Male Coho Salmon (\textit{Oncorhynchus kisutch}) Exposed to a Time-Course of Urban Sewage Effluent Exhibit a Sporadic Low Incidence of Sex Reversal and Intersex

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Developing coho salmon alevin (\textit{Oncorhynchus kisutch}) were exposed to concentrations of 1 to 10\% municipal wastewater effluent (MWWE) during the labile period of sexual differentiation. MWWE was collected at regular time intervals from a municipal wastewater treatment plant during a 4-week period that coincided with the latter part of the spawning season of coho salmon. The salmon were exposed, from just prior to hatch until 28 d post hatch, to MWWE collected at 3 a.m., 8 a.m., 3 p.m. or 8 p.m. Subsequent gonadal development was assessed by histology and compared to genetic sex as determined by Y-chromosomal DNA markers. The MWWE (analyzed using GC-HRMS) had relatively high concentrations of steroidal estrogens and other endocrine active substances at some time points during the exposure period. A low incidence of intersex and sex reversal was noted in the coho salmon exposed to MWWE collected at only two time points; 3 a.m. (1\% MWWE) and 8 p.m. (10\% MWWE), and ≤19\% of the male fish in these exposure groups were affected. The coho salmon utilized were of wild genetic backgrounds and thus the sporadic nature of the response observed may be due to a significant genetic influence on the ability to respond to exogenous hormones at this early stage of development.

\textbf{Key words:} municipal wastewater, endocrine, sex reversal, intersex, Pacific salmon

\section*{Introduction}

Previous studies have shown that various fish species exposed to estrogenic substances and effluents in laboratory and field studies can show variable levels of disrupted gonadal development, including intersex and sex reversal (e.g., Gimeno et al. 1996; Gray and Metcalfe 1997; Jobling et al. 1998; Rodgers-Gray et al. 2001; Afonso et al. 2002). In some species, treated municipal wastewater effluents (MWWE) have been shown to cause dose-dependent disruption in gonadal duct development (Rodgers-Gray et al. 2001). Previous studies from our laboratory have indicated that Pacific salmon exposed to relatively high concentrations (i.e., 100–30\%) secondary MWWE can exhibit gonadal disruption including intersex and sex reversal (Afonso et al. 2002). In this study we have examined the responses of coho salmon to more environmentally relevant concentrations of MWWE to determine if the responses observed previously are relevant to real-world exposures. In recognition of the fact that diurnal, seasonal and year-to-year variations in temperature, rainfall, fluctuating inputs from domestic, commercial and industrial sources, amongst other factors, can profoundly impact the characteristics of MWWE, we have also examined whether diurnal variation in the composition of MWWE can impact the response of Pacific salmon to MWWE exposure.

\section*{Material and Methods}

\subsection*{Fish}

Coho salmon eggs were obtained from Chehalis River Hatchery (B.C.) at the eyed stage of development. Approximately 3000 eggs were obtained (4.7 eggs per gram) from mixed parentage derived from multiple adult coho salmon that had spawned at the hatchery on January 20, 2003. The eggs were transported to the West Vancouver Laboratory and placed in Heath incubation trays supplied with well water at 10°C.

