Y-Chromosomal DNA Markers for Discrimination of Chemical Substance and Effluent Effects on Sexual Differentiation in Salmon

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Chinook salmon alevins were exposed during their labile period for sex differentiation to different concentrations of bleached kraft mill effluent (BKME), primary sewage effluent, secondary sewage effluent (SE), 17β-estradiol, testosterone, and nonylphenol. After exposure for 29 days post hatching (DPH), fish were allowed to grow until 103 and 179 DPH, at which time their genetic sex was determined using Y-chromosomal DNA markers and their gonadal sex was determined by histology. Independent of treatment, all fish identified as genetic females (XX) in these experiments possessed normal female gonads. Only the highest dose of some treatments affected the development of gonads in genetic XY males. At 103 DPH, some genetic males treated with 1 µg estradiol/L, BKME 100% ,and SE 30% developed as physiological females, presenting ovaries identical to genetic females in the control group. The physiological female condition in XY fish was also observed in these treatments groups at 179 DPH, which suggests that the effect is permanent, whereas in other groups the effect changed between sampling periods. Identification of the genetic sex of individual animals using sex-linked DNA markers provides a useful tool for investigating environmental factors influencing sex determination and differentiation. *Key words:* **DNA markers, effluent, endocrine disruption, feminization, pulp mill, salmon, sewage, sex differentiation, testis, Y chromosome.** *Environ Health Perspect* **110:881–887 (2002). [Online 23 July 2002]**
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Natural and synthetic chemicals present in discharged effluents have been shown to mimic natural hormones and disrupt the endocrine systems of several animal species (*1*). Some of these endocrine-disrupting chemicals can mimic estrogens, hormones with several important functions in vertebrates including stimulation of female reproductive development (*2*).

Estrogenic chemicals present in effluents include natural and synthetic estrogens, pesticides, alkylphenol compounds, and plant sterols (*2–5*). The estrogenic activity of these compounds in oviparous vertebrate species has been examined by measuring their ability to stimulate vitellogenin synthesis, an egg protein normally secreted in response to endogenous estrogen (*6*).

Studies have demonstrated that sewage effluent and bleached kraft mill effluent (BKME) may contain a mixture of chemicals that can be estrogenic to fish (*2,7*) and affect reproductive development (*8*). Others studies in fish have demonstrated that endocrine-disrupting chemicals, when tested in high concentrations, can affect sexual differentiation in fish (*9–13*), and it has been shown that paper mill effluent and treated sewage effluent can disrupt gonadal development (*14–16*). In these studies, fish were treated during the period of sexual differentiation, and, based on histopathology of the gonads, males were found to be feminized in some cases.

In several species of fish the physiological sex can be altered by administering androgens or estrogens before or during early sex differentiation (*17*). Sex differentiation of the male and female gonads normally begins several days after hatching, depending on the species (*18*). In salmonids the labile period, when the sex of fish can be altered, is between hatching and first feeding (*19,20*).

We hypothesized that substances present in effluents might disrupt the normal process of sex differentiation in fish and used chinook salmon (*Oncorhynchus tshawytscha*) as a model species for investigation. Chinook salmon possesses an XY sex determination system (*21*), and we have previously isolated DNA markers from the Y chromosome that allow unambiguous determination of genetic sex in this species (*22–24*). In the present study, we investigated the utility of Y-chromosomal DNA markers as tools to examine whether fish exposed to effluents and test chemicals would express phenotypic sexes different from their genetic sex.

Materials and Methods

Fish. Chinook salmon eggs were obtained from Big Qualicum River Hatchery (BC), brought to the West Vancouver Laboratory (Fisheries and Oceans Canada, West Vancouver, BC) and placed in incubation trays, supplied with well water at 10°C. Ten days before hatching, groups of 100 eggs were transferred to small, perforated plastic chambers.

Test substances. The substances and concentrations tested were sex steroid hormones (17β-estradiol, testosterone) as positive controls: 10 µg/L, 1 µg/L, 100 ng/L; an alkylphenol (nonylphenol) at the same concentrations as the steroids; bleach kraft mill effluent (BKME): 100%, 30%, 10%; secondary sewage effluent (SE) and primary sewage effluent (PE), both at the same concentrations as BKME. The BKME was obtained from a softwood pulp mill operation located in southern British Columbia, and the primary and secondary effluents from a sewage treatment plant within the Greater Vancouver Regional District, Vancouver, British Columbia.

