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Multivariate analysis of biochemical responses using non-invasive methods to evaluate the health status of the endangered blackfin goodeid (*Girardinichthys viviparus*)

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ABSTRACT

The study of endangered fish species is quite a complex process that involves, in the worst case, the sacrifice of specimens. To solve this ethical problem, some laboratory studies have been conducted in the skin mucus layer (SML) on fish species with valuable results. However, to date research evaluating a panel of biomarkers in the SML of wild fish does not exist. In the current study we assessed the effects of pollutants (E_1, E_2, E_3, EE_2, BPA, NP, OP, Cu, Fe, Mn, Pb, Zn, $C_{10}H_8$, $C_{16}H_{10}$, $C_{18}H_{12}$, B[a]P $C_{20}H_{12}$, B[k]F $C_{20}H_{12}$, $C_{22}H_{12}$) upon a panel of biomarkers [O_2•, H_2O_2, lipid peroxidation (as TBARS), carbonyl proteins (RC═O), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), vitellogenin (VTG) and metallothionein (MT)] evaluated in the SML of the wild endangered *Girardinichthys viviparus* inhabiting two polluted lakes in Mexico Valley. Possible relationships were analyzed by principal components analysis (PCA) and redundancy analysis (RDA). The main finding was a clear induction of pro-oxidant forces (ROS) in the SML, probably related to biotransformation of estrogenic and phenolic compounds, in addtion to a redox process of Cu and Fe. As a consequence, oxidative tissue damage (TBARS and RC═O) and increases in antioxidant defenses were observed. In male fish, VTG was associated with bisphenol A (BPA) apparently potentiated by Cu and Fe water concentrations; meanwhile, in female fish VTG was linked to estrogens. By PCA, MT was correlated with Fe and Cu; however, it was not linked with diminution of oxidative stress. For the first time have demonstrated the useful of the SML using a panel of biomarkers for monitoring the health of wild fish.

1. Introduction

Conservation biologists have been increasing the use of novel techniques to study wild vertebrates. In particular, non-invasive approaches for sampling have become the main strategy in wildlife research, allowing the protection of wild specimens (Garshelis, 2006; MacKay et al., 2008). These methods are suitable for protecting endangered species whose existence cannot be further jeopardizing by the use of destructive methods (Esteban and Castaño, 2009; Dzul-Caamal et al., 2013). In the aquatic ecosystem, organisms are exposed to a variety of environmental stressors that generally are present in complex mixtures (Ben-Khedher et al., 2013). During the absorption process, the toxicants released in aquatic ecosystems are carried by the blood or lymph to a storage point, or to the liver for biotransformation and/or storage, and for excretion through the bile (Needham et al., 2005). However, these substances are likely also translocated to other organs such as the gills or kidney for excretion or could be stored in the fatty tissues (Nussey et al., 2000). As a consequence of these toxicokinetic and toxicodynamic processes, measurements of biomarkers or body burdens on the fluids, cells or tissues detect more the presence of some xenobiotics more quickly and specifically, allowing an early estimation of the damage (Monserrat et al., 2007; Tlili et al., 2010). The skin mucus layer (SML) is the interface between the fish and external environment. It is present on the skin and gill surfaces, but also in the gut lining; indeed, on all mucosal surfaces (Benhamed et al., 2014). The SML plays an important role in immune functions, respiration, ionic and osmotic regulation, reproduction, excretion, and protection against microorganisms, toxicants and hydrolytic enzymes (Cone, 2009; Macpherson et al., 2005).

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In environmental risk assessment, some studies have been conducted in the SML of fish species that found interesting relationships with certain toxicants (Moncaut et al., 2003; Meucci and Arukwe, 2005; Trenzado et al., 2006; Arukwe and Røe, 2008; Rey Vázquez et al., 2009; Maltais et al., 2010; Dzul-Caamal et al., 2013; Nigam et al., 2012, 2014; Maltais and Roy, 2014). However, a wide panel of biomarkers that can be evaluated in the SML of wild fish species has not been available.

On the one hand, Mexico is a privileged land in terms of biodiversity but it is also a country with great vulnerability in its freshwater ecosystems. This phenomenon has historically augmented the degree of disturbance caused by anthropogenic activities, increasing the risk of extinction of native ichthyofauna particularly in Central Mexico (Dzul-Caamal et al., 2012). The blackfin goodeid (*Girardinichthys viviparus* Bustamante, 1839) is a critically endangered fish species according to the IUCN (www.iucnredlist.org) and is included as an endangered species in Mexican guidelines (NOM-059-SEMARNAT-2010). In a previous report, the blackfin goodeid exposed in the laboratory to water from its extant localities suffered a noticeable lipid peroxidation in the liver as well as a variation in the antioxidant defenses according to the sex of the fish were observed (Vega-López et al., 2008a). In the same way, in the liver of the male blackfin goodeid elevated levels of vitellogenin and metallothionein were detected suggesting a greater sensitivity to endocrine disrupting compounds and metals regarding to female fish (Olivares-Rubio et al., 2015). Interestingly, during the estrogen metabolism carried out by CYP450 isoenzymes ROS induction could be occurring (Cavalieri et al., 2000) and in the goldfish scale cell line some genes involved in the estrogenic response are present such as the osteonectin gene (Lehane et al., 1999). Due to the conservation status of this fish species, the loss and degradation of its habitat and the lack of non-invasive methods for monitoring the health of the blackfin goodeid, the aim of this study was to assess a battery of biomarkers in the SML of *G. viviparus* inhabitants from two polluted lakes in the Valley of Mexico. Biomarkers of the estrogenic response (VTG) and the response to metals (MT) as well as broad-spectrum biomarkers such as pro-oxidant forces ($O_2^{\bullet}$ and H_2O_2), oxidative damage (TBARS and RC=O) and antioxidant enzymes (SOD, CAT and GPX) in the SML of *G. viviparus* of both sexes were related to the water concentration of endocrine disrupting compounds (estrogenic and phenolic compounds), heavy metals and polycyclic aromatic hydrocarbons using two multiparametric analyses (principal component analysis and redundancy analysis).

2. Materials and methods

2.1. Sampling and fish collection

Three sampling campaigns were performed in the summer (July), autumn (October) and winter (December) in 2012. Surface and bottom water samples collected with a Van Dorn bottle were placed in 4-L amber glass bottles previously washed and rinsed with HPLC-grade hexane for polyaromatic hydrocarbons (PAH) and estrogen analysis; heavy metals analysis in 1-L plastic bottles.

The collection of specimens was performed in accordance with Mexican protocols for the production, protection and welfare of experimental animals (SAGARPA, 2001); meanwhile, the handling was done in accordance with national and international protocols (http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=CELEX:31986L0609:EN:NOT). Thirty *Girardinichthys viviparus* (15♂, 15♀) were collected from each lake using a 3 mm-mesh-opening dip net. The skin mucus layer (SML) was obtained by gently scraping the body surface with a sterile stainless steel spatula into micro-centrifuge tubes. The mucus of the ventral skin was not collect to avoid intestinal contamination and/or intestinal damage (Dzul-Caamal et al., 2013). After sampling, the fish were placed in situ for observation during 2 h in an 80-L tank filled with aerated water from the lakes before their release. A group of parent fish was maintain in the laboratory for at least one year until reproduction to obtain unexposed fish for use as controls. For maintenance, a glass aquarium of 140 L with synthetic water (0.22 g $MgSO_4$, 0.18 g $NaHCO_3$, 0.08 g KCl and 0.13 g $CaSO_4{\cdot}2H_2O$ per L), photoperiods of 18 h light and 8 dark, at $20 \pm 2\,^{\circ}C$, with aeration and constant filtration were used. The eight-month-old control specimens had a mean length of 47.13 ± 0.27 mm and a mean weight of 0.91 ± 0.03 g.