\subsection*{Treatment Protocol}

Approximately seven days prior to hatch (hatching date was predicted based on 430–530 ATUs) the coho salmon eggs were transferred to plastic perforated boxes
(100 eggs per box) and placed in glass aquaria in a temperature-controlled room at 10°C. The coho were exposed in these aquaria to various treatments until 28 d post hatch. This exposure period was chosen based on previous work that has shown that the critical period of sexual differentiation in Pacific salmon occurs around the time of hatching (Baker et al. 1988). Each aquarium contained 4 L of test solution and a static renewal system was used with test solutions being completely renewed twice a week. 17β-estradiol (E2) at a final concentration of 1 µg/L was used as a positive control. A stock of 200 µg E2/mL was prepared in 95% ethanol and 20 µL (4 µg E2) of this stock mixed into 4 L of water for the positive control exposures (1 µg E2/L). The final concentration of ethanol in the positive control exposures was 5 µL/L (<0.001%). Two types of negative controls were used: water-only controls and solvent controls; the fish in the latter control groups being exposed to 5 µL/L of 95% ethanol to control for the solvent exposure in the positive controls. MWWTP was collected weekly from Annacis Island municipal wastewater treatment plant (MWWTP) (Greater Vancouver Regional District) at regular time intervals over 24 h. The collection times were: 3 a.m., 8 a.m., 3 p.m. and 8 p.m. At each collection time 8-L aliquots of each effluent sample were reserved for chemical analyses. This MWWTP is a trickling filter/solid contact (TF/SC) biological secondary treatment plant. It has an average daily flow rate of 455 million litres per day and services a population of >740,000. Average levels of suspended solids in the effluent are 10 mg/L and average BOD levels are 8 mg/L. Mean hydraulic retention time of the bioreactor ranges from 0.3 to 2 h. Effluents were diluted in the laboratory in well water for the exposures. Following the 28-d exposure period the alevins were initially transferred to Heath hatchery trays supplied with well water at 10°C, and then moved to larger tanks supplied with well water at Rosewall Creek Hatchery, Vancouver Island (B.C.) for grow-out until they were sampled on July 3, 2003. A random subsample of 24 fish from each replicate tank was removed by dip netting and sacrificed using an overdose of the anaesthetic MS-222 (tricaine methanesulfonate; Syndel Laboratories, Vancouver, B.C.). Blood samples were taken by caudal puncture for analysis of genetic sex, and weights and lengths were recorded.

**Molecular Biology**

For determination of genetic sex a Y-linked marker, GH-P, was used (Du et al. 1993; Devlin et al. 2001). Two microlitres (2 µL) of blood from each fish was added to 100 µL of 0.01 M NaOH to lyse cells and yield template DNA. The samples were then frozen at -20°C until they were analyzed. Each sample was boiled for 7 min at 100°C to denature the template. The primers used were GH5 (5'-AGCCTGGATGACAATGACTC-3') and GH6 (5'-TACAGAGTGCAGTTGGCCT-3') and the PCR reaction was the same except that 1.2 µL of MgCl2 was added, thereby reducing the deionized water to 20.45 µL. 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 7 min. Samples were analyzed in 2% agarose gels. In each PCR reaction series known male and female positive controls were utilized.

**Histology**

A whole body cross section was cut from each sampled fish and preserved in Davidson’s preservative (300 mL ethanol, 200 mL 37% formaldehyde, 100 mL acetic acid and 300 mL deionized water) for histological sex examination. The preserved samples were then transferred to 95% ethanol and subsequently embedded in paraffin. Sections (4–5 µm) were cut from the whole body cross sections and then stained with hematoxylin and eosin. One section from each fish was examined.

**Recombinant Yeast Assay (RYA)**

Two hundred millilitres of each sample were extracted onto a 500-mg Oasis HLB 6 cc SPE cartridge column (Waters Corp., Milford, Mass., U.S.A.) after glass wool filtration. Columns were stored at -20°C prior to elution which was performed using 5 mL of 25% methanol (MeOH) in double Milli-Q deionized (DMQ) water, followed by 50% MeOH, 100% MeOH, diethyl ether and cyclohexane for a total of five fractions. To assess the amount of estrogenic substances bound to the particulate fraction of the wastewater samples, the glass wool plugs were rinsed with 5 mL of DMQ water followed by 5 mL of anhydrous ethanol that was collected for RYA. Additionally, 0.2-µm filters used to sterilize aqueous fractions were rinsed with a few millilitres of ethanol to test for estrogenic substances lost in filter sterilization. Diethyl ether and hexane fractions were taken to dryness under N2 (<30°C) and resuspended in MeOH prior to RYA analysis.

The recombinant yeast strain used in this work was obtained from J.P. Sumpter from Brunel University, Middx, U.K. This yeast was modified to contain the DNA sequence of the human estrogen receptor (hER) on the main chromosome as well as an expression plasmid carrying the reporter gene Lac-Z that encodes for the enzyme β-galactosidase. The procedure was carried out as specified in Routledge and Sumpter (1996). For calculation of β-galactosidase activity, we used the ratio of 540 nm to 650 nm (optical density) minus the same ratio from an appropriate blank well. Dose-response curves (DRCs) were plotted as the blank subtracted β-galactosidase activity for
all twelve E₂ standards run in triplicate versus the log of the concentration in grams per litre. The curves were fitted using a sigmoidal dose-response curve (variable slope), Marquardt-Levenberg algorithm in SigmaPlot version 8.0 (SPSS, Chicago, Ill., U.S.A.). Coefficients of determination ($r^2$) of 0.998 were observed for our does-response curves.

