs in each pond).

Fry measurements

Complete sets of largemouth bass fry collected from the hatching jars and the spawning mats were saved in 10% formalin for future measurements. From all sets, total length was measured in 30 fry, and fry weights were estimated by weighing up to four groups of 25 fry after removing excess water with a paper towel. Yolk sac length, width, and volume (the latter estimated by using the equation of Hoyt [23] of $0.524 \times \text{yolk length} \times \text{yolk width}^2$) also were measured in 30 3-d-old fry collected from hatching jars. The frequency of gross abnormalities to the head, vertebral column, and yolk sac also were quantified by evaluating up to 300 fry per clutch.

Experimental design and data analysis

Five concentrations of effluent were evaluated for effects on fish (0, 10, 20, 40, and 80% of full strength). For each concentration, two 1,500-L tanks were used for test chambers (10 tanks in total). The two tanks per concentration were connected to each other with a polyvinyl chloride pipe, so that one member of each pair of tanks received the effluent overflow from the other member of the pair. Thus, replicate tanks (per concentration) were not truly independent. Approximately 60 fish were put into each tank. The sex ratio of the fish added to the tanks was about 1:1.

At the end of each exposure period, approximately 10 female fish and 10 male fish were selected randomly from each tank. Because only two tanks per concentration were used, we elected to use individual fish from the tanks as the basic unit of measurement. Thus, the experimental design involved pseudoreplication. Measurements were made on about 20 female fish and 20 male fish (10 fish of each gender from each tank) per exposure duration.

We analyzed male and female fish from the tanks for effects separately with two-way analysis of variance (SAS PROC GLM) [24] to determine whether effluent concentration or duration of exposure affected body measurements, organosomatic indices, sex steroids, or VTG.

Differences in fecundity, egg size, percentage of live eggs, hatchability, fry production, and fry measurements were evaluated by one-way analysis of variance. Data sets that did not meet the criteria of normality and homogeneity of variance (SAS PROC UNIVARIATE) [24] were log- or arcsin-transformed before analysis. A Dunnett's multiple comparison test was used to determine differences in means, compared to fish from the control group. The relationship between stages of gonadal development was compared among treatments by a Kendall's tau test of association (SAS PROC FREQ) [24]. In this test, a 95% confidence interval (CI) that does not include 0 indicates a significant positive or negative relationship between treatment and stage of gonadal development. For purposes of statistical comparisons, ovaries and testes were classified as either low to moderate (stages 1 and 2 for both sexes) or high gametogenesis (stage 3 for males and stages 3 and 4 for females). The frequency distributions of fry abnormalities were compared among treatments by a χ^2 test (SAS PROC FREQ) [24]. Statistical significance was assessed at $p \leq 0.05$.

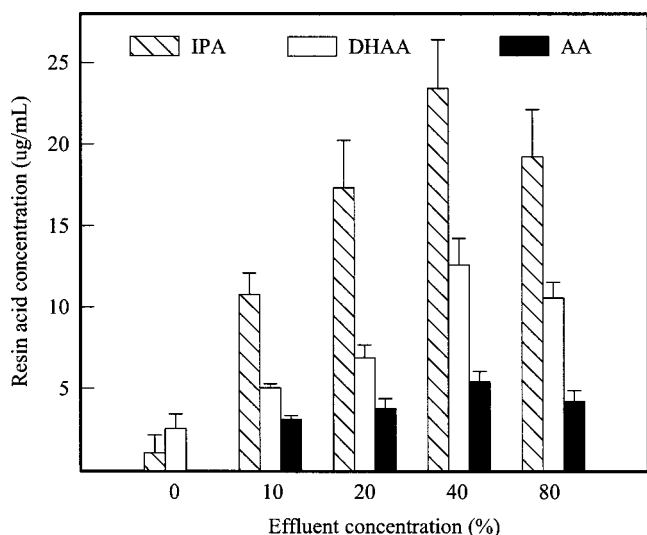


Fig. 1. Mean \pm standard error for total (free and conjugated) resin acid concentrations in bile of largemouth bass exposed to different concentrations of pulp and paper mill effluent. Each bar represents pooled samples from five females and five males, from the 28- and the 56-d exposures. IPA = isopimaric acid; DHAA = dehydroabietic acid; AA = abietic acid.

