Gayana 83(1): 10-20, 2019.
Original Article
ISSN 0717-652X

Mucus phosphoproteins as an indirect measure of endocrine disruption in native small-bodied freshwater fish, exposed to wastewater treatment plant and pulp and paper mill effluents

Fosfoproteínas en mucus como una medida indirecta de disrupción endocrina en peces nativos dulceacuícolas de pequeño tamaño, expuestos a efluentes de plantas de tratamientos de aguas domésticas y de celulosa

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ABSTRACT

Environmental monitoring programs commonly use fish to study the health of aquatic ecosystems. Nevertheless, lethal sampling techniques are often employed, resulting in ethical considerations. This issue is magnified in Chilean rivers, which contain various endemic fish with conservational concern, according to the International Union for Conservation of Nature Red List of Threatened Species™. Therefore, the aim of this study was to determine if mucosal vitellogenin levels in a native Chilean fish could be used to accurately assess the endocrine disruption potentials of wastewater treatment plant and pulp and paper mill effluents (WWTPEs and PPMEs, respectively). For this, Carmelita de Concepción (*Percilia irwini*) specimens were exposed WWTPEs and PPEs for 12 days, and mucosal vitellogenin-like phosphoprotein concentrations were determined with a colorimetric assay. Increased VTG-like phosphoproteins and hepatic ethoxyresorufin-o-deethylase induction levels (widely used as biomarker for exposure) were detected in effluent-exposed individuals. This study supports the endocrine disruption potentials of WWTPEs and PPEs in *P. irwini*. Notably, this is the first study to use non-lethal biomarkers to determine the effects of industrial effluents in a native Chilean freshwater species, thus presenting an alternative vitellogenin-like protein detection method. Nevertheless, additional population and toxicity studies of fish native to Chilean rivers are needed. Further investigation is also required on xeno-oestrogen compounds and on methods for mitigating potential effects on biodiversity.

Keywords: endocrine disruption, fish, mucus phosphoproteins, vitellogenin.

RESUMEN

Los programas de monitoreo ambiental comúnmente usan peces para estudiar la salud de los ecosistemas acuáticos. Sin embargo, a menudo se emplean técnicas de muestreo letal, lo que resulta en consideraciones éticas. Este es mayor en ríos chilenos, donde habitan peces endémicos en alguna categoría de conservación preocupante, según la Unión Internacional para la Conservación de la Naturaleza™. Así, el objetivo de este estudio fue determinar si los niveles de vitelogenina en mucus externo de peces nativos podrían usarse para evaluar el potencial de disrupción endocrina de efluentes de plantas de tratamiento de aguas residuales y de las plantas de celulosa y papel (WWTPE y PPME, respectivamente). Para esto, ejemplares de Carmelita de Concepción (*Percilia irwini*) se expusieron a WWTPE y PPE durante 12 días, y se determinaron las concentraciones de fosfoproteínas de tipo vitelogenina (VTG) el mucus mediante un ensayo colorimétrico. Se evidenció un aumento de fosfoproteínas tipo VTG e inducción de la etoxiresorufina-o-deetilasa hepática (biomarcador de exposición) en individuos expuestos a efluentes. Este estudio respalda el potencial de disrupción endocrina de los WWTPE y los PPE. Cabe destacar que este es el primer estudio que utiliza biomarcadores no letales para determinar los efectos de los efluentes industriales en una especie nativa de agua dulce chilena, por lo que presenta un método alternativo de detección de proteínas similares a la vitelogenina. Sin embargo, se necesitan estudios poblacionales y de toxicidad adicionales. Se requiere investigación adicional sobre los compuestos de xenoestrógenos y mitigación de los posibles efectos sobre la biodiversidad.

Palabras clave: disrupcion endocrina, fosfoproteinas, mucus, peces, vitelogenina.

INTRODUCTION

Fish are commonly utilized in environmental monitoring programs to study the effects of effluents on water systems (Roach & Walker 2017). There is a general consensus that fish status is a reliable indicator of environmental health, but most data endpoints are obtained through invasive and, many times, lethal techniques precisely in places where fish are scarce. Since the 90's, the ethical considerations are a primary motivation for developing techniques compatible with species protection (Fossi & Leonzio 1994). However, lethal techniques are the one used for monitoring the health of the ecosystem.

Non-lethally collected samples such as blood, urine, and biopsy tissues can be used to monitor specimens without sacrificing the animal, which is particularly relevant for endangered species or in studies with limited sample sizes (Fossi et al. 2003). Indeed, the last decade has seen increased research in developing non-lethal sampling techniques (Cerveny et al. 2016; Alves et al. 2016; Madeira et al. 2017; Veldhoen et al. 2014).

