REPRODUCTIVE, PHYSIOLOGICAL, AND BIOCHEMICAL RESPONSES IN JUVENILE FEMALE RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) EXPOSED TO SEDIMENT FROM PULP AND PAPER MILL INDUSTRIAL DISCHARGE AREAS

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Abstract—Four pulp and paper mills discharge their effluents in the same section of the Biobío River in central southern Chile. Pulp mill effluents are a very complex mixture with characteristics that depend on the type of raw material, the process technology, and the effluent treatment. To investigate the effect of pulp mill effluent discharges, immature *Oncorhynchus mykiss* were exposed to river sediments in the laboratory for 29 d. Three sampling areas were defined in a spatial gradient in the river: Preimpact, impact, and postimpact zones relative to the pulp and paper mill discharge areas. Ethoxyresorufin-O-deethylase activities were significantly higher in fish exposed to impact and postimpact sediments when compared to those exposed to preimpact sediments, and higher levels of vitellogenin were observed in the plasma of female fish exposed to impact and postimpact sediments. Histological analysis of the gonadal tissue showed an induction of gonadal maturation in fish exposed to sediment coming from the impact and postimpact zones (oocytes in a vitellogenic state). No site differences were observed in erythrocytes, although differences were noted in the leukocytes in the exposure areas. Finally, the biomarker approach showed evidence that the sediment associated with pulp mill effluent discharges produces some effects in fish under laboratory conditions.

Keywords—Pulp mill effluent Ethoxyresorufin-O-deethylase Gonadal maturation Vitellogenin Leukocytes

INTRODUCTION

During the last few years, many of the reproductive alterations reported in fish have been related to disruption of the endocrine system, the causal agents of which can be classified as natural (e.g., steroids, phytoestrogen, and micotoxins) or synthetic (e.g., industrial chemicals, pesticides, and their metabolites) compounds [1]. In aquatic environments, effluents from pulp and paper industries have been associated with a variety of effects observed in the laboratory and the field [2]. One of the main environmental problems is the capacity of many compounds derived from cellulose production to behave as endocrine-system modulators, interfering with the synthesis, secretion, transport, binding, or excretion of hormones widely described as being endocrine-disrupting compounds or xenoeestrogens [3].

Among the alterations observed, particularly in fish, are effects on sexual differentiation [4], effects on normal gonadal development in males and females [5,6], reduction of plasma steroid levels [7–9], induction of plasma vitellogenin (VTG; yolk precursor protein) in adult males as well as juvenile gonadal males and females [10,11], induction of the cytochrome P450–dependent monoxygenase detoxification system [12–15], reduced fertility, reduced viability of offspring, disruption of sexual development and behavior, low rates of metabolic activity, decreased growth, and occurrence of atrophy [16,17].

In Chile, and specifically in the Biobío River, the pulp and paper mill industry is very important; 83% of the national pulp mill production is generated by industries located in this river basin [18]. Each year, these industries produce more than 1,000,000 tons of pulp from pine and eucalyptus. They discharge their treated liquid effluents into the river, causing potential pollution problems [19]. Although during the last decade the pulp and paper mill industries in Chile have implemented treatment systems for their effluents (primary and secondary), information regarding the potential for endocrine-disruptor effects is unavailable. Endocrine impacts of pulp mill effluents have been established thoroughly in countries such as Canada [20].

At first, some studies identified organochlorine compounds as the agents responsible for the observed toxic effects in some organisms [21]. Recent studies have found that new intermediate products are formed or released during normal operation of the pulp mills; these are compounds such as chlorocimenes, retene, chloroethenes, lignins, and resin acids [22]. Many of these compounds are easily eliminated by the secondary treatment and have not been implicated in endocrine effects. However, phytoestrogens and flavonoids appear to be compounds that are capable of jeopardizing the reproductive capacity and producing toxic effects in aquatic organisms [23].