Treatment protocol. Chinook salmon alevins were exposed for 29 days post hatching to different concentrations of the tested substances. We chose to expose fish for 29 days starting after hatching because it has been demonstrated that for chinook salmon, the labile period when the sex of fish can be altered begins around hatching (*19*). Treatments were performed in duplicate, and each replicate consisted of 100 alevins. Fish were held in perforated plastic chambers within glass aquaria containing 4 L of the test solution, which was completely renewed every second day. The steroid hormones and the alkylphenol were diluted in 95% ethanol, and the concentration of ethanol in water was less than 0.1%. Effluents were diluted in well water. In addition to the positive controls, there were two more control groups: one was immersed in water and the other in water containing 0.1% ethanol. We used the latter group to assess whether ethanol affects sexual differentiation. During the period of drug administration, fish remained in a temperature-controlled environment chamber at 10°C. The solution in each glass aquarium was aerated using an air stone connected to an aquarium air pump. We recorded mortalities and the general condition of the fish before each renewal of the solutions during the period of treatment.

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Molecular biology and histology. To evaluate the effects of the treatments on sex differentiation, we examined both the phenotypic and genetic sex of individuals. We used a Y-chromosomal DNA marker for chinook salmon to determine the genetic sex of the fish at any stage of development. We used histologic analyses to determine the gonadal sex. The appearance of a genetic male showing female or intersex gonads, or vice versa, would demonstrate an effect of the treatments.

Fish were sampled at 103 and 179 days post-hatching (DPH). At each sampling time, fish were randomly collected from each replicate. They were killed by an overdose of the anesthetic.

They were killed by an overdose of MS-222 (tricaine methanesulfonate; Syndel Laboratories, Vancouver, British Columbia, Canada) and we collected blood for determination of genetic sex. We determined the genetic and histologic sex of approximately 10 fish from each replicate of the control and positive control groups and of 20 fish from each replicate from the effluent-treated groups. A whole-body cross-section was cut (5–7 mm), just posterior to the operculum, and fixed in Davidson's preservative solution (300 mL ethanol, 200 mL 37% formaldehyde, 100 mL acetic acid, and 300 mL deionized water) for histological sex identification. The preserved sample was dehydrated in ethanol, embedded in paraffin, and sections (4–5 µm) were stained with hematoxylin and eosin. We analyzed all groups (genetic and histological sex determined) at 103 DPH. Whenever the effects observed in each group at 103 DPH were seen in a low percentage, we did not examine the samples from 179 DPH.

For determination of genetic sex (*23*,*24*), we used detection of a Y-linked growth hormone pseudogene. We added 2 µL of blood from each fish to 100 µL of 0.01 N NaOH to lyse cells and yield template DNA, and then froze the samples at –25°C until analyzed. Before polymerase chain reactions (PCR), samples were boiled for 10 min at 100°C to denature the template. PCR reactions were performed in a total volume of 50 µL, using 2 µL of template DNA, 5 µL of 10x PCR buffer (Bethesda Research Labs, Bethesda, MD), 5 µL 2mM dNTP, 1.5 µL 50 mM $MgCl_2$, 1 µL 25 pmol/µL primers GH5 (5′-AGCCTGGATGACAATGACTC-3′) and GH6 (5′-TACAGAGTGCAGTTGGCCT-3′) (*25*), 34.25 µL of deionized water, and 0.25 µL of 5 U/µL Taq DNA polymerase (Bethesda Research Labs). In some cases, we also confirmed genetic sex (determined with GH5 and GH6) using another Y-linked marker, OtY1 (*24*), in which case primers OtY1 (5′-GATCTGCTGGCTGGATTTGG-3′) and OtY2 (CCAGCGATGGTTTGTTTGAG-3′) were used in PCR reactions. All genetic sexing data using the two PCR assays were concordant. The PCR reaction conditions were initial denaturation of DNA at 95°C for 3 min, followed by 35 cycles of amplification (denaturation, 94°C for 1 min; annealing, 52°C for 1 min; and extension, 72°C for 1 min), and a final extension at 72°C for 10 min. Samples were analyzed on 2% agarose gels. In each PCR reaction series, we used known male and female positive controls.

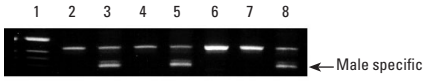

Figure 1. Polymerase chain reaction detection of Y-linked GH pseudogene sequences from chinook salmon blood DNA. Lane 1: template from 1 kb marker DNA; lanes 2–4: template from control female (2–4) and control male (3); lanes 5 and 8: template from genetic male treated with 17β-estradiol and BKME, respectively, presenting physiological female condition; lanes 6–7: template from genetic female treated with 17β-estradiol and BKME, respectively.

Measurement of alkylphenolic chemicals in the full-strength BKME and treated sewage effluent. We collected samples of full-strength BKME and treated sewage effluents (2 L) once a week, during the experimental period, and stored them at –20°C. The effluents were analyzed for nonylphenol (NP) and nonylphenol ethoxylates (NPEOs; n = 1–19). The analytic methodology used was based on the work described by Shang et al. (*26*), with modifications in the sample cleanup steps that were necessary to handle these types of samples; these modifications are described in detail below.

