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TIMING OF EXPOSURE TO A PULP AND PAPER EFFLUENT INFLUENCES THE MANIFESTATION OF REPRODUCTIVE EFFECTS IN RAINBOW TROUT

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Abstract—Rainbow trout were exposed to a secondary treated, thermomechanical/bleached kraft pulp and paper effluent in 12,000-L, flow-through exposure tanks at an environmental research facility located at a pulp and paper mill in Kawerau, New Zealand. Trout (age, 2+ years) were obtained from a local hatchery and exposed either to upstream river water or a nominal concentration of 12% (v/v) effluent diluted in upstream river water. Three treatment groups were used: Effluent exposure that started approximately three months before gonadal growth (eight-month total exposure), effluent exposure that started approximately halfway through gonadal development (two-month total exposure), and trout exposed to reference water alone for the total duration of the experiment. Trout were sacrificed just before spawning; exposure, growth, and reproductive endpoints were assessed during and at the termination of the experiment. Reduction in growth was observed in both sexes in the eight-month treatment group relative to the river water reference treatment group. No differences were observed in condition factor or liver size in either treatment. Females in the eight-month exposure group also had significantly lower ovary weight. The two-month exposure group showed no differences from the reference group in growth or somatic indices. Estradiol and testosterone were reduced in blood samples taken from the eight-month exposure group by four months into the experiment as compared to the reference treatment. Steroid and vitellogenin levels in individual female trout from this treatment were significantly correlated with gonadosomatic indices (GSI) measured at the termination of the experiment. The GSI was not correlated strongly or consistently with pregnenolone, nor were any treatment-related pregnenolone differences observed, indicating that the steroid hormone reductions likely were not related to cholesterol side-chain cleavage. Male trout showed significant induction of vitellogenin and lower 11-ketotestosterone during the experiment (only the eight-month group was examined), but this did not result in any significant differences in testes development. Thus, this study has shown an impact of pulp mill effluent exposure on the reproductive physiology of female trout that appeared to be hormonally mediated. Furthermore, the effect could only be manifest when the exposure was initiated before the start of gonad development.

Keywords—Pulp Paper Reproduction Fish Bleached kraft mill effluent

INTRODUCTION

The biological effects of pulp and paper wastewaters have been the subject of intense scientific scrutiny during the past three decades. In response, the wood-fiber industry has undergone improvements in pulping and bleaching processes and wastewater treatment technologies to reduce environmental impacts. Foremost among these improvements are the widespread use of aerobic treatment systems and the conversion to elemental-chlorine-free (ECF) bleaching. These changes have eliminated acute lethality in fishes and led to dramatic improvements in the quality of receiving environments. However, despite these enormous improvements in effluent quality, some pulp and paper effluents still show the potential to alter the reproductive physiology of fishes.

Reproductive impacts observed in fishes exposed to pulp and paper mill effluents include altered secondary sexual characteristics [1] and reduced gonad size [2–9], which are both thought to be associated with impaired biosynthesis of sex steroid hormones [10–13]. Pulp and paper mill effluent has also induced expression of the female yolk precursor protein, vitellogenin, in the livers of juvenile and male fish [14,15]. Estrogenic nonylphenol ethoxylates are known components of some effluents [16], whereas an estrogenic isoflavone, genistein, has been identified in one effluent [17]. Pulp mill effluent may also possess androgenic qualities [18–20]. The potent androgen androstenedione has been implicated as the main compound responsible for this activity [19].

Studies performed using a secondary treated, thermomechanical/bleached kraft pulp and paper mill effluent in New Zealand demonstrated that the effluent was not estrogenic as measured by plasma vitellogenin concentrations in juvenile rainbow trout [18]. Exposure of sexually mature rainbow trout to the same effluent during the last two months of gonadal development did not produce any measurable effects on plasma steroid hormone concentrations or on gonad growth [21]. Female mosquitofish exposed to similar environmentally relevant concentrations of this effluent showed significant gonapodial morphogenesis, or masculinization [18]. The contradiction between androgenicity in mosquitofish and lack of reproductive effects in trout as shown in these studies could not be explained. An unrelated study found that the reproductive influences of pulp and paper mill effluent on fathead minnows were greater when exposure was initiated before sexual maturation [22], which is a possible reason why trout may not have responded to the aforementioned effluent.

The goal of the present study was to determine if effluent from a modernized pulp and paper mill could elicit reproductive responses in rainbow trout if exposure was initiated well before the period of gonadal growth started. This experiment was paired with a repeat of a previous study that started exposure midway through active reproductive development. Thus, three treatment groups were used: An eight-month exposure, a two-month exposure, and a reference group. These

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experiments utilized environmentally relevant exposure concentrations and controlled for confounding environmental variables, particularly energy intake, through use of large experimental holding tanks and controlled ration levels.

MATERIALS AND METHODS

Mill description

The Tasman Mill is located adjacent to the Tarawera River in the Bay of Plenty region of the North Island, New Zealand. The Tasman mill uses both kraft and thermomechanical pulping (TMP) processes (760 and 1,010 air-dried tons·d^{-1}, respectively). The mill furnish is largely softwood (*Pinus radiata*), with the occasional pulping of *Eucalyptus* sp. Kraft pulp is chlorine bleached at either of two bleach plants with sodium hypochlorite (HH) or chlorine dioxide (DEopDnD or DEopPD). The Tasman Mill has been ECF since April 1998. The TMP effluent is pretreated in-mill using an aerobic, moving-bed bioreactor system. This pretreated effluent and the effluent from the remainder of the mill operations are collected into a single drain and treated in an aerated lagoon system with 5- to 6-d retention time before release to the Tarawera River. The Tasman Mill has had secondary treatment since 1972. The mean total effluent flow of the Tasman Mill is approximately 180,000 $m^3 \cdot d^{-1}$. The initial dilution of this effluent in the Tarawera River ranges between 5% and 12%.

Exposure system

Rainbow trout exposures were performed in an experimental facility located at the Tasman Mill. The geographical location and the description of the facility have been described in detail elsewhere [21]. The system used in this experiment consisted of six 12,000-L, epoxy-coated fiberglass tanks. Each tank received gravity flow through individual pipes that were split off from the mixing tanks. Effluent composition of replicate tanks was identical, because flows were split off from the same mixing tank. Water flow into exposure tanks was a continuous 20 L/min (95% replacement time of 30 h).

Reference water was pumped directly from the Tarawera River at a point upstream of any influences of the two pulp and paper mills that release wastewater into the river. Full-strength (100% v/v) effluent was transported by tanker truck on a weekly basis from the point just before the effluent enters the river to an 80,000-L, concrete holding reservoir adjacent to the experimental ponds. Effluent in the holding reservoir was continually recirculated using submersible pumps to prevent solids from settling and to stop the effluent from becoming anaerobic. Effluent and dilution water flow in the exposure treatments were measured using digital in-line flow meters (Great Plains Industries, Wichita, KS, USA), and flow was adjusted daily using stainless-steel globe valves. The nominal dilution rate in the exposure tanks was 12% (v/v). This value was chosen because it approximates the upper level of the effluent dilution in the Tarawera River.

