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Estrogenicity and intersex in juvenile rainbow trout (*Oncorhynchus mykiss*) exposed to *Pine*/*Eucalyptus* pulp and paper production effluent in Chile

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ABSTRACT

Pulp and paper mill effluents (PPMEs) have been shown to increase gonad size, cause early maturation, and disrupt hormone functions in native and non-native Chilean fish. In this study, we assessed reproductive (plasma vitellogenin; VTG, gonad development) and metabolic (ethoxyresorufin-*O*-deethylase activity; EROD) end points, relative liver size (LSI) and condition factor (*K*) of juvenile female and male rainbow trout exposed to effluents. Unlike previous studies, which have focus either on the specific effects of effluent on fish in laboratory exposures or biotic population statuses downstream of discharge sites, we simultaneously assessed the impacts of PPMES on trout using two approaches: (1) laboratory exposures of tertiary treated PPME produced from processing *Eucalyptus globulus* or *Pinus radiata*; and (2) in situ bioassay downstream of the combined discharge of the same pulp mill. Despite an increase in the average gonadosomatic index (GSI) in exposed fish, no statistical differences in gonad size between exposed and unexposed individuals was detected. However, both female and male fish exposed to effluents showed significantly higher concentrations of plasma VTG, so more in fish exposed to *Eucalyptus*-based effluent when compared to *Pinus* PPME. In addition, male fish showed intersex characteristics in all exposure assays (*Eucaliptus* and *Pinus*) and, despite the low concentration of effluent in the river (<1% [v/v]), similar responses were observed in the caged fish. Finally, EROD activity was induced in both in situ exposures and laboratory assays at the higher PPME concentration (60–85% PPME). This study confirms estrogenic effects in Chilean fish exposed to PPME and the necessity for biological effects monitoring in addition to the assessment of physical–chemical endpoints as required in current government regulations.

1. Introduction

Fish are considered a sentinel species in environmental monitoring programs designed to assess the impacts of pulp and paper mill effluents (PPMEs) in Canada (Munkittrick, 2004; Lowell et al., 2005; McMaster et al., 2006), the United States (Sepúlveda et al., 2002; Theodorakis et al., 2006; Yeom and Adams, 2007), Sweden (Larsson et al., 2000; Sandström and Neuman, 2003), Finland (Donald, 2003; Karels and Oikari, 2000) and New Zealand (Van den Heuvel et al., 2007). In general, less emphasis has been placed on fish-based environmental monitoring in South America. Over the past decade interest in monitoring PPME quality in Chile (Orrego et al., 2005, 2006, 2009, 2010; Chiang et al., 2010, 2011) increased with new industrial development, improving treatment of wastewater, and increasing environmental requirements by national and international agencies, as well as foreign pressure from trade markets.

Modern mills have implemented advanced wastewater treatment technologies, including the use of elemental chlorine to elemental chlorine-free ECF, total chlorine-free or TCF processes, to improve the pulp-production process. These production changes have been shown to decrease developmental alterations in exposed fish within the receiving water bodies in North America (Munkittrick et al., 1997). However, endocrine disruption (ED)

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is still detected at many of these sites (Scott et al., 2011; Costigan et al., 2012; Simmons et al., 2012).

PPMEs affect fish reproduction (McMaster et al., 1991; Munkittrick et al., 1992; Orrego et al., 2005, 2006, 2009, 2010; Chiang et al., 2011; Scott et al., 2011; Costigan et al., 2012). Mill effluents are complex mixtures, containing various compounds related to the raw materials used and processing techniques (Hewitt et al., 2008). In the Northern hemisphere, fish responses to PPMEs include various reproductive impairments, such as reduced gonad size, female reduced fecundity with aging, male decreased secondary sexual characteristics, and changes in the hormones levels related to reproduction (McMaster et al., 1991; Munkittrick et al., 1992; Gibbons et al., 1998; Mellanen et al., 1999; Tetreault et al., 2003). Contrariwise, juvenile rainbow trout exposed to PPME in Chile showed increased gonad size, induction of maturation and estrogenicity when exposed to sediments from discharge zones and when injected directly with PPME extracts (Orrego et al., 2005, 2006; 2009). Responses of the native fish populations downstream of PPME discharges included increased gonad size in males, increases in in vitro production of 17β-estradiol (E2) in females and decreased in vitro production of 11-ketotestosterone (11-KT) in males (Chiang et al., 2011). Moreover, Chamorro et al. (2010) showed 3 times higher estrogenic activity (as 17α- ethynylestradiol (EE2) equivalents) from pulp production using *Eucalyptus* (*E. globulus*) when compared to *Pine* (*P. radiata*). However, Orrego et al. (2010) have shown that secondary and tertiary effluent treatment at Chilean pulp mills reduced the induction of 17β-estradiol, testosterone and CYP19a activities in triploid rainbow trout. Despite these past studies, it has been hard to establish the cause of these reproductive and growth effects using only laboratory or field based approachs in fish exposed to PPME discharges.

To assess the effects of PPME, laboratory exposures to a various effluents can be an appropriate strategy to study the specific effects on fish with a greater capacity to establish potential causal relationships. However, the ecological relevance of laboratory studies is frequently debated due to a limited ability to extrapolate resulting information to true environmental conditions (Crane et al., 2007). Thus, integrating laboratory exposures with in situ bioassays (caging fish in the field) offer advantages, including controlling effluent exposures while maintaining environmental relevance. Furthermore, these in situ bioassays allow researchers to control the number, size, age and reproductive status of the fish tested, as well as various experimental parameters such as spatial gradients, number replicates, and time of exposure (Oikari, 2006; Orrego et al., 2006; Crane et al., 2007). Controlling these factors also reduce various uncertainties which often complicate results from field studies assessing fish responses to PPME exposure, while maintaining vital environmental relevance.