RESULTS

Assessment of effluent exposure

Total concentrations of three resin acids (isopimaric, dehydroabietic, and abietic acids) were measured in bile of bass as a method to evaluate exposure to different concentrations of B/UKME. Compared to controls, fish exposed to effluents had higher concentrations of all resin acids in bile (Fig. 1). In exposed fish, the overall concentrations of isopimaric acid were 17.3 ± 1.7 g/ml, nearly two and 4.5 times the concentration of dehydroabietic (8.5 ± 0.9 g/ml) and abietic acids (4.0 ± 0.3 g/ml), respectively. Concentrations of resin acids in bile of control fish were 1.1 ± 1.1 , 2.5 ± 0.9 , and 0.01 g/ml for isopimaric, dehydroabietic, and abietic acids, respectively. Thus, these results would suggest that measuring resin acids in bile of largemouth bass provides a good way for evaluating exposure to B/UKME.

Effects on reproduction

The reproductive parameters for female largemouth bass exposed to different concentrations of B/UKME are summarized in Table 1. Concentrations of sex steroids were lower after exposure to pulp and paper mill effluents. Plasma concentrations of E_2 decreased after exposures to 20% and higher (56 d) and 80% effluents (28 d), with no changes of 11-KT across treatments. Vitellogenin decreased in a dose-response manner for both lengths of exposure, with changes beginning at lower concentrations with increasing lengths of exposure. The stage of ovarian activity (expressed as level of oogenesis) was inversely related to effluent concentration, but only in bass exposed to effluents for 28 d (Kendall's tau 95% CIs of -0.51 to -0.25 and of -0.31 to 0.44 for the 28- and 56-d groups, respectively).

The reproductive parameters for male largemouth bass exposed to different concentrations of B/UKME are presented in Table 2. For both exposure durations, 11-KT decreased in a dose-dependent manner after exposure to 20% and higher effluent. On the other hand, E_2 increased in male fish exposed to effluents ($\geq 20\%$) for 56 d. The VTG concentrations in males were more variable (in many treatments, concentrations were below the detection limit), and the overall mean was only 0.14 ± 0.03 mg/ml (all fish in the study). Similarly to what was observed in females, the stage of testicular development in males (expressed as degree of spermatogenesis) was inversely related to effluent exposure, but only in the 28-d exposure group. We did not observe histological changes for the 56-d group (Kendall's tau 95% CIs of -0.58 to -0.28 and of -0.15 to 0.19 for the 28- and the 56-d groups, respectively).

Because of the seasonal nature of all of the reproductive parameters measured in this study, many or all of them were expected to increase with the progression of the reproductive season and thus the study (i.e., from late December to late February). This usually was translated into increases in reproductive biomarkers in bass exposed to effluents for 56 d, compared to bass exposed to effluents for 28 d, regardless of treatment. For example, in female bass, both sex hormones increased with increasing length of exposure (from 522 ± 20 to 968 ± 37 pg/ml, and from 217 ± 8 to 602 ± 17 pg/ml, from days 28 to 56, for E_2 and 11-KT, respectively). Hormone

Table 1. Reproductive parameters from captive female largemouth bass exposed to various concentrations of pulp and paper mill effluents for 28 or 56 d. Values presented are means \pm standard error (sample size). Asterisks indicate differences in relation to control group (analysis of variance, Dunnett's multiple comparison test; $\alpha = 0.05$)