We processed BKME and effluent samples in batches of six that contained one procedural blank and five real samples. Depending on the type of effluent, the sample volume

Table 1. Effects of 17β-estradiol (EST), testosterone (TST), nonylphenol (NON), bleached kraft mill effluent (BKME), primary sewage effluent (PE), and secondary sewage effluent (SE) on sex differentiation of chinook salmon alevins.

Group	Sampling DPH	No.	Genetic sex	Effects NGD	PF	INT	AT
Control	103	20	9 M	9			
			11 F	11			
Control	179	20	10 M	10			
			10 F	10			
Control alcohol[a]	103	20	8 M	8			
			12 F	12			
Control alcohol[a]	179	18	9 M	9			
			9 F	9			
NON 10 µg/L	103	20	11 M	11			
			9 F	9			
TST 1 µg/L	103	18	10 M	10			
			8 F	8			
EST 1 µg/L	103	18	9 M	5	3	1	
			9 F	9			
EST 1 µg/L	179	18	6 M	4	2		
			12 F	12			
EST 100 ng/L	103	19	10 M	9		1	
			9 F	9			
BKME 100%	103	36	20 M	1	2	4	13
			16 F	16			
BKME 100%	179	35	14 M	12	2		
			21 F	21			
BKME 30%	103	23	9 M	9			
			14 F	14			
BKME 10%	103	35	17 M	16		1	
			18 F	18			
SE 100%[b]	103	20	6 M	2		1	3
			14 F	14			
SE 30%	103	38	20 M	17	2	1	
			18 F	18			
SE 30%	179	34	14 M	14			
			20 F	20			
SE 10%	103	36	20 M	20			
			16 F	16			
PE 30%	103	35	13 M			1	12
			22 F	22			
PE 30%	179	36	14 M	14			
			22 F	22			
PE 10%	103	39	17 M	17			
			22 F	22			

Abbreviations: AT, affected testis; INT, intersex; NGD, normal gonadal development; PF, physiological female.
[a]Only fish from replicate two were analyzed because all fish from replicate 1 died at the beginning of the experiment.
[b]Fish from SE 100% group at 179 DPH in both replicates died after first sampling.

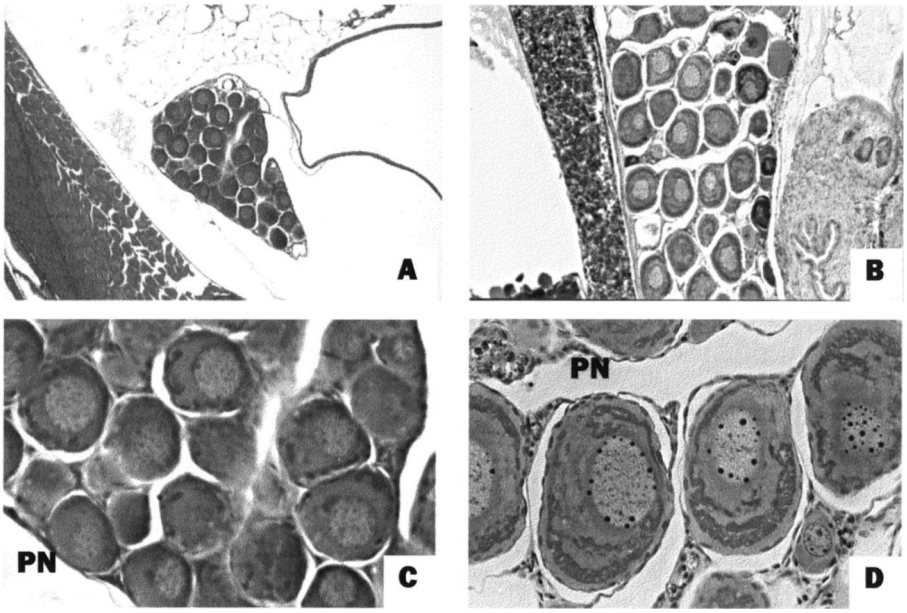

Figure 2. Cross-section of genetic female chinook salmon gonads at 103 (*A,C*) and 179 DPH (*B,D*) at 10× (*A,B*) and 40× (*C,D*) hematoxylin–eosin. Note the difference between sample times in size of the oocytes, which are synchronously at the perinucleolar stage (PN).