The goal of this study was to assess reproductive (plasma VTG, gonad development, relative gonad size) and metabolic (EROD activity, relative liver size and condition factor) responses of juvenile female and male rainbow trout exposed separately to tertiary treated PPME from *E. globulus* and *P. radiata* pulp production, along with an in situ bioassay downstream the discharge of the same pulp mill.

2. Materials and methods

2.1. Fish exposure and sampling

Juvenile (305 days post-hatch) rainbow trout (weight: 95 ± 11 g) obtained from a commercial fish farm were used as the experimental model for the current study. Due to the fish age, there were no obvious external sex differentiation characteristics, so gender proportion was uneven (and randomly assigned) for all the experiments. Rainbow trout were exposed independently in two bioassays to tertiary treated *E. globulus* PPME and to *P. radiata* PPME in four different concentrations (10, 35, 60 and 85% [v/v]) during a period of 21 days (d) at controlled temperature (13 ± 1 °C). Control treatments with fish individuals in dechlorinated water were assessed. Each treatment was completed in triplicate in 70 L tanks with a fish density of 33.9 g L^{-1}. Exposures were static, with 100% renewal every 2 d with fresh effluent and 12:12 h photoperiod. Samples were obtained at the end of exposure.

The in situ bioassay (caging experiment), was performed with a fish density of 26 g L^{-1} in two 90 L cages (2 per site) at three sites, preimpact (PRE, 3 km upstream to the PPME discharge), impact (IMP, 300 m downstream to the PPME discharge) and postimpact (POST, 10 km downstream to the PPME discharge). For the caging experiment, rainbow trout samples were taken after 11, 21 and 31 d of exposure. The river temperature during the experiment ranged from 13–15 °C and PPME discharge was <1% [v/v] of the river flow at the IMP site.

Fish blood was collected with heparinized syringes. Between 1–2 ml of blood was centrifuged at 7000 g for 10 min at 4 °C. The resulting plasma stored at −80 °C until VTG analysis. Fish were sacrificed by spinal severance and total length (±1 cm), total weight (±0.1 g) and gonad and liver weight (±0.0001 g) were recorded. Gonad tissues were kept in Bouin solution until histological procedures and liver tissues were frozen in liquid nitrogen and later in the lab stored at −80 °C until EROD analysis. Using this information, the condition factor ($K = 100 \times$ (weight (g)/length^3 (cm)); Fulton, 1902; Environment Canada, 2010), gonad somatic index (GSI = 100 × (gonad weight (g)/ total weight (g)); Environment Canada, 2010) and liver somatic index (LSI = 100 × (liver weight (g)/ total weight (g)); Environment Canada, 2010) were calculated to assess the general condition of the fish.

2.2. Plasma Vitellogenin

VTG plasma concentrations were analyzed by an indirect Enzyme-Linked ImmunoSorbent Assay (ELISA). The analysis was performed using 96 wells polystyrene microplates, according the procedure described by Tyler et al. (2002), following modifications from Orrego et al. (2010). A specific polyclonal antibody (rabbit anti-vitellogenin trout, PO-2 Biosense Laboratories, Bergen, Norway) was the primary antibody and the secondary antibody was conjugated with peroxidase (goat anti-rabbit IgG Sigma–Aldrich, St Louis, USA), developed with OPD (*o*-phenylenediamine dihydrochloride) solution. The reaction was measured at 490 nm in an ELISA microplate reader (Baush & Lomb, DNM, 9602G). Final VTG concentrations were calculated according to a standard calibration curve (1–1000 ng ml^{-1}) using purified rainbow trout vitellogenin (Biosense Laboratories, Norway) (Tyler et al., 2002; Orrego et al., 2005, 2006, 2010). The detection limit for the assays was 6.5 ng mL^{-1}.

2.3. Ethoxyresorufin-O-deethylase (EROD) activity.

EROD activity was analyzed following the protocol of Lubet et al. (1985). The floating post-mitochondrial fraction (S9) was obtained from livers homogenized in Tris–HCl (50 mM)/$MgCl_2$ (25 mM) buffer (pH 7.5), and centrifuged at 9000 × *g* for 20 min at 4 °C. The analysis was performed using an LS 50B spectrofluorometer (PerkinElmer, Beaconfield, UK) for 5 min at 25 °C. Protein analysis was performed in a 96 well microplate reader (Baush & Lomb, DNM, 9602G) using a Bradford method with bovine serum albumin (Sigma–Aldrich, St. Louis, USA) as a reference material. EROD activity was expressed as ρ mol min^{-1} mg $protein^{-1}$.

2.4. Gonad histology

A subsample of gonad tissue was taken from both female and male individuals. The gonad tissue was fixed in Bouin solution (48 h) and washed in 70% [v/v] ethanol three times for 15 min each. The tissue was subsequently dehydrated with a series of ethanol solutions (70–99% [v/v]) and chloroform, and infiltrated two times in liquid paraffin at 58 °C for 2 h. After, the samples were embedded in paraffin at room temperature (16 °C) for 24 h. The embedded tissue was sectioned 7 μm thick and stained with a solution of hematoxylin and eosin (0.5% [w/v]) each. The proportion of cells in the distinct maturation stages for the different treatments was assigned according to a defined scale (Orrego et al., 2006) using a Zeiss Axioplan 2 microscope with a coupled digital camera for photography (Gottingen, Germany; Digital Nikon DXM 1200, Thornwood, NY, USA).