| Parameters | Effluent concentration (%) | | | | |
|--------------------------------------|----------------------------|----------------------|----------------------|-----------------------|-----------------------|
| | 0 | 10 | 20 | 40 | 80 |
| Females, 28 d | | | | | |
| Hepatosomatic index (%) ^a | 1.4 ± 0.07 (20) | 1.6 ± 0.06 (20)* | 1.6 ± 0.07 (20)* | 1.6 ± 0.08 (20)* | 1.6 ± 0.07 (21)* |
| Gonadosomatic index (%) ^b | 3.0 ± 0.17 (20) | 3.0 ± 0.17 (20) | 2.8 ± 0.11 (20) | 2.4 ± 0.08 (20)* | 2.4 ± 0.06 (21)* |
| 17 β -Estradiol (pg/ml) | 570 ± 53 (26) | 522 ± 37 (31) | 609 ± 54 (34) | 491 ± 38 (27) | 408 ± 28 (29)* |
| 11-Ketotestosterone (pg/ml) | 151 ± 7.8 (26) | 178 ± 15 (31) | 267 ± 17 (34) | 265 ± 21 (28) | 214 ± 17 (29) |
| Vitellogenin (mg/ml) | 1.3 ± 0.13 (26) | 1.5 ± 0.13 (31) | 1.4 ± 0.16 (34) | 0.73 ± 0.09 (28)* | 0.21 ± 0.04 (30)* |
| Females, 56 d | | | | | |
| Hepatosomatic index (%) | 1.8 ± 0.09 (20) | 2.2 ± 0.09 (19)* | 2.1 ± 0.1 (20)* | 1.9 ± 0.08 (19)* | 2.0 ± 0.08 (19)* |
| Gonadosomatic index (%) | 2.7 ± 0.13 (20) | 2.9 ± 0.19 (19) | 2.7 ± 0.13 (20) | 2.6 ± 0.09 (19) | 2.4 ± 0.11 (19)* |
| 17 β -Estradiol (pg/ml) | $1,298 \pm 70$ (21) | $1,135 \pm 78$ (18) | 921 ± 84 (20)* | 712 ± 35 (18)* | 737 ± 33 (19)* |
| 11-Ketotestosterone (pg/ml) | 589 ± 40 (21) | 569 ± 42 (18) | 558 ± 49 (20) | 644 ± 29 (18) | 653 ± 21 (19) |
| Vitellogenin (mg/ml) | 3.0 ± 0.30 (23) | 2.6 ± 0.27 (19) | 1.5 ± 0.15 (20)* | 1.1 ± 0.13 (18)* | 0.35 ± 0.01 (19)* |

^a Hepatosomatic index = $100 \times$ liver weight/(body wt - liver wt).

^b Gonadosomatic index = $100 \times$ gonad weight/(body wt - gonad wt).

Table 2. Reproductive parameters from captive male largemouth bass exposed to various concentrations of pulp and paper mill effluents for 28 or 56 d. Values presented are means \pm standard error (sample size). Asterisks indicate differences in relation to control group (analysis of variance, Dunnett's multiple comparison test; $\alpha = 0.05$)

| Parameters | Effluent concentration (%) | | | | |
|--------------------------------------|----------------------------|----------------------|----------------------|------------------------|------------------------|
| | 0 | 10 | 20 | 40 | 80 |
| Males, 28 d | | | | | |
| Hepatosomatic index (%) ^a | 1.4 \pm 0.06 (20) | 1.3 \pm 0.07 (19) | 1.2 \pm 0.06 (16) | 1.3 \pm 0.06 (20) | 1.3 \pm 0.06 (20) |
| Gonadosomatic index (%) ^b | 0.78 \pm 0.03 (20) | 0.75 \pm 0.04 (19) | 0.75 \pm 0.05 (16) | 0.67 \pm 0.03 (20) | 0.54 \pm 0.03 (20)* |
| 17 β -Estradiol (pg/ml) | 314 \pm 22 (24) | 227 \pm 21 (19) | 240 \pm 16 (16) | 358 \pm 27 (21) | 369 \pm 23 (20) |
| 11-Ketotestosterone (pg/ml) | 653 \pm 58 (24) | 552 \pm 53 (19) | 498 \pm 63 (16)* | 466 \pm 38 (21)* | 332 \pm 29 (20)* |
| Vitellogenin (mg/ml) | 0.19 \pm 0.06 (24) | 0.02 \pm 0.01 (19) | 0.09 \pm 0.08 (16) | 0.008 \pm 0.004 (22) | 0.002 \pm 0.002 (20) |
| Males, 56 d | | | | | |
| Hepatosomatic index (%) | 1.7 \pm 0.07 (20) | 1.8 \pm 0.09 (21) | 1.6 \pm 0.14 (20) | 1.8 \pm 0.09 (20) | 1.7 \pm 0.14 (20) |
| Gonadosomatic index (%) | 0.95 \pm 0.04 (20) | 1.0 \pm 0.08 (21) | 1.2 \pm 0.11 (20)* | 0.75 \pm 0.03 (20) | 0.58 \pm 0.04 (20)* |
| 17 β -Estradiol (pg/ml) | 407 \pm 41 (20) | 482 \pm 59 (20) | 563 \pm 30 (19)* | 657 \pm 19 (21)* | 739 \pm 15 (21)* |
| 11-Ketotestosterone (pg/ml) | 1,052 \pm 42 (20) | 1,056 \pm 50 (20) | 815 \pm 53 (19)* | 750 \pm 28 (21)* | 735 \pm 39 (21)* |
| Vitellogenin (mg/ml) | 0.25 \pm 0.07 (20) | 0.32 \pm 0.17 (21) | 0.25 \pm 0.12 (19) | 0.18 \pm 0.06 (21) | 0.09 \pm 0.05 (21) |