Effluent dilution was measured every hour in one of the exposure tanks. Electrodeless conductivity probes (model 3600E; Great Lake Instruments, Milwaukee, WI, USA) were placed in one of the reference tanks, in one effluent exposure tank, and in a flow cell at the end of the 100% effluent line just before the mixing tanks. Temperature-corrected conductivity was measured using three Great Lakes Instruments model 672E conductivity analyzers and was logged every hour using a Hobo data-logger (Onset Computer, Bourne, MA, USA). Because of the high conductivity of the effluent relative to the reference river water, conductivity could be used to calculate dilution. The water temperature in two reference tanks and two effluent tanks was recorded every hour using Onset Tidbit temperature loggers.

Fish and exposure

Age 2+ years rainbow trout were obtained from Eastern Fish and Game New Zealand, Ngongotaha Hatchery, located near Rotorua. Trout were transported from the hatchery to Kawerau (~80 km) in early September 1999 (Austral winter) and placed in Tarawera River water in experimental tanks for a 16-d acclimation period before effluent exposure started. No transport-related mortality was observed. Trout were held under ambient light conditions at both the hatchery and the experimental facility in Kawerau, New Zealand.

Immediately before the initiation of exposure, trout were anesthetized using MS-222 (100 mg·L^{-1}), weighed, and measured. Each trout was implanted with an individually numbered T-bar-type tag (HallPrint, Holden Hill, SA, Australia). Exposure density was 80 trout per 12,000-L tank. A subsample of 20 immature males and 20 immature females was sacrificed at this time to obtain time-zero blood for steroid analysis and initial organ weights.

Trout were fed commercial salmon food (Pacific Lite, 6-mm pellets, 14% lipid, 43% crude protein; NRM Feed Mills, Hope-Nelson, New Zealand). Ration level was set at 0.7% of wet body weight per day as based on the total biomass of trout in each tank. Based on known growth trajectories for this ration level, the quantity of food was adjusted monthly to reflect the change in biomass. Growth estimates were within 5% of actual measured growth.

The longer-term exposure began in two replicate tanks on September 24, 1999. The duration of this exposure was approximately eight months; thus, this treatment is referred to here as the eight-month treatment. Trout would be expected to start gonad growth in January at the earliest, so fish in this treatment were exposed to effluent while in a sexually immature state for at least three months. The shorter-term exposure was started on March 25, 2000, and is referred to here as the two-month exposure. In general, by late March male trout have completely developed testes, and female trout are halfway through the vitellogenic period of sexual development.

Two interim measurements were performed on December 28 to 30, 1999, henceforth referred to as 93 d, and on March 31 to April 2, 2000, henceforth referred to as 188 d. The latter sampling date was one week after the two-month exposure started. Trout were recaptured, anesthetized, weighed, and measured, and 2 ml of blood were removed by caudal puncture. Trout were sacrificed on May 29 to 31, 2000, for a minimum exposure of 247 d for the eight-month treatment and 64 d for the two-month treatment. Because of the duration of the sampling procedure, it was not possible to perform either the interim or the final sampling in 1 d. Replicate tanks were sampled on different days, and where possible, at least one reference and one treated tank were sampled during the same day. The order of sampling of the treatment and reference tanks was reversed on consecutive days.

During the necropsy procedure, each trout was rendered unconscious by a blow to the head, and 5 ml of blood were removed by caudal puncture, placed in a heparin-coated vacutainer, and stored on ice. Blood was spun at 500 *g* in a refrigerated centrifuge to obtain plasma. Trout gender, weight,

length, gonad weight, liver weight, and spleen weight were recorded. In female trout, a subsample of approximately 5 g of ovarian tissue was frozen at −20°C pending fecundity analysis (determined by direct count). The average weight of each ovarian follicle, hereafter called the calculated egg weight, was derived by dividing the total weight of each ovary by the total number of eggs calculated for each ovary.

Ovarian, liver, and muscle tissue for chemical analysis were placed on prefired (450°C) aluminum foil in individually labeled bags and archived at −20°C. The gallbladder was removed intact, placed in a 2-ml cryovial, and quick-frozen in liquid nitrogen vapor phase in a liquid nitrogen dry-shipper. Liver for mixed-function oxygenase (MFO) analysis (0.5–1.0 g) was placed in a 2-ml cryovial and also frozen in liquid nitrogen vapor phase. Liver, bile, and plasma samples were stored at −85°C pending analysis.

Steroid hormone and vitellogenin analysis

Steroid hormones were measured according to the method of McMaster et al. [23]. Plasma samples were thawed, and steroid hormones were extracted with diethyl ether. The steroids, testosterone, estradiol, pregnenolone, and 11-ketotestosterone were obtained from Sigma (St. Louis, MO, USA). Testosterone and estradiol antibodies were obtained from ICN (Costa Mesa, CA, USA), 11-ketotestosterone antibody from Helix Biotech (Vancouver, BC, Canada), and pregnenolone antibody from Medicorp (Montreal, PQ, Canada). Tritiated testosterone and estradiol were obtained from Amersham Life Science (Little Chalfont, Buckhamshire, UK), and tritiated pregnenolone was obtained from NEN Life Science Products (Boston, MA, USA). Tritiated 11-ketotestosterone was the gift of G. Van Der Kraak (University of Guelph, Guelph, ON, Canada). The plasma extract from female trout was analyzed for estradiol, testosterone, and pregnenolone, whereas that from male trout was analyzed for 11-ketotestosterone and testosterone using standard radioimmunoassay procedures. Plasma vitellogenin was measured with a specific rainbow trout enzyme-linked immunosorbent assay kit (Biosense Laboratories, Bergen, Norway).

*7-Ethoxyresorufin-*O*-deethylase analysis*

Hepatic MFO enzyme activity was estimated in postmitochondrial supernatant (PMS) as 7-ethoxyresorufin-*O*-deethylase (EROD) activity using a modification of the fluorescence plate-reader technique outlined by van den Heuvel et al. [24]. Liver extracts were homogenized in a cryopreservative buffer (0.1 M phosphate, 1 mM EDTA, 1 mM dithiothreitol, and 20% glycerol, pH 7.4 overall) and spun at 9,000 *g* to obtain the PMS. The EROD reaction mixture contained 0.1 M *N*-2-hydroxyethylpiperazine-*N*′-2-ethane-sulfonic acid (HEPES) buffer (pH 7.8; Sigma), 5.0 mM Mg^{2+}, 0.5 mM NADPH (Applichem, Darmstadt, Germany), 1.5 μM 7-ethoxyresorufin (Sigma), and approximately 0.5 mg·ml^{-1} of PMS protein. The EROD activity was determined kinetically in 96-well plates using one reading every minute for 10 min on a BMG Polarstar Galaxy microplate fluorometer (BMG Labtechnologies, Offenburg, Germany). Resorufin was determined using 544-nm excitation and 590-nm emission filters. Protein content was estimated from fluorescamine (Sigma) fluorescence (390-nm excitation filter, 460-nm emission filter) against bovine serum albumin (Sigma).