**Chemical Analysis**

The method used to determine the levels of endocrine-disrupting compounds (EDCs) in wastewater samples was previously developed and reported (Ikonomou et al., submitted for publication). In brief, 40 mL of sample was extracted with approximately 3 times 10 mL dichloromethane using sonication to break up emulsions. Extracts were reduced, dried over sparing amounts of sodium sulphate and cleaned up using 5% (w/w) deactivated Florisil. Extracts were derivatized using 50 µL of anhydrous pyridine and 50 µL of fresh BSTFA:TMCS (99:1) for 3 h at 90°C. Five $^{13}$C-labelled, deuterated or non-naturally occurring surrogate standards were used as internal standards for recovery correction and performance evaluation. Quality control samples including procedural blanks, spikes and duplicates were run with each batch of 10 samples. Results are reported as recovery corrected and blank subtracted values (ng/L). Internal standard recoveries, assessed using one of three deuterated polynuclear aromatic hydrocarbons (PAHs) performance standards, were generally >70% and never outside of 40 to 120% as a quality control criterion. Table 1 illustrates the means and range of method detection limits (MDLs) obtained for the compounds measured in this work.

**Statistics**

Statistical analysis was performed with Statistica 7.0 software (Statsoft, Tulsa, Okla., U.S.A.). All data were tested for homogeneity and normality with the Levene test and normal probability plots of the residuals. If these assumptions were met, analysis of variance (ANOVA) followed by an appropriate multiple comparison test was performed on the results. The differences described were statistically significant at $p < 0.05$. Non-parametric Pearson product moment correlation was performed for the correlation analysis.

**Results**

**Fish Condition**

There were no significant differences in final sampling weights, lengths and condition factors \([CF = \frac{W(\text{g/L/cm}^3)}{L}]\) of the fish among treatment groups except for the fish exposed to effluent collected at 3 a.m. (see Table 2). Factorial ANOVA was used and determined that condition factor was not significantly different based on sex and treatment levels. The fish exposed to MWWE collected at 3 a.m. had significantly larger condition factors than both 8 a.m. and 3 p.m. (Tukey $p = 0.04$). The control data showed too much variability to be included in the ANOVA as determined by the Levene test for variance homogeneity.

**Sexual Differentiation**

In the untreated control groups, the genetic sex of the fish corresponded to the cellular phenotype of the gonads. Fish identified as genetic females showed normal gonadal development, presenting ovaries with synchronous oocytes at the perinucleolar stage. Fish identified as genetic males in the negative control groups also exhibited normal testes development and presented testes with normal spermatogonia and spermatocytes surrounded by sertoli-like cells. Some treatments impacted the gonadal development of a proportion of the genetic males (Table 3). In the time-course effluent exposures the only male fish to be sex reversed were a small proportion of those exposed to 10% MWWE collected at 8 p.m. In this group, 15% (4 out of 26) of genetic males were sex reversed, i.e., they presented as physiological females, and 1 genetic male was intersex at the time of sampling (Fig. 1). In addition, two genetic males exhibited intersex following exposure to 1% MWWE collected at 3 p.m. In the positive control group exposed to 17β-estradiol (1 µg/L) 38% of the genetic males (9 of 24) were intersex at the time of sampling and 1 genetic male was sex reversed, i.e., presented as a physio-

<table>
<thead>
<tr>
<th>Compound</th>
<th>MDL in ng/L</th>
<th>Compound</th>
<th>MDL in ng/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estrone</td>
<td>7.6(5.0–11)</td>
<td>Cholesterol</td>
<td>25(5.8–44)</td>
</tr>
<tr>
<td>Equilin</td>
<td>18(9.3–28)</td>
<td>Coprostan-3-one</td>
<td>28(17–49)</td>
</tr>
<tr>
<td>17α-Estradiol</td>
<td>6.9(4.5–11)</td>
<td>β-Sitosterol</td>
<td>23(12–39)</td>
</tr>
<tr>
<td>17β-Estradiol</td>
<td>7.1(1.6–12)</td>
<td>Nonylphenol</td>
<td>172(115–219)</td>
</tr>
<tr>
<td>d-Equilenin</td>
<td>17(4.1–31)</td>
<td>Bisphenol A</td>
<td>2.1(1.7–2.4)</td>
</tr>
<tr>
<td>17α-Ethinylestradiol</td>
<td>7.1(6.1–9.0)</td>
<td>Pinosylvin</td>
<td>14(1.8–27)</td>
</tr>
<tr>
<td>(-)-Norgestrel</td>
<td>84(74–98)</td>
<td>DEHP</td>
<td>20(13–25)</td>
</tr>
<tr>
<td>Estriol</td>
<td>1.5(1.1–2.3)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
logical female. In all other treatments there was no evidence of intersex or sex reversal.