The weight/size ratio or Fulton's condition factor in wild fish ($W = aSL^x$), where W = weight and SL = standard length, were obtained through a potential curve fitting using Microsoft Excel® (Microsoft, Redmond, WA, USA), as previously reported (Le Cren, 1951). The constants "a" and "x" are derived from the mathematical model. The relative condition factor (K) of the fish was calculated as $K = W/aSL^x$ based on Le Cren (1951).

2.2. Sample treatment

The SML was diluted 1:1 (w:v) by the addition of phosphate buffered saline solution (10 mM Na_2HPO_4, 138 mM NaCl, 2.7 mM KCl, pH7.4) containing aprotinin (0.04 IU/ml) and 4-(2-aminoethyl) benzenesulphonyl fluoride (2 mM). After homogenization with a hand-held pestle homogenizer the mixture was centirfuged at $9000 \times g$/30 min/4 °C. The supernatant (S9 fraction) was stored at −20 °C until analysis of antioxidant enzymes and for metallothionein and vitellogenin quantification, except for ROS quantification in which case analysis was performed immediately. A uncentrifuged fraction was re-suspended on a vortex with 1 mL phosphate buffered saline solution for the evaluation of oxidative damage biomarkers, this fraction was chosen because the oxyradicals involved in the initiation of lipid peroxidation in the cellular membranes as well as oxidation and damage to proteins are present in this fraction, not only in the soluble fraction (Hermes-Lima, 2004).

2.3. Quantification of ROS ($O_2^{\bullet}$ and H_2O_2)

The evaluation of ROS ($O_2^{\bullet}$ and H_2O_2) was performed by the specific oxidation of dihydroethidium (DHE) and dihydrofluorescein diacetate (DHF-DA) to determine $O_2^{\bullet}$ and H_2O_2, content, respectively following the Dzul-Caamal et al. (2013) report. Results were expressed per g tissue.

2.4. Lipid peroxidation (TBARS) and protein carbonyl contents (RC=O)

Lipid peroxidation evaluated as thiobarburic acid reactive substances (TBARS) was assessed using the method of Buege and Aust (1978) and results were presented as μmol TBARS/g tissue.

The oxidation of proteins (RC=O) was evaluated by the method of Levine et al. (1994) with some modifications according to previous report (Dzul-Caamal et al., 2013) expressed as mM RC=O/mg protein/g tissue.

2.5. Antioxidant enzymes

The activities of the enzymes involved in antioxidant defense were evaluated in the S9 fraction of the SML. SOD (EC 1.15.1.1) activity was assayed according to the method of Misra and Fridovich (1972). Catalase (CAT) (EC 1.11.1.6) activity was assessed by the method of Radi et al. (1991). The activity of glutathione peroxidase (GPx) (EC 1.11.1.9) was quantified by the method of Lei et al.

(1995). The results for the antioxidant enzymes were expressed as mM/min/mg protein/g of fish.

All tests were performed in triplicate including ROS, oxidative damage biomarkers and antioxidant enzymes.

2.6. Metallothionein (MT) and vitellogenin (VTG) evaluation by enzyme-linked immunosorbent assay (ELISA)

The contents of metallothionein (MT) and vitellogenin (VTG) in the SML were measured by ELISA following the procedure of Vega-López et al. (2006, 2007a) using polyclonal serum (anti-VTG and anti-MT produced in rabbits in the lab) diluted 1:7000. The VTG was quantified by comparison with a standard curve (0.3–6.0 ng) with a purified VTG from *G. viviparus* as standard (Vega-López et al., 2006). MT induction and purification were performed as in Pedersen et al. (1994) and were quantified by a calibration curve (1–30 ng) of *G. viviparus* MT. Results were expressed as ng/mg^{-1} protein.

2.7. CYP1A expression in the SML

In an independent sampling campaign (August 2013), we evaluated CYP1A1 mRNA in the SML of some male and female wild fish from the lakes under study by RT-PCR because we found statistical relationships between ROS levels with estrogen concentration in the water. Total RNA was isolated using Trizol method, the cDNA synthesis was performed with reverse transcriptase. The first-strand cDNA was employed as the template for RT-PCR with specific CYP1A1 primers (Nogueira et al., 2010) amplifying a product of 500 pb. As an internal control of RNA integrity and cDNA quality, the 18s rRNA gene was used amplifying a product of 453 pb (Vega-López et al., 2007a).

2.8. Physicochemical water assay

2.8.1. Heavy metal analysis

Concentrations of Cu, Fe, Mn, Pb and Zn were determined in acid digestion (HNO_3 AA grade, Sigma–Aldrich^{TM}) followed by A.A. using the direct air-acetylene flame method (APHA et al., 1998). Standard curves for Cu, Fe, Mn and Pb were performed from 1.0 to 5.0 mg/L, and for Zn from 0.2 to 1.5 mg/L.

2.8.2. Analysis of estrogenic and phenolic compounds

Estrogen extraction and method for quantification was performed following the protocol of Wang et al. (2006). Curves were constructed from 1–5 ng/L, using Sigma–Aldrich standards and methanol (HPLC grade) as diluent. Retention times were the following: E_1 = 19.9403; E_2 = 16.8064; E_3 = 18.4182; EE_2 = 19.7961 min. The concentration of estrogens was evaluated by the percentage recovery of spiked samples added to standard solutions containing, 1, 10, 50 ng mL^{-1} of E_3, E_2, E_1 and EE_2, respectively.

The measurement of total phenol (TP) was performed following spectrophotometric EPA method 420.1 by chloroform extraction. Nonyl phenol (NP) was measured by an Alkylphenol ELISA kit (Ecologiena^{TM}; Tokiwa Chemical Industries CO., Ltd) with 100% reactivity with NP and 96% with octyl phenol (OP). Bisphenol A (BPA) was measured with a BPA ELISA kit (Ecologiena0^{TM}; Japan EnviroChemicals, Ltd). These measurements were performed according to the procedures of the manufacturers.

2.8.3. Analysis of polycyclic aromatic hydrocarbons (PAH)

The analysis of PAHs was conducted in a Biotek Synergy MX spectrofluorometer using a Sulpeco^{TM} standard PAH mixture according to a previous report (Vega-López et al., 2013). PAH

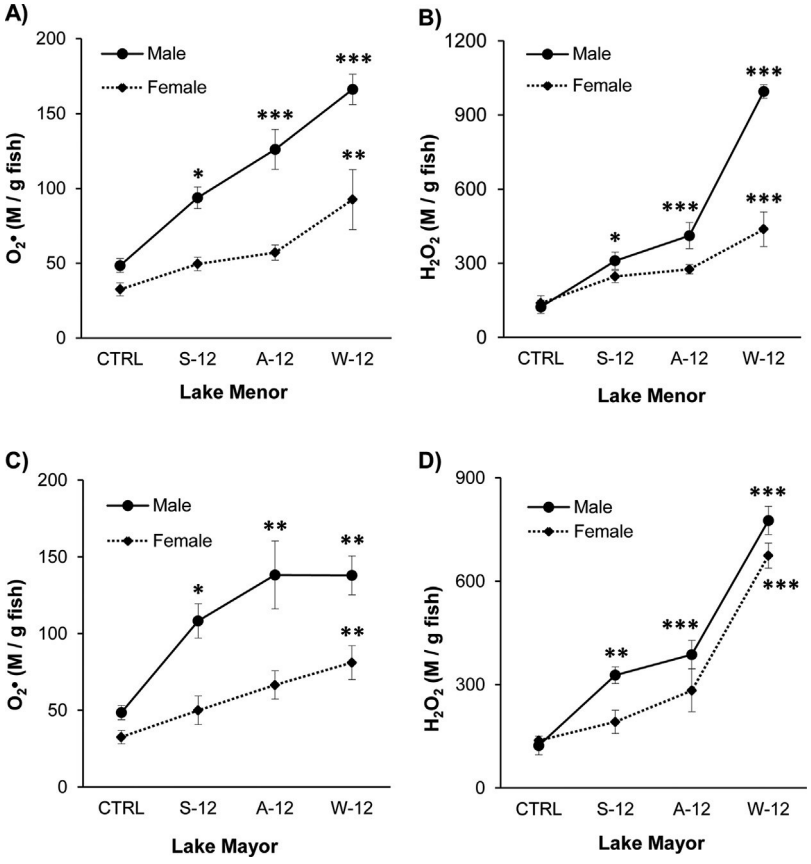

Fig. 1. Content of ROS ($O_2^{\bullet}$ and H_2O_2) in the skin mucus layer (SML) of wild *Girardinichthys viviparus*. Panel (A) and (B) male and female fish from Lake Menor, respectively. Panel (C) and (D) male and female fish from Mayor Lake, respectively. Significant differences with regard to control fish: $^{*}p \leq 0.05$, $^{**}p \leq 0.01$ and $^{***}p \leq 0.001$.