As a growing threat in aquatic environments, endocrine disrupting chemicals (EDCs) disturb the endocrine system, which subsequently upsets the reproductive and developmental functions of organisms (Sumpter & Jobling 1995; Jobling & Tyler 2003). Among EDCs, xenoestrogens have received considerable attention due to natural oestrogen mimicry, good marker availability, and a demonstrated disruption of reproductive cycles that threatens population sustainability (Kidd et al. 2007). Point source pollution, such as wastewater treatment plant effluents (WWTPEs) and pulp and paper mill effluents (PPMEs), is the primary source of oestrogens and oestrogen-like compounds in aquatic ecosystems. While most well-operated secondary treatment systems effectively mitigate the impacts of oxygen demand, suspended solids, and eutrophication on aquatic ecosystems, specific classes of chemical compounds, such as xenoestrogens, are not always targeted in treatment systems (Metcalfe et al. 2001; Xu et al. 2014; Yu et al. 2013). In particular, ECDs from WWTPEs affect fish reproduction (Bahamonde et al. 2015; Garcia-Reyero et al. 2011; Jobling et al. 1998; Jobling et al. 2002). Furthermore, WWTPE exposure is associated with altered steroid hormone levels (Rodgers-Gray et al. 2001; Hoger et al. 2006; Adeogun et al. 2016), vitellogenin (VTG) induction, and histological alterations in the gonads (e.g. intersex) (Rodgers-Gray et al. 2001; Hoger et al. 2006; Bahamonde et al. 2014, 2015). Since the early 1980s, studies have also assessed fish exposure to PPMEs, with detrimental effects detected at different reproductive levels. In North America, described effects include an increased age of sexual maturity, decreased gonad size, reduced female fertility with age, and reduced male secondary sex characteristics (Munkittrick et al. 1991; Milestone et al. 2012). Conversely, in South America the effects of industrial effluents include enlarged gonad maturation and oestrogenicity in juvenile rainbow trout *Oncorhynchus mykiss* (Walbaum, 1792) (Orrego et al. 2005, 2006, 2009) and oestrogenicity in wild fish populations (Chiang et al. 2011). Specifically, Orrego et al. (2005, 2006) reported increased gonad size, an induction of gonadal maturation, and increased plasma VTG in juvenile *O. mykiss* exposed to sediment taken from discharge areas for PPMEs, and Chiang et al. (2015) reported intersex characteristics in rainbow trout caged near PPME discharge sites. Furthermore, oestrogenic and anti-androgenic effects have been reported in two Chilean fish species, the common Carmelita *Percilia gillissi* Girard 1855 and *Trichomycterus areolatus* Valenciennes, 1846 (Chiang et al. 2011). Chamorro et al. (2010) demonstrated that, despite discharge pre-treatment, pine and eucalyptus wood pulp effluents are highly oestrogenic. As with WWTPs, modern PPMs have improved waste treatment processes, and, in North American waters, this has led to decreased alterations in effluent-exposed individuals (Munkittrick et al. 1997). Nevertheless, some negative impacts remain, particularly in association with endocrine disruption (ED) (Scott et al. 2011; Simmons et al. 2012).

Vitellogenin is typically synthesized in female livers through endogenous 17-β-oestradiol (E2) regulation, transported via the blood stream to the ovaries, and absorbed by maturing oocyte s (Arukwe & Goksøyr 2003). Concentrations of vitellogenin (VTG), which are typically very low or absent in male and juvenile fish, can increase with xenoestrogen exposure, thereby providing a sensitive indicator of oestrogenic EDC contamination in aquatic environments (Arukwe & Goksøyr 2003; Maltais & Roy 2009; Genovese et al. 2012). Vitellogenin levels have been studied in fish blood and mucus with different analytical techniques (Moncaut et al. 2003; Arukwe & Røe 2008; Rey-Vázquez et al. 2009; Pollino et al. 2009). Studies in *Cichlasoma dimerus* (Heckel, 1840) detected VTG in skin mucus and blood plasma samples utilizing a Western blot with a monoclonal VTG antibody after an 11-day hormonal treatment with E2 (Moncaut et al. 2003). Similarly increased VTG levels in *C. dimerus* skin mucus and blood plasma samples occurred after a 60-day exposure to 4-tert-octilphenol (Rey Vázquez et al. 2009). In turn, Arukwe & Røe (2008), through immunohistochemistry analyses, found that VTG levels in Atlantic salmon *Salmo salar* Linnaeus, 1758 skin mucus and blood plasma were similar after nonylphenol exposure, thereby validating skin mucus as a sensitive, non-invasive biomarker. Finally, Western blotting and lipovitellin-based enzyme-linked immunosorbent assays (ELISA) were used to detect VTG induction in the surface mucus and plasma of E2-induced goldfish *Carassius auratus* (Linnaeus, 1758) (Wang et al. 2015).

Many endemic South American fish do not have

available monoclonal or polyclonal antibodies. Therefore, simple, rapid, and affordable methods are needed to assess the effects of EDCs in wild, and often threatened, fish species exposed to xenoestrogens (Connon et al. 2012). Analytical techniques using colorimetric phosphoprotein detection can identify VTG in blood plasma. For example, Kramer et al. (1998) exposed fathead minnows *Pimephales promelas* Rafinesque 1820 to environmentally relevant E2 concentrations and showed a positive correlation between this colorimetric assay and ELISA.