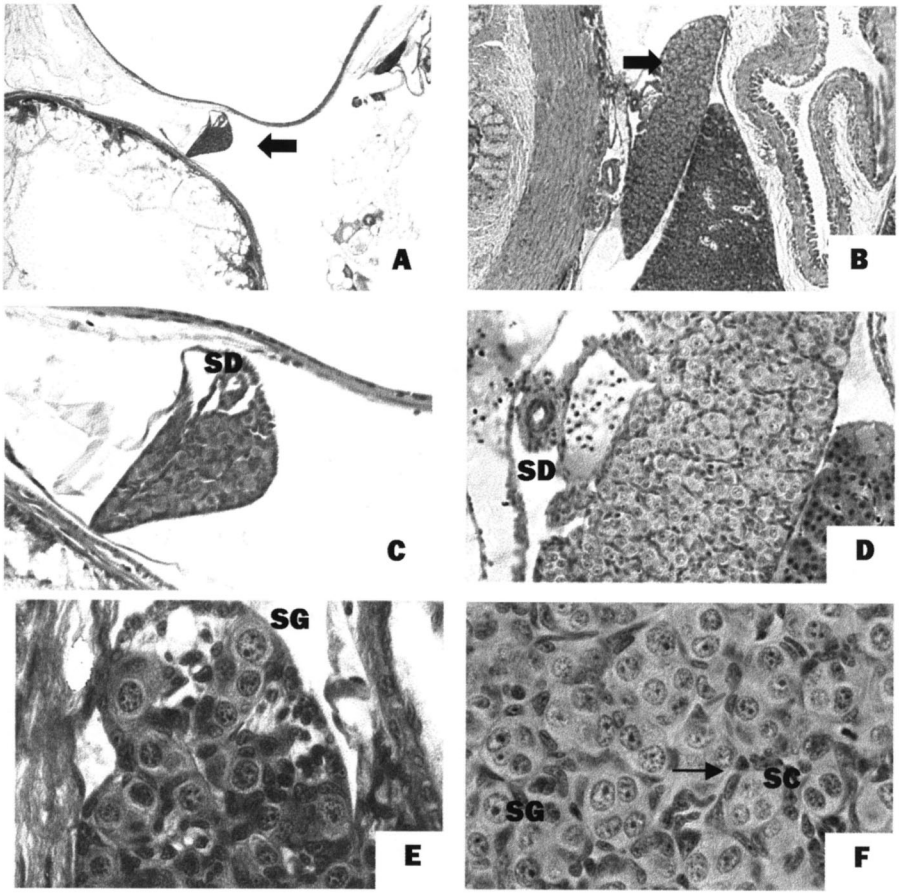

Figure 3. Cross-section of genetic male chinook salmon gonads at 103 (*A,C,E*) and 179 DPH (*B,D,F*) at 10× (*A,B*), 40× (*C,D*), and 100× (*E,F*) hematoxylin–eosin. Note the difference between sample times in size and germ cells. (*C*) and (*D*) show the sperm duct (SD), and (*E*) and (*F*) show spermatogonia (SG), spermatocytes (SC), and Sertoli cells (arrow). Thick arrows indicate testis (*A,B*).

analyzed varied between 50 and 100 mL. Samples were placed in a 500-mL separatory funnel, the sample volume was made up to a total volume of 100 mL with double milli-q water, spiked with the methods surrogate internal standard (polyoxyethylene-6-myristyl ether), and a few drops were added to the sample to adjust the pH to 2 and then extracted three times with 100-mL aliquots of DCM (dichloromethane). The extracts were evaporated down to a few milliliters and transferred with DCM to a 20-mL vial, where they were evaporated to dryness under a gentle stream of nitrogen. The samples were reconstituted in a few milliliters of hexane and loaded onto solid-phase extraction (SPE) columns with 5x 1-mL aliquots of hexane. The vial was further rinsed with 5x 1-mL aliquots of DCM/acetone (1:1), which were also loaded onto the SPE column. The hexane eluent was discarded, but the DCM/acetone eluent was collected. The SPE column was further eluted with another 5 mL of DCM/acetone. The SPE columns used were 10 cm^3, 500 mg NH_2 sorbent (Varian part no. 1211-3040), and the final cleaned-up extract collected was in 10 mL of DCM/acetone. The final extracts were then spiked with ^{13}C-NP (method performance standard used in our NP analyses) and polyoxyethylene 3-lauryl ether (method performance standard used in our NPEOs analyses). Extracts were finally analyzed by liquid chromatography electrospray ionization mass spectrometry using the same conditions as described in detail by Shang et al. (*26*).

Results

Survival. Survival of fish in various treatments varied between 97% and 100%, except for the 10 µg/L estradiol treatment group, in which all fish died between the 27th and 29th days of treatment, and in the 100% primary effluent treatments, in which all fish died between days 5 and 9. The mortalities in the 100% primary effluent treatments are suspected to have occurred due to low O_2 levels in the primary effluent. Accidental losses also occurred when aeration failed in two groups at 5 and 22 days after the beginning of the treatments, killing all fish in replicate 1 of the control ethanol group and all fish from the testosterone 10 µg/L group, respectively. Probably due to water quality problems, all fish in both replicates of the SE 100% group died after first sampling.