Water and bile chemistry

Water samples for determining organic extractives were taken on a weekly basis on the day immediately after the delivery of effluent. A subsample of 100% effluent was filtered within 10 min of collection through 15-cm glass fiber-C filters. Both the filter paper and the filtrate were stored frozen at −20°C pending analysis.

Filtrate samples were thawed, and 125 ml of filtrate were adjusted to pH 9.0 with NaOH. Surrogate standards (2,4,6-tribromoanisole, 2,4,6-tribromophenol, D_{10}-anthracene, D_{31}-palmitic acid, 8[14]-abietenic acid, and dihydrocholesterol) were introduced immediately before extraction. Samples were continuously extracted with dichloromethane using glass liquid-liquid extractors. Extracts were passed through sodium sulfate and concentrated with nitrogen using a Zymark Turbovap (Hopkinton, MA, USA). The final extract was silylated with bis(trimethylsilyl)-trifluoroacetamide plus 1% trichloromethylsilane and analyzed by gas chromatography with mass-selective detection (GC-MSD). All analyte concentrations were corrected for extraction blanks and adjusted for recovery of the appropriate surrogate standard.

Trout bile for extractives analysis was pooled from at least 30 individual trout per treatment. Bile from male and female trout was pooled, because a previous study had shown no gender-related differences in the accumulation of bile metabolites [21]. Bile samples were hydrolyzed with ethanolic potassium hydroxide. Hydrolyzed bile was extracted at pH 9.0 with methyl-*tert*-butyl ether, and the extract was dried with sodium sulfate. Extracts were derivatized and analyzed by GC-MSD as described for water samples above. Samples were corrected for surrogate recovery and blank determinations.

Statistics

Condition factor, liver size, gonad size, and fecundity data were analyzed using analysis of covariance (ANCOVA) on base-10 logarithmically transformed variables, with body size (length or wt) as the covariate. Although statistical comparisons using ANCOVA were completed on body weight, liver weight, spleen weight, gonad weight, and total fecundity, data are presented as somatic indices for ease of comparison.

Fulton's condition factor was calculated as body weight ÷ $length^3$·100. The gonadosomatic index (GSI) was calculated as gonad weight ÷ (body wt − gonad wt)·100. The liver-somatic index was calculated as liver weight ÷ (body wt − liver wt)·100, and the spleen somatic index was calculated as spleen weight ÷ (body wt − spleen wt)·100. Only maturing trout were used in the calculation of GSI. For comparison purposes, fecundity was calculated as number of eggs per kilogram of wet body weight.

Growth data and steroid data were compared independently using analysis of variance (ANOVA) after log-transformation of data in which significant heteroscedasticity or departures from normality were observed. Initially, replicate tanks were tested as a variable in all ANOVAs. Replicate tanks did not add significant variability for any of the endpoints; thus, individuals from replicate tanks were pooled for all further analyses. Dunnett's test was used for post-hoc comparisons of treatment groups to the reference group. The EROD data and vitellogenin data would not conform to the assumptions of parametric analysis after log transformations and were instead compared using a nonparametric Kruskal-Wallis one-way ANOVA with Bonferroni adjustment for multiple comparisons. All statistical testing was completed using the SYSTAT® soft-

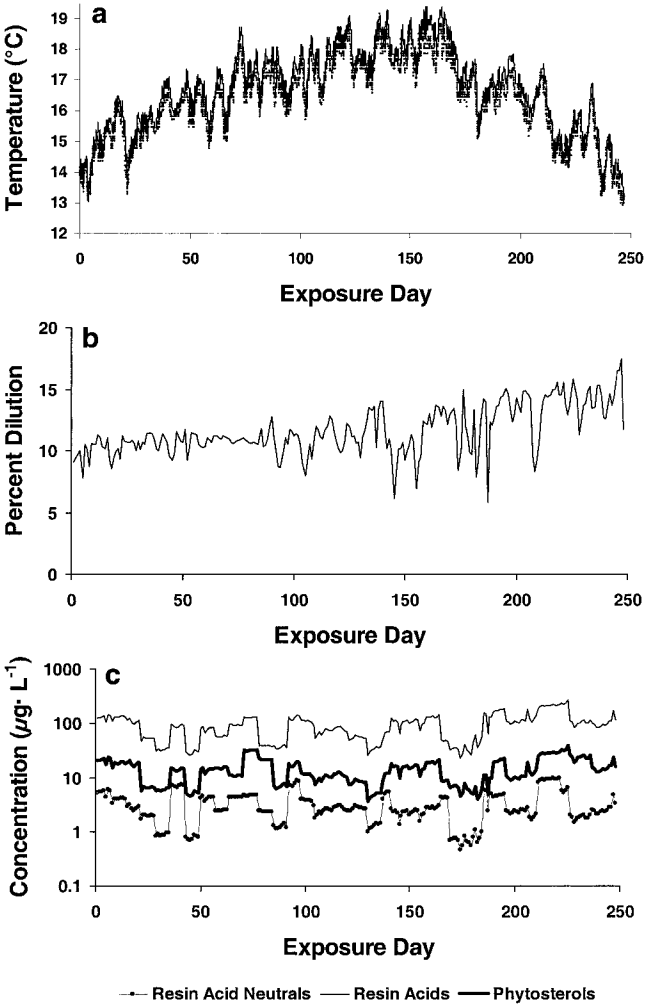

Fig. 1. Water-quality characteristics during an eight-month exposure of rainbow trout to a pulp and paper mill effluent. **a.** Temperature. The black line represents the mean of two effluent tanks and the dotted line the mean of two reference tanks. **b.** Daily averages of the dilution of effluent (% v/v) in the eight-month exposure as calculated from conductivity measurements. **c.** Calculated total concentrations of the three main families of extractives (resin acid neutrals, resin acids, and phytosterols) in the exposure tanks.

ware package [25]. The critical level of statistical differences for all analyses in the present study was assessed at $\alpha = 0.05$.

RESULTS

Bulk chemistry parameters on the final effluent were measured continually at the outfall to the Tarawera River by mill environmental staff. During the period encompassed by this experiment, mean (standard error of the mean, *n*) BOD was 27.9 mg·L^{-1} (0.6, 250), total suspended solids were 40.2 mg·L^{-1} (1.2, 250), and pH was 7.43 (0.03, 174). The mean water temperatures in the reference and eight-month effluent treatments were 16.36°C (0.02, 5,928) and 16.71°C (0.02, 5,928), respectively. Water temperature ranged from a high of 19.6°C during the middle of the experiment in February to a low of 12.9°C at the end of the experiment in late May. The temperature profile closely followed the seasonal temperature changes in the Tarawera River (Fig. 1a). The effluent used was typically warmer than the river water, and as a result, a mean temperature difference of 0.3°C was observed throughout the exposure period.