2.5. Statistical analysis

Normal distribution was assessed using the Shapiro–Wilkís test. Due to the nature of the study, analyses examined individual data within sex/experiment. Fish sex was confirmed by histological analysis. For *Pinus* and *Eucalyptus* bioassays, data analysis was performed to evaluate differences versus respective controls, while for the caging experiment, data analysis compared IMP/POST sites with PRE site independently for each sampling period (11, 21 and 31 d after exposure). For the laboratory bioassays and in situ bioassays with PPME, statistical differences between exposure concentrations/sites and their control/PRE site were assessed using an analysis of variance (ANOVA, $p < 0.05$) including plasma VTG levels, K, GSI, LSI and EROD activity, followed by Tukey's post hoc to test differences in individuals. All statistical analysis was performed with the raw data, except for female gonadal development; oocyte percentage of each developmental stage per treatment was assessed using Chi-square test. EROD and VTG data are shown as fold change as recommended by Feswick et al. (2014) for inter-laboratory comparisons purposes. ANOVA and normality assumptions were performed using SYSTAT 11 software (SYSTAT Software, Inc. USA). To characterize overall significance of the responses between experiments and sexes, a meta-analysis following Glass (1976) and Rosenberg et al. (2000) procedures was performed. Briefly, meta-analysis uses Hedgeís *d* as a measure for the data to compare and combine the results from the three different experiments. We assess each treatment endpoint mean against its control, along with the pooled within experiment variability (δ^2; Comprehensive Meta-Analysis V2®, USA).

3. Results

No differences between triplicates (bioassays) and duplicates (cages) were observed for any of the analyzed endpoint. Individual

Table 1
Summary statistics for female and male juvenile rainbow trout (*O. mykiss*) by sex and treatment. Values of condition factor (*K*), gonadosomatic index (GSI) and liversomatic index (LSI) are reported as mean ± S.E. (*n*).

Sex	Experiment	Treatment	*K*	GSI	LSI
Female	Bioassay with PME*Eucaliptus* sp. pulpproduction	Control	0.93 ± 0.05 (35)	0.14 ± 0.01 (25)	1.22 ± 0.10 (35)
		10%	0.84 ± 0.08 (38)	0.13 ± 0.01 (30)	1.08 ± 0.10 (38)
		35%	0.89 ± 0.03 (30)	0.12 ± 0.01 (28)	0.90 ± 0.05 (30)
		60%	0.93 ± 0.03 (31)	0.16 ± 0.02 (30)	0.91 ± 0.05 (31)
		85%	0.94 ± 0.04 (33)	0.16 ± 0.01 (27)	1.19 ± 0.15 (33)
	Bioassay with PME*Pinus* sp. pulp production	Control	0.88 ± 0.01 (38)	0.15 ± 0.01 (30)	1.03 ± 0.05 (38)
		10%	0.87 ± 0.03 (36)	0.08 ± 0.01 (33)	0.90 ± 0.09 (36)
		35%	0.91 ± 0.02 (34)	0.15 ± 0.01 (32)	1.00 ± 0.06 (34)
		60%	0.94 ± 0.01* (31)	0.14 ± 0.01 (30)	1.07 ± 0.03 (31)
		85%	0.91 ± 0.02 (32)	0.14 ± 0.01 (30)	0.95 ± 0.04 (32)
	In situ bioassay	Pre day 11	0.98 ± 0.02 (8)	0.08 ± 0.01 (8)	1.36 ± 0.03 (8)
		Imp day 11	0.92 ± 0.01 (12)	0.11 ± 0.01* (11)	1.42 ± 0.12 (12)
		Post day 11	0.95 ± 0.02 (10)	0.14 ± 0.01* (10)	1.34 ± 0.08 (10)
		Pre day 21	0.93 ± 0.01 (8)	0.12 ± 0.01 (7)	1.25 ± 0.07 (8)
		Imp day 21	0.94 ± 0.03 (10)	0.14 ± 0.02 (10)	1.06 ± 0.06 (10)
		Post day 21	0.91 ± 0.02 (10)	0.14 ± 0.01 (10)	1.02 ± 0.07 (10)
		Pre day 31	0.92 ± 0.01 (12)	0.11 ± 0.01 (12)	0.93 ± 0.03 (12)
		Imp day 31	0.93 ± 0.03 (10)	0.12 ± 0.01 (10)	0.97 ± 0.04 (10)
		Post day 31	0.86 ± 0.01* (13)	0.13 ± 0.01 (13)	1.05 ± 0.06 (13)
Male	Bioassay with PME*Eucaliptus* sp. pulpproduction	Control	0.98 ± 0.02 (33)	–	1.26 ± 0.03 (33)
		10%	0.70 ± 0.02 (35)	–	1.14 ± 0.05 (35)
		35%	0.90 ± 0.01 (38)	0.04 ± 0.01 (25)	0.89 ± 0.05 (38)
		60%	0.96 ± 0.02 (34)	0.04 ± 0.003 (30)	0.91 ± 0.03 (34)
		85%	0.85 ± 0.07 (36)	0.04 ± 0.003 (22)	0.95 ± 0.03 (36)
	Bioassay with PME*Pinus* sp. pulp production	Control	0.93 ± 0.02 (36)	0.03 ± 0.003 (26)	1.00 ± 0.05 (36)
		10%	0.93 ± 0.02 (35)	0.06 ± 0.01 (24)	0.82 ± 0.06 (35)
		35%	0.91 ± 0.03 (33)	0.03 ± 0.002 (29)	1.24 ± 0.07* (33)
		60%	0.92 ± 0.01 (36)	0.03 ± 0.004 (30)	1.10 ± 0.05* (36)
		85%	0.94 ± 0.02 (33)	0.03 ± 0.002 (27)	1.04 ± 0.03* (33)
	In situ bioassay	Pre day11	1.02 ± 0.07 (8)	0.04 ± 0.01 (8)	1.48 ± 0.30 (8)
		Imp day 11	0.93 ± 0.03 (4)	0.07 ± 0.01 (4)	1.36 ± 0.10 (4)
		Post day 11	0.95 ± 0.03 (4)	0.11 ± 0.02 (4)	1.47 ± 0.07 (4)
		Pre day 21	1.01 ± 0.03 (8)	0.04 ± 0.02 (8)	1.22 ± 0.02 (8)
		Imp day 21	0.98 ± 0.01 (4)	0.05 ± 0.003 (4)	1.00 ± 0.03 (4)
		Post day 21	0.91 ± 0.03 (6)	0.04 ± 0.01 (6)	0.95 ± 0.12 (6)
		Pre day 31	–	–	–
		Imp day 31	–	–	–
		Post day 31	0.84 ± 0.03 (6)	0.02 ± 0.004 (6)	0.89 ± 0.04 (6)

Mortality rates for the experiments: (a) Bioassay with PME *Eucaliptus* sp. pulp production, 2.6–13.3%; (b) bioassay with PME *Pinus* sp. pulp production, 1.3–13.3%; (c) in situ bioassay 10.7% (Pre), 18.8% (Imp), 12.7% (Post). (–) No samples were available.