^a Hepatosomatic index = $100 \times \text{liver weight}/(\text{body wt} - \text{liver wt})$.

^b Gonadosomatic index = $100 \times \text{gonad weight}/(\text{body wt} - \text{gonad wt})$.

concentrations almost doubled in males from the 56-d group, compared to males from the 28-d group, regardless of treatment (11-KT increased from 506 ± 24 to 880 ± 24 pg/ml, and E_2 increased from 306 ± 12 to 572 ± 20 pg/ml). Vitellogenin in males also increased, from a mean of 0.07 ± 0.02 mg/ml to a mean of 0.2 ± 0.05 mg/ml in the 28- and 56-d groups, respectively.

Effects on hepatosomatic index, gonadosomatic index, length, weight, and condition factor

In females exposed to at least 10% effluent, HSI were higher compared to controls, regardless of length of exposure, and GSI were decreased after exposure to high effluent concentrations ($\geq 40\%$ and 80% for 28 and 56 d, respectively) when compared to controls. Both HSI and GSI values in males exposed to 80% effluent for 28 and 56 d were generally lower than those of controls. However, an unexpected increase occurred in GSI in males exposed to 20% or higher effluent for 56 d.

In males, HSI increased from an overall mean of $1.3 \pm 0.03\%$ in the 28-d group to $1.7 \pm 0.05\%$ in the 56-d group. Effects of exposure duration on GSI were larger for the 0, 10, and 20% treatment groups, with a higher index for the 56-d group ($0.9 \pm 0.04\%$) compared to the 28-d fish ($0.7 \pm 0.02\%$). The HSI increased from an overall mean of $1.5 \pm 0.03\%$ in the 28-d group to a mean of $2.0 \pm 0.04\%$ in the 56-d group.

The mean \pm standard error length, weight, and condition factor for female bass in this study were 260 ± 0.78 mm, 212 ± 2.11 g, and 1.19 ± 0.005 , respectively. The corresponding values for males were 266 ± 1.01 mm, 223 ± 2.48 g, and 1.17 ± 0.005 . For both sexes, length, weight, and condition factor did not differ among treatments.

Spawning study

A summary of spawning activity and number of fry produced is presented in Table 3. The number of mats collected for the indoor hatchability study averaged four across ponds (range one to six), which corresponded to approximately 25% of the total number of mats present in each pond. Originally, we intended to collect approximately one half of the mats (i.e., 10 mats) seen with eggs for these studies, but because of water visibility problems, the number of mats that actually were seen with eggs in each pond was reduced. This was particularly evident in the case of the pond stocked with bass exposed to 40% effluents, from which only four mats were seen with eggs (and one collected) at some point during the study (Table 3).

Indoor hatchability study

Fecundities, egg sizes, percentage of live eggs, and hatchabilities did not differ much among treatments, and averaged $7,307 \pm 980$ eggs, 1.33 ± 0.01 mm, $77 \pm 4.3\%$, and $46 \pm 4.0\%$, respectively (Fig. 2). The average number of fry pro-

Table 3. Summary of spawning mat activity and total number of fry collected from hatching jars (indoor hatchability study) and from spawning mats in ponds (outdoor hatchability study). Values presented are means \pm standard error. Clean-water ponds were stocked with 15 males and 20 females that had been exposed to different concentrations of pulp and paper mill effluent for 56 d, and were left to spawn for 42 d. Asterisks indicate differences in relation to control group (analysis of variance, Dunnett's multiple comparison test; $\alpha = 0.05$)

| Parameters | Prior exposure concentration (%) | | | | |
|---|----------------------------------|-----------------|------------------|----------------|-----------------|
| | 0 | 10 | 20 | 40 | 80 |
| Indoor hatchability study (jars) | | | | | |
| Number of mats with eggs collected | 6 | 5 | 3 | 1 | 6 |
| Average fry produced/spawned female | 2,589 \pm 496 | 3,881 \pm 947 | 2,263 \pm 967 | 2,683 | 1,978 \pm 675 |
| Outdoor hatchability study (ponds) | | | | | |
| Number of mats left in ponds | 13 (8) ^a | 14 (8) | 17 (11) | 18 (9) | 13 (10) |
| Average fry produced/spawned female | 2,472 \pm 1,287 | 855 \pm 239* | 1,510 \pm 311* | 911 \pm 468* | 57 \pm 50* |