**In vitro Estrogenic Activity**

SPE extracts of the wastewater were subjected to screening using a hERα recombinant yeast screen (i.e., ER transcriptional assay) to determine the net in vitro estrogenic activity of each sample (Routledge and Sumpter 1996). SPE extracts were used rather than whole effluent which was also examined but not reported, to provide greater sensitivity and precision due to the 8 times concentration factor obtained by extracting 200 mL and eluting into 5×5 mL fractions via SPE. The highest hER activity was associated with the effluent samples collected at 8 a.m. corresponding to the period of lowest flow at the MWWTP (Fig. 2). In this case the activity in the hER assay was significantly higher (Tukey p < 0.05) at 8 a.m. when compared to the 3 a.m. sample based on a randomized complete block design (RCBD) ANOVA where blocking by week of collection was significant and justified (p < 0.05). Samples were analyzed in triplicate assays, and in triplicate within each assay. Additionally, re-extraction of the original effluent (stored at -20°C) was performed to confirm these results.

**Effluent Chemistry**

The levels of 15 substances were measured in the effluent samples (Fig. 3). There were no significant differences (RCBD ANOVA p > 0.05) between time points for any of the 15 substances measured in the effluent samples as well as the multivariate profile as measured by MANOVA (Wilks Lambda, p > 0.05). Although blocking by sampling week was determined to be significant, suggesting that the chemical profiles were indeed significantly different between the weekly sampling dates. Visual examination of the effluent chemical profiles showed that the effluent samples collected on April 3 and 10 were elevated in steroidal estrogens compared to the other sampling weeks. Simple correlation analyses showed no significant correlations between chemistry or in vitro hER activity, and sex-specific biological effects (n = 4; R < 0.95). The levels of steroidal estrogens and weakly estrogenic chemicals, e.g., nonylphenol, bisphenol A, in the MWWE varied depending on the date and time of sampling. For example, the levels of 17α-ethinylestradiol were non-detectable in the samples of MWWE taken in the first two weeks of exposure regardless of the time of collection. However, in the last two weeks of exposure the levels

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**TABLE 2.** Fish lengths, weights and condition factors at the time of sampling

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Weight (g)</th>
<th>Length (mm)</th>
<th>Condition factor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>Mean</td>
</tr>
<tr>
<td>Negative control (water)</td>
<td>1.13</td>
<td>0.35</td>
<td>48.0</td>
</tr>
<tr>
<td>Positive control (17β-estradiol)</td>
<td>1.03</td>
<td>0.27</td>
<td>46.4</td>
</tr>
<tr>
<td>10% MWWE collected at 3 a.m.</td>
<td>1.12</td>
<td>0.28</td>
<td>48.1</td>
</tr>
<tr>
<td>1% MWWE collected at 3 a.m.</td>
<td>1.00</td>
<td>0.02</td>
<td>46.4</td>
</tr>
<tr>
<td>10% MWWE collected at 8 a.m.</td>
<td>0.95</td>
<td>0.22</td>
<td>46.1</td>
</tr>
<tr>
<td>1% MWWE collected at 8 a.m.</td>
<td>0.98</td>
<td>0.23</td>
<td>46.1</td>
</tr>
<tr>
<td>10% MWWE collected at 3 p.m.</td>
<td>0.89</td>
<td>0.24</td>
<td>45.0</td>
</tr>
<tr>
<td>1% MWWE collected at 3 p.m.</td>
<td>0.94</td>
<td>0.20</td>
<td>45.8</td>
</tr>
<tr>
<td>10% MWWE collected at 8 p.m.</td>
<td>0.97</td>
<td>0.21</td>
<td>46.4</td>
</tr>
<tr>
<td>1% MWWE collected at 8 p.m.</td>
<td>0.97</td>
<td>0.21</td>
<td>46.3</td>
</tr>
</tbody>
</table>