* Indicates significant differences between the treatment and its respective reference ($p < 0.05$).

data of all each treatment was used to assess differences between treatments for each individual experiment.

Mortality was observed in all the experiments. *Pinus* and *Eucaliptus* PPME exposure showed similar total mortality rates (8.2 and 8.5%, respectively), but *Pinus* exposure showed an increasing mortality with the PPME concentration, ranging from 1.3% (Control) to 13.3% mortality rate at 85% effluent exposure. *Eucaliptus* pulp effluent showed mortality rates from 9.3% in the control to 13.3% in the higher concentration treatment. Mortality rates in the other treatments were scattered and had values ranging between those observed in the control and 85% treatments (Table 1).

3.1. Condition factor, liversomatic index, gonadsomatic index

Juvenile female fish exposed to *Eucalyptus* sp. pulp mill effluent had no significant differences between treatments in the ANOVA analysis for *K* (ANOVA $p = 0.492$), LSI (ANOVA $p = 0.103$), or GSI (ANOVA $p = 0.477$). At the *Pinus* pulp mill effluent exposure, a significant increase in *K* in female fish (Table 1) at higher concentration was detected (60%, ANOVA $p = 0.022$) compared with their control. The caging experiment showed a significant decrease in *K* of females at the end of the exposure time in the POST site compared with the PRE site (31 d exposure, $p = 0.006$). There was a small increase in the GSI of females at higher effluent concentrations treatments in bioassays and downstream the effluent outfall sites of caging exposure, but ANOVA analysis only showed significant increase in GSI for female fish at IMP and POST sites after 11 d of caging experiment ($p < 0.001$). No changes were observed at LSI between the different treatments and their controls on females (ANOVA $p = 0.756$, 11 d, $p = 0.063$, 21 d; $p = 0.180$, 31 d). Overall, male sample sizes were smaller in the three experiments, especially in the *Eucalyptus* bioassay. No statistical significant changes were observed for *K* (ANOVA $p > 0.05$) and GSI (ANOVA $p > 0.05$) for any of the treatments. However, a significant increase was observed in LSI for male fish exposed to the higher concentrations of *Pinus* pulp production effluent (ANOVA, $p = 0.013$) (Table 1).

3.2. Liver EROD activity

Liver EROD activity showed no statistical differences between sexes ($p > 0.05$), therefore females and males were pooled by treatment or site for comparing purposes. Increased responses of the average liver EROD activity were shown at the higher concentrations of pulp mill effluent (*Pinus* sp. and *Eucaliptus* sp.; Fig. 1A). Fish exposed to *Eucalyptus* and *Pinus* PPME showed higher EROD activities starting at 35% of effluent (1.63 and 1.79 fold change, respectively; Fig. 1A). ANOVA analysis showed significant differences in exposed fish to 60% and 85% effluents to control on *Eucalyptus* ($p < 0.001$) and *Pinus* pulp production effluent ($p < 0.001$). Average EROD activity for fish at 60% of effluent concentration was a 2.13 (*Eucalyptus*) and 2.21 (*Pinus*) fold change higher than the respective control. The highest EROD activity (2.92 fold change) was at 85% effluent concentration of *Eucalyptus* pulp production. Fish exposed to *Pinus* effluent had 2.12 fold change higher activity that their control (Fig. 1A).

Despite the high variability and the lack of statistical differences between treatments on each exposure time at the in situ experiment, fish caged in the IMP site showed a higher average EROD activity (>1.5 fold change, Fig. 1B). Along with this, EROD activity variability increase between individual fish exposed to higher effluent concentrations for all the experiments. During the caging experiment, EROD activity average was higher in IMP site, but highly variable. Average EROD activity in IMP site was >1.5 fold change higher after 21 and 31 d of exposure, but due to high within-group variability, ANOVA analysis showed no significant differences between sites in different times of exposure (11 d, $p = 0.164$; 21 d, $p = 0.098$; 31 d, $p = 0.220$) (Fig. 1B).