WWTPEs and PPMEs have showed endocrine disrupting activity (oestrogenic) in Chilean aquatic ecosystems, but due to conservation purposes and technological issues, there is no monoclonal or polyclonal antibodies for VTG (main biomarker of oestrogenic exposure in fish) to assess the impact of those effluents. Indirect analytical procedures to assess VTG (as phosphoproteins) are a novel approach that will increase the available data on EDC´s in aquatic ecosystems. The aim of the present study was to develop a non-lethal sampling methodology to study the effects of EDCs in the Chilean fish Carmelita de Concepción *Percilia irwini* Eigenmann, 1928. In particular, mucus samples were assessed using the colorimetric phosphoprotein assay to determine the possible oestrogenic effects of WWTPEs and PPMEs on native, wild fish from the Biobio River Basin of Chile.

MATERIALS AND METHODS

Fish exposure to 17β-ethinyl oestradiol (EE2)

To validate indirect vitellogenin determinations, juvenile *O. mykiss* (44.2 ± 9.3 g) were exposed to EE2 concentrations known to induce vitellogenesis. Fish were obtained from a fish farm (Salmones Pangue, 21 km Bulnes Road, Biobio Region, Chile) and acclimated for one month in glass tanks (70 L) with dechlorinated water at the Bioassay Laboratory, Universidad de Concepción, Chile. Tank water was maintained at 16 °C with a 12:12 light:dark photoperiod and was replaced every two days. Fish were fed to satiety every three days. After acclimation, 15 fish per tank (in triplicate) were exposed for 10 days to a constant EE2 concentration (10 ng L^{-1} Kidd et al. 2007). Fish kept in dechlorinated water were used as the control. Just prior to initial exposure and on days 3, 5, 7 and 10, five fish were randomly selected from the three replicates and were anesthetized; blood samples were taken from the caudal vein. Then, the same sampled fish were sacrificed by cervical dislocation (according to the protocols of Canada Department of Fisheries and Oceans, 2004 and approved by bioethical committee) to obtain liver and gonad tissues. Blood plasma, for VTG and phosphoprotein analyses, and liver tissue, for assessments of ethoxyresorufin-O-deethylase (EROD) activity, were frozen in liquid nitrogen and maintained at -80 °C until analyses.

P. irwini exposure to WWTP and PPM effluents

In June 2011, 100 wild adult *P. irwini* (48.0 ± 8.2 mm, 1.13 ± 0.87 g) were collected upstream of the Biobio River (37.684428° S, 71.953966° W). Fish were captured by electrofishing in shallow riffles using a backpack unit (Halltech Aquatic Research Inc., Guelph, ON, Canada). Collected fish were transported to the Bioassay Laboratory at the Universidad de Concepción, Chile and acclimated for one month in glass tanks (70 L) containing dechlorinated water maintained at 16 °C and replaced every two days. A 12:12 light:dark photoperiod was implemented, and fish were fed worms (to satiety) every three days. To prevent infections, fish were treated during the first week of acclimatization with increasing salt doses (0.3 to 1 g L^{-1}). On week two of acclimatization, some fish showed signs of protozoal infection (*Ichthyophthirius multifiliis)* and were retreated with another round of the same increasing salt doses for two weeks.

To expose the fish to EDCs, water was collected from the plumes of PPM and WWTP discharge zones, 100-150 m downstream the effluent discharge (37.580620°S, 72.535325W and 37.6691118° S, 72.051039° W respectively). To evaluate the status of the river, water was also collected upstream of Santa Barbara, a town proximal to the largest point source of pollution discharge near Biobio River Basin headwaters (37.582844° S, 72.545173° W). Fish (n = 10 per tank, in duplicate) were exposed for 12 days to WWTP, PPM, or river water. Furthermore, negative (i.e. dechlorinated water) and positive (i.e. 10 ng L^{-1} EE2) controls were used. Fish mucus was sampled from all the available fish on days 0, 4, 7, and 12. To minimize manipulation and possible stress, fish mucus was obtained by gently scraping the sides of the individuals with a previously sterilized steel spatula. The collected mucus (≈0.2-0.3 mL) was stored in a 0.5 mL Eppendorf tube and kept -20 °C until analysed. Just prior to initial exposure and on days 7 and 12 of the experimental period, *P. irwini* (10) were sacrificed to collect gonad and liver samples. Due to small fish size, a sufficient quantity of blood could not be obtained. Physiological indices were calculated as previously described.

Biochemical analyses

Plasma vitellogenin

An indirect ELISA was conducted using 96-well polystyrene microplates to assess rainbow trout VTG plasma concentrations, following the procedure described by Tyler et al. (2002) and with modifications from Orrego et al. (2010). The polyclonal rabbit anti-vitellogenin trout PO-2 specific antibody (Biosense Laboratories, Bergen, Norway) was used as the primary antibody. In turn, goat anti-rabbit IgG conjugated to peroxidase (Sigma-Aldrich, St. Louis, MO, USA) was used as a secondary antibody, with *o*-phenylenediamine dihydrochloride applied as the substrate solution. The reaction was measured at 490 nm

in a DNM-9602G microplate reader (Baush + Lomb, Rochester, NY, USA). Final VTG concentrations were calculated according to a standard calibration curve (1-1000 ng mL^{-1}) using purified rainbow trout vitellogenin (Biosense Laboratories) (Tyler et al. 2002; Orrego et al. 2005, 2010, 2006).