Table 1
Population features of male and female *Girardinichthys viviparus* from Lake Menor and Lake Mayor of Chapultepec Park (Mexico).

Population features	Menor lake		Mayor lake	
	Males	Females	Males	Females
W (g)	0.35 ± 0.09^a	0.70 ± 0.34	0.41 ± 0.08^b	0.71 ± 0.30
L-U (g)	0.2–0.56	0.30–1.72	0.25–0.60	0.37–1.87
SL (mm)	26.58 ± 1.64	36.21 ± 4.41	26.58 ± 1.64	34.91 ± 7.76
L-U (mm)	24–31	25.9–48	23.1–30.4	26.7–57.1
K	1.02 ± 0.25^a	1.08 ± 0.43^a	0.99 ± 0.17^b	1.04 ± 0.31^b

$n = 45$ fish of each sex and locality. Mean ± standard deviation. *W* = weight. SL = standard longitude. L = lower value. U = upper value. *K* = relative condition factor. Different letters denote statistical differences between localities whiting the same sex at $p \leq 0.05$. The relative condition factor (*K*) of the fish was calculated as $K = W/aSL^x$ using the weight/size ratio or Fulton's condition factor ($W = aSL^x$), where *W* = weight and SL = standard length based on Le Cren (1951).

standard curve samples (0.002–0.012 μg/L) were placed in 96 well plates (polymer base optical button black treated N/SterPS; Nunc) for fluorescence. The excitation and emission wavelengths were as follows: naphthalene ($C_{10}H_8$) 273 and 360 nm; pyrene ($C_{16}H_{10}$) 327 and 385 nm; benzo[a]anthracene ($C_{18}H_{12}$) 277 and 376 nm; benzo[a]pyrene ($C_{20}H_{12}$) 380 and 430 nm; benzo[k]fluoranthene ($C_{20}H_{12}$) 255 and 420 nm; indeno[1,2,3-*c,d*]pyrene ($C_{22}H_{12}$) 250 and 495 nm.

2.9. Statistical analysis

The results were expressed as mean ± standard deviation ($n = 15$, per sex, lake and sampling campaign). Data on the biomarkers was compared using a one-way ANOVA followed by Dunnet post hoc test considering $p \leq 0.05$ to represent a significant difference. In addition, the data was subjected to multiparametric analysis. Principal component analysis (PCA) is based in a linear response model relating species and environmental variables (Van den Brink et al., 2003), which in the current study was used to relate the biomarkers in the SML to environmental variables. PCA results were considered significant with a factorial weight >0.7. To determine which factors were responsible for the structure or groupings obtained in the PCA, a redundancy analysis (RDA) was carried out. The RDA is an ordination method of direct gradient analysis (Ter Braak and Prentice, 1988) able to find both specific patterns of biological parameters and to assess the relationships between biological (response variables) and environmental data (explanatory variables) using XLSTAT software for Excel. The Monte Carlo permutation was used to evaluate the statistical significance of the canonical axes.

3. Results

3.1. Physiological features of Girardinichthys viviparus

The mean weight and standard longitude of wild female fish from both lakes were similar. In contrast, in male fish from Lake Mayor a higher weight with regard to specimens from Lake Menor was documented, whereas the mean standard longitude was similar. However, the specimens of both sexes from Lake Menor presented a higher relative condition factor (K) compared with fish from Lake Mayor (Table 1).

3.2. Content of ROS

The concentration of H_2O_2 was greater than that of $O_2^{\bullet}$ in the SML of both sexes of wild fish, as observed also in control fish. In the SML of male fish from both lakes (Fig. 1A and C), the concentrations of $O_2^{\bullet}$ and H_2O_2 were higher in all sampling campaigns than in control fish, with a peak in the winter (December 2012). In the SML of male fish from Lake Menor, maximum levels of $O_2^{\bullet}$ and H_2O_2 in the SML were 2.8-fold higher and 6.3-fold higher, respectively, than in the control group. In contrast, in female fish in both lakes, these pro-oxidant forces were significantly greater than in female control fish only in the winter ($p < 0.001$) probably could be explained by high standard deviation observed during the summer and autumn (Fig. 1A–D).

3.3. Oxidative damage biomarkers

In wild fish of both sexes, oxidation of polyunsaturated fatty acids and proteins was greater in all sampling campaigns than in control fish ($p \leq 0.05$). In the SML of male fish, lipid peroxidation evaluated as TBARS increased as ROS concentration increased, particularly in Lake Menor specimens ($p < 0.001$). In females from both lakes, the maximum TBARS level was detected in the autumn ($p < 0.001$) (Fig. 2A). Protein oxidation quantified as carbonyl group (RC=O) content in the SML was higher in fish from both lakes than in control fish, except for male fish from Lake Mayor during the summer. A peak of RC=O was noticeable in the autumn in fish of both sexes (Fig. 2B).

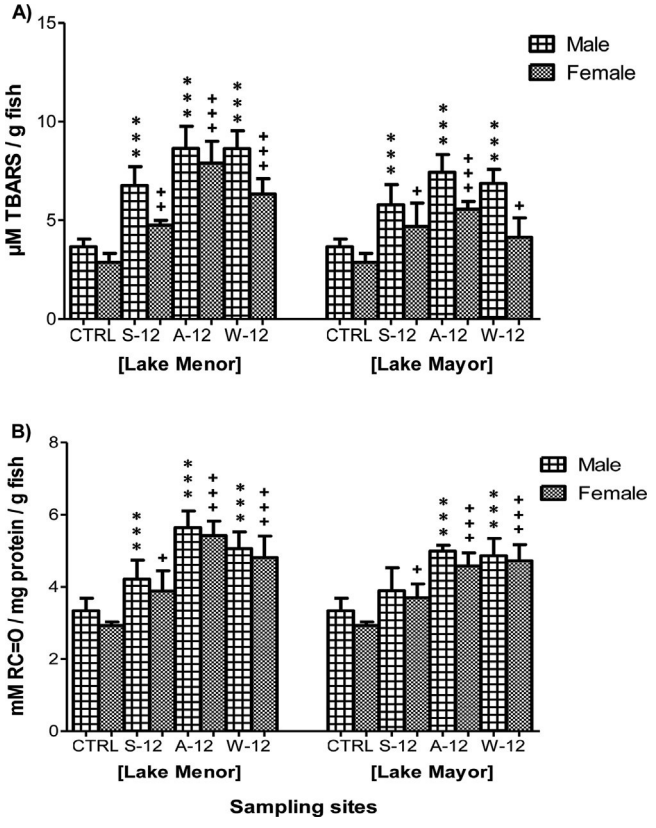

Fig. 2. Levels of lipid peroxidation (A: TBARS) and protein oxidation (B: RC=O) in the skin mucus layer (SML) of wild *Girardinichthys viviparus*. Significant differences with regard to control fish: $^*p \leq 0.05$, $^{**}p \leq 0.01$ and $^{***}p \leq 0.001$.