Because some of these compounds are highly lipophilic, the expected concentrations in river water may be much lower than those in sediments. Recent studies from the United Kingdom have shown that some endocrine-disrupting compounds are accumulating in sediments and are biologically available [24]; therefore, the testing of sediment toxicity is suggested near pulp mill discharges. The objective of the present study was to evaluate the effects of exposure to sediments affected by pulp and paper mill effluent discharge in the Biobío River...
in central southern Chile. Biochemical, physiological, and reproductive biomarkers were evaluated for statistically significant differences among the different treatments using a model species, *Oncorhynchus mykiss*.

**MATERIALS AND METHODS**

**Study area**

The Biobio River is located between 36°45′S; 72°59′W and 38°20′S; 71°15′W, with a length of 380 km, and it has a drainage basin of 24,260 km². It has a marked seasonal hydrological profile, with mean flow rates fluctuating between approximately 1,600 m³/s during the winter and close to 200 m³/s in the summer. This river originates in the Icalma and Galletue lakes in the Andes mountains but also receives inputs from the spring precipitation [25].

**Sediment samples**

Three stations were selected following a gradient from the effluent discharges of the pulp and paper mill industries present in the Biobio River (Fig. 1). The preimpact zone (PRE) was located in the Santa Barbara sector (37°27′25″S, 72°45′29″W) and corresponds to the reference station (free of cellulose industry discharge effluents). The impact zone (IMP) was located in the Talcamavida sector (37°11′25″S, 72°42′29″W), 15 km downriver from the last discharge (area directly influenced by cellulose industrial effluent discharge). The postimpact zone (POST) was located in the Chiguayante sector (36°58′05″S, 72°56′44″W), near the city of Concepción (corresponding to an area less influenced by the discharges). Sediment samples from each site were taken with a Petite-Ponard (Wildco®, Saginaw, MI, USA) dredge and were transported (refrigerated at 4°C) to the laboratory for toxicity testing.

**Experimental design**

The exposure experiment was performed in the bio-testing laboratory of the Faculty of Biological and Oceanographic Sciences of the University of Concepción. The 50 specimens of *O. mykiss* (rainbow trout) juveniles (age, one year) were obtained from the Polcura Cultivation Center and had an average size of 23.6 ± 1.7 cm and an average weight of 121.3 ± 14.4 g. These trout were acclimated for two weeks in 90-L tanks under a controlled temperature of 11 ± 1°C, a 12:12-h light:dark photoperiod, and constant oxygenation (7–8 mg/L). The water was changed and the fish fed every 2 d. Once this period was finished, 10 specimens were killed (to obtain samples for time zero). The remaining fish were placed in four 70-L cages at a density of 10 individuals per aquarium, containing sediment coming from the three sampling areas (PRE, IMP, POST), in a 1:10 w/v proportion. A control aquarium contained potable dechlorinated water. Before fish were added, the sediments were allowed to decant in aquariums with dechlorinated water for 48 h while the oxygenation level was...
maintained constant (7–8 mg/L). No mortality was observed during the entire experiment.

Exposure time was 29 d under conditions identical to those of the acclimation period (the fish were fed every 2 d, and water was changed every 7 d, carefully avoiding sediment resuspension). Once this period was over, the fish were killed and their morphometric parameters (weight and length) obtained to calculate the somatic indexes: Condition factor, gonadosomatic index (GSI), and liver somatic index (LSI). Subsequently, blood samples were collected using heparinized syringes for plasma VTG analysis (1 ml of whole blood) and syringes containing ethylene diamino tetraacetic acid for qualitative and quantitative hematological analysis (1 ml of whole blood). These samples were analyzed in the Department of Clinical Biochemistry and Immunology of the Pharmacy, Faculty of the University of Concepción. Finally, gonad samples were weighed and set in Buoin solution, and the livers were stored in liquid nitrogen until their analysis in the Department of Molecular Biology or Biomarker Laboratory of the EULA–Chile Center of the University of Concepción.