Table 1. Dissolved organics concentrations (μg·L^{-1}) in 100% (v/v) effluent during the experiment ($n = 34$)

Compound	Mean total concentration	Standard error of the mean
Fichtelite	15.8	1.6
Dehydroabietin	0.3	0.1
Tetrahydroretene	6.9	1.3
Retene	5.9	1.2
Methyldehydroabietin	0.2	0.1
Total resin acid neutrals	29.2	3.1
Pimaric acid	55.2	5.0
Sandaracopimaric acid	4.9	0.5
Isopimaric acid	27.0	2.3
Palustric acid	6.3	0.9
Dehydroabietic acid	97.1	8.1
Abietic acid	82.5	8.1
Neoabietic acid	7.6	1.2
Pimarenic acid	19.6	1.8
Sandaracopimarenic acid	31.7	3.0
Isopimarenic acid	44.6	4.4
13-Abietenic acid	153.8	17.0
Dihydroisopimaric acid	14.8	1.6
Pimaranic acid	13.6	1.3
Isopimaranic acid	8.0	0.8
Abietanic acid	151.0	13.8
Seco-1-dehydroabietic acid	54.5	6.9
Seco-2-dehydroabietic acid	28.2	4.4
12-Chlorodehydroabietic acid	1.7	0.2
14-Chlorodehydroabietic acid	7.7	0.8
12,14-Dichlorodehydroabietic acid	0.4	0.1
7-Oxodehydroabietic acid	1.8	0.4
Total resin acids	812.0	68.4
Campesterol	2.9	0.3
Stigmasterol	11.1	1.5
β-Sitosterol	80.0	7.7
β-Sitostanol	29.5	2.6
Total phytosterols	123.5	11.1

Daily average rates of dilution were estimated from the conductivity measurements (Fig. 1b). The mean effluent dilution during the experiment was 11.6% v/v (0.1, 249). The average composition of the major extractives in the full-strength (100% (v/v)) effluent supplied to the exposure tanks is shown in Table 1. Based on the average daily dilution and on the weekly extractives measured, the total concentrations of these extractives to which fish were exposed was calculated on a daily basis (Fig. 1c). Much of the variability in the concentrations to which trout were exposed occurred in the week-to-week variability of the effluent composition. The mean total resin acid neutrals during the exposure period were estimated at 3.3 μg·L^{-1}, the mean phytosterols (dominated by β-sitosterol) were estimated at 14.6 μg·L^{-1}, and resin acids at 94.8 μg·L^{-1}. No increased mortality rate was observed in the trout exposed to effluent. The reference tanks had a survival rate of 92.1%, whereas the eight-month and two-month treatment tanks had 95% and 92.9% survival, respectively.

Analysis of bile extractives (Table 2) from trout at the termination of the experiment revealed the excretion of resin acids, which is diagnostic of exposure to pine softwood furnish pulp and paper mill effluents. No resin acid neutrals (present in effluent) were found in the bile. Resin acid neutrals are generally presumed to be metabolized, and parent compounds are seldom detected in bile. Phytosterols in bile showed no obvious differences related to effluent exposure. The EROD activity, a typical biochemical indicator of exposure to pulp

Table 2. Extractives concentrations ($\mu g \cdot g^{-1}$ dry wt) in pooled, hydrolyzed trout bile and mean (SE) hepatic 7-ethoxyresorufin-*O*-deethylase activity (EROD, $pmol \cdot mg^{-1} \cdot min^{-1}$)[a]

Compound/parameter	Eight-month	Two-month	Reference
Pimaric acid	97.5	257.0	<0.1
Sandaracopimaric acid	<0.1	<0.1	<0.1
Isopimaric acid	20.5	51.4	<0.1
Palustric acid	<0.1	<0.1	<0.1
Dehydroabietic acid	132.8	239.6	34.9
Abietic acid	82.5	205.5	36.6
Neoabietic acid	<0.1	<0.1	<0.1
Pimarenic acid	42.3	24.7	<0.1
Sandaracopimarenic acid	88.7	53.1	<0.1
Isopimarenic acid	74.7	52.6	<0.1
13-Abietenic acid	43.1	21.9	8.1
Pimaranic acid	13.5	12.7	<0.1
Isopimaranic acid	6.6	4.8	<0.1
Abietanic acid	242.7	591.1	<0.1
Seco-1-dehydroabietic acid	103.6	69.8	<0.1
Seco-2-dehydroabietic acid	76.7	54.0	<0.1
12-Chlorodehydroabietic acid	<0.1	<0.1	<0.1
14-Chlorodehydroabietic acid	9.6	30.5	<0.1
12,14-Dichlorodehydroabietic acid	<0.1	<0.1	<0.1
7-Oxodehydroabietic acid	<0.1	<0.1	<0.1
Total resin acids	1,068.0	1,668.9	79.7
Campesterol	8.7	70.0	17.3
Stigmasterol	11.1	36.7	<0.1
β-Sitosterol	<0.1	8.0	20.5
β-Sitostanol	<0.1	<0.1	<0.1
Total phytosterols	8.7	114.7	37.8
EROD activity			
Males	9.09[b] (0.89)	5.20 (1.36)	3.21 (0.55)
Females	0.32 (0.06)	0.07 (0.01)	0.22 (0.09)

[a] Bile was pooled from at least 20 individual trout, and triplicate determinations were averaged.
[b] $p < 0.05$.

and paper effluents, was significantly elevated in male trout in the eight-month exposure group (threefold over reference), but not in male trout in the two-month exposure group (Table 2). The EROD activity in female trout was depressed during the late reproductive period, and no treatment-related differences were observed.

This particular strain of rainbow trout typically has both males and females that do not reach sexual maturity by age three years. Rates of immaturity for male trout were 13.8%, 32.2%, and 36.1% for the reference, two-month, and eight-month treatments, respectively. The corresponding rates in females were 20.9%, 23.0%, and 18.4%, respectively. The difference between the reference and the treatment males could not be attributed to effluent exposure, because the male trout in the two-month effluent exposure group would have had nearly complete testes development at the initiation of exposure. No obvious differences existed in the maturation rate of the females because of effluent exposure.