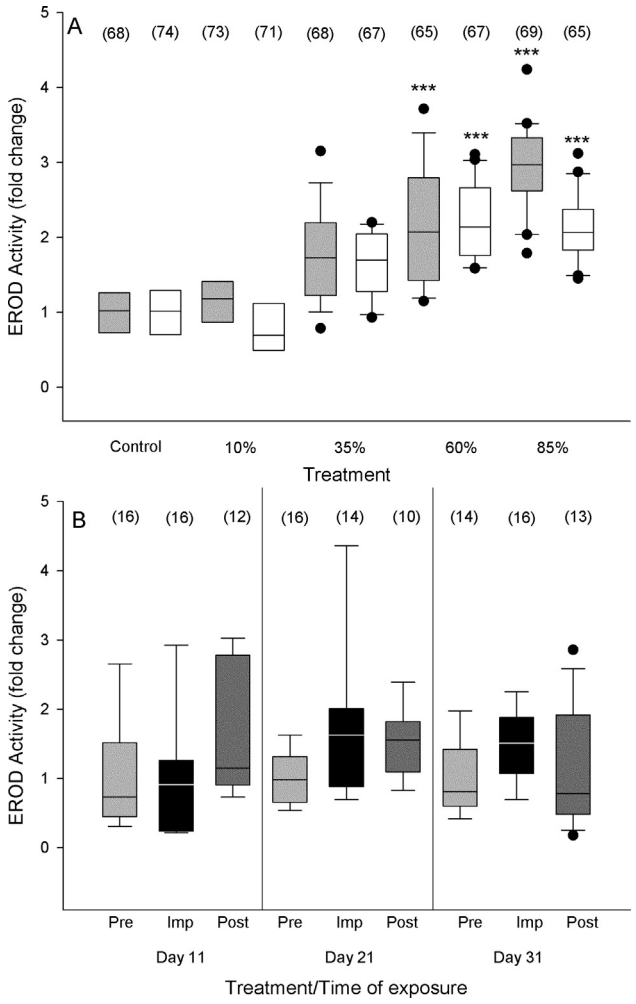

Fig. 1. Boxplot of the liver ethoxyresorufin-*O*-deethylase (EROD) activity (mean ± S.E.) fold change for juvenile rainbow trout by treatment. (A) Juvenile rainbow trout exposed to *Eucalyptus* pulp production effluent (grey bars) and to *Pinus* pulp production effluent (white bars); (B) Caged juvenile rainbow trout exposed to pulp mill effluent for 11, 21 and 31 d. PRE = preimpacted site, IMP = Impacted site, POST = postimpacted site. Values marked with asterisks (***) presented significant differences in the ANOVA analysis ($p < 0.001$) between effluent concentration and the respective control. The horizontal line in the box plots is the median, the lower boundaries of the box represent the 25th and 75th percentiles, respectively, minimum and maximum data points are represented by the whiskers. The black dots (•) are outliers.

3.3. Plasma vitellogenin

Plasma VTG levels were detected in both juvenile female and male rainbow trout. Increasing levels of VTG in plasma were observed at *Eucalyptus* PPME concentration from 10% (>50 fold change) to 85% (>200 fold change) in female fish (Fig. 2A, ANOVA $p < 0.001$). The increasing levels were less dramatic in female fish exposed to *Pinus* effluent compared to the fish exposed to *Eucalyptus* effluent. No change were detected at VTG levels in female fish exposed to *Pinus* effluent at the lower percent of exposure (10% and 35% effluent); however an increased ≈1.5 fold changes was detected at the 65 and 85% effluent concentration. Significant differences were observed in female fish exposed to 60 and 85% effluent concentration (Fig. 2B, ANOVA $p < 0.002$) compared with the control fish.

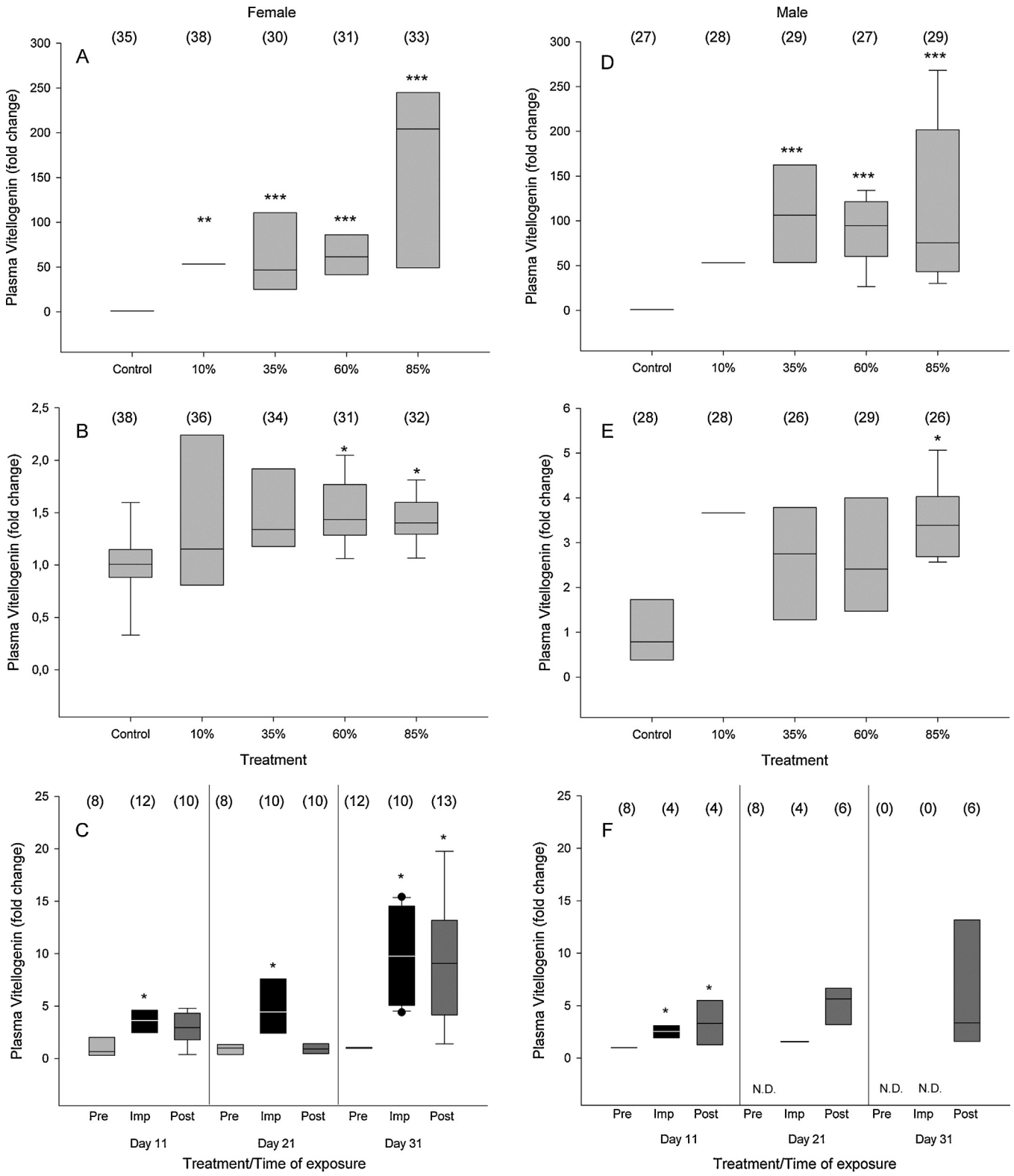

Fig. 2. Boxplot of plasma vitellogenin levels (VTG; mean ± S.E.) for female (A–C) and male (D–F) juvenile rainbow trout, exposed to *Eucalyptus* pulp production effluent (A, D), *Pinus* pulp production effluent (B, E) and caged fish for 11, 21 and 31 d (C, F). PRE = preimpacted site, IMP = impacted site, POST = postimpacted site. Values marked with asterisks present significant differences in the ANOVA analysis ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$) between effluent concentrations or sites. Data in the boxplots are as in Fig. 1.