Physiological indexes

Physiological indexes of condition were calculated based on the morphometric information, such as the condition factor (100-weight/length²), gonadosomatic index (GSI) (100-gonad wt/total organism wt), and liversomatic index (LSI) (100-liver weight/total wt of fish).

Blood samples

Blood samples were obtained by a puncture of the caudal vein and preserved in ethylideninitrilo tetraacetic acid. Determination of the hematological parameters [26] was conducted using hemoglobin measurement performed following the Cyanmetahemoglobin method (Valtek Diagnostic Drabkin, Santiago, Chile) at 546 nm in a spectrophotometer (Microlab 100; Merck By Vital Scientiﬁcs, Amsterdam, The Netherlands). Hematocrit and leukocrit were determined by the microhematocrit method, and immature erythrocytes were determined using a Rees Ecker solution in Neubauer chambers (on a total of 1,000 erythrocytes counted by smear) [27].

Induction of liver detoxification enzymes

Cytochrome P450 enzyme activity was evaluated as 7-ethoxyresorufin-O-deethylase (EROD) [28] in the floating (fraction S9) obtained from livers homogenized in a sucrose buffer (0.1 M, pH 7.5) and centrifuged at 9,000 × g for 20 min at 4°C. Its final value was expressed as pmol/min/mg of protein. Protein analysis was performed using a Bio–Rad Protein Kit (Hercules, CA, USA), which uses bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA) as a reference material.

Plasma VTG

Blood samples were obtained for each individual and preserved in heparin, later separating the plasma (500 µl) from the cells by centrifugation at 7,000 g for 10 min. The concentrations of plasma VTG were measured using an enzyme-linked immunosorbent assay obtained from different protocols [29–31]. Briefly, the microplates were incubated with rainbow trout VTG (V01004301-001; Biosense Laboratory, Bergen, Norway) or trout plasma samples, and then plates were blocked with bovine serum albumin after those plates were coated with rabbit anti-Sea Bream VTG polyclonal antibody (V01402201-100; Biosense Laboratories). A goat anti-rabbit immunoglobulin G peroxidase was conjugated as a secondary antibody (Product A 6154; Sigma-Aldrich). Finally, the plates were measured at 490 nm in an enzyme-linked immunosorbent assay (Microplate Autoreader EL311; Bio-Tek Instruments, Winoosky, VT, USA), and the VTG concentration was determined based on a standard calibration curve (1–1,000 ng/ml) [29].

Gonad histology

The histological analyses were performed in the Department of Molecular Biology of the Faculty of Biological Sciences of the Universidad de Concepción. The gonads were weighted and set in Buoin solution (48 h) and then washed in 70% alcohol. Subsequently, they were dehydrated with a series of ethanol solutions (70–99%). Finally, they were embedded in liquid paraffin at 58°C (24 h) for later sectioning (thickness, 7 µm) and staining with hematoxylin and eosin (0.5%).

Maturity states were assigned according to Table 1, as adapted from Matsubara et al. [32] and from Sokolowska and Warszawy [33], in addition to measurements of cell and nuclear diameter using a 100× magnification (Zeiss Axioplan 2, Digital Nikon DXM 1200, Thornwood, NY, USA) and the proportion of cells in the distinct maturation stages (I, II, III, and IV).

Statistical analysis

Data normalization was first verified, and then the levels of the each parameter were evaluated using the analysis of variance (ANOVA) parametric test (p < 0.05) to determine if statistically significant differences existed between the measurements of the distinct treatments. Those that were considered to be statistically significant were later confirmed by means of the Tukey post-hoc test (p < 0.05).

RESULTS

Physiological indexes

Statistically significant differences were not observed in condition factor or LSI, with only a slight decrease following 29 d of exposure (Fig. 2A). The GSI values (Fig. 2B) showed a significant increase in the fish group exposed to IMP sediments, with an increase in gonad size (ANOVA, p < 0.05). No other groups showed a significant difference from the control group or the fish sacrificed at time zero.