Phosphoprotein analysis in blood and mucus

Subsamples of mucus and plasma (10 µL) were stored overnight at 4 °C with trichloroacetic acid (10%, 2 mL). Then, samples were centrifuged at 4,500 rpm for 10 min; trichloroacetic acid (5%, 2 mL) was added to the precipitate and incubated at 90 °C for 30 min. Samples were successively washed with ethanol (0.5 mL), a mix of chloroform:ether:ethanol (1:2:2), acetone, and ether (0.5 mL). The precipitate was dissolved, incubated at 80 °C in NaOH (2 N, 0.5 mL), neutralized with an equivalent amount of concentrated HCl, and incubated at 100 °C for 30 min.

The protocol described by Thermo Fisher Scientific Inc. (2004) was used to precipitate proteins from mucus. Briefly, acetone (240 µL, -20 °C) was added to the samples (10 µL), which were then vortexed, incubated for 60 min at -20 °C, and centrifuged for 10 min at 14000 rpm. The supernatant was disposed of, and the remaining acetone was allowed to evaporate from the uncapped tube at room temperature for 30 min. To dissolve the protein pellet, a mix of 400 µL of tris-HCl (0.5 M) and Triton X-100 (2%) was added to the samples.

Phosphoprotein concentrations in plasma and mucus samples were determined by a modified colorimetric assay using the commercial Phosphoprotein Phosphate Estimation Assay kit (No.23270; Thermo Fisher Scientific Inc., Waltham, MA, USA). Each microplate used phosphoprotein standards (i.e. phosvitin) as the positive control and Tris-buffered saline as the blank. First, NaOH (2 N, 50 µl) was added to each well, and the microplate was mixed for 30 s and incubated at 65 °C for 30 min. Then, HCl (4.7 N, 50 µL) was added to each well, and the microplate was mixed for 30 s. A phosphate reagent (50 µL; ammonium molybdate solution with three volumes of malachite green) was added to each well and mixed for 30 s. The microplate was incubated at room temperature for 30 min, and absorbance was read at 650 nm. To determine the amount of phosphorylation, test sample absorbance values were calculated using a standard curve of phosvitin (2.5-100 µg mL^{-1}).

Protein analysis

Total proteins were analysed using 10 µL of plasma and mucus previously stored at -20 °C. Protein analysis was performed in a DNM-9602G microplate reader (Baush + Lomb) using a Biuret Microplate Assay. Final protein concentrations were calculated according to a standard calibration curve (0-20 mg mL^{-1}) using bovine serum albumin (Sigma-Aldrich) as a reference material.

EROD activity

The protocol described by Lubet et al. (1985) was used to analyse EROD activity. Briefly, the floating post-mitochondrial fraction (S9) was obtained from whole livers homogenized in a Tris-HCl (50 mM) and $MgCl_2$ (25 mM) buffer (pH 7.5) and centrifuged at 9,000 *x* g for 20 min at 4 °C. Analyses were performed using an LS 50B fluorescence spectrometer (PerkinElmer, Beaconsfield, UK) for 5 min at 25 °C. Protein analyses were performed using a 96-well DNM-9602G microplate reader (Baush + Lomb) following the Bradford Method and with bovine serum albumin (Sigma-Aldrich) as a reference material. EROD activity was expressed as ρmol min^{-1} mg $protein^{-1}$.

Sex steroid hormone production

The protocol described by McMaster et al. (1995) was used to determinate sex steroid production. After fish sampling, 12-30 mg of gonad tissue were kept in a M199 medium at 4 °C (< 6 h). The M199 medium contained Hank's salts without bicarbonate (Gibco, Carlsbad, CA, USA) complimented with 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 4.0 mM sodium bicarbonate, 0.01% streptomycin sulphate, and 0.1% bovine serum albumin (pH 7.4). Sex steroids were cultured *in vitro* in pasteurized, Falcon 3047 24-well plates (Thermo Fisher Scientific). The tissues were incubated for 24 h at 16 °C in M199; after incubation, the supernatant was drawn off and maintained at -80 °C until analysis. Testosterone (males, total 41) and 17-β-oestradiol (females, total 38) were quantified at the National Water Research Institute (Burlington, ON, Canada) using a radioimmunoassay procedure according to a standardized protocol.

Statistical analysis

Fish sex was confirmed by histological analysis. Due to the nature of the study and the lack of external secondary sex characteristics on *P. irwini*, analyses examined individual data within sex, with the exception of mucus phosphoproteins. Normal distribution was assessed using the Shapiro-Wilk's test. All biological endpoints were assessed using an analysis of variance (ANOVA, $p < 0.05$), followed by Tukey's *post hoc* test to establish differences between treatments. Normality assumptions and ANOVA were performed using the SYSTAT 11 software (SYSTAT Software, Inc., Chicago, IL, USA). All biological endpoints were separately assessed for each exposure sampling time between treatments and, simultaneously, temporal changes were analysed using day 0 as a control.

RESULTS

O. mykiss EE2 exposure

After juvenile rainbow trout exposure to EE2, physiological indices were evaluated at days 0 and 10 (data not shown).

There were no significant differences in k ($p = 0.424$) or HSI ($p = 0.063$) compared to day 0 or between control and exposed fish at day 10. GSI was evaluated according to sex; however, no significant differences were observed for either females ($p = 0.166$) or males ($p = 0.160$).