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**TABLE 3.** Effects of MWWE time-course exposure on sexual differentiation of coho salmon alevins

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total number of fish sampled</th>
<th>Percent genetic males</th>
<th>Genetic males (% intersex)</th>
<th>Genetic males (% sex reversed)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control (water)</td>
<td>47</td>
<td>47</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Negative control (solvent)</td>
<td>48</td>
<td>50</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Positive control (17β-estradiol)</td>
<td>47</td>
<td>51</td>
<td>38</td>
<td>4</td>
</tr>
<tr>
<td>10% MWWE collected at 3 a.m.</td>
<td>47</td>
<td>62</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1% MWWE collected at 3 a.m.</td>
<td>46</td>
<td>48</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10% MWWE collected at 8 a.m.</td>
<td>46</td>
<td>54</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1% MWWE collected at 8 a.m.</td>
<td>47</td>
<td>51</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10% MWWE collected at 3 p.m.</td>
<td>47</td>
<td>53</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1% MWWE collected at 3 p.m.</td>
<td>35</td>
<td>46</td>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td>10% MWWE collected at 8 p.m.</td>
<td>45</td>
<td>58</td>
<td>4</td>
<td>15</td>
</tr>
<tr>
<td>1% MWWE collected at 8 p.m.</td>
<td>48</td>
<td>54</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
of 17α-ethinylestradiol were high (>170 ng/L; reproduced in duplicates) in MWWE samples collected at 3 a.m. and 8 a.m. on April 10, 2003, and in all samples collected on April 16, 2003, except the 8 a.m. sample. Levels of 17β-estradiol were also low in the first week of exposure when only the 8 a.m. sample had detectable levels (3.1 ng/L), whereas levels in weeks 2 and 3 were 20 to 25 ng/L at all sample times. Levels of 17β-estradiol were lower (3.4–5.7 ng/L) in samples taken at 3 a.m., 3 p.m. and 8 p.m. in week 4 but still elevated in the sample taken at 8 a.m. (25 ng/L). Levels of estrone varied from <2 ng/L at all sample times in week 1 to >40 ng/L in weeks 2 and 3. However, levels of estrone were only detectable in the 8 a.m. sample at week 4 (39.5 ng/L) (Fig. 3A). The levels of nonylphenol in the MWWE samples ranged from >7 to >22 µg/L depending on the date and time of sampling, and the levels of Bisphenol A in the MWWE ranged from 58 to >1000 ng/L (Fig. 3B). Low levels of DEHP were also detected in the effluent (it should be noted that DEHP is not considered an estrogen agonist).

**Discussion**

Confirming the results of our previous study (Afonso et al. 2002) we found that male Pacific salmon exposed to MWWE during the critical period of sex determination exhibit a sporadic low incidence of intersex and sex reversal. This response is not clearly dose-dependent and appears to be driven primarily by individual susceptibilities to estrogenic compound exposures. The mixed parentage of the exposed fish indicates that offspring of only a few of the genetic crosses had a low enough response threshold to develop an intersex condition or to undergo physiological sex reversal. Afonso et al. (2002) found that 40% of the male chinook salmon exposed to E2 (1 µg/L) during sexual differentiation were sufficiently sensitive to this exposure to result in intersex or physiological sex reversal. Similarly, in this study we found that 42% of the male coho salmon exposed to E2 (1 µg/L) exhibited intersex or sex reversal. Afonso et al. (2002) also found that only 0 to 9% of genetic male chinook salmon exposed to 30 to 100% secondary treated MWWE exhibited either

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**Fig. 1.** Cross-section of genetic male coho salmon gonads. A and B: intersex phenotype with oocytes within testicular tissue. C: complete sex reversal. D: normal testes.
intersex or sex reversal. In this study, we found that 0 to 19% of the genetic male coho salmon exposed to 1 to 10% secondary treated MWWE exhibited either intersex or sex reversal depending on the treatment group. These results indicate that most genetic males in a population of coho salmon exposed to 1 to 10% secondary treated MWWE could be expected to exhibit no gross reproductive pathology (e.g., intersex or sex reversal) if exposed for this duration within this critical developmental period. However, the impacts of MWWE exposure will depend greatly on the susceptibility of the populations, subpopulations and individuals exposed. Previous studies using gynogenetic clones of carp (all normally female) found that the response to exogenous androgen exposure varied significantly between families (Komen et al. 1993). In this study, we examined the sensitivity of a particular stock of coho salmon that returns to spawn relatively late in the season. It would be interesting to determine whether genetically distinct coho stocks (e.g., those that return earlier and/or to different natal streams) have different ranges of susceptibility.