Hematological analyses

Hematological parameters did not present significant differences between the fish exposed to PRE, IMP, and POST sediments. In the blood, parameters such as hematocrit and hemoglobin (Fig. 3A) increased with respect to the fish exposed to control conditions, although without significant differences. The hematological constants (secondary erythrocytic indexes) of the fish exposed to PRE, IMP, and POST sediments (Fig. 3B) did not present significant differences with respect to the control group for the quantity of immature erythrocytes, although they reached close to 10% of the total erythrocytes analyzed per fish. In the leukocytes, all fish exposed to the distinct sediments demonstrated a statistically significant diminishment of leukocytes and thrombocytes (ANOVA and Tukey, p = 0.026) with respect to the control, but no significant differences were found between groups (Fig. 3C).

EROD activity

Fish exposed to IMP and POST sediments presented a clear induction of EROD activity (Fig. 4), which was statistically
Table 1. Oocyte maturation states for rainbow trout (*Oncorhynchus mykiss*)

<table>
<thead>
<tr>
<th>State</th>
<th>Cell diameter (μm)</th>
<th>General appearance</th>
<th>Follicular cells</th>
<th>Cortical alveoli</th>
<th>Yolk</th>
<th>Nucleus</th>
<th>Nucleoli</th>
</tr>
</thead>
<tbody>
<tr>
<td>Previtellogenics</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I: Nucleolocromatina</td>
<td>20–200</td>
<td>Cytoplasm is evenly tinted; nucleus is large, surrounded by a thin layer of cytoplasm</td>
<td>A large quantity of prefollicular cells surrounds the oocyte</td>
<td>No</td>
<td>No</td>
<td>Large; occupies 3/4 of the cell</td>
<td>Single, large nucleolus</td>
</tr>
<tr>
<td>II: Perinucleolar</td>
<td>200–400</td>
<td>Oocyte and nucleus both increase in size, a large quantity of nucleolus appears in the periphery of the nucleus; cytoplasm is uniform, although vacuoles may be present in advanced stages</td>
<td>Follicular cells surround the oocyte</td>
<td>No</td>
<td>No</td>
<td>Large, with a large quantity of nucleoli</td>
<td>Numerous nucleoli appear in the periphery of the nucleus</td>
</tr>
<tr>
<td>Vitellogenics</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>III: Early (cortical–alveolar)</td>
<td>400–500</td>
<td>Yolk vesicles, observed as hollow spheres, appear in cytoplasm</td>
<td>Corium begins to form</td>
<td>Numerous cortical alveoli begin forming in the periphery of the cytoplasm</td>
<td>Strictly speaking, it doesn’t exist in this state</td>
<td>Begins to diminish in size</td>
<td>A small number, evenly distributed in the periphery of the nucleus Disappear into the nucleus</td>
</tr>
<tr>
<td>IV: Medium</td>
<td>0.5–1</td>
<td>Yolk granules appear and fuse, forming a great flowing mass at the end of vitellogenesis</td>
<td>Completely formed corium</td>
<td>Disappear; contents merge into the cytoplasm</td>
<td>Numerous granules begin to appear in the periphery of the cytoplasm</td>
<td>Occupies a small portion of the cytoplasm</td>
<td></td>
</tr>
<tr>
<td>V: Late</td>
<td>2–3</td>
<td>Nucleus is present but small; yolk begins to form a great fluid mass</td>
<td>No</td>
<td>Yolk granules begin to coalesce</td>
<td>Small</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>Maturation VI</td>
<td>3–5</td>
<td>Nucleus migrates to the periphery of the cytoplasm; their membranes coalesce</td>
<td>No</td>
<td>A large, transparent, flowing mass</td>
<td>Migrates toward the periphery of the cytoplasm</td>
<td>No</td>
<td></td>
</tr>
</tbody>
</table>