^a Number of mats seen with eggs or with fry is given in parentheses.

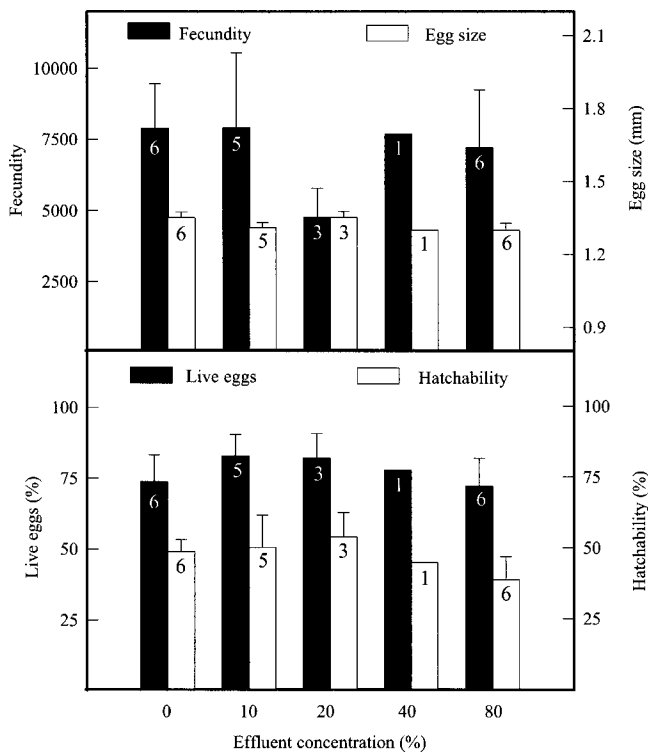


Fig. 2. Results of the indoor hatchability study showing mean \pm standard error of fecundity, egg size, percentage of live eggs, and hatchability of eggs spawned by largemouth bass in clean fish ponds after an in vivo exposure to different concentrations of pulp and paper mill effluent for 56 d. Eggs were collected from the ponds and brought indoors for controlled hatchability studies. Hatchability was determined 3 d after hatch. Numbers inside histograms indicate sample sizes (n = number of mats collected from ponds and brought indoors). No differences were found from the control group (analysis of variance, $p > 0.05$).

duced per spawned female was similar across treatments and ranged from $1,978 \pm 675$ for the 80% treatment, to $3,881 \pm 947$ for the 10% group (age 3 d) (Table 3). Fry produced from effluent-exposed bass had yolk sacs that were of similar length (11.3 ± 0.06 mm), but had widths that decreased with effluent exposure (from a mean of 7.7 ± 0.09 mm in the control group to a mean of 7.3 ± 0.1 mm in the 80% group). However, this slight decrease in yolk sac width did not result in changes in calculated yolk sac volume (overall mean of 339 ± 6.1 mm³). Although fry measured at day 3 were of similar length across ponds (5.6 ± 0.01 mm), body weights were lower at the 40 and 80% effluent treatment groups (1.3 ± 0.03 and 1.1 ± 0.03 mg for the 0 to 20% and 40 and 80% groups, respectively) (Fig. 3a). The frequency of fry abnormalities also increased, from an average of 10.5% in the 0 through 40% effluent groups, to almost 17% in the 80% effluent group. The distribution of fry abnormalities was similar in the three lower-concentration groups, but the abnormalities of the head were greater in the 40 and 80% treatment groups (mean of 5.3% vs 0.73% in the control, 10, and 20% effluent groups) (Fig. 3a).