The total growth for each trout was calculated as a percentage of the original weight and length of tagged individuals (Fig. 2a, b, d, and e). In both females and males in the eight-month treatment, a small but statistically significant reduction in growth was observed by the end of the experiment. These differences were observed when either length or weight was used as the growth variable. In only one case (length in males) did the two-month exposure differ significantly from the reference, and this was well before effluent exposure had begun in that treatment. However, despite the differences in growth, no significant differences were found in condition factor. Condition factor in all sexes and treatments showed a steady increase throughout most of the experiment, but it tended to taper off as trout approached gonadal maturity. The condition factors during interim measurements and at the end of the experiment indicated no effect of effluent exposure on the accumulation of body mass relative to length (Fig. 2c and f). Similarly, no changes in liver size relative to the reference trout were observed (Fig. 3a). Female trout in the eight-month treatment group did show significantly elevated spleen size (Fig. 3b).

In females, effluent exposure for eight months resulted in significantly lower gonad weights relative to those in the reference trout (Fig. 3c). However, two months of continuous effluent exposure did not impact on the latter half of vitellogenesis in females in the two-month treatment (Fig. 3c). No treatment-related differences were observed in fish fecundity. The mean fecundity was 2,596 (96, 24), 2,414 (116, 30), and 2,742 (87, 28) $eggs \cdot (kg\ body\ wt)^{-1}$ for the eight-month, reference, and 2-month treatments, respectively. However, the mean calculated egg weight (53 mg) in the eight-month exposure was significantly lower ($p < 0.001$) than egg weights of trout in the reference and 2-month exposure groups (68 and 63 mg, respectively). Thus, it can be concluded that the smaller ovary size in females resulted from smaller ovarian follicles and not from fewer eggs. In males, no significant differences were observed in testes development over the exposure period (Fig. 3c). Male rainbow trout showed typical secondary sex characteristics in all treatments, including hooked jaw, altered coloration, smaller scale size, and modified body shape. No male secondary sex characteristics were observed in either female trout or trout that did not mature.

The steroid hormone analysis was performed on a subset of trout such that the same individuals were used for every time period. Steroid hormones followed a typical seasonal trend, with sexual maturation beginning at approximately 100 d of effluent exposure (January). Testosterone in males and females and 11-ketotestosterone in males rose from the onset of maturation until the experiment was terminated (Fig. 4b, c, and d). Estradiol in females plateaued during the last 64 d of the experiment (Fig. 4a). In female trout, estradiol and testosterone in the eight-month exposure group were significantly lower than reference concentrations after 93 d of exposure, whereas estradiol alone was significantly depressed at 188 d of exposure (Fig. 4a and b). In males, 11-ketotestosterone in the eight-month exposure group was significantly lower than in the reference group at 93 d of exposure (Fig. 4c). Pregnenolone was measured in females at 93 and 188 d of exposure; no treatment differences were observed at either of these sampling periods. After 93 d of exposure, mean pregnenolone concentrations were 4.3 (0.2, 12), 4.3 (0.2, 12), and 4.2 (0.2, 12) $ng \cdot ml^{-1}$ for trout in the reference, two-month, and eight-month treatments, respectively. After 188 d of exposure, mean pregnenolone concentrations were 7.4 (0.7, 12), 9.1 (0.5, 12), and 7.2 (0.5, 23) $ng \cdot ml^{-1}$ for trout in the reference, two-month, and eight-month exposures, respectively. Pregnenolone remained relatively constant as compared to testosterone and estradiol, which increased dramatically during the period of active sexual maturation.

To determine whether lower ovary size could have resulted from reduced vitellogenin deposition in the ovaries, plasma vitellogenin in females was measured at 93 and 188 d of ex-

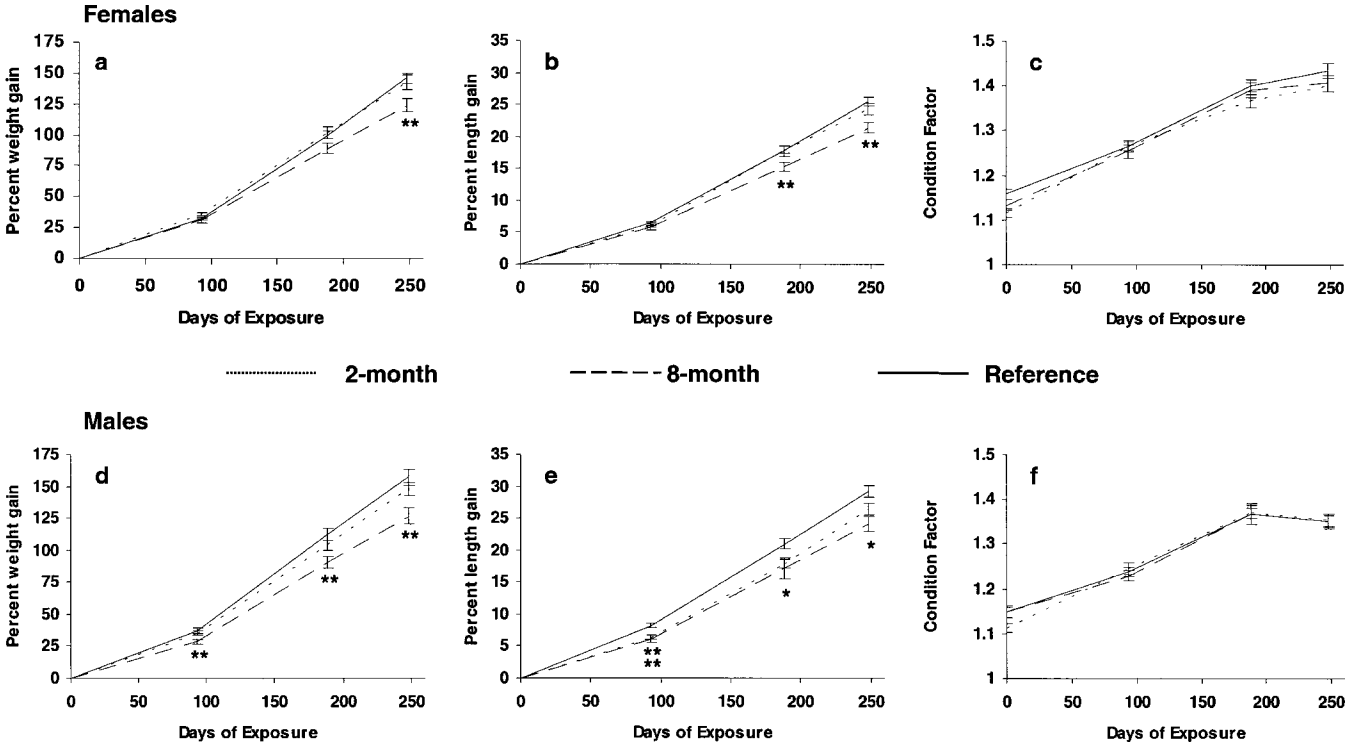

Fig. 2. Mean growth and condition parameters. **a.** Weight gain in females. **b.** Length gain in females. **c.** Condition factor in females. **d.** Weight gain in males. **e.** Length gain in males. **f.** Condition factor in males. Error bars indicate standard error of the mean. Sample sizes range from 45 to 51 for each data point. $*p < 0.05$, $**p < 0.01$.

posure (only in the reference and eight-month treatment groups). Reduced levels of circulating vitellogenin were found at both time periods (Fig. 5a). As expected, a large rise in vitellogenin occurred in both treatments during the 93- to 188-d exposure period, reflecting the increases in vitellogenesis associated with gonadal growth. Vitellogenin was measured in male trout from the reference and eight-month exposure groups as an indicator of exposure to estrogenic compounds in the effluent. At 93 d of exposure, a 25-fold increase in plasma vitellogenin was observed in effluent-exposed male trout (Fig. 5b). Male effluent-exposed trout also showed a fourfold elevation of vitellogenin at 188 d of exposure, but levels were 1,000-fold lower than those measured at 93 d of exposure.