* Adapted from Matsubara et al. [32] and Sokolowska and Warszawy [33].
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Fig. 2. Physiological indexes in trout exposed to sediments (29 d) from the distinct areas impacted by pulp and paper mill industrial discharge. (A) Condition factor (K) and liversomatic index for the distinct treatments. (B) Values for the gonadosomatic index (GSI) of the distinct treatments compared with the experimental control. Error bars represent the mean ± standard error. *Statistically significant difference (p < 0.05). IMP = impact; LSI = liver somatic index; POST = postimpact; PRE = preimpact.

significant (ANOVA and Tukey, p = 0.013) with respect to fish exposed to PRE sediments or experimental control fish.

Plasma VTG and gonad histology

Plasma VTG concentrations (Fig. 5) presented statistically significant differences (ANOVA and Tukey, p = 0.017) between the fish exposed to IMP and POST sediments with respect to the levels observed in fish exposed to PRE sediments and the control group. Moreover, only one specimen from both IMP and POST sediments was male. In this case, the plasma VTG levels reached 0.097 μg/ml with IMP sediments and 0.048 μg/ml with POST sediments (Fig. 6). Testes morphology in both fish presented normal development.

The average diameters of the oocytes of rainbow trout (Table 1) permitted the description of three principal oocyte-maturation states: Previtellogenic (stages I and II), with a diameter in the range of 20 to 400 μm; vitellogenic (stages III–V), for the range of 0.4 to 3 mm; and mature (stage VI), with an average diameter range of 3 to 5 mm. Micrographic analysis showed a significant increase in stage III and IV eggs in the IMP sediments and a significant increase in stage III eggs in the POST sediments. Fish exposed to IMP sediments showed 5% of total eggs in stage IV (medium vitellogenesis).

Figure 6A presents the characteristic gonadal maturation state observed in the trout exposed to PRE sediments and the control trout, for which the presence of oocytes in stages I and II is observed. The average diameter of these oocytes in stage II is 177 μm, with a nucleus dominating the cytoplasm. Notable differences were observed in the trout exposed to IMP sediments (Fig. 6B), which presented a large quantity of vitelline granules and a substantially reduced nucleus size, because it was the only group that presented oocytes in stage IV. The stage III oocytes presented a large quantity of vitelline vesicle (cortical alveoli) in the periphery of the cytoplasm. Moreover, among the vitellogenic oocytes, a large quantity of primary oocytes, although much smaller than the stage III and IV oocytes, was observed. Finally, Figure 6C corresponds to
the ovaries of trout exposed to POST sediments, in which only stage III oocytes with vitelline vesicles were observed in the periphery of the cytoplasm, with an average diameter of 300 μm. In addition, asynchronous ovarian development was observed when compared with oocytes of stages I–III.

DISCUSSION

Endocrine disruption has emerged as one of the more important sublethal effects of xenobiotic exposure. The fish endocrine system regulates an immense series of processes, including reproduction, growth, development, metabolism, behavior, and several homeostasis-regulating mechanisms. A wide consensus exists that the compounds derived from cellulose production have the capacity to alter the endocrine system [38], indicating that the observed effect occurred only at the reproductive physiology level. Usually, field studies reflect an increase in LSI downstream of discharges (IMP and POST) and the reference area upstream of discharges. It is necessary to highlight that just as for the reproductive response, consistent, statistically significant differences were observed among areas downstream of discharges (IMP and POST) and the reference area upstream of discharges, which was defined as an objective of the present experiment.