In this study we found no correlation between effluent chemistry or an in vitro screening assay for estrogenic activity, and the ultimate responses of intersex/sex reversal in the fish exposed. This is perhaps not surprising given the highly sporadic nature of the biological response observed. These results serve to underline the importance of combining effluent chemistry, in vitro screening assays, and in vivo studies using environmentally relevant effluent concentrations and exposures at appropriate life history stages when determining the potential ecological relevance of hormonally active substances in MWWE. In this regard, Murk et al. (2002) reported discrepancies as high as 80% in wastewater effluents using an ER-CALUX bioassay versus chemical analysis determined E2 equivalents. Potential explanations for the large discrepancy between chemical estimation of estrogenicity (assuming an additive response) and observed net estrogenic activity in vitro include unidentified chemical species which need to be incorporated into the targeted chemical analysis, synergistic effects between estrogens and matrix components which would invalidate the method of additive response used in chemical based E2 equivalence (Silva et al. 2002), and also, several weak or non-estrogens may be bio-activated in vitro (Petit et al. 1997). Additionally, the presence of any anti-estrogens in the test mixture will lower the net estrogenic response for that mixture using any ER assay.

**Fig. 2.** Four-week averaged daily flow (small diamonds) and effluent hER activity (large squares) (sum of all 5 SPE fractions) for TF/SC Plant (* greater than 3 a.m. sample p < 0.05 Tukey). Error bars show 95% confidence intervals.

**Fig. 3.** (A) Levels of steroidal estrogens in MWWE samples collected at 3 a.m., 8 a.m., 3 p.m. and 8 p.m. weekly for 4 weeks; (B) levels of NP, β-sitosterol, cholesterol, coprosten-3-one, DEHP and Bisphenol A, in MWWE samples collected at 3 a.m., 8 a.m., 3 p.m. and 8 p.m. weekly for 4 weeks.
For some exposure groups, there was a lack of biological response despite levels of potent steroidal estrogens in the MWWE being very high at some time points. For example, the male coho exposed to 1 to 10% collected at 8 a.m., were exposed to >0.2 to 2 ng/L 17β-estradiol in week 2 and >1.7 to 17 ng/L 17α-ethinylestradiol (EE2) in weeks 3 and 4 but none of the genetic males in this exposure group exhibited intersex or sex reversal. In contrast, other species exposed to EE2 throughout the period of sexual differentiation to levels as low as 0.1 to 1 ng/L, e.g., Japanese medaka (Metcalfe et al. 2001) and 0.3 to 4 ng/L (fathead minnow) (Pawlowski et al. 2004; Parrott and Blunt 2005; Länge et al. 2001), have resulted in skewed sex ratios and intersex fish. It should be noted that these exposures were with pure chemicals and were not levels of EE2 in effluent. This may indicate that most of the genetic male coho exposed in this study were not susceptible to intersex/sex reversal at this level of exposure, that the bioavailability of the estrogens in the effluent was limited, or that the timing of these steroidal estrogen spikes occurred too late in the exposure period to influence sexual differentiation. It is important to note that the levels of some potent estrogens such as EE2 were nondetectable in the samples of MWWE taken in the first two weeks of exposure regardless of the time of collection and were only elevated in the last two weeks of exposure. The levels of potent steroid estrogens were therefore low during the earlier stages of sexual differentiation. In this regard, studies in Japanese medaka have found that the exact timing of exposures to estrogenic chemicals is critical in the development of intersex with the highest rate of intersex being developed when the fish were exposed at 3 d post hatch (Gray et al. 1999). The timing and duration of exposure to endocrine active substances is of critical importance for influencing sex determination in fish and, because of large differences in developmental rates, the labile stage occurs at different chronological times in different species.