During the in situ experiment, caged female juvenile rainbow trout showed a significant increase of VTG levels in plasma at IMP site, exposure day 11 (≈4 fold change, ANOVA $p = 0.006$), day 21 (≈5 fold change, ANOVA $p = 0.011$) and day 31 (>10 fold change, ANOVA $p = 0.039$). At POST site, a significant increase in VTG levels was observed after 31 d exposure (≈9 fold change, ANOVA $p = 0.039$), compared to PRE site for each time of exposure (Fig. 2C).

Male fish exposed to the same treatments showed similar changes in plasma VTG as female trout. The *Eucalyptus* PPME exposure indicated that male fish had significant increased levels of VTG

at 35% (>100 fold change), 60% (>90 fold change) and 85% (>60 fold change) effluent concentration (Fig. 2D, ANOVA $p < 0.001$). Male fish treated with *Pinus* PPME showed a similar pattern to the female trout from the same bioassay (i.e., increasing plasma VTG levels and reduced variability between individuals with higher effluent concentrations). Male fish showed higher changes in plasma VTG compared to female, from ≈2 to >3 fold change compared with the control. A significant increase was observed in male fish exposed to 85% *Pinus* effluent (Fig. 2E, ANOVA $p < 0.006$).

Due to the lack of enough male showing detectable levels of VTG at 21 d and 31 d of exposure, all the results were expressed as fold-of-change from PRE site at 11 d. Male fish showed higher levels of VTG downstream pulp mill discharge during day 11 of exposure in IMP (>3–4 fold change) and POST sites (>5 fold change; ANOVA $p = 0.041$; Fig. 2F). No detectable levels of VTG were found in fish from PRE site during 21 d and 31 d of exposure. Finally, only one fish showed detectable VTG levels in IMP site during day 21 of exposure (7.5 fold change; Fig. 2F).

3.4. Gonad histology

Histological analysis of gonads in PPME-exposed fish showed an increase in advanced maturing stages of oocyte development (with an increment in Stage V oocytes, from 9% in controls to >25% in exposed fish) in female fish exposed to higher *Eucalyptus* PPME exposure (Chi square, $p < 0.001$, Fig. 3A). Meanwhile, for *Pinus* PPME-treated fish, higher frequency of Stage IV oocytes was observed in fish treated to 35% (v/v) effluent and an increased frequency of oocytes in stage III in fish exposed to *Pinus* PPME concentrations of 60 and 85% (v/v), but no statistical differences between control and exposed fish (Chi square, $p > 0.05$, Fig. 3B). During the in situ experiment, an increase of advanced maturing stages of oocytes in female fish was observed at the IMP site, compared to PRE site. Higher frequencies of maturing oocyte stages (Stage IV) were observed at day 11 in the IMP and POST sites (Chi square, $p < 0.001$, Fig. 3C), while proportions of advanced stages of maturation were statistically significant after 21 d (Stage V) and 31 d (Stage IV) of exposure in IMP site (Chi square, $p < 0.001$) and after 21 d of exposure on POST site (Chi square, $p < 0.001$) (Fig. 3C).

Gonads from some male fish showed intersex characteristics. Oocytes at different stages of development were observed in one male fish exposed to *Pinus* effluent at 85% (v/v) effluent concentration; two males exposed to *Eucalyptus* PPME at 60% and 85% (v/v), and one male at the in IMP site at day 11 of exposure during the in situ experiment (Fig. 3A–C) No Intersex characteristic were observed in fish from the control groups.

3.5. Overall responses to different experiments

Meta-analysis of all data obtained in the present study of PPME-exposed, indicated that juvenile rainbow trout showed significant direct or indirect impacts on metabolic or reproductive endpoints (Fig. 4). The Hedgeís *d* value indicated no critical effect of *K* in males or females for the whole study. Juvenile female fish exposed to PPME showed a significant increase in Hedgeís *d* value for EROD activity (1.05), VTG plasma levels (0.9), and in GSI (0.45). Juvenile male PPME-exposed fish showed an increase in Hedgeís *d* values for LSI (0.85), EROD activity (1.45), and plasma VTG levels (2.2). According to the meta-analysis, fish exposed to PPME would show an increase in plasma VTG levels and EROD activity, with a potential impact on GSI or LSI, but no effects on *K*.

4. Discussion

The current study shows that, despite substantial improvements in pulp production and PPME treatment, there are still detectable estrogenic effects in exposed freshwater fish. These effects, including increased age at sexual maturity, decreased gonad size, reduced fertility with female age and reduced male secondary sex characteristics, have been detected in fish from the northern hemisphere (McMaster et al., 1991; Munkittrick et al., 1992; Hewitt et al., 2008). Other studies also show estrogenic effects in fish from PPME with secondary/tertiary treatments from different raw materials (Hewitt et al., 2008; Orrego et al., 2009, 2010; Chamorro et al., 2010) and at different levels of biological organization (Chamorro et al., 2010; Chiang et al., 2011). In Chile, estrogenic effects in fish due to PPME were first reported in juvenile female rainbow trout exposed to contaminated sediments (Orrego et al., 2005) and caged fish near primary-treated PPME discharge sites(Orrego et al., 2006). These studies showed high plasma VTG concentrations and premature maturation of juvenile female gonads (Orrego et al., 2005, 2006), which differ greatly from the effects that have been reported in North American systems.