Because trout were individually tagged, it was possible to correlate plasma steroid hormones and vitellogenin at the different bleeding times with the GSI measured at the termination of the experiment. Male trout showed no GSI differences or any significant GSI-steroid correlations. Statistically significant correlations were found between GSI and both estradiol and testosterone in female trout at all time periods, with the exception of estradiol at 248 d (Table 3). This is thought to reflect the observation that estradiol synthesis reduces or ceases as spawning approaches. These correlations show that female trout with a lower ratio of ovary weight to body weight at the end of the experiment had a tendency toward low estradiol and testosterone levels from the prematurational state and maintained lower steroid levels throughout the experiment. Vitellogenin showed similar correlations with GSI, as was observed with testosterone and estradiol. Pregnenolone showed a weaker correlation with GSI, which was significant only at 188 d of exposure. These observations, together with strong steroid correlations between time periods (data not shown), also indicated that an individual fish with low sex steroid concentrations tended to maintain low levels over time.

DISCUSSION

This study demonstrated that exposure to effluent from an integrated bleached kraft/TMP mill was capable of reducing ovarian development in rainbow trout. This observation was only manifest in trout exposed during the prematurational state; it was not observed when fish exposure was initiated during midmaturation. Correlations between steroid hormones and gonad size strongly suggest that a hormonal mechanism, resulting in decreased vitellogenin transport, was responsible for reduced gonad size. The observed gonad size effects were accompanied by decreased growth, but not by decreased condition factor.

Evidence is insufficient to conclude that reduction of growth in trout of the eight-month exposure group was caused by the direct metabolic effects of effluent exposure. The amount of diet offered to trout was constant between treatments; however, the exact amounts consumed cannot be easily quantified. Visual feeding was probably more difficult for trout in the effluent exposure tanks because of the darker color and reduced water clarity. Throughout the experiment, the temperature of the effluent could not be reduced to the point at which the exposure tanks were identical in temperature to the reference tanks. A temperature differential of approximately 0.3°C was present. The temperature optimum for rainbow trout growth is expected to be between 15.5 and 16.5°C [26], though this may vary with the size and strain of fish. In this experiment, river water temperature was below and above the optimum range for approximately equal amounts of time. Thus, it seems unlikely that the observed growth differences were caused by the temperature differential, because it would pro-

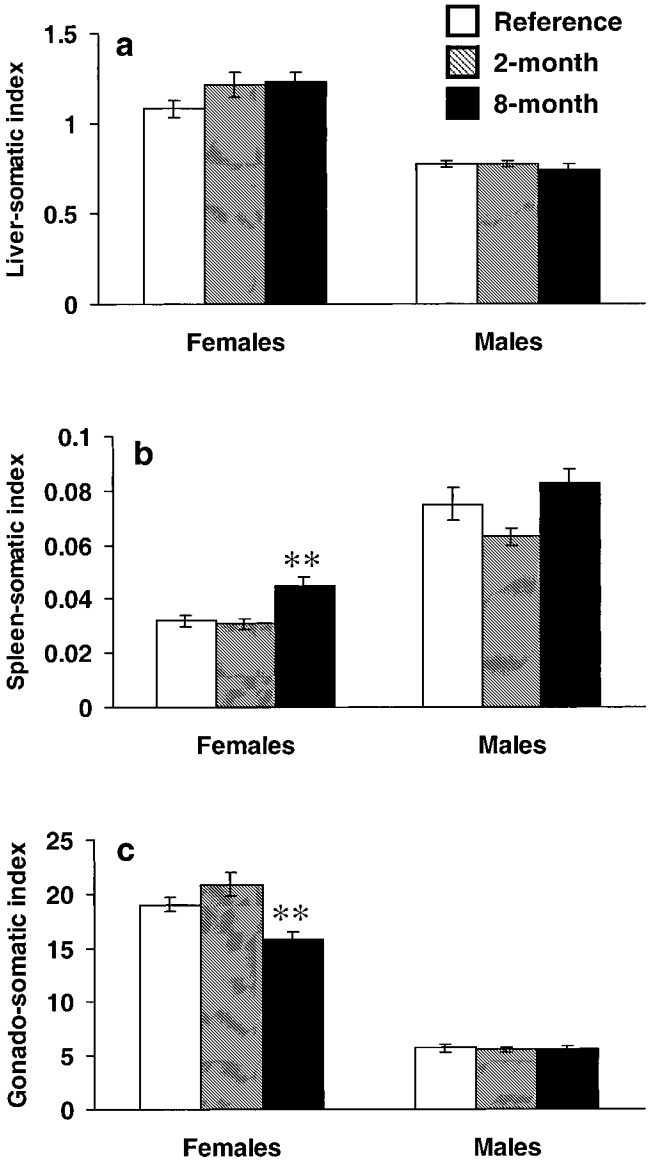

Fig. 3. Mean somatic parameters. **a.** Liver-somatic index. **b.** Spleen somatic index. **c.** Gonadosomatic index. Error bars indicate standard error of the mean. Sample sizes range from 24 to 30 for each bar. Asterisks indicate significant differences between a treatment and the reference. $^{**}p < 0.01$.

vide growth stimulus at lower-than-optimum temperatures and growth inhibition at higher-than-optimum temperatures.

Relatively few of the studies examining the effects of effluent on fish growth have used controlled rations. Those that did found decreased growth [27–29], increased growth [30], or no change in growth [31,32]. As with our study, many of these studies did not quantify consumption, only the quantity of food offered to the fish. Only the studies by Stoner and Livingston [27] and by Webb and Brett [28] calculated food conversion efficiency, and those authors found it to be reduced. To our knowledge, these studies are the only direct evidence (using controlled laboratory studies) of metabolism-based growth effects in fish exposed to pulp mill effluent. However, the nature of the poorly or untreated effluents tested in these older studies is dramatically different from the secondary treated effluent used in this study. Thus, the most likely cause of the growth differences observed in our study was reduced consumption of food rather than reduced conversion efficiency.