To validate the technique, phosphoprotein levels were measured in rainbow trout plasma and mucus. Phosphoproteins were measured at days 0, 3, 5, 7, and 10 of EE2 exposure. A significant correlation was found between the fold changes of plasma VTG and mucus phosphoproteins ($r^2 = 0.658$, $p < 0.001$; Fig. 1). A similar correlation was found for VTG concentration values (ng mL^{-1}; figure not shown). Although, the r^2 was lower (0.404), the p-value indicated a significant correlation ($p = 0.011$).

P. irwini bioassays: Mucosal VTG and phosphoprotein measurements

Bioassays were carried out on the endemic *P. irwini*, a Biobio River fish exposed to WWTP and PPM point source pollution discharges near the town of Santa Bárbara, Chile. The water quality parameters for collected WWTP, PPM, and river waters are indicated in Table 1. There were differences in water quality data from the studied sites. Conductivity and chloride increased in both WWTP and PPM waters, but WWTP water displayed increases in five-day biochemical oxygen demand, total phosphorous, nitrate, silicon dioxide, and turbidity, indicating lower water quality than PPM water.

Percilia irwini were also exposed to two negative controls (i.e. dechlorinated water and river water collected upstream of point source pollutions) and a positive control (i.e. EE2). This positive control was chosen as EE2 is known to induce VTG increases in the plasma and mucus (Kidd et al. 2007; Wang et al. 2015). At days 0, 4, 7, and 12, mucosal phosphoproteins were measured ($n = 10$ per treatment). While no significant differences were observed in the phosphoprotein concentrations between the dechlorinated water controls at days 4 ($p = 0.944$) and 7 ($p = 0.235$), there was an unexpected significant increase at day 12 in this negative control group ($p < 0.001$; data not shown). Considering this, the data were analysed as fold changes compared to the negative control at every sampling point (Fig. 2).

Significant phosphoprotein increases occurred between the river and dechlorinated water groups. Significant fold change increases were observed at days 4 (2.55 ± 1.19, $p = 0.023$) and 12 (2.52 ± 1.33, $p = 0.013$), but not day 7 ($p = 0.360$). Phosphoprotein concentrations also significantly increased in the EE2 exposure group on days 4, 7, and 12 ($p = 0.009$, $p = 0.032$, and $p = 0.015$, respectively). Fish exposed to WWTPEs displayed significantly increased fold changes on days 4 (11.71 ± 4.95, $p < 0.01$) and 12 (8.38 ± 82.23, $p < 0.01$). Due to protozoal infections, fewer fish were available for WWTP water treatments, and day 7 sampling was omitted in this group to prevent further stress. Similar to the WWTPE group, fish exposed to PPMEs displayed significant fold change increases in mucosal phosphoproteins by days 4 (9.70 ± 1.77, $p < 0.01$) and 12 (11.04 ± 4.54, $p < 0.01$). On day 7, PPME fish evidenced significantly decreased phosphoprotein concentrations as compared to the control (0.57 ± 0.23; $p = 0.03$).

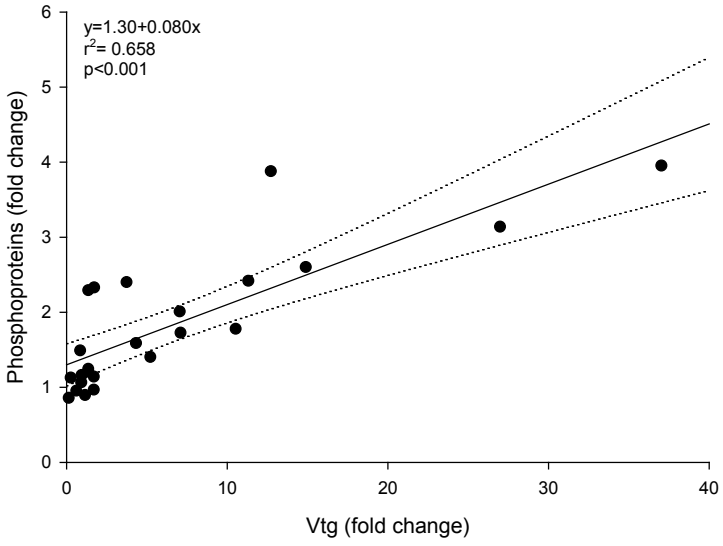

Figure 1. Correlation between the fold change of plasmatic VTG and plasma phosphoproteins in *O. mykiss* exposed to 10 ng ml^{-1} of EE2 for 10 days. / Correlación entre *fold change* de los niveles de VTG plasmático y las fosfoproteínas plasmáticas en *O. mykiss* expuestas a 10 ng ml^{-1} de EE2 por 10 días.

EROD activity

EROD activity was measured in the *P. irwini* liver. Samples were not sacrificed on day 4 to preserve individuals for later study. In dechlorinated water controls, EROD activity significantly decreased between days 0 and 7 (0.63 ± 0.26, p = 0.01; Fig. 3), whereas there was a significant fold change increase between days 0 and 12 (1.37 ± 0.42, p = 0.02). At day 7 of exposure, EROD activity significantly increased in fish exposed to river water ($p < 0.001$), EE2 ($p < 0.001$), and PPMEs ($p = 0.002$). No WWTP fish were collected for EROD activity assessments. Finally, at day 12 of exposure, significant differences were observed only in the PPME exposure group ($p = 0.03$).