Studies have shown that compounds (such as nonsteroidal cyclic diterpenes-triterpenes, fatty acids, phytosterols, phenols, isoflavonoids; Hewitt et al., 2008; Chamorro et al., 2010; Scott et al., 2011) from pulp production with varying levels of effluent treatment (primary, secondary and tertiary; Hewitt et al., 2008; Orrego et al., 2009, 2010; Milestone et al., 2012) have different degrees of impact on fish reproduction, despite treatment improvements. PPME treatment may be responsible for the delayed induction in the responses observed in our study (EROD, VTG and oocyte development). Caged male and female fish showed increasing plasma VTG levels downstream of the effluent discharge, and fish exposed directly to the effluents showed increasing levels of VTG in proportion of the higher effluent concentrations. Furthermore, Orrego et al. (2009, 2010),) showed a similar response pattern, with higher EROD activity and plasma VTG concentrations in fish exposed to higher effluent concentrations and downstream of the PPME discharge in caged fish. Orrego et al. (2009, 2010),) also showed that different effluent treatments had differential ED effects on fish; effects from tertiary treatment were lower or delayed compared to untreated and secondary treatment. These differences in the magnitude of ED effects relative to treatment-level might be related to experimental design, test media, and the endpoints assessed. In the present study, EROD activity in caged fish showed no significant difference upstream or downstream from the PPME discharge, but it showed an average increase and higher variability in downstream sites; this could be related to variability in effluent quality and dilution over time. Similar response patterns were observed for whole body endpoints (e.g., condition factor) and few endpoints showed differences from controls after 21 or 31 d of exposure, indicating that a longer exposure time is needed to detect reductions in EROD variability and to induce changes in the whole body endpoints in the fish from the receiving water body. Alternatively, fish may be able to recover after exposure to low concentrations of PPME. The raw material used in the pulping process may also play a role in the differential reproductive responses in fish, as stated in several previous studies (Hewitt et al., 2008; Chamorro et al., 2010; Milestone et al., 2012). Differences in plasma VTG were higher in fish exposed to *Eucalyptus* (>50 to >200 fold change from reference levels) versus *Pinus* PPME (>1 to >4 fold change from reference levels). Caged fish showed an increasing VTG plasma concentration over time downstream of the effluent discharge, and this change was higher than the one observed in fish exposed to the *Pinus* PPME bioassay. As stated by Milestone et al. (2012), few studies found estrogenic activity in wood or effluent from South American pulp mills. Nevertheless, studies utilizing yeast estrogen screen (YES) have shown that *Eucalyptus* PPME has higher estrogenic activity than *Pinus* PPME (Chamorro et al., 2010; Milestone et al., 2012).

On the other hand, intersex characteristics were observed in male fish exposed to PPME and not in control groups. Despite the