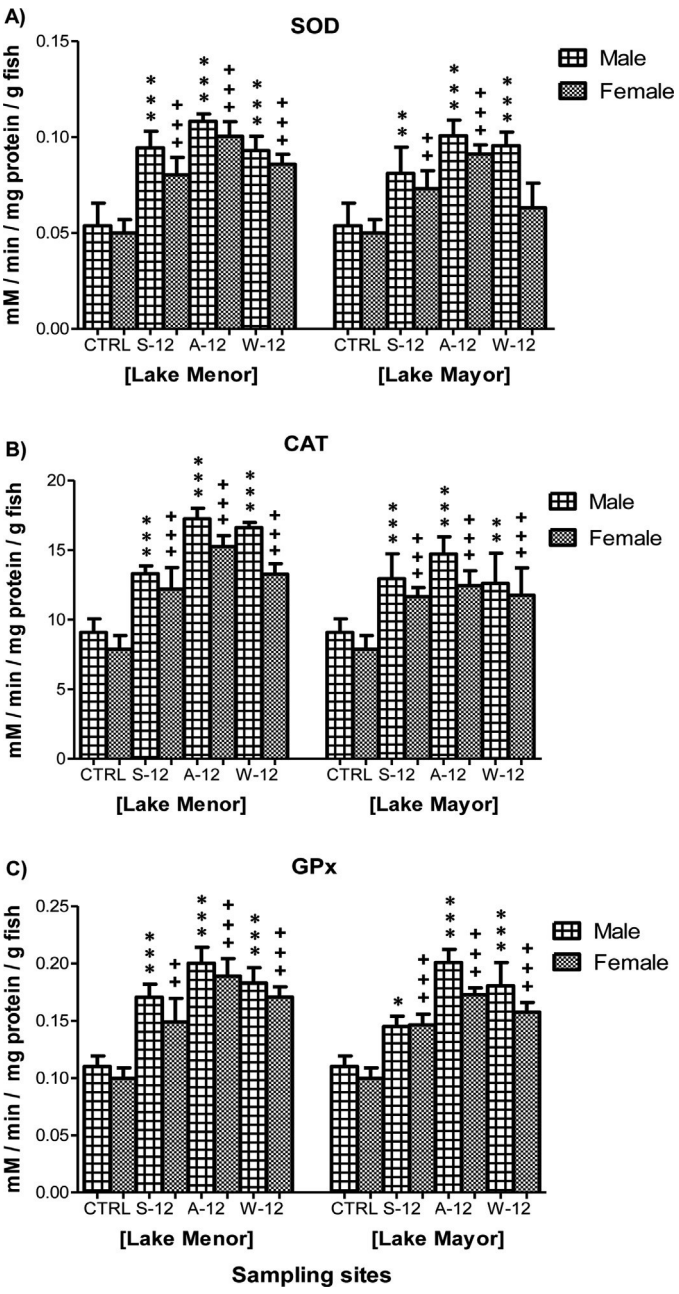

Fig. 3. Activities of enzymes involved in antioxidant defense in the skin mucus layer (SML) of wild *Girardinichthys viviparus*. Panel (A) activity of superoxide dismutase (SOD). Panel (B) activity of catalase (CAT). Panel (C) Activity of glutathione peroxidase (GPx). Significant differences with regard to control fish: $^{*}p \leq 0.05$, $^{**}p \leq 0.01$ and $^{***}p \leq 0.001$.

3.4. Activity of antioxidant enzymes

In all cases, the activities of the enzymes involved in antioxidant defense in the SML of the wild fish were greater than those in control fish ($p \leq 0.01$). The SOD activity of specimens of both sexes and lakes was elevated statistically ($p \leq 0.01$) compared to control fish except in female fish collected in Lake Mayor during the winter (Fig. 3A). CAT activity was close to two fold higher in wild fish ($p \leq 0.01$) than in control fish. Maximum CAT activity was detected in fish of both sexes from Lake Menor during the autumn, being greater in males than in females (Fig. 3B). GPx showed a higher activity in wild male fish than in females, particularly in the autumn ($p < 0.001$). In addition, the activity of GPx (Fig. 3C) displayed a similar pattern as lipid peroxidation. Interesting, the metabolic rate of GPx was similar to SOD activity, but substantially lower than CAT activity, suggesting the importance of CAT in antioxidant defense in the SML of *G. viviparus*.

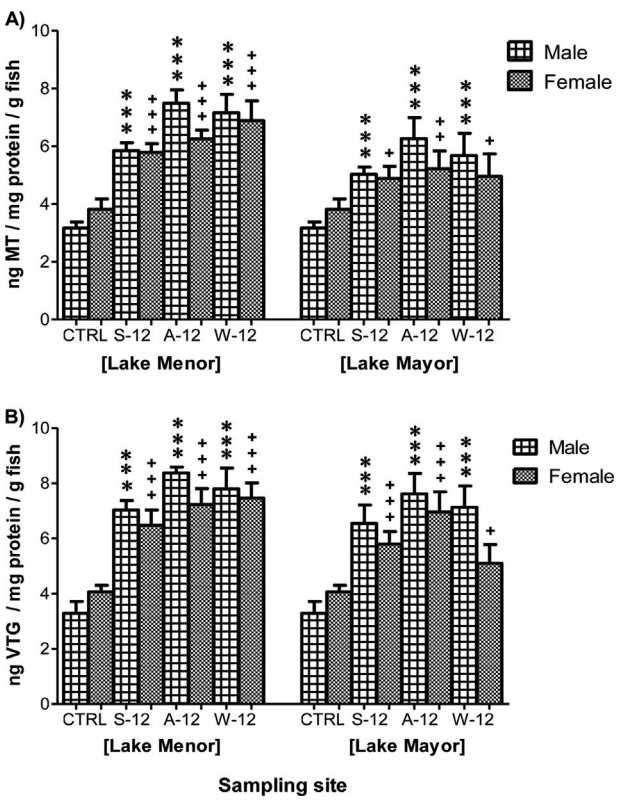

Fig. 4. Levels of metallothioneins (MTs) and vitellogenin (VTG) in the skin mucus layer (SML) of wild *Girardinichthys viviparus*. Panel (A) MT. Panel (B) VTG. Significant differences with regard to control fish: $^{*}p \leq 0.05$, $^{**}p \leq 0.01$ and $^{***}p \leq 0.001$.

3.5. Metallothionein (MT) and vitellogenin (VTG) assays

Levels of metallothionein (MT) in the SML of wild fish of both sexes were greater than in the control group. In male fish from both lakes, a high MT content ($p < 0.001$) with regard to control fish was detected peaking in the autumn, particularly in specimens from Lake Menor. However, in female fish from Lake Menor increased levels of MT reached a peak in the winter ($p < 0.001$); but, in females from Lake Mayor only a slight induction in this biomolecule ($p \leq 0.05$) was found in all sampling campaigns (Fig. 4A).

In wild fish, the amount of vitellogenin (VTG) in the SML was about two-fold higher than in control fish. During the autumn and winter, a noticeable VTG induction in male fish from both lakes and in females from Lake Menor was documented ($p < 0.001$). However, in the winter the amount of VTG in female fish from Lake Mayor decreased with regard to the previous sampling campaigns and was different ($p < 0.05$) from control females (Fig. 4B).