Outdoor hatchability study

The percentage of mats used by females was similar among ponds and ranged from 50 to 76% (Table 3). We noted what appeared to be a dose-related decline in fry production (Table 3). The average number of fry produced per spawned female (age 14 d) decreased with effluent exposure from $2,472 \pm 1,287$ fry in the control group, to 57 ± 50 in the 80% group. With the exception of fry produced by bass exposed to 20% effluents, fry weights and lengths decreased from an average of 3.8 ± 0.27 mg and 7.8 ± 0.07 mm in the control group to 2.7 ± 0.15 mg and 7.1 ± 0.03 mm in fry produced by adult bass exposed to B/UKME (Fig. 3b). We did not detect an association between effluent exposure and the frequency of abnormalities in 14-d-old fry collected from ponds (overall mean of 1.5% abnormalities; about eight times lower than in the 3-d-old fry; Fig. 3b).

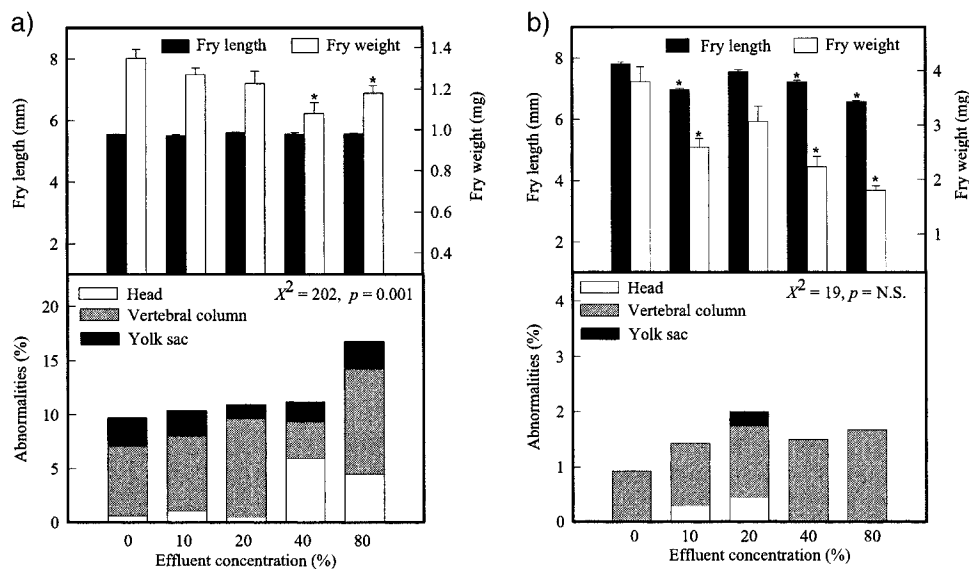


Fig. 3. Results from the indoor (a) and outdoor (b) hatchability studies showing mean \pm standard error of yolk-fry measurements (total length and weight) and percent abnormalities measured from fry produced by largemouth bass in clean fish ponds after an in vivo exposure to different concentrations of pulp and paper mill effluent for 56 d. Eggs were left to hatch in ponds, and measurements were taken at an average age of 3 d (jars) or 14 d (ponds). For fry length and weight, asterisks indicate significant differences with the control group (analysis of variance, Dunnett's multiple comparison test; $\alpha = 0.05$). Differences in the frequency distribution of abnormalities were analyzed with a χ^2 test.

DISCUSSION

In the experiment reported here, we were able to estimate exposure to B/UKME through the measurement of total resin acids in bile. Except for a decline in the 80% effluent group, concentrations of isopimaric and dehydroabietic acids in bile of largemouth bass increased in relation to the mean percentage dilution of B/UKME. Abietic acid, on the other hand, did not follow this trend; instead, it increased to similar levels for all effluent concentrations. A decrease in isopimaric and dehydroabietic acids in bass exposed to high concentrations of B/UKME suggests impaired metabolism of these compounds. Similar declines have been reported in brown trout exposed to high concentrations of wood sterols [22,25–27]. Resin acid concentrations in bile of fish have been used as a biomarker of exposure to pulp and paper mill effluents, and the values observed in our investigation fall within the range of those reported from whitefish (*Coregonus larvaretus*) and rainbow trout caged at increasing distances from pulp and paper mills [27]. Although fish can readily build up body burdens of resin acids after waterborne exposures, depuration rates also are fast, with half-lives that are 4 d or less [28]. These results suggest that measuring certain resin acids (isopimaric and dehydroabietic acids) in bile of free-ranging bass might be a useful indicator of recent exposure to pulp and paper mill effluents, but that the measurement of more persistent compounds (e.g., chlorinated organics) may be better for assessing historical exposures.