Reported effects of exposure to cellulose effluents in the reproductive physiology in Micropterus salmoides include a reduction in VTG levels (20% in females after 29 d of exposure) and a 40% reduction in GSI (in both sexes) [34]. This differs from the results of the present experiment, in which VTG levels increased approximately 50% in the fish exposed to IMP and POST sediments (after 29 d of exposure). It is important to note that the present experiment involved immature fish, whereas previous exposures used sexually mature individuals. In the early phases of gonadal maturation, steroid hormones provide a positive feedback, whereas in mature individuals, steroid regulation is via negative-feedback loops. The increased VTG in the circulation also was associated with increased gonad size and the presence of more mature egg follicles. These physiological indexes provide a good approximation of physiological alterations in the exposed fish [38], and the fact that no statistically significant differences were found in the other analyzed indexes (condition factor and LSI) indicates that the observed effect occurred only at the reproductive physiology level. Usually, field studies reflect an increase in LSI downstream of the discharges. With the present experimental design, we made a partition of chemicals released in a pulp mill effluent, considering only those chemicals that were able to bind particles and be attached to sediments; in that way, we were able to isolate the chemicals that were capable to producing VTG induction but not increasing LSI.
However, in this type of laboratory experiment, a slight weight loss in the fish can be expected, and this could alter the condition factor, the baseline value of which for this species in the laboratory was described as one [39].

In fish exposed to sediments affected by pulp and paper mill industrial discharge effluents, normal gonadal development appears to have been altered, because ovaries preparing for maturation were observed only in fish exposed to IMP and POST sediments. Previous studies have shown only a negative relationship between exposure to endocrine-disrupting compounds and gonadal development [4], appearance of ovo-testis [5], and negative effects on gonadal growth [6].

The present study showed that an induced response to sediments coming from areas associated with pulp and paper mill industry discharge is translated into an induction of early maturation of the gonadal tissue (appearance of oocytes in vitellogenic state) (Fig. 6B). This is not normal for female trout juveniles; during this stage of the salmonid life cycle, the individual directs all its energy to somatic growth rather than to gonadal growth. Under normal conditions, gonadal maturation usually is reached at three years of age and a weight greater than 600 g [40]. Consequently, the present results, related with the observed GSI in the fish exposed to IMP sediments, present a larger gonadal size than observed in the other treatments and, possibly, are caused by VTG incorporation by the oocytes (not observed in the other treatments). Here, we have shown that immature females exhibit significant plasma VTG levels compared with control groups of fish derived from the PRE area. Moreover, two male specimens coming from IMP and POST areas showed detectable plasma VTG.
levels, although no changes in gonadal development were observed.

The induction of EROD activity observed in fish exposed to IMP and POST sediments compared with those observed in fish exposed to PRE sediments is in agreement with experiments concerning the exposure of the same species to sediments collected near cellulose industrial discharge in the Saimaa Lake (Finland) [41]. This suggests a potential bioavailability from the sediments of chemicals that would be capable of altering the endocrine system in the exposed organisms [24]. This effect was reproduced in the laboratory from sediments collected in the field, and the laboratory bioassay represents a good way to study both these effects and potential causative compounds.

Somatic-type answers expressed during a short exposure to xenobiotics coincide with the changes in the gonadal structure of fish exposed during 28 d to cellulose industrial effluents [34], an effect that does not repeat in a longer exposure time, which could result, as mentioned earlier, from the fact that the organisms tend to re-establish their homeostasis through the alteration of other paths, such as somatic growth reduction.

The present study presents evidence that sediments collected in the IMP and POST areas of pulp and paper industry discharges are potentially responsible for the adverse effects observed in the fish exposed to laboratory conditions, such as the induction of EROD activity and the clear increase in gonadal maturation, which was observable in the appearance of oocytes in the vitellogenic stage and the increase in plasma VTG levels. The effects should be confirmed with in situ experiments, perhaps by caging in a similar gradient, as well as a physicochemical characterization of sediments and effluents, which would permit the identification of possible causal agents and direct future research studies. This is our next step. It also will be important to examine the response of reproductively mature individuals to these same exposures.

The results of the plasma VTG analysis are consistent with the hypothesis that the alternation in gonadal development observed in female rainbow trout is the consequence of exposure to both IMP and POST sediments.

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