3.6. CYP1A expression

Interestingly, in the SML of male and female fish from Lake Menor and Lake Mayor CYP1A1 expression was observed (SAMEA3420836 ERS739729) (http://www.ebi.ac.uk/ena/data/view/ERS739729)

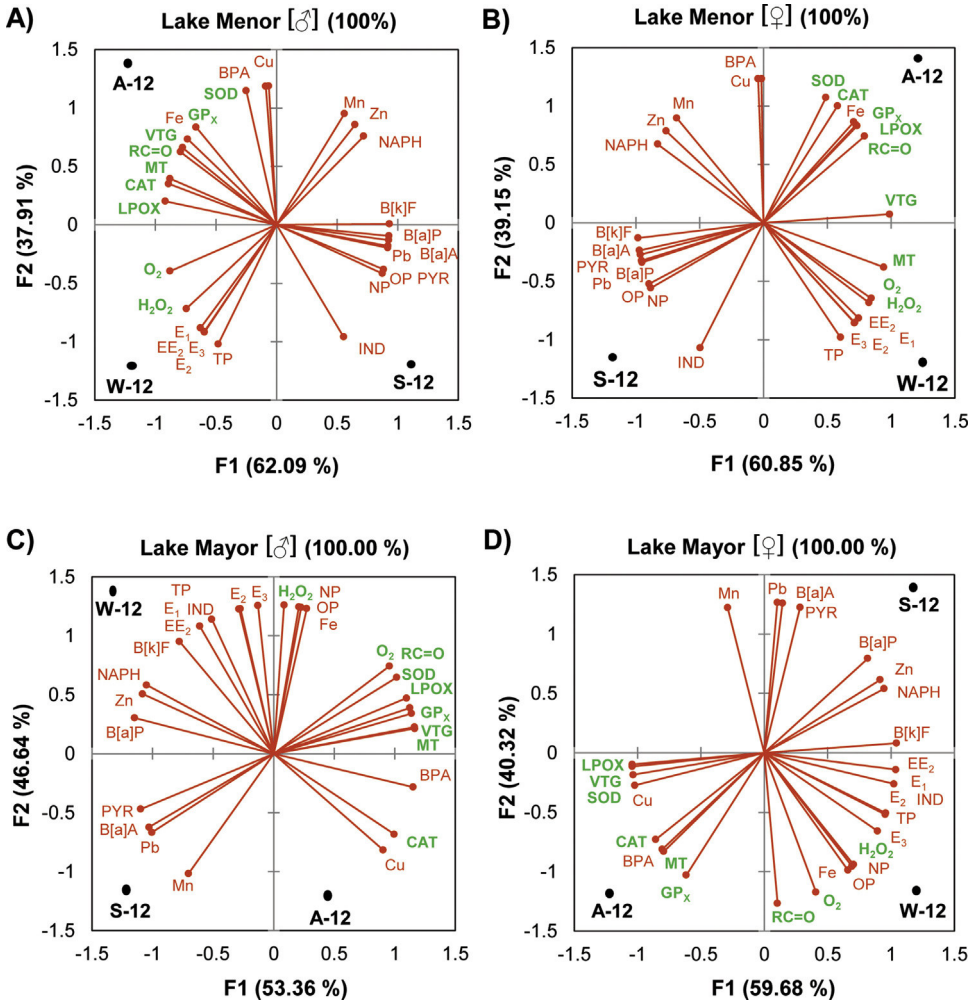

Fig. 5. Principal component analysis (PCA) showing 100% of the explained variance in all cases taking into account the first (F1) and second components (F2) of the panel of biomarkers ($O_2^{\bullet}$, H_2O_2, TBARS, RC=O, SOD, CAT, GPx, VTG and MT) evaluated in the SML of wild *Girardinichthys viviparus* and their relations with water levels of pollutants (E_1, estrone; E_2, 17β-estradiol; E_3, estriol; EE_2, ethinyl estradiol; TP, total phenols; NP, nonyl phenol; OP, octyl phenol; BPA, bisphenol A; IND, indeno[1,2,3-*c*,*d*]pyrene; NAPH, naphthalene; PYR, pyrene; B[a]A, benzo[a]anthracene; B[k]F, benzo[k]fluoranthene; B[a]P, benzo[a]pyrene; Fe, Cu, Mn, Pb and Zn). S-12: summer 2012; A-12: autumn 2012; W-12: winter 2012.

3.7. Multivariate analysis of biochemical responses

The obtained results of the principal component analysis (PCA) showed integration of the biomarker responses in the SML of *G. viviparus* between sexes, sites, sampling campaigns and toxicants levels, indicating that discrimination between locations occurs. Taking the first and second components into account explained 100% of the variance in the PCA in all cases (Fig. 5).

Relationships of biomarkers evaluated in the skin mucus layer (SML) were found with phenolic compounds (Fig. 6A), metals (Fig. 6B), estrogens (Fig. 6C), and in a few cases with polyaromatic hydrocarbons (Fig. 6D).

A redundancy analysis plot (RDA) was also constructed with response variables (biomarkers) and explanatory variables (environmental data) (Fig. 7). In fish from Lake Menor the first bi-plot (RDA1) explained a high correlation between the response variables and explanatory variables, both in male (75.01%) and female fish (66.50%). In blackfin goodeid from Lake Mayor the ordination (RDA1) explained 81.28% and 66.50% of the variation in the data in male and female fish, respectively. The triplot (2-dimensional biplots; RDA1 and RDA2) explained 100% of the correlation between the response variables and explanatory variables. Also the biomarkers in the SML were related with environmental data.

4. Discussion

The useful of the skin mucus layer (SML) as a target in environmental monitoring represents a significant advance in the protection of endemic or endangered species without requiring their sacrifice. Using multiparametric analysis a sex-linked response in the SML biomarkers of *G. viviparus* with certain environmental pollutants were detected. During the winter, levels of ROS ($O_2^{\bullet}$ and H_2O_2) were related to water total phenol and to estrogenic compounds (E_1, E_2, E_3 and EE_2) in male fish from Lake Menor and in female fish from both lakes using PCA and RDA, suggesting true relationships. In male fish from Lake Mayor, ROS content was dependent on high water levels of certain estrogens (E_2 and E_3) and on the concentration of TP in water using RDA. The present results suggest that during estrogen metabolism in the skin of the blackfin goodeid, ROS could be generated. During the hydroxylation of E_1 and E_2 carried out by CYP450 isoenzymes, estrogen-derived metabolites including 2-, 4-, and 16α-hydroxyoestradiol (Bui and Weisz, 1989; Shou et al., 1997; Zhu and Conney, 1998; Chourasia and Joy, 2010) allow ROS generation (Cavalieri et al., 2000). The hydroxyl groups in a vicinal position on the 2- and 4-hydroxylated catechols could suffer further oxidation by semiquinones with consequent formation of $O_2^{\bullet}$ (Nutter et al., 1994; Tabakovic et al., 1996;