Similarities and differences in the reproductive responses of female and male bass exposed to effluents for 28 and 56 d were observed. In both sexes, exposure to B/UKME did not appear to influence body weight, length, or condition factor. For both exposure durations, females and males exposed to high concentrations of the effluents (40 and 80%) had lower GSI values (overall declines of 22 and 35% for female and male bass, respectively). Histological evaluation of gonads revealed changes in both ovaries and testes (negative relationship between effluent exposure and gonadal development), but only in fish exposed for 28 d. This apparent discrepancy between histological effects seen at 28 d but not at 56 d is interesting, and would suggest that these effects might be transitory or that some acclimation is possible in bass under these exposure conditions.

Plasma concentrations of sex steroids and VTG in male and female bass varied differently after exposure to B/UKME effluent. In males, we observed a dose-related decline in 11-KT, starting at 20% effluent, whereas E_2 increased in the 56-d group. In females, VTG and E_2 decreased in a dose-related manner after exposures to 20% or higher B/UKME effluent. However, we did not observe changes in plasma concentrations of 11-KT. These results are consistent with those reported for several field studies that have evaluated the impacts of bleached kraft mill effluent (BKME) on fish reproductive physiology. From these studies, investigators have shown that free-ranging fish exposed to pulp mill effluents have lower concentrations of plasma sex steroids, lower GSI values, reduced expression of secondary sex characteristics, and greater age to maturation [5–7,29–37].

We had presumed that exposure to B/UKME effluents would result in delayed or absence of spawning, decreased fecundities and egg sizes, decreased hatchabilities, or reductions in fry growth or survival. However, despite the reductions in sex plasma steroids and VTG, bass from all B/UKME ex-

posure regimes began spawning approximately 10 d after they had been moved to the clean-water ponds. Males showed aggressive territorial behavior (i.e., biting and chasing snorklers away) regardless of prior exposure conditions, although this was not quantified. Additionally, almost two months of effluent exposure did not affect subsequent fecundity, egg size, percentage of live eggs, or hatchability. However, fry produced by bass exposed to high concentrations of effluent (40 and 80%) tended to be smaller, and they had a greater frequency of deformities. We also were interested in measuring some of these parameters in older fry (average age of 14 d) hatched and grown under more natural conditions. Results from this portion of the study revealed that the most significant negative effects of effluent exposure were translated on reduced fry growth and survival.

Relatively few studies have been conducted on the effects of BKME on egg parameters, and the results from these studies are conflicting. In contrast to what we observed, fertility (as indicated by the percentage of spawned eggs that hatched) was lower in zebrafish (*Danio rerio*) after exposure to chlorinated phenolics from a bleach plant effluent [38] and in brown trout after exposure to BKME [39]. On the other hand, McMaster et al. [40] found equal or greater fertilization rates and no effect on hatchability of eggs of white sucker after BKME exposure, despite reductions in concentrations of sex steroids, gonad and egg size, and sperm motility. Additionally, fecundity or hatchability were not altered by exposure to BKME in several other field [32,41,42] and laboratory studies [43].

Plasma concentrations of E_2 and VTG declined in female bass exposed to pulp and paper mill effluents, as did GSI values. However, these declines were not associated with reduced fecundity, egg size, or hatchability. A possible explanation for this lack of association could be related to the timing of exposure. Vitellogenesis in Florida largemouth bass starts in late October and peaks in January [44], so by the time our experiments started, females already had allocated a considerable amount of VTG in the developing oocytes. A more prolonged period of exposure, long enough to include more of the oocyte growth phase, might have affected one or more of the egg parameters measured in this study. The lack of effect on fecundity could also be explained by the relatively high variance observed in this parameter (overall standard deviation of 4,698 eggs), coupled with the relatively small number of clutches examined. In addition, because largemouth bass are capable of spawning several times during a spawning season, alterations in fecundity might go undetected during the first spawn, but become evident later on in the season.