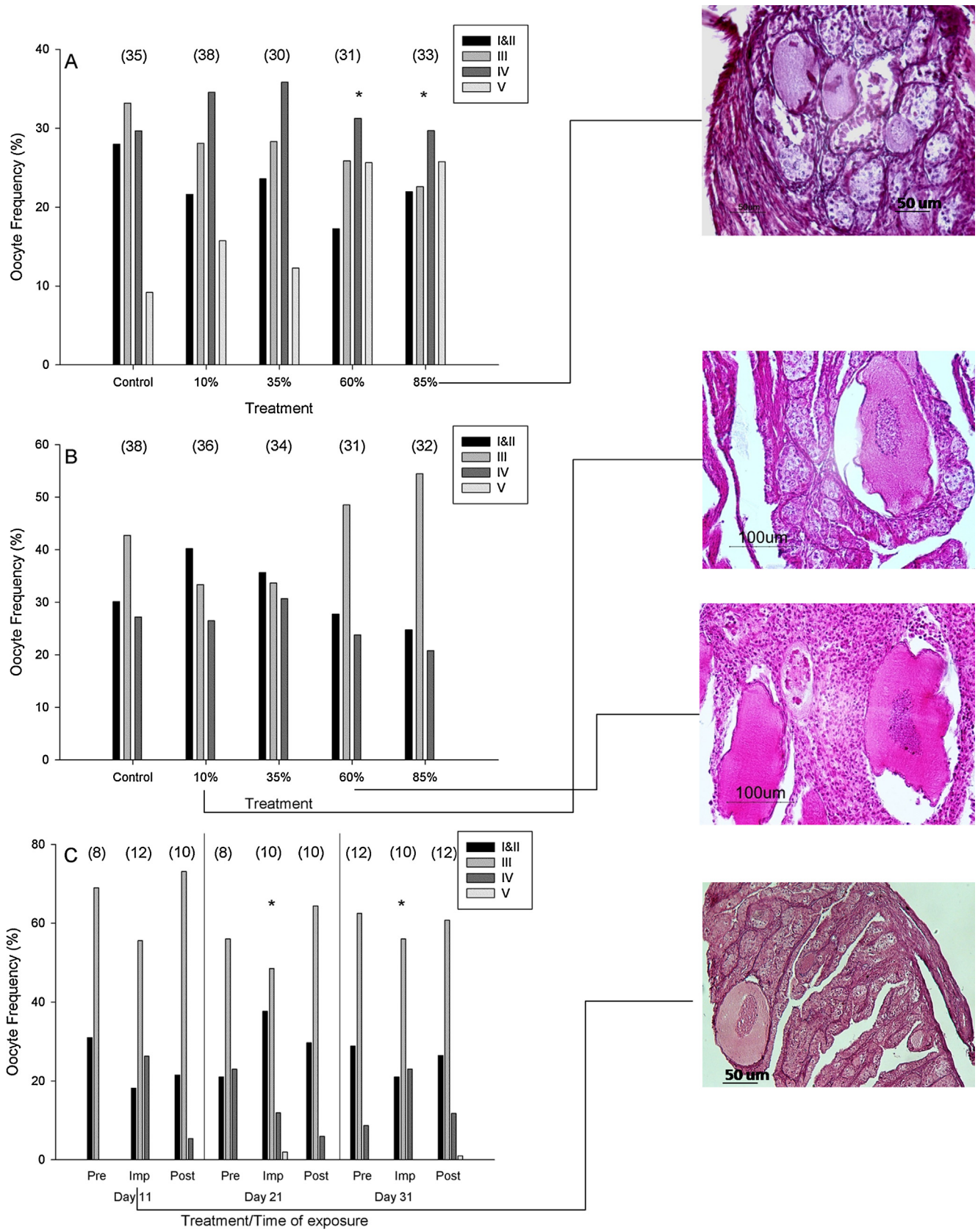

Fig. 3. Percentage of the different oocyte maturation stages for female juvenile rainbow trout. Fish were exposed to *Eucalyptus* pulp production effluent (A), *Pinus* pulp production effluent (B) or caged for 11, 21 and 31 d (C). PRE = Preimpacted site, IMP = impacted site, POST = postimpacted site. At right, microphotographs show intersex gonad tissue found in male juvenile rainbow trout for each treatment. Values marked with asterisks (*) presented significant differences in the Chi-square analysis ($p < 0.05$).

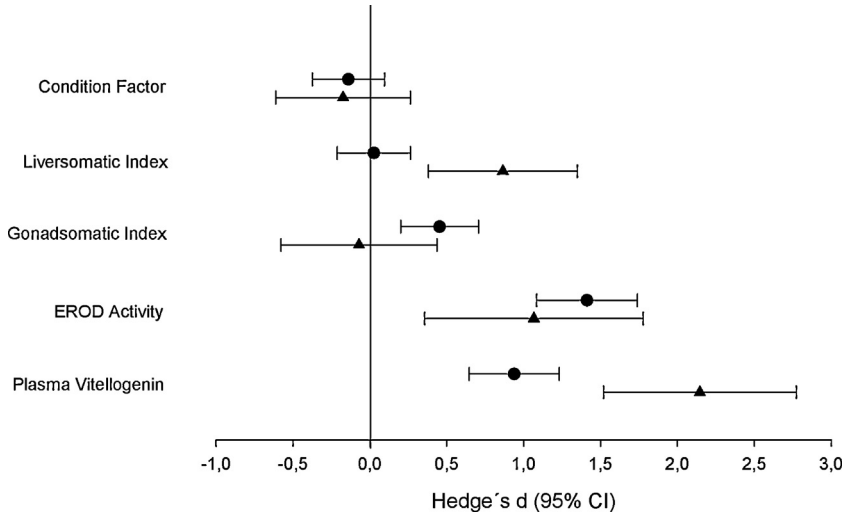

Fig. 4. Meta-analysis/synthesis of data (i.e., condition factor, liversomatic index. gonadosomatic index, EROD activity and plasma vitellogenin), from the three independent experiments in the current study. Hedgeís *d* values ±95% confidence interval for female (●) and male fish (▲) responses to direct and indirect exposure to pulp mill effluent.

higher VTG concentration in exposed males, there is not enough data to test if there is a relationship between VTG and intersex, though Bahamonde et al. (2013) suggest that no such relationship. At the same time, females showed increased VTG levels and proportions of late maturing oocytes in fish exposed to PPME. Other authors have suggested that juvenile trout and salmon treated with estrogenic steroids and aromatizable androgens could lead to an accumulation of Gonadotropin hormone (GtH) in the pituitary, resulting in a release of Luteinizing hormone (LH) which is responsible for the changes in gonad maturation and VTG production (Crim and Peter, 1978; Crim and Evans, 1979; Peter, 1983; Crim et al., 1986). At the same time, a change in Gonadotropin-releasing hormone (GnRH) in the hypothalamus could lead to changes in gonad activity (Crim and Evans, 1979). Thus, GnRH or active analogues in PPME could stimulate the release of GtH in trout, possibly explaining the changes in VTG levels and oocyte development that we observed.

As several authors have suggested and stated, the different ED effects reported to be induced by PPME could be related to the different raw materials, processes, or effluent treatments (Hewitt et al., 2008; Orrego et al., 2009, 2010; Scott et al., 2011; Milestone et al., 2012). Treatment could play a fundamental role, diminishing the concentration of phytosterols with aromatizable androgens. According to Orrego et al. (2009, 2010),), additional levels of treatment could reduce the estrogenic effects of PPME on Chilean fish. These authors stated that androgens present in PPMEs could be aromatized via CYP450 activity (which is responsible of converting C19 androgens into C18 estrogens in the steroid pathway) explaining the increase in EROD activity and thus the increase in VTG levels and high GSI in fish exposed to different degrees of PPME treatment. Analyzing the female fish responses, VTG levels, EROD activity and GSI responses very closely related with evidence results from the work of Orrego et al. (2009). That data is supported by the meta-analysis (assessed using Hedgeís *d*) as well, showing that main responses for female juvenile rainbow trout to the exposure to PPMEs are the three endpoints mentioned above. Male fish showed similar tendencies toward increased EROD and VTG levels as the meta-analysis showed.

In conclusion, this study not only confirms the potential of estrogenic effects due to PPME in Chile on juvenile female rainbow trout (Orrego et al., 2005, 2006) and in triploid rainbow trout (Orrego et al., 2009, 2010), but also it showed PPME-induced estrogenic effects on juvenile male fish. Furthermore, this is the first report showing the potential of *Eucalyptus* and *Pinus* pulp production to induce intersex characteristics in juvenile male rainbow trout in the PPME receiving water body. Finally, this study shows that high plasma VTG levels and EROD activity are the main responses of fish exposed to PPME and that there is a potential impact on LSI and GSI. These effects showed a sex-specific differential effect of both *Pinus* and *Eucalyptus* PPME on juvenile trout growth and development, showing that *Eucaliptus* PPME has a stronger estrogenic effect. Our results indicate that current PPME treatment in Chile is not able to eliminate all reproductive and growth effects in exposed fish.

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