Effects on sex differentiation. Genetic sex identification was based on the amplification of a male-specific band, which is approximately 300 bp (Figure 1). All samples of physiological males (i.e., those possessing a normal testis) from all groups examined in this experiment amplified this specific band.

For control groups, the genetic sex of all fish corresponded to the cellular phenotype of the gonads independent of the period of sampling (103 and 179 DPH; Table 1). In controls, all fish identified as genetic females showed normal gonadal development, presenting ovaries filled with synchronous oocytes at the perinucleolar stage (Figure 2). The gonadal sex of males in the control group (determined histologically) also corresponded 100% to the genetic sex. Males from the control group presented testes well packed with spermatogonia and spermatocytes that were surrounded by sertoli-like cells (Figure 3). It was also possible to identify the sperm duct in most of the gonads of genetic males.

Independent of the treatment, all fish identified as genetic females (n = 292) presented normal gonadal development and the germ cells were in the same stage of development as the ones observed in the females from the control group. These results indicate that none of the treatments affected the expression of the physiological sex of genetic females.

For genetic males, some treatments did affect gonadal development (Table 1), and, in some groups, the effect changed between sampling periods. Males treated with testosterone (1 µg/L) and nonylphenol (10 µg/L) presented normal gonadal development, and the genetic sex coincided 100% to the histological sex (Table 1). Therefore, we did not examine fish from the lower doses tested. However, some genetic males (three out of nine) treated with estradiol (1 µg/L) at 103 DPH developed as physiological females, presenting normal female gonads (Figure 4A, B). One genetic male treated with estrogen presented intersex gonads (Figure 4C), with oocytes at the perinucleolar stage among testicular tissue. At 179 DPH we did not observe any intersex gonads, but two out of six genetic males were physiological females. In the group treated with 100 ng estradiol/L, one of nine males presented intersex gonads, but only at 103 DPH.

Treatment with BKME 100% affected the gonadal development of the genetic males at 103 DPH in different ways (Table 1). Some genetic males (2 of 20) developed as physiological females, showing ovaries with normal appearance (Figure 5A). Other fish (4 of 20) presented intersex gonads, and several (13 of 20) showed abnormal testes with a low number of gonocytes scattered among the interstitial tissue (Figure 5B), or even absence of gonocytes (Figure 5C). We noticed that the intersex condition found in the genetic males, independent of the treatment, was in most of cases restricted to one of the gonads (Figure 5D, E). In contrast, at 179 DPH, only the full physiological female condition was observed in affected genetic males (2 of 14). Neither intersex gonads nor abnormal testicular development were found.

There was no abnormal testis development or intersex condition in genetic males exposed to BKME 30%. In the treatment group BKME 10%, one genetic male (of 17) presented an intersex gonad at 103 DPH,