Coho salmon are differentiated gonochorists and early gonadal development proceeds via an undifferentiated gonad directly to testis or ovary. Primordial germ cells (PGCs) are formed very early in the embryonic development of teleosts and they remain undifferentiated and undetermined until exposed to hormonal and other influences from the developing gonad (Devlin and Nagahama 2002). Prior to differentiation, all somatic cells appear similar in males and females until the PGCs migrate into the germinal ridge and subsequent cell division results in the formation of oogonia or spermatogonia. In salmonids, PGCs can be identified in the germinal ridge at the time of hatching; however, histological analysis cannot identify any differences between gonadal cells between males and females at this stage. The first clear histological evidence of a definite difference between males and females has been noted at prophase 3 with the differentiation of oocytes at approximately 3 weeks post hatch. Similarly, in coho salmon clear differentiation of ovarian tissue has been observed as early as 27 d post hatching (Pifferrer and Donaldson 1989; Devlin and Nagahama 2002). Growth and differentiation of the gonad requires complex neuroendocrine control and involves multiple biochemical, neurological and physiological pathways. This complexity also provides many levels at which gonadal development and reproduction can be disrupted. Sex-specific differences in sex steroids, and mRNAs for steroidogenic enzymes such as aromatase, are present in the embryonic gonad before any histological evidence of sex differentiation is apparent, indicating that the endocrine cascade leading to sex determination is expected to be initiated well before 27 d post hatch in Pacific salmon and hence vulnerable to disruption from an early stage. In differentiated gonochoristic species, hermaphroditism (intersex) and spontaneous sex reversal are very rare although 4 intersex Onchorhynchus keta (Pacific chum salmon) individuals have been reported since 1978 (Devlin and Nagahama 2002). The causes of these rare cases of intersex are unknown although environmental effects (e.g., exposure to exogenous hormones), normal variances in endogenous sex-determination physiology (e.g., variable hormone production or reception), occasional germline mutations that alter sex determination processes in descendants, or genetic mutations in cells during development that alter the generation or interpretation of sex determination signals for a subset of cells within the gonad (e.g., genetically mosaic individuals) are all possible explanations. There is evidence that genetic mutations can be important in this regard as gametes collected from the feminized portion of the intersex gonad of one genetic male chum salmon segregated the sex-reversal mutation to the next generation (Devlin and Nagahama 2002).

The levels of EE2 measured in some of the effluent samples in this study are higher than a number of others reported in the literature. For example, in Rodgers-Gray et al. (2001) levels in EE2 secondary MWWE were consistently found to be <1 ng/L whereas in this study the levels of EE2 in some samples were >170 ng/L. The EE2 levels detected in some of the samples in this study are much higher than would be expected based on comparisons of typical per capita consumption of EE2 and average daily flow rates for this MWWTP. Clearly further research is required to determine whether these surprisingly high levels of EE2 can be consistently detected in effluent from this MWWTP. The levels of 17β-estradiol in Rodgers-Gray et al. (2001) were 4 to 8.8 ng/L, estrone levels ranged from 27 to 56 ng/L, and NP levels from 2700 to 1200 ng/L. In addition, Bennie et al. (1998) found mean levels of 2.3 µg/L for NP and 3.8 µg/L NP2EO in Canadian MWWTP effluents, with maximum NP levels being 62.08 µg/L for primary effluents and 4.79 µg/L for secondary effluents (Bennie et al. 1998). The maximum levels of these substances in the MWWE studied in this case were generally higher—with maximum 17β-estradiol levels exceeding 25 ng/L, estrone levels ranging from 1 to 54 ng/L, and with mean NP levels
ranging from 14,242 ± 4890 ng/L (8 a.m. samples) to 37,265 ± 32620 ng/L (3 a.m. samples). In the case of Rodgers-Gray et al. (2001) the MWWE samples were obtained from a MWWTP with activated sludge and biological filter secondary treatments in the U.K. serving a population of 138,000 with 86% of its influent load from domestic sources. Indeed most of the values in the literature are based on activated sludge MWWTPs. In contrast, the MWWTP in this study was a large trickling filter MWWTP—a type of plant much less studied than activated sludge MWWTPs. Published levels of steroidal estrogens in effluents from trickling filter MWWTPs are typically higher than those detected in effluents from activated sludge MWWTPs indicating that the trickling filter is less efficient at removing estrogenic substances than activated sludge processing. For example, in a survey of MWWE from 18 MWWTPs in Germany, a composite effluent sample from a trickling filter MWWTP serving 226,000 people equivalents was found to contain 18 ng/L estrone, 5.4 ng/L E2 and 12 ng/L EE2, whereas the median values for all the MWWTPs studied (which were predominantly activated sludge) were between 0.4 ng/L (EE2) and 1.6 ng/L (E2) (Spengler et al. 2001). The trickling filter plant studied here was upgraded from primary to secondary treatment in 1999 and serves >740,000 people in fourteen communities in the Greater Vancouver Regional District (GVRD) before discharging to the Fraser River. As such, this trickling filter MWWTP serves a much larger population base than many of the trickling filter plants cited in the literature. The trickling filter process is an important part of the current wastewater treatment profile of British Columbia since this province has 4 trickling filter MWWTPs that collectively serve >1 million people out of a total population of <3 million, i.e., approximately 40% of the population served (Environment Canada 2006). These plants are not designed to remove steroidal estrogens from effluents as they are primarily designed to remove TSS and BOD and indeed there has been a dramatic decrease in effluent TSS and BOD levels (>80% improvement) with the upgrade of the MWWTP to secondary treatment. This study confirms that there are significant levels of steroidal estrogens, and other endocrine active substances, in MWWE from this trickling filter MWWTP, and these are sufficient to be detected in an in vitro bioassay, and to cause intersex and sex reversal in a few genetic male coho exposed to 1 to 10% effluent during the labile period of sexual differentiation. The ecological significance of these results is unknown at this time and requires further study on several aspects, not the least of which is furthering our understanding of the basis for individual differences in response, chemical analysis of the effluent over a longer time period to study wide variations in levels of endocrine active substances particularly potent steroidal estrogens and, although complex to conduct with this species, exposure of Pacific salmon in short pulses to environmentally relevant effluent concentrations at appropriate life history stages and full life cycle assessment. Given the sporadic nature of biological response observed it would be premature to judge trickling filter plants against activated sludge processing until further studies have been conducted to address their comparative ecological impacts.