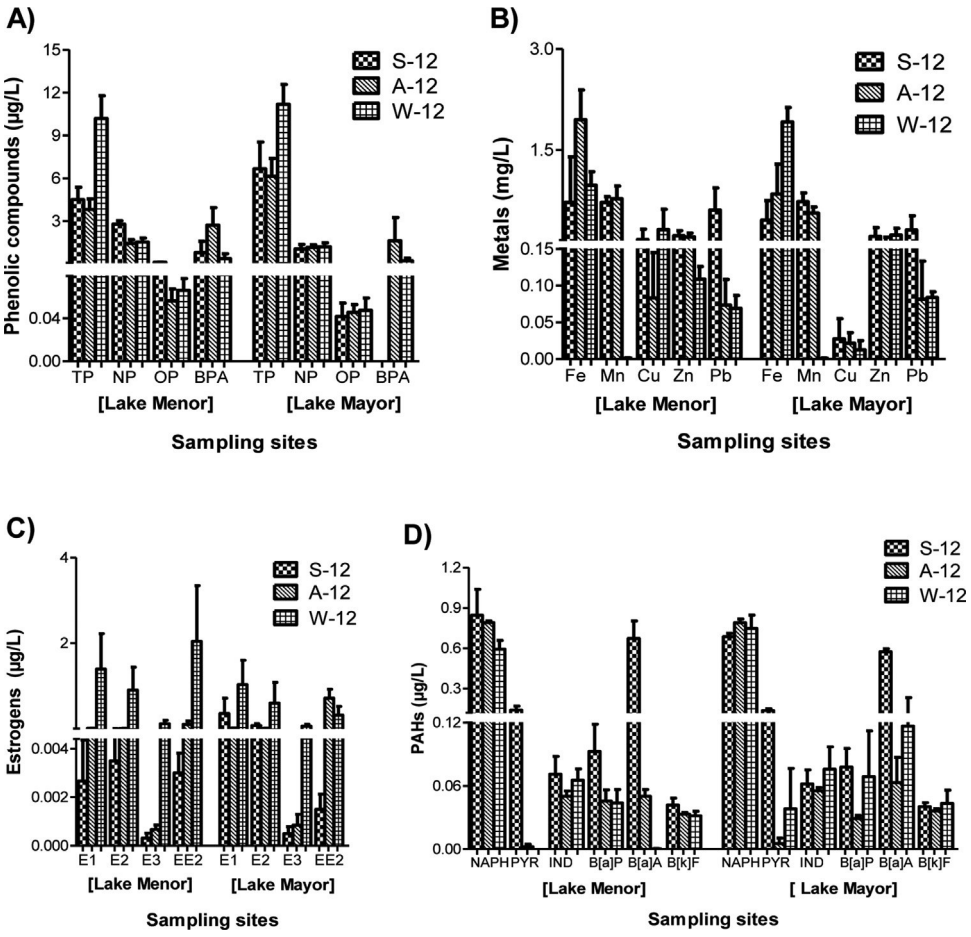

Fig. 6. Water levels of environmetal pollutants in the lakes under study. Panel (A) phenolic compounds, TP, total phenols; NP, nonyl phenol; OP, octyl phenol; BPA, bisphenol A. Panel (B) metals. Panel (C) estrogens (E_1, estrone; E_2, 17-β-estradiol; E_3, estriol; EE_2, ethinyl estradiol). Panel (D) PAHs (IND, indeno[1,2,3-*c,d*]pyrene; NAPH, naphthalene; PYR, pyrene; B[a]A, benzo[a]anthracene; B[k]F, benzo[k]fluoranthene; B[a]P, benzo[a]pyrene).

Farinati et al., 2002; Roy et al., 2007; Okoh et al., 2011). The $O_2^{\bullet}$ can achieve autodismutation by non-enzymatic via or dismutation by enzymatic via producing H_2O_2 (Hermes-Lima, 2004). Considering the role of CYP450 isoenzyme activities as a source of ROS and the alteration of CYP450 mRNAs by exposure to estrogenic compounds, we evaluated CYP 1A1 expression in the SML of wild blackfin goodeid, finding positive results. In the liver of *Carassius auratus* treated with EE_2, alterations in CYP 1A1 mRNA (Yan et al., 2012) and on CYP 1A1 protein expression in the liver of *Moxostoma hubbsi* (Maltais and Roy, 2014) were documented. A similar response was noted in male goldfish exposed to E_2 (Yan et al., 2013). CYP1A expression was also detected on the skin scales of *Carassius auratus* treated i.p. with β–naphthoflavone (Quirós et al., 2007) and in the scales of Atlantic salmon (*Salmo salar*) exposed to polychlorinated biphenyls (PCBs) and polyaromatic hydrocarbons (PAHs) (Rees et al., 2005; Van Beneden et al., 2010). The results of this study indicate that hydroxylation of exogenous estrogen in the SML mediated by CYP450 isoenzymes is likely to be a source of ROS. Differences with regard to male blackfin goodeids from a less estrogen-polluted lake (Lake Mayor) indicate that endogenous levels of these compounds in female fish from this lake can potentiate the toxicity of these endocrine disrupting compounds. However, these relationships were found only in the winter, suggesting that during the summer and autumn the concentration of estrogen in the water was below a threshold needed to induce CYP450 isoenzymes in the skin of the blackfin goodeid. In this regard, a concentration-dependent response was shown between E_2 concentrations in water and ROS generation in the liver of Japanese sea bass *Lateolabrax japonicus* (Thilagam et al., 2010). These authors concluded that hepatic ROS levels might serve as a biomarker to indicate estrogen contamination. This response could be augmented by cross-talk between the estrogen receptor (ER) and the aryl hydrocarbon receptor (AhR) agonist (Andersson et al., 2007; Mortensen and Arukwe, 2007; Vega-López et al., 2008b; Gräns et al., 2010; Yan et al., 2012), as is the case with exogenous estrogens and PAHs. Findings of PCA and RDA showed that the metabolism of certain PAHs such as benzo[k]fluoranthene and indeno[1,2,3-*c,d*]pyrene was also a source of ROS in the skin of the female blackfin goodeid from Lake Mayor. Despite there being no preceding studies about ROS generation mediated by CYP450 isoenzymes in the SML of fish species, in fish liver a potential impairment of electron flow during the redox process of these isoenzymes may generate ROS in the presence of molecular oxygen (Schlezinger et al., 2006; Vega-López et al., 2009; Arzuaga and Elskus, 2010). Independent of the source of ROS in the SML of the wild blackfin goodeid of both sexes, the greater levels of ROS in wild fish than in control specimens confirms the useful of evaluating ROS in the SML of *G. viviparus* in monitoring the metabolism of exogenous estrogens and some PAHs carried out in the fish skin, in addition to other compounds such as water total phenols (TP).

Relations between ROS levels with TP suggest that these compounds are metabolized in the osteoblast and osteoclast scales of the blackfin goodeid, generating ROS, which were diffusible to the mucus layer. In this regard, phenol could produce hydroquinones mediated by the activity of liver CYP450 isoenzymes. Further, the hydroquinones could suffer redox reactions by NADPH-cytochrome