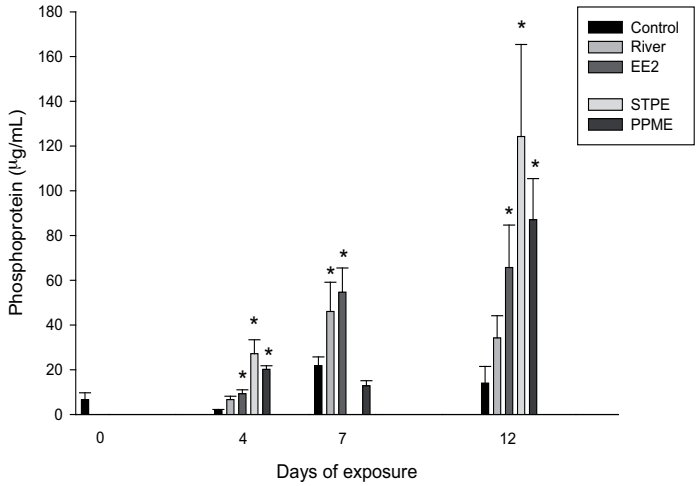

Figure 2. Phosphoprotein (Fold Change) compared to day 0 in *P. irwini* exposed to WWTPE and PPME after 4, 7, and 12 days of exposure. Control: Tank with dechlorinated water. River Water: Pond with Biobio River water collected upstream of point source pollutions. EE2: Pond with EE2 (10 ng L^{-1}). WWTP: Pond with WWTP water. PPM: Pond with PPM water. Asterisks (*) indicate significant differences between treatments and respective day 0 controls. / Fosfoproteínas (*fold change*) comparado al día 0 en *P. irwini* expuesta a WWTPE y PPE después de 4, 7 y 12 días de exposición. Control: tanque con agua declorinada. Agua de río: estanque con agua colectada del río Biobío, agua arriba de las fuentes de contaminación puntual. EE2: estanque con EE2 (10 ng L^{-1}). WWTP: estanque con efluentes de WWTP. PPM: estanque con efluentes de PPM. Asteriscos (*) indican diferencias significativas entre tratamientos y respectivo a los controles del día 0.

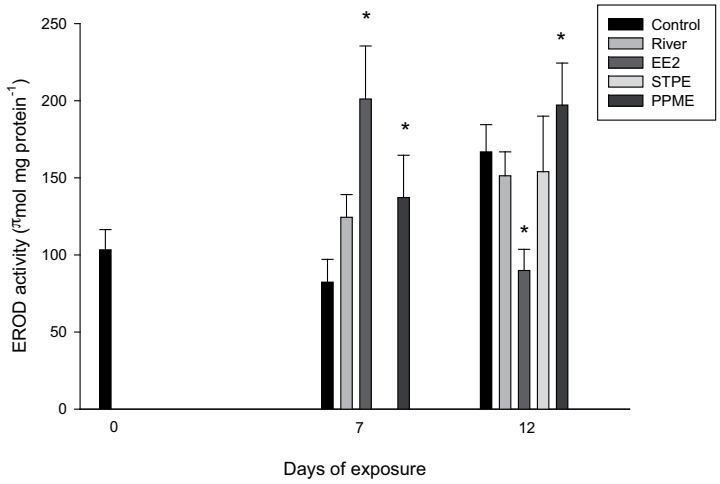

Figure 3. Liver EROD activity (Fold Change) in *P. irwini* exposed to different bioassay effluents over 12 days. Values are shown as the average ± standard error. Control: Tank with dechlorinated water. River Water: Pond with Biobio River water collected upstream of point source pollutions. EE2: Pond with EE2 (10 ng L^{-1}). WWTP: Pond with WWTP water. PPM: Pond with PPM water. Asterisks (*) indicate significant differences between treatments and respective day 0 controls. / Actividad EROD hepatica (*fold change*) en *P. irwini* expuesta a diferentes efluentes en bioensayos de 12 días. Se muestras valores en promedio ± error standard. Control: tanque con agua declorinada. Agua de río: estanque con agua colectada del río Biobío, agua arriba de las fuentes de contaminación puntual. EE2: estanque con EE2 (10 ng L^{-1}). WWTP: estanque con efluentes de WWTP. PPM: estanque con efluentes de PPM. Asteriscos (*) indican diferencias significativas entre tratamientos y respectivo a los controles del día 0.

Hormone levels and somatic indices

Steroid hormone production was evaluated in the gonads. Oestradiol was measured in females and testosterone in males. No statistical tests were applied due to low detection limits in several samples and, therefore, low sample sizes. Females exposed to EE2 and PPM water exhibited increased oestradiol production at day 7, whereas females exposed to river water presented no differences with the dechlorinated water control (Fig. 4). At the day 12 endpoint, all females exposed to EE2 and WWTP and PPM waters were below the detection limit for oestradiol. Testosterone production was only detected in control males on days 0 and 7. Additionally, on day 7, testosterone levels were measured in males exposed to all treatments, with all other sampling points below detection limits. Somatic indices showed scarce differences between the experimental and control treatments. Only the GSI in females exposed to PPM water displayed a higher average at day 7 as compared to the control, but no significant difference was observed (data not shown).