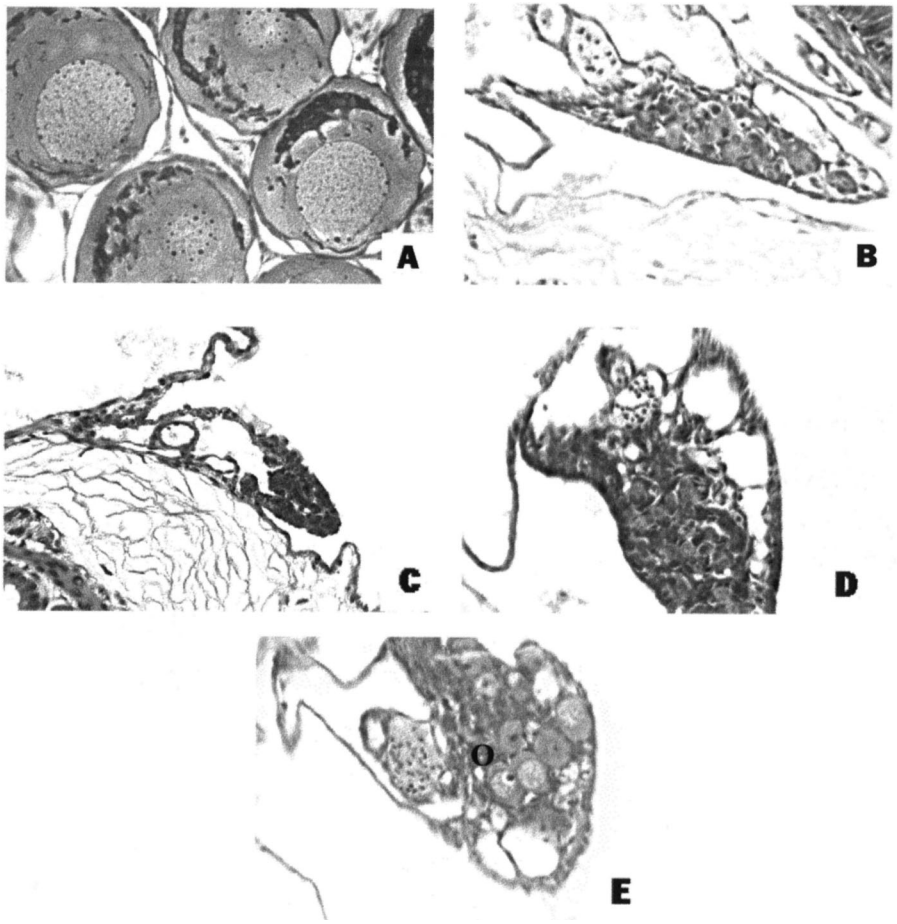

Figure 5. Cross-sections of genetic male chinook salmon at 103 DPH treated with BKME 100% showing affected testis, hematoxylin–eosin, 40×. Phenotypic female (*A*). Disorganized testis presenting scattered germ cells (*B*) or absence of them (*C*). Left (*D*) and right (*E*) gonads of the same fish showing intersex condition. Note oocytes (O) among testicular tissue only in the right gonad.

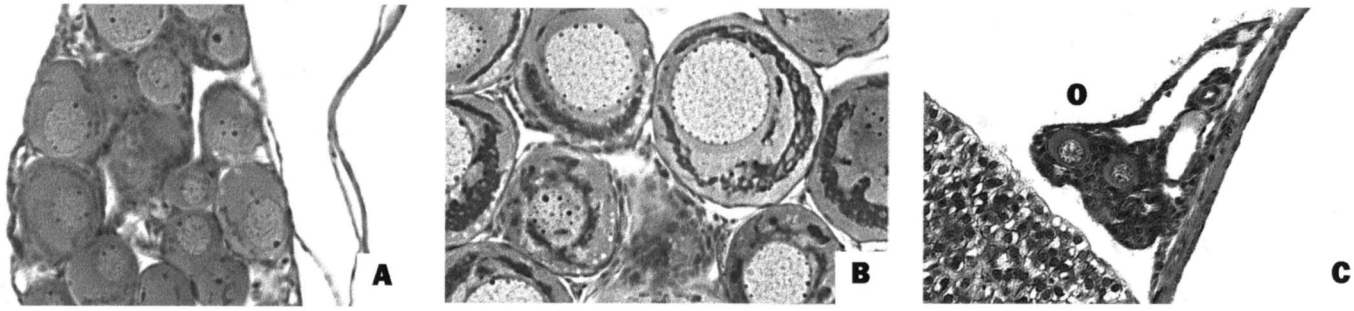

Figure 4. Cross-section of genetic male chinook salmon gonads treated with 17β-estradiol (1 µg/L), hematoxylin–eosin, 40×. (*A,B*) Phenotypic female at 103 and 179 DPH, respectively, showing synchronous development of the oocytes, as observed in genetic females (Figure 2). (*C*) Intersex fish at 103 DPH presenting oocytes (O) among testicular tissue.

whereas the remaining fish had normal testes (Table 1).

Some genetic males (three of six) treated with SE 100% presented abnormal testes (as described above for effects of BKME), and one genetic male presented intersex gonads (Table 1). In the treatment group SE 30%, 2 of 20 genetic males developed as physiological females, and 1 of 20 showed intersex gonads. None of these effects were observed at 179 DPH (Table 1). Treatments with SE 10% and PE 10% did not affect the normal testis development of genetic males. However, treatment with PE 30% affected the gonadal development of 100% of the genetic males at 103 DPH. Twelve (of 13) genetic males showed abnormal testes and one presented intersex gonads (Table 1). No effects were observed at 179 DHP.

In cases where XY females were identified using GH5 and GH6, a second Y-chromosomal DNA marker, OtY1, confirmed that these individuals were indeed genetic males.

Alkylphenolic chemicals in the effluents. Alkylphenolic chemicals were found in higher concentrations in the treated sewage effluents than in BKME. Nonylphenol per se was 2.5- and 3.9-fold higher in PE than in BKME and SE, respectively (Table 2). The ethoxylate monomers were detected with different concentration profiles in all three matrices (PE, SE, and BKME) as well. For all matrices the total ethoxylate concentration (i.e., the sum of all monomers), was higher than that of nonylphenol.

Discussion

In the current experiments, gonadal differentiation of male chinook salmon was affected by high (SE 30% and 100%, PE 30%, and BKME 100%) but not low concentrations (10%) of sewage and pulp mill effluent. Fish from control groups expressed their phenotypic sex in accordance to their genetic sex, as did genetic females from all groups. However, some treatments feminized or disrupted sexual differentiation of genetic males. Not all individuals within a treatment group showed the same gonadal alterations, suggesting that substantial individual genetic variation may exist regarding sensitivity to endocrine disruptors.