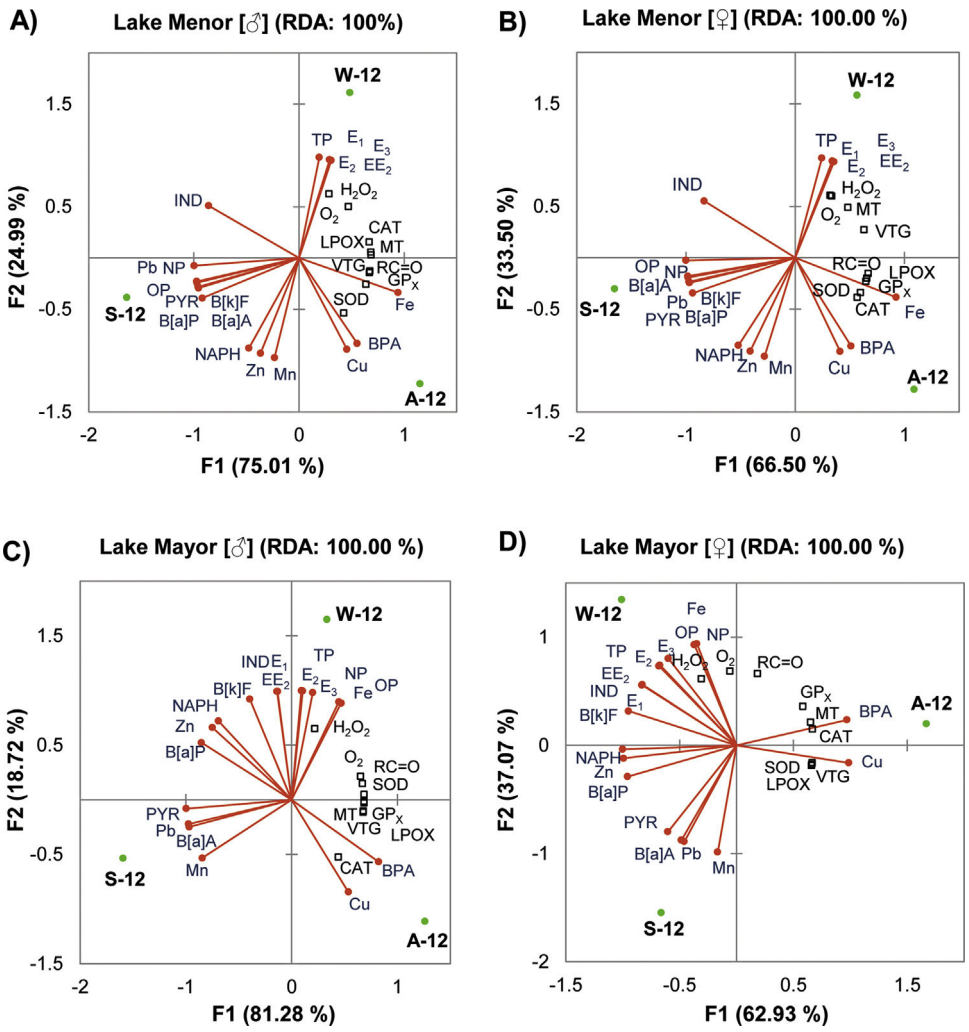

Fig. 7. Redundance analysis (RDA) showing the relationships between biological response variables: $O_2^{\bullet}$, H_2O_2, TBARS, RC=O, SOD, CAT, GPx, VTG and MT, and environmental data (explanatory variables: E_1, estrone; E_2, 17β-estradiol; E_3, estriol; EE_2, ethinyl estradiol; TP, total phenols; NP, nonyl phenol; OP, octyl phenol; BPA, bisphenol A; IND, indeno[1,2,3-*c*,*d*]pyrene; NAPH, naphthalene; PYR, pyrene; B[a]A, benzo[a]anthracene; B[k]F, benzo[k]fluoranthene; B[a]P, benzo[a]pyrene; Fe, Cu, Mn, Pb and Zn). In fish from Lake Menor the first bi-plot (RDA1) explained a high correlation between the response variables and explanatory variables, both in male (75.01%) and female fish (66.50%). In blackfin goodeid from Lake Mayor the ordination (RDA1) explained 81.28% and 66.50% of the variation in the data in male and female fish, respectively. The triplot (2-dimensional bi-plots; RDA1 and RDA2) showed 100% correlation between the response variables and explanatory variables. S-12: summer 2012; A-12: autumn 2012; W-12: winter 2012.

P450 reductase activity forming semiquinones, which under aerobic conditions may generate $O_2^{\bullet}$ (Joseph and Jaiswal, 1998; Roy et al., 2007; Okoh et al., 2011; Ramírez et al., 2013). Similar relationships were detected with nonyl phenol (NP) and octyl phenol (OP) only in the winter proposing that activities of enzymes involved in phenol metabolism in the SML of blackfin goodeid are subject to a self-regulation modulated by substrate concentration. By exposure to halogenated phenolic compounds ROS induction in other fish tissues was detected (Luo et al., 2008; Dong et al., 2009; Li et al., 2013). However, others studies under controlled conditions are needed to clarify these findings.

The oxidative stress response can be considered as a balance between pro-oxidant forces (i.e. $O_2^{\bullet}$, H_2O_2), biomarkers of oxidative damage (i.e. lipid peroxidation and protein oxidation), the activities of enzymes involved in antioxidant defense (SOD, CAT, GPx), and the content of unspecific antioxidant biomolecules (i.e. Hermes-Lima, 2004; Valavanidis et al., 2006; Vega-López et al., 2007b). In this way, it is important to explore the interrelations among these factors in order to reach a better understanding of the biological response and its relationship with toxicants able to induce this damage.

The current study showed a sex-linked response of the antioxidant enzymes that was related with ROS levels in the SML of male blackfin goodeid. Despite the importance of gender related differences in these enzymes, only a few studies have documented this phenomenon (McFarland et al., 1999; Meyer et al., 2003; Vega-López et al., 2007b). In an earlier study assessing a complete panel of antioxidant defenses, CAT activity was higher in male than in female rats (Pinto and Bartley, 1969) as were seen in the goby (*Zosterisessor ophiocephalus*) (Livingstone et al., 1995). In male *G. viviparus* (Vega-López et al., 2007b) and in male *Ameca splendens*, another member of the Goodeidae Family (Vega-López et al., 2009), hepatic SOD and CAT activities were greater than in female fish. These results are in agreement with the current study despite these studies being conducted in the liver; in contrast, in fresh plasma samples of the urodele amphibian *Pleurodeles waltl*, SOD and CAT activities were higher in females than in males (Alekhova et al., 2001). However, the relation to antioxidant defenses with ROS in the male fish SML could be indirect because some toxicants are able to induce oxidative stress on *G. viviparus* SML of both sexes.

During the seasons under study, the concentrations of toxic compounds able to oxidize lipids and proteins in the SML of

the blackfin goodeid showed fluctuations that were related with the biological response. In agreement with the current results, in *Acipenser ruthenus* spermatozoa an increase in SOD activity has been detected after exposure to BPA (Hulak et al., 2013), as in several mouse tissues (Kabuto et al., 2003). In contrast, diminution of these enzymes in zebrafish embryos was observed after short-term exposure to BPA (Wu et al., 2011), as was also the case in the testes of Sprague-Dawley rats (Chen et al., 2012). The results of the present study and preceding reports seem to be contradictory about the toxic effects of BPA elicited on antioxidant defenses; however, it is important to stress that the target tissue, species and exposure were not the same. Additionally, toxicants may overwhelm detoxification processes, particularly when the toxic effects involve an agent's adsorption, distribution, readsorption and toxication in the entire organism (Gregus, 2008). In this way, the relevance of the SML of fish in the context of an environmental monitoring program stands out because fish skin is a metabolically active site for certain compounds so it is unnecessary for all the processes cited above to occur.

It is well known that imbalances in electron flux during redox processing of metals (e.g. Fe and Cu) induce ROS by Fenton and Habber-Weiss reactions (Hermes-Lima, 2004). The relation of SOD and peroxidases in the SML of the blackfin goodeid with these divalent/polyvalent metals suggests that the high metabolism of the enzymes involved in antioxidant defenses is able to diminish ROS levels generated by redox processing of metals during the autumn compared to the winter. Similar results have been found in other tissues and fish species (Livingstone et al., 1995; McFarland et al., 1999; Meyer et al., 2003; Vega-López et al., 2012), even though the seasonality of the biological responses was a not clearly documented.

In female fish SML the median Cu concentration in the water and the highest BPA content were linked with the highest TBARS levels and with RC=O in specimens of both sexes from Lake Menor as observed also in the SML of female fish from Lake Mayor during October. These results suggest a probable synergism between Cu and BPA or potentiating the effects of BPA. To our knowledge previous reports about Cu and BPA interactions on oxidative stress in fish do not exist; however, in some studies increased damage of this type was found in fish exposed to BPA. By the other hand, in the SML of Lake Mayor male specimens relationships of TBARS and RC=O with the NP and OP content of the water without seasonality were found. In sterlet (*Acipenser ruthenus*) spermatozoa (10^8 cells) treated with BPA or with NP an increase in TBARS and RC=O was observed from 2.5 to 10 μg/L (Hulak et al., 2013). Lipid peroxidation accompanied by decreases in SOD and CAT activities in a concentration-dependent pattern above 10 μg/L in zebrafish embryos exposed to BPA has also been found (Wu et al., 2011). The unfertilized eggs of Chinese rare minnow (*Gobiocypris rarus*) treated with NP from 50 to 200 μg/L showed a positive correlation between ROS, TBARS and protein carbonyl induction accompanied by SOD inhibition (Zhang et al., 2008). Oxidative damage elicited by NP and BPA in the blackfin goodeid can be explained by ROS generation and inhibition of antioxidant enzymes, as occurs in mammals (Choi et al., 2014). Using molecular docking it was documented that mammalian CAT and SOD were maximally inhibited by BPA and NP, respectively (Jayakanthan et al., 2015). However, during the summer the lower ROS content, TBARS and RC=O levels and low activities of antioxidant defenses in the SML of male blackfin goodeid from Lake Mayor coincided with the high NP concentration. These findings suggest that oxidative damage by exposure to NP and OP is a discrete process, probably affecting the fish when other alkyl phenols such as BPA reach threshold levels needed to induce oxidative stress. It stands out that the metabolism of phenolic compounds is surely a source of $O_2^{\bullet}$ and H_2O_2 in the SML of *G. viviparus* despite differences in the chemical structure of these toxicants. In addition, the present results provide greater evidence about the environmental relevance of phenolic compounds and show a greater sensitivity of the skin of adult *G. viviparus* to oxidative stress elicited by alkyl phenols than did zebrafish embryos, sterlet spermatozoa and eggs of Chinese rare minnow. Further studies under controlled conditions are needed to reinforce or refute the current supposition about the role of phenol metabolism as a source of ROS and oxidative damage in the SML of *G. viviparus*.