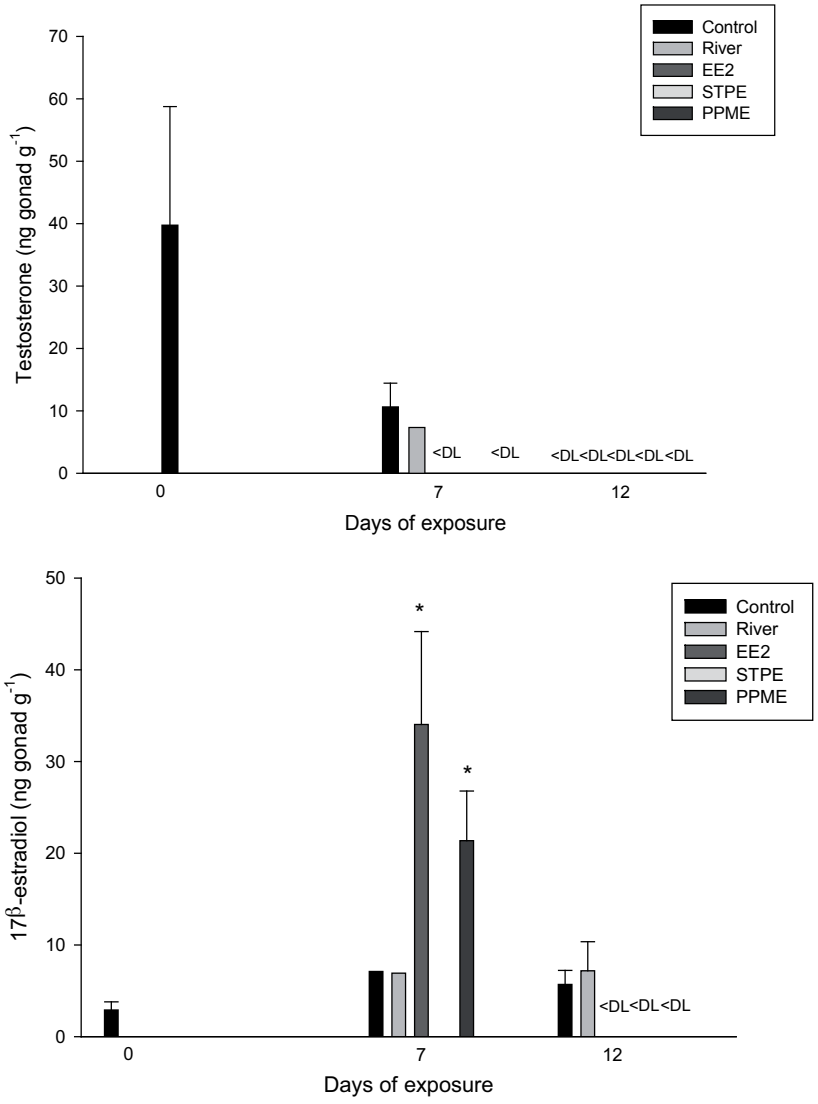

Figure 4. *In vitro* production of testosterone and oestradiol (ng gonad g^{-1}) by *P. irwini* tested tissues (n = 94) after exposure to river, WWTP, and PPM waters on days 7 and 12. Control: Tank with dechlorinated water. River Water: Pond with Biobio River water collected upstream of point source pollutions. EE2: Pond with EE2 (10 ng L^{-1}). WWTP: Pond with WWTP water. PPM: Pond with PPM water. Asterisks (*) indicate significant differences between treatments and respective day 0 controls. <LD= below detection limit. / Producción *in vitro* de testosterona y estradiol (ng g de gonada $^{-1}$) en tejido testeado de *P. irwini* (n = 94) a aguas de río, WWTP y PPM después de 7 y 12 días de exposición. Control: tanque con agua declorinada. Agua de río: estanque con agua colectada del río Biobío, agua arriba de las fuentes de contaminación puntual. EE2: estanque con EE2 (10 ng L^{-1}). WWTP: estanque con efluentes de WWTP. PPM: estanque con efluentes de PPM. Asteriscos (*) indican diferencias significativas entre tratamientos y respectivo a los controles del día 0. <LD = bajo el límite de detección.

DISCUSSION

Numerous research teams have focussed efforts on developing tools that assess EDC effects while maximizing monitoring program outcomes, minimizing lethal sampling, and preventing additional negative impacts on native, and often endangered, fish populations (Gray et al. 2002). One alternative method uses fish mucus as a viable, non-lethal tool for monitoring VTG levels and, through this, for determining the oestrogenic effects of urban and industrial EDC effluents on fish populations.