Differences in growth were not accompanied by differences in condition factor. In many field studies, condition factor and liver size in fish increased with pulp mill effluent exposure [2,9,34,35]. These observations likely stem from changes in food quantity or quality created by nutrient enrichment of the receiving environment. The use of mesocosms and controlled rations in this experiment effectively removed the variability caused by food supply that may be encountered in field studies, and the resultant lack of effect on fish condition favors the hypothesis that increases in condition factor in the field are caused by enrichment effects. The liver is a major energy-storage organ. However, interpreting elevated liver size is more complicated, because it may change with factors other than energy mobilization. Changes in the size of the spleen in the eight-month exposed females cannot be easily explained. One possibility is that the fish in the eight-month exposure became more susceptible to infection, resulting in an enlarged spleen caused by melanomacrophage proliferation. Alternatively, a stress on the respiratory system could result in the proliferation of erythrocytes in the spleen. The responses mentioned can occur quite quickly, so the two-month exposure group must not have been exposed to the factor that caused this observation.

Energy storage, as indicated by condition factor and liver size, was similar in all treatments, yet the utilization of this energy as female gonadal tissue was reduced in the eight-month exposure group. It appears that the transfer of energy from somatic tissue to gonad was either impaired or reduced in these fish. The observation that trout in the eight-month exposure group grew less as compared to trout in the other groups may lead to the conclusion that lower food consumption resulted in smaller ovaries. We know of three arguments against this: Similar condition factors mean that energy per unit length was similar in the reference and eight-month groups and that energy was converted into gonad in one group but not in the other; subsequent experiments have shown that greatly reducing the ration does not, in fact, decrease the GSI (M. van den Heuvel, unpublished data); and strong evidence is presented here of a hormonal mechanism of impaired gonad growth.

All the reproductive effects manifest in the present study were only seen in trout exposed to effluent for the full eight months. Identical to a previous study [21], trout exposed two months before spawning showed absolutely no reproductive effects. This suggests that the critical period when trout may be responsive to this effluent is before the onset of gonad maturation. This may only represent a short delay in the onset of gonad growth, but trout do not appear to catch-up or recover from this delay. A similar phenomenon has been observed in fathead minnows exposed to a pulp mill effluent [22]. Reproductive effects in fathead minnows were more severe when exposure began in the juvenile stage. From the data presented here, the specific mechanism for such a timing-induced effect appears to be hormonally mediated. Because of the insensitivity of trout that are already maturing, it can be postulated that the relatively low level of steroid hormones during the period of immaturity allows exogenous compounds to compete for the site(s) of action, thus delaying the onset of maturation. The very high level of steroid hormones during active maturation may prevent this simply because of the higher concentration ratio of endogenous to exogenous hormones. If the relative concentration of steroid hormones is important, then this also has implications for differences in species sensitivity.

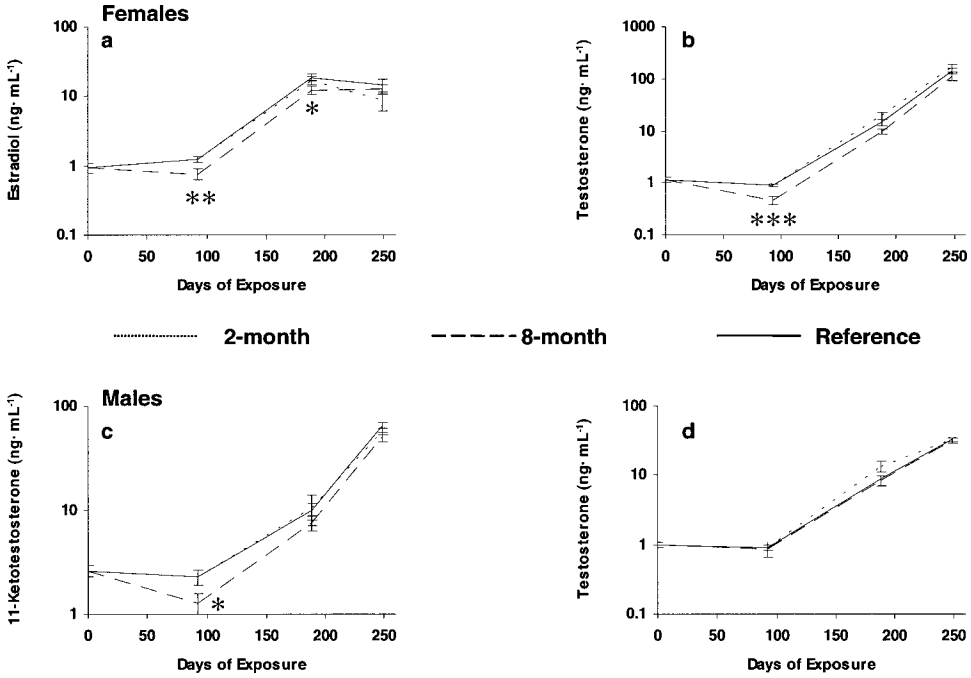

Fig. 4. Mean circulating steroid hormones during the period of exposure. **a.** Estradiol in females. **b.** Testosterone in females. **c.** 11-Ketotestosterone in males. **d.** Testosterone in males. Error bars indicate standard error of the mean. Sample size is 12 individuals for all points, with the exception of 93-d females, for which the sample size is 24. Asterisks indicate significant differences between a treatment and the reference within a time period. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

Reduced gonad size appears to have been mediated by lower levels of the steroid hormone estradiol. A number of field studies have identified reduced steroid hormones as being associated with impaired ovary development [12,13,35], but the present study may be the first to demonstrate such a relationship (correlation) on an individual-fish basis. Estradiol acts on the liver to mediate vitellogenin synthesis; here, lower levels of estradiol were accompanied by reduced circulating vitellogenin. Testosterone was also reduced, indicating that the specific lesion was not the conversion of testosterone to estradiol. Some evidence suggested reduced conversion of testosterone to 11-ketotestosterone in males early during the experiment, but this did not result in any observable difference in testes development. The measurement of pregnenolone in female trout, to determine whether cholesterol conversion was impaired, indicated no apparent reduction in conversion. Although no treatment-related pregnenolone differences were found, a weak correlation during the midphase of gonad growth leaves doubt as to whether impaired conversion of cholesterol to pregnenolone can be ruled out. A number of enzymatic steps occur between pregnenolone and estradiol, all of which could add further sources of variability to the pregnenolone-GSI correlation. The conversion of cholesterol to pregnenolone is one of the acutely regulated steps in the synthesis chain and, thus, still appears to be the likely reaction for impairment by chemical exposure. McMaster et al. [12,35] performed extensive experimentation using in vitro incubations of ovarian follicles in white sucker to determine possible sites of disruption of the steroid biosynthetic pathway. Multiple sites of disruption were observed, with the main lesion thought to be the conversion of androstenedione to testosterone. Results from these studies were equivocal, because different lesions were observed with different sampling periods, effluent sources, seasons, and fish species.