From the pond experiment, the average number of fry produced per spawned female declined with increasing exposure to B/UKME, starting at the 10% concentration (Table 3). Declines in the numbers of fry produced probably were not due to decreased fecundity or hatchability, because the indoor study did not reveal effects of effluent exposure on these parameters. More likely, the declines in fry production resulted from decreased survival due to increases in fry deformities or delayed growth rates. Such impairments may be critical for survival, for the susceptibility of fry to environmental factors may be increased, and it is essential for larvae to be able to swim properly to obtain food. Thus, the transition period between internal and external feeding during the early yolk sac stage is generally recognized as one of the most sensitive with respect to vulnerability to toxicants [45]. Feeding by largemouth bass larvae starts at around 8 d of age [46], which

corresponds to the age range at which decreased survival was observed in this study.

Little information exists on the developmental effects of BKME on fish. In the laboratory, survival from larvae to adult, and growth of fathead minnows (*Pimephales promelas*) were not affected until the exposure concentration of BKME exceeded 20% effluent [43,47]. Additionally, in one of these studies, BKME effluent did not cause morphological or histopathological abnormalities in hatched fish. In an investigation with perch (*Perca fluviatilis*), Karås et al. [42] reported no effects on fecundity and egg mortality values in fish from a BKME-exposed area, but fry hatched from this site were smaller and had a greater frequency of abnormalities, which resulted in fewer fry and fewer young-of-the-year fish.

Developing bass embryos exposed to chemicals present in the B/UKME we tested could explain the increased frequency of deformities and retarded growth observed. Chemicals in BKME that can be translocated from the mother to the developing oocyte include chlorinated organics such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin [48,49], as well as naturally occurring wood-derived compounds such as phytosterols [18]. These studies also reported declines in fry survival due to retarded growth, and increased prevalence of deformed larvae, after exposure of adult fish to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin [48,49], and phytosterols [18]. Furthermore, retene, a breakdown product of abietic acid, may reduce fry growth, cause yolk sac edema, and increase the mortality of larval fish [34]. We did not measure chemicals in either eggs or fry in the present study, but evidence indicates that aqueous uptake of dioxins and furans is not as important as ingestion via the food chain [50,51]. Clearly, more studies are needed to better understand the chemical(s) responsible for the observed effects, and their mode(s) of action.

Another factor that could have contributed to the observed changes in fry is the quality of the yolk deposited in the developing oocyte during the course of the *in vivo* exposures. Vitellogenesis involves the production of egg yolk, and includes the mobilization and transport of lipids, metals, ions, vitamins, and hormones to the fish ovary [52]. Decline in the production of VTG by livers of B/UKME-exposed females could have resulted in concomitant decreases in the amounts and types of essential nutrients (and possibly hormones) being transported into the developing egg, which could have negatively affected the normal development of fry.

A common objective of controlled laboratory studies is to estimate threshold concentrations of chemicals capable of causing specific alterations. Once effluent concentrations in the receiving streams are known, it is sometimes possible to extrapolate the laboratory results to predict impacts in the field. However, these extrapolations are subject to many uncertainties. In this respect, a limitation from this study was the lack of exposure of fish throughout the complete gametogenic cycle. However, based on our exposure conditions, results from our spawning study suggest potential negative effects of B/UKME on fry growth and survival, with a threshold concentration of approximately 10%. Although this threshold concentration falls within the 60% average yearly concentration of effluent that exists in the stream near the point of discharge (Rice Creek), it is above the concentrations reported at the confluence of Rice Creek with the St. Johns River. Currently, field studies are needed that quantify exposure of bass to these effluents, while evaluating their potential effects on reproductive success.

Acknowledgement—We wish to express our gratitude to Georgia-Pacific for having funded this study. David Spraley and Myra Carpenter were responsible for the construction and maintenance of the treatment tank system. Shane Ruessler, Carla Wieser, Jon Wiebe, Nicola Kernaghan, and Kelly McDonald assisted in the treatment and processing of fish and in the analysis of sex hormones. Kevin Kroll and Marjorie Chow conducted the vitellogenin analyses, and Galin Jones assisted in the statistical analyses. Dieb Birkholz assisted in the analyses of resin acid in bile. We wish to thank two anonymous reviewers for their extensive comments, which greatly improved the quality of this manuscript. Finally, we would like to thank Richard Krause and Bob DeMauro for their assistance in the development of the methodology employed for spawning and hatching of largemouth bass in this study.

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