Testosterone at the concentration used (1 µg/L) did not affect sex differentiation, although natural androgens can have weak masculinizing effects in fish at higher concentrations (*27*,*28*). However, 17β-estradiol (1 µg/L) affected the phenotypic sex of genetic males, producing both completely sex-reversed XY fish (with normal ovaries) or fish with intersex gonads. In carp (*Cyprinus carpio*), a monosex male population exposed to 17β-estradiol (10 and 100 µg/L) for 20 days during the period of sexual differentiation resulted on the development of oviduct in all fish (*11*), a condition that persisted after fish had returned to clean water for 59 days (*29*). Exposure of a monosex population of male carp to 17β-estradiol (10 µg/L) for 90 days resulted in phenotypic female gonad containing oviduct and oocytes (*10*), and in Japanese medaka (*Oryzias latipes*), exposure to 17β-estradiol (1 µg/L) for 90 days beginning 1 DPH resulted in complete feminization (*30*). Similarly, Nakamura (*31*) induced complete feminization in masu salmon (*Oncorhynchus masou*) by administering 0.5 µg/L 17β-estradiol for 18 days, starting 5 days after hatching (fish were examined 90 DPH).

In our study we sampled the fish 103 and 179 DPH, approximately 73 and 150 days after exposure. We noticed that, in some treatments, the physiological female condition observed in genetic males tended to be reproducible between different sampling times within a treatment, which suggests a permanent effect. However, the intersex or affected testis conditions observed in the first sampling, which have also been shown in male carp exposed to 1 mg/L 4-*tert*-penthylphenol (*10*), seems to be transient because we did not identify these disruptions at the second sampling. A transitory sex-reversal effect has also been observed in androgen-treated rainbow trout (*32*). A longer period of exposure may be required to definitely alter the cellular pathway of sexual differentiation. This points out the importance of long-term studies to clearly define whether the effects of endocrine disruptors on sex differentiation on reproductive development are transient or permanent. If the physiological female condition persists in XY genetic males and the fish reach sexual maturation and spawn, it may alter the natural genetic sex ratio (50:50 ratio). There have been laboratory studies demonstrating that feminized XY salmon can reproduce, and offspring sex ratio is 75% males (*21*,*33*,*34*). Nonylphenol, an alkylphenol present in some sewage effluents, did not influence sex differentiation in this study (at 10 µg/L). This compound has been shown to be weakly estrogenic to fish (*7*) and to mammals (*35*), and an intersex condition has been induced in Japanese medaka (*Oryzias latipes*) exposed to nonylphenol (50–100 µg/L) during 90 days post hatch (*13*). An all-female rainbow trout (*Oncorhynchus mykiss*) population exposed to nonylphenol (30 µg/L) for 35 days from hatch had significantly lower body weight than a control group at 84 days after the start of the exposure (*36*). As indicated by the above studies, it may be possible that in the current study the dose of nonylphenol as well as the period of exposure tested were too low to elicit an effect.

Some genetic males exposed to BKME 100% (2 out of 20) developed as physiological females in the current study. Although there is no previous report showing whether BKME affects sexual differentiation in salmon, it has been well documented that BKME affects reproductive development in fish (*37*,*38*,*39*). Several compounds present in pulp mill effluents, such as β-sitosterol, lignans, resin acids, and stilbenes have been shown to have estrogenic activity (*40*,*41*). Further, it has been shown that β-sitosterol, the predominant plant sterol in pulp mill effluents (*8*), can induce the expression of the vitellogenin gene in the liver of juvenile rainbow trout (*41*) and induce vitellogenin synthesis in male goldfish (*42*).

Exposure of chinook salmon to sewage effluent (30% and 100%) also affected phenotypic sex in genetic males. Sewage effluent has

Table 2. Alkylphenolic chemicals concentration in the 100% BKME, PE, and SE.