The specific compounds in pulp mill effluents that cause impaired ovary development or depressed steroid hormone concentrations in fish have not been identified. In light of the diverse and variable nature of such effluents and their effects on fish, it is unlikely that only one compound or class of compound is responsible. Phytosterols were initially implicated as impacting reproduction, but that seems to be very unlikely in this particular study. Fish in these experiments were probably exposed to more phytosterols from the commercial salmon food than from the effluent. Diet analysis revealed significant quantities of the same phytosterols as those present in this effluent [21]. Bile analysis in the present and a previous study [21] indicated that reference fish were exposed to similar quantities of phytosterols as effluent-exposed fish. In general, the effects observed in fish exposed to phytosterols do not match those of pulp and paper effluent exposure [36].

Effluent from the same mill used in the present study caused masculinization of female mosquitofish [18]. Extracts of that same effluent produced an in vitro androgen-receptor-binding response [18]. Similar androgenic effects have been observed with pulp mill effluents since the 1970s [37]. Recently, androstenedione has been implicated as a potential androgen in pulp mill effluent [19], and very strong evidence of androgen-receptor binding has been found [20]. By contrast, no direct evidence of any specific mechanism (androgenic, estrogenic, or other) was found in the present trout experiments. Female trout did not develop male secondary sexual characteristics, as has been seen in mosquitofish. The observation that trout may be more sensitive to reproductive alterations during the previtellogenic state does suggest a steroid hormone-like mechanism (agonist or antagonist). During the prematurational state, levels of steroids are low, but they increase dramatically during vitellogenesis. Thus, the relative ratios of an exogenous steroid-like structure to endogenous steroids would favor more severe effects during the previtellogenic state. Trout exhibit remarkably high levels of steroids during maturation as com-

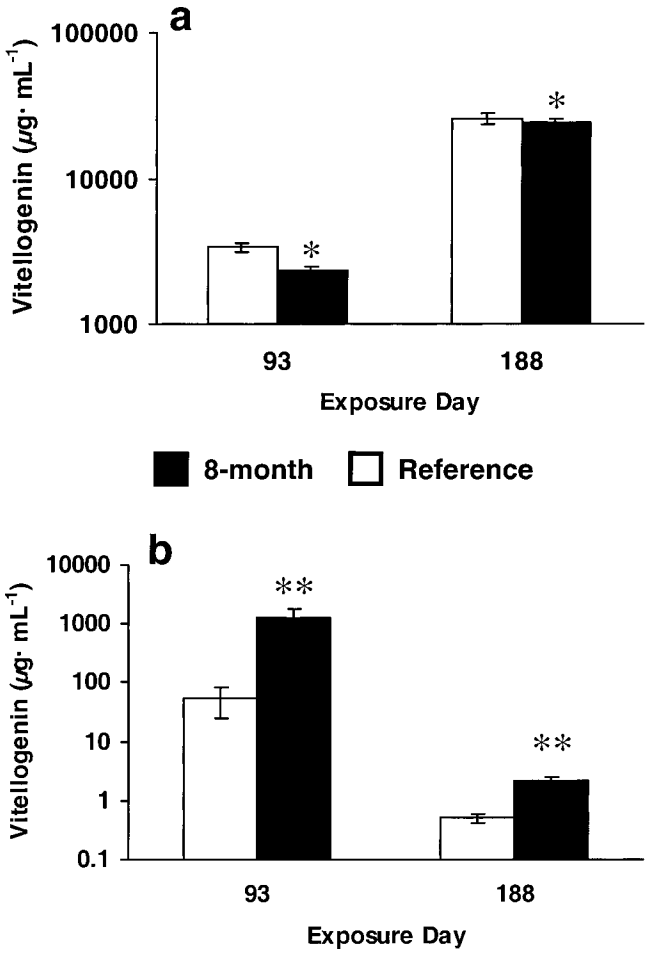

Fig. 5. Mean plasma vitellogenin concentrations at 93 and 188 d of effluent exposure. **a.** Females. **b** Males. Error bars indicate standard error of the mean. Sample size is 12 individuals for all points, with the exception of 188-d males, for which the sample size is 5. Asterisks indicate significant differences between eight-month treatment and reference fish within a time period. $*p < 0.05$; $**p < 0.01$.

Table 3. Correlations between circulating steroid hormones and vitellogenin measured in female trout during the course of the exposure with gonadosomatic index measured at the termination of the experiment[a]

Steroid	Exposure day	Pearson correlation coefficient (r)	n
Estradiol	93	0.547[b]	48
	188	0.425[c]	47
	248	−0.26	36
Testosterone	93	0.537[b]	48
	188	0.482[c]	47
	248	0.606[b]	36
Pregnenolone	93	0.09	36
	188	0.363[d]	47
Vitellogenin	93	0.405[d]	35
	188	0.500[c]	35

[a] Correlations were tested with Bonferroni adjusted probabilities.
[b] $p < 0.001$.
[c] $p < 0.01$.
[d] $p < 0.05$.

pared to other fish, and this may, in part, be why actively maturing trout are unresponsive to this effluent as compared to mosquitofish.

Exposure to estrogenic compounds in males was indicated by the induction of vitellogenin. Similar observations have been reported with exposure to bleached kraft mill effluent [14,15]. The exposure had no subsequent effect on testes development or secondary sexual characteristics in males. Four previous experiments with juvenile [18] and male rainbow trout [21] failed to show induction of vitellogenin. The presence of estrogenic compounds likely is sporadic in pulp mill effluents, and they could possibly be sourced from cleaning agents used from time to time within the mill. These results demonstrate the difficulty of trying to characterize a very complex effluent that is constantly changing.

Another curious observation about the reproductive effects was the pattern or distribution of response. The magnitude of observed mean steroid hormone differences would appear to be relatively minor, yet highly significant correlations were found between steroids and GSI. These observations reflect the fact that some individuals respond adversely to effluent whereas others do not respond at all. This discrete pattern of response can be seen in the vitellogenin and EROD data in male trout as well. The reasons for these apparent differences in sensitivity are unknown, but they are not unique to this study. Tremblay and Van Der Kraak [15] illustrated a similar pattern of response with rainbow trout exposed to pulp and paper mill effluent.

Aerobically treated pulp and paper mill effluents continue to demonstrate subtle and complex effects on fish reproductive physiology. In many cases, dilution of pulp mill effluents in the environment are below the threshold for observation of these effects [10]. However, the potential for environmental effects remains at sites with insufficient or seasonally low dilution rates. Precisely what the ecological relevance of these subtle effects are, if any, will continue to be controversial. However, continued study will ultimately elucidate mechanisms that can result in practical wastewater treatment solutions or process steps to prevent the formation of bioactive compounds.

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