The modified protocol outlined by Pollino et al. (2009) and applied in the present assessments performed well. Rainbow trout exhibited a significant correlation between measurements of VTG levels in the plasma and mucus. The same significant correlation between plasma and mucus VTG concentrations have been described in goldfish *C. auratus* (Wang et al. 2015), white bass *Morone chrysops* Rafinesque 1820 (Barkowski & Haukenes 2014), copper redhorse *Moxostoma hubbsi* Legendre, 1952, and shorthead redhorse *Moxostoma macrolepidotum* (Lesueur, 1817; Maltais & Roy 2009). Therefore, fish mucus is supported as a viable, non-lethal matrix for quantifying VTG levels, one of the most common endocrine disruption indicators.

Measurements of VTG in *P. irwini* mucus were also found to be a reliable test of endocrine disruption induced by WWTPEs and PPMEs. VTG-like phosphoprotein concentrations increased over prolonged effluent exposure times in fish (as fold change to control). In particular, *P. irwini* appears highly sensitive to EDCs (Chiang et al. 2011), and VTG-like phosphoprotein levels increased very rapidly compared to other blood and mucus studies that utilized different analytical techniques and specific EDCs (Moncaut et al. 2003; Arukwe & Røe 2008; Rey Vázquez et al. 2009; Pollino et al. 2009). Consequently, mucus and VTG-like phosphoproteins are a promising non-lethal tool for assessing EDCs in wild fish. Importantly, a non-lethal sampling methodology is needed for minimally-invasive environmental monitoring, particularly concerning threatened species (Rees et al. 2011; Veldhoen et al. 2014, 2013).

Although this study focused on VTG-like phosphoproteins in mucus as a substitute to lethal sampling for assessing endocrine disruption in an endemic wild fish, other responses have also been closely associated with different endocrine disruption responses in Chilean fish (Orrego et al. 2009, 2011; Chiang et al. 2011, 2015). While previous studies have shown that *P. gillissi* (the only congeneric specie from *P. irwini*) EROD activity and sex hormone levels are highly dependent on season (Chiang et al. 2011), these factors were isolated and different responses were observed over the presently assessed treatment period.

Orrego et al. (2009, 2010) stated that androgens present in PPMEs could be aromatized via CYP19a activity, and this could explain the delayed increase in EROD activity in relation to VTG induction. Orrego et al. (2009) further propose that aromatizable androgens and oestrogenic compounds possibly present in PPMEs and WWTPEs could result in a gonadotropin hormone accumulation, leading to a release of the luteinizing hormone, which, in turn, is responsible for VTG production. Although there are few studies on the chemicals present in WWTPEs in Chile, these chemicals could have the mechanisms or/and estrogenic compounds similar to those reported by other studies (Green et al. 2013; Arlos et al. 2015; Jobling et al. 2009; Orrego et al. 2009, 2010). However, further studies on sex steroids are required to fully clarify this situation.

This study recorded an increase in 17β-oestradiol during the first week of effluent exposure, but this later fell under detection limits. Testosterone levels behaved similarly. These results contradict evidence of ED in *P. gillissi* from a previous study. Specifically, Chiang et al. (2011) reported induced 17β-oestradiol production and no change in testosterone levels in the gonads of *P. gillissi* downstream of PPME discharge sites. Additionally, all previous related research indicates that female fish downstream of PPME discharge sites in Chile undergo reproductive system stimulation and an induction of gonadal maturation. The contrasting results obtained in the present study could be due to differences in effluent exposure time, particularly when considering that the adult fish used in the current bioassays were only exposed to effluents for 12 days. The reported effects were observed after 7 days of exposure and it could be due to compensatory physiological and metabolic mechanisms, not know so far for this native fish. Despite the time taken to show effects, the present analyses of fish responses produced results similar to Orrego et al. (2009), with higher mucosal VTG-like phosphoprotein levels and EROD activity found in correlation with effluent exposure and time.

Since different results could be obtained in field vs. laboratory studies, further comparative tests and assessments of possible seasonal influences on mucosal VTG levels should be performed. Despite this limitation, the present study provides evidence for differential responses to PPMEs and WWTPEs in an endemic Chilean fish species. Both effluents showed endocrine-disrupting potentials, but higher mucosal VTG levels were found following exposure to PPMEs. In Chile, PPMEs have been described as oestrogenic-like stressors, with WWTPEs presenting lesser oestrogenic effects. Nevertheless, little is known about the possible effects of WWTPEs on aquatic life in Chile. Although a primarily oestrogenic effect was observed in the present study, the presence of other endocrine-disrupting androgenic and anti-androgenic compounds requires additional research as they could possibly play a role in the responses of *P. irwini* to point source pollution.

ACKNOWLEDGEMENTS

This article was funded by an Idea Wild Research Grant to G. Chiang and NSERC Strategic Grant to K.R. Munkittrick. R. Barra would like to thank funds from FONDAP-CONICYT Grant 15130015 (CRHIAM) that supported the study. The authors are highly appreciative of field support provided by F. Tucca and R. Sanchez, in addition to being grateful the equipment made available by the Biomarkers Lab at the EULA-Center and by the Bioassays Lab at the Faculty of Natural Sciences and Oceanography, Universidad de Concepción. The authors have no conflict of interest to declare. Paulina Bahamonde is supported by Nucleo Milenio INVASAL funded by Chile's government program, Iniciativa Científica Milenio from Ministerio de Economía, Fomento y Turismo.

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Recibido: 27.11.2017
Aceptado: 22.11.2018