Chemical	BKME 100% (µg/L)	PE 100% (µg/L)	SE 100% (µg/L)
Nonylphenol	8.22 ± 4.74	20.50 ± 4.08	5.20 ± 1.19
NP1EO	2.67 ± 1.48	19.70 ± 6.70	19.68 ± 1.65
NP2EO	0.72 ± 0.12	13.30 ± 3.67	34.85 ± 6.34
NP3EO	0.52 ± 0.04	7.68 ± 1.18	7.98 ± 1.60
NP4EO	1.51 ± 0.96	5.22 ± 0.98	4.10 ± 0.52
NP5EO	2.18 ± 1.13	4.38 ± 0.72	2.98 ± 0.51
NP6EO	0.60 ± 0.14	6.29 ± 1.28	1.47 ± 1.04
NP7EO	0.32 ± 0.05	9.03 ± 2.16	1.77 ± 1.31
NP8EO	0.32 ± 0.13	11.29 ± 3.45	1.94 ± 1.43
NP9EO	0.27 ± 0.12	13.24 ± 4.22	2.02 ± 1.57
NP10EO	0.27 ± 0.11	14.00 ± 4.85	1.81 ± 1.33
NP11EO	0.22 ± 0.10	13.84 ± 4.74	1.38 ± 0.99
NP12EO	0.19 ± 0.09	12.90 ± 4.27	1.04 ± 0.74
NP13EO	ND	10.43 ± 3.37	0.64 ± 0.42
NP14EO	ND	7.14 ± 2.46	0.42 ± 0.19
NP15EO	ND	4.99 ± 1.68	0.25 ± 0.20
NP16EO	ND	3.43 ± 1.21	0.20 ± 0.16
NP17EO	ND	1.77 ± 0.97	0.10 ± 0.13
NP18EO	ND	1.13 ± 0.43	0.05 ± 0.10
NP19EO	ND	0.50 ± 0.28	ND
Total	18.01	180.76	87.88

Abbreviations: ND, not detected; NPEO, nonylphenol etoxylate. Values for each chemical correspond to the mean ± SD of the four samples collected during the experimental period.

been reported to contain estrogenic substances (*2*,*43*,*44*). Those studies demonstrated that male rainbow trout exposed to sewage effluent synthesized vitellogenin, a protein found in blood plasma of sexually mature female fish produced in response to estradiol. Recently, Rodgers-Gray et al. (*16*) demonstrated that juvenile roach (*Rutilus rutilus*) exposed to treated sewage effluent (100%) developed feminized ducts. They also showed that all fish still presented feminized ducts after 150 days in clean water, indicating a permanent effect.

The gonadal development of genetic females was not affected by the treatments, suggesting that the effluents primarily have estrogenic activity. However, treated females may have had altered physiologies undetected in the present experiments (i.e., increased vitellogenin gene expression and synthesis), warranting further investigation.

It is important to note that our study used concentrations of substances that were in some cases very high and are unlikely to correspond to field situations in nature. For example, 17β-estradiol concentration in treated sewage effluent in the UK ranged from 4 to 88 ng/L, depending on the period of the year (*16*,*45*), some 11- to 250-fold less than one of the concentrations that we tested (1 µg/L). Further, the main effects we observed with effluents were found only at the highest concentrations tested. Several chemicals with estrogenic activity have been identified in effluents discharged into aquatic environments, and it is possible that the effects we have observed are due to the additive effects of all of them. Sumpter and Jobling (*2*) demonstrated that a mixture of five estrogenic chemicals present in discharged effluents was a much more potent stimulator of vitellogenin synthesis by cultured hepatocytes of rainbow trout than was each chemical individually.

The chemical analysis of the effluents revealed the presence of nonylphenol and several ethoxylates. Most of the studies dealing with the estrogenicity of effluents in fish concentrate on the effects of steroids (estrogens) and nonylphenol mono- and diethoxylates (*16*,*30*,*46*,*47*). This study also raises the question whether the higher nonylphenol ethoxylates play a role, as individual substances or synergistically with the other estrogenic chemicals, on fish health. A more extensive analysis of the chemical composition of these effluents will be required to provide a clearer picture of possible endocrine-modulating compounds.

In summary, we used a Y-chromosomal DNA marker for chinook salmon that allows determination of genetic sex to examine the effect of different chemical and effluent treatments on gonadal development. The use of this technique indicated that exposure of chinook salmon alevins during the labile period for sex determination to estrogens, sewage effluent, or BKME could affect sexual differentiation. In most previous studies examining the effect of chemicals on sexual differentiation in fish, the genetic sex of individual fish has not been known with certainty. These studies have relied upon a shift in sex ratio (50:50), and the phenotypic sex proportion in the control group was compared with the phenotypic sex proportion in the treated groups (*17*). However, an alternative approach to using sex-linked DNA markers to determine genetic sex is to generate monosex populations that can be used for experimental treatments. For some fish species, all-male populations can be produced by crossing homogametic males (YY) with normal females (XX) (*10*,*11*), whereas all females are produced by crossing sex-reversed females (XX; physiological male) with normal females (XX) (*28*). Such approaches cannot be used for examining wild fish but are very useful for laboratory investigations and for field studies using caged sentinel animals. Recently, Nagler et al. (*48*) used genetic sex markers (*24*) to identify sex-reversed chinook salmon collected from the Hanford Reach region of the Columbia River in Washington state, USA, demonstrating the utility of genetic sexing technology for surveying wild populations of fish for incidences of sex reversal.

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