Environmental levels of Fe and Cu contributed to lipid peroxidation and protein oxidation in the SML of the blackfin goodeid of both sexes. However, the greatest oxidative damage in the SML of Lake Menor specimens coincided with the higher levels of these metals found during the autumn, contrasting with a less differentiated seasonality in the SML of blackfin goodeid from Lake Mayor. The coincidence in the peaks of SML oxidative stress biomarkers from Lake Menor specimens with the highest heavy metal contents (Fe and Cu) suggest a true statistical relationship, contrasting with the findings of Lake Mayor specimens, where the observed relations could be considered a coincidence fact attributable to other toxic compounds as discussed above.

The role mixtures of metals play as well as environmental exposure in oxidative stress induction in the gill, liver and kidney of some fish species is well known (i.e. Oakes and Van Der Kraak, 2003; Valavanidis et al., 2006; Monserrat et al., 2007; Sevcikova et al., 2011; Mahboob, 2013). The current results evaluated as TBARS and RC=O in the SML of the blackfin goodeid are in agreement with previous results showing that this target is suitable for monitoring oxidative stress. However, there have been few reports about mucus production as a resistance mechanism in fish exposed to metals (McDonald and Wood, 1993; Andreji et al., 2005). The brown trout (*Salmo trutta*) treated with Cu produces a significant amount of mucus to reduce the uptake of Cu into the gills (Hansen et al., 2007). These authors proposed that acclimation to chronic metal exposure involve different strategies such as mucus production as well as the activation of metal-related stress gene transcription (metallothioneins). The concentration of sialic acid as a measurement of mucus production in the intestine of the rainbow trout (*Oncorhynchus mykiss*) pretreated with Zn in the diet was increased by a single dose of Cd (Khan and McGeer, 2013). However, in the liver of *Oreochromis* sp. exposed a significant induction of MT was detected; nevertheless, the density and size of producing-mucus cells on the gills were not changed (Wu et al., 2007). This result showed that there is not a direct correlation between MT and mucus content in fish exposed to metals. Regarding the possible role of MT in diminution of oxidative damage, the current results suggest that MT in the SML does not protect the skin of the blackfin goodeid against the oxidative stress elicited by metals because the peak of MT production in this fish species coincided with greater oxidative damage. However, the increase in mucus production in fish exposed to metals (Hansen et al., 2007; Khan and McGeer, 2013) is crucial considering the extent of the skin surface as the first target organ and by the role of the skin in innate immunity (Ellis, 2001; Tasumi et al., 2004; Tsutsui et al., 2005; De Veer et al., 2007; Esteban, 2012). This stresses the need for more studies to clarify the MT content in the mucus of fish exposed to metals.

For first time it was found by PCA and RDA that VTG levels in the SML of male blackfin goodeid from both lakes and female fish from Lake Menor were related to the concentration of BPA in the water during the autumn. To our knowledge there are no preceding reports about VTG induction in the SML of fish species by exposure to BPA; however, the estrogenicity of this compound is wide recognized (Larsen et al., 2006; Crain et al., 2007; Shanle and Xu, 2011). The estrogenicity of BPA has also been documented in the liver of females of this fish species using canonical correspondence analysis (Olivares-Rubio et al., 2015). Vitellogenin, a phosphoglycolipoprotein precursor of oocyte yolk, is synthesized in the liver

of egg-laying vertebrates under the control of 17-β estradiol (Tata and Smith, 1979). Nevertheless, many sorts of toxicants can activate the estrogen receptor (ER) due to its high promiscuity (Shanle and Xu, 2011), inducing VTG synthesis (Van der Oost et al., 2003). VTG in the SML of male fish from Lake Mayor was related to the lower concentrations of NP and OP in the water as documented in the SML of other fish species (Meucci and Arukwe, 2005; Arukwe and Røe, 2008; Rey Vázquez et al., 2009; Maltais and Roy, 2014). The lack of a relationship of NP and OP concentrations in the water with VTG in the SML of male blackfin goodeid from Lake Menor and in female fish from both lakes probably confirms the discrete process of oxidatition elicited by NP and OP affecting the fish response as discussed above.

Independent of the toxic relationships, after VTG synthesis and its post-transcriptional modification, this protein is released to the bloodstream to reach its target tissue, which in female fish is the oocyte (Tata and Smith, 1979). During the circulation of VTG in the bloodstream it is possible that this protein can reach the SML due to the elevated vasculature in the skin; however, previous studies about VTG transfer to fish skin do not exist. Despite the foregoing it has been proposed that VTG confinement in mucus vacuoles of the skin serves as an excretory pathway in male fish (Moncaut et al., 2003). Nevertheless, in the SML of female fish VTG could have a pheromonal function as has been proposed for female garter snakes (Garstka and Crews, 1981), as in the South American cichlid fish *Cichlasoma dimerus* (Moncaut et al., 2003).

Interestingly, VTG levels in the SML were related with the Fe and Cu content of the water, both in male and female blackfin goodeid, using PCA or RDA. However, the relationships apparently indicate that these metals potentiate the endocrine disrupting effects of alkylphenols and estrogens. Nevertheless, further studies under controlled conditions are required on this topic.

The results of the current study showed a larger condition factor (K) for the Lake Menor specimens with regard to fish from Lake Mayor. This response probably could be explained by the amount of available food in Lake Menor, where a greater diversity of phytoplankton was reported (Vega-López et al., 2013). It is considered that a K value greater than 1.0 denotes a better condition of the fish (Le Cren, 1951; Jin et al., 2015). Although this is true for practical purposes in fisheries (Jennings et al., 2014), anthropogenic alterations could decrease the body size of fishes (Audzijonyte et al., 2013). In a program to monitor fish health as in our study, the K value does not reflect the impact of toxicants on the fish skin of both sexes of blackfin goodeid. Another feature commonly used to describe a fish population is its weight (Jin et al., 2015). Male fish from Lake Mayor attained a greater weight, which was accompanied by low levels of damage in the SML regarding male fish from Lake Menor. A comparison between the weights of fish with the biomarkers evaluted in the SML did not provide confident findings because this factor could be influenced by the liver weight.

5. Conclusion

The current results corroborate that the skin mucus layer (SML) of wild *G. viviparus* is a sensitive target for monitoring the impacts of complex mixtures of toxicants found in aquatic ecosystems from the Valley of Mexico. The metabolism of estrogenic and phenolic compounds such as alkylphenols is involved with the biological response in the skin of this fish species, possibly mediated by ROS generation. This statement is based on the number of the relationships between ROS ($O_2^{\bullet}$, H_2O_2), oxidative stress biomarkers and the activities of enzymes involved in antioxidant defense such as SOD, CAT and GPx. In this regard, it is possible to propose the following toxicological order of importance: BPA > E_1, E_2, E_3, EE_2 > total phenols > NP and OP in addition to Fe and Cu. VTG content in the SML of the blackfin goodeid showed a sex-linked response, i.e. in male fish levels of this biomolecule were influenced by BPA during the autumn and apparently potentiated by Cu and Fe. In contrast, in the SML of female fish, the endocrine disrupting effect was due to the influence of estrogens during the winter. On the other hand, MT was correlated with Fe and Cu; however, it was not possible to affirm that MT in the SML played a protective role in reducing oxidative stress elicited by metals. What stands out is a sex-linked response in the SML of *G. viviparus* that depends on the nature of the toxicants, their concentrations, bioavailability and interactions, in addition to the season of the year. Further studies under controlled conditions are needed to clarify these assumptions with the aim of validating this approach to monitoring of fish health.

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