Conclusion

Following exposure to MWWE, some genetic male coho salmon become sex reversed, others exhibit intersex, and other males appear to be relatively unaffected. It is important to remember that complete sex reversal and intersex are gross effects and, as such, these profound alterations in development can be easily identified using whole body histological cross sections of juvenile fish. Subtle changes in gene expression, and steroid hormone production and effect, must precede these profound histological changes—but in only a few individuals do these subtle changes push a fish to a ‘tipping point’ or threshold where a cascade of effects is initiated resulting in the dramatic result of complete sex reversal. Since all individuals in a treatment group were exposed to the same level of effluent and its hormonally active components, then it is possible that fish exhibiting intersex or sex reversal have ‘sensitive genotypes’ and those not responding at the same level of exposure have ‘resistant genotypes.’ It is important that we increase our understanding of the genetic and physiological basis for these significant individual differences in response. This knowledge would be useful for selecting and monitoring appropriate populations and individuals for the standardized testing of endocrine disrupters (e.g., optimization of OECD test methods), and for the appropriate design and interpretation of field studies on the effects of endocrine disrupters on wild fish populations. Genetic polymorphisms are well recognized as having important implications in the field of human toxicology and risk assessment and, when sufficient data are available, chemical-specific adjustment factors (CSAFs) can be developed to account for variability in toxicokinetics and toxicodynamics replacing default values for human variability and interspecies differences (Dorne and Renwick 2005). However, genetic polymorphisms and their implications for ecotoxicology and ecological risk assessment, particularly for fish, have yet to be thoroughly explored. Further understanding in this area would assist in the development and refinement of appropriate uncertainty factors for risk assessment of endocrine active substances.

Acknowledgements

This work was supported by Fisheries and Oceans Canada and the Greater Vancouver Regional District (GVRD). Sincere thanks to Ellen Teng, Dionne Sakhrani, Mitch Uh, Carlo Biagi, Doug Swanston (Seacology), Ben Goh, Robert Ng and Theresa Gregonia (GVRD) for their
invaluable assistance with effluent collection, PCR assays, fish husbandry and sampling. In addition, we would like to thank J.P. Sumpter for the recombinant yeast strain.

References


Ikonomou MG, Sheng-Suan C, Fernandez MP, Blair JD, Fischer M. Submitted for publication.


Received: January 9, 2006; accepted: June 20, 2006.