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claudio.amescua@atmosfera.unam.mx

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Olivares-Rubio, Hugo F.; Dzul-Caamal, Ricardo;
Nájera-Martínez, Minerva; Vega-López, Armando
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**DISRUPTION OF HYPOTHALAMUS-PITUITARY-LIVER-GONADS AXIS IN THE
ENDANGERED *Girardinichthys viviparus* EXPOSED TO ENVIRONMENTALLY RELEVANT
CONCENTRATIONS OF A MIXTURE OF METALS AND WITH 17 α -ETHYNIL ESTRADIOL**

Hugo F. OLIVARES-RUBIO, Ricardo DZUL-CAAMAL, Minerva NÁJERA-MARTÍNEZ and
Armando VEGA-LÓPEZ*

Laboratorio de Toxicología Ambiental, Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional.
Avenida Wilfrido Massieu, Unidad Profesional Zacatenco, Ciudad de México, México, C. P. 07320
*Corresponding author: avegadv@yahoo.com.mx

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Key words: gonadotropins, estradiol, vitellogenin, metallothionein, sex-linked response

ABSTRACT

Girardinichthys viviparus is an endemic and endangered Mexican fish species with matrotrophic viviparity that only inhabits in some polluted water bodies in the Valley of Mexico. In the current study, *G. viviparus* of both sexes were exposed for 21 days to a mixture of metals with relevant environmental concentrations (T1) and to the same mixture spiked with 25 ng of 17 α -ethynil estradiol (EE₂)/L (T2). Some biomarkers involved in endocrine disruption of the hypothalamus-pituitary-liver-gonads axis such as gonadotropins I and II (GtH I and GtH II, respectively), and estradiol (E₂) concentrations in the head and gonads were measured. Vitellogenin (VTG) in the liver and gonads, and metallothionein (MT) in the head, liver and gonads were assessed. Increases of GtH I and decreases in GtH II, and alterations of E₂ in the head and gonads were found in fish treated with T1 and T2. Higher content of hepatic and gonadal VTG only in fish treated with T2 was detected. MT was notably induced by T2; however, a time-dependent MT reduction was observed. In both treatments, the hypothalamic-pituitary control point was most affected and their alterations were documented by gonadal and head content of E₂. In female fish, it is most likely that endogenous levels of E₂ diminished the alterations elicited by EE₂ on this control point of the axis in contrast with male fish. The endocrine disruption of this fish species is a dynamic and complex process.

Palabras clave: gonadotropinas, estradiol, vitelogenina, metalotioneínas, respuesta ligada al sexo

RESUMEN

Girardinichthys viviparus es un pez mexicano endémico y en peligro de extinción con viviparidad matrotrófica que sólo habita en algunos cuerpos de agua contaminados del Valle de México. En este estudio, especímenes de *G. viviparus* de ambos sexos fueron expuestos durante 21 días a una mezcla de metales a concentraciones ambientalmente relevantes (T1) y a la misma mezcla enriquecida con 25 ng de 17 α -etnil estradiol (EE₂)/L (T2). En la cabeza y las gónadas se midieron biomarcadores implicados en el eje endocrino sexual hipotálamo-pituitaria-gónadas-hígado, así como las concentraciones de estradiol (E₂), gonadotropina I y II (GtH I y GtH II, respectivamente). En el

hígado y las gónadas se evaluó la vitelogenina (VTG) y en la cabeza, el hígado y las gónadas los niveles de metalotioneínas (MT). En la cabeza y las gónadas de los peces expuestos a ambos tratamientos se observaron aumentos de la GtH I, disminuciones de GtH II y alteraciones de E_2 . Solamente en los peces expuestos al T2 se encontraron incrementos hepáticos y gonadales de VTG. Las MT fueron inducidas particularmente por el T2. Sin embargo, se observó una reducción dependiente del tiempo. En ambos tratamientos, el punto de control hipotálamo-pituitaria fue el más afectado y sus alteraciones fueron evidenciadas por los contenidos de E_2 . En contraste con los machos, en hembras es probable que los niveles endógenos de E_2 disminuyeran por las alteraciones provocadas por EE_2 en este punto de control del eje. La disrupción endocrina de esta especie de pez es un proceso dinámico y complejo.

INTRODUCTION

In teleost fish, the reproduction is regulated by the hypothalamus-pituitary-liver-gonads (HPLG) axis (Janz and Weber 2000). In response to external and internal stimuli, the gonadotropin-releasing hormone (GnRH) is liberated from hypothalamus to the gonadotropic cells stimulating the release of gonadotropins (GtHs) (Bailhache et al. 1994, Rempel and Schlenk 2008). Both gonadotropins GtH I and GtH II could bind to specific receptors in the testis and ovaries during the final stages of gamete maturation for the synthesis of steroid hormones as 17β -estradiol (E_2) in females and 11-ketotestosterone in males (Janz and Weber 2000). Steroid hormones are responsible for most biological activities such as the involvement in the control of sexual differentiation, maturation and reproduction (Diotel et al. 2010). The process of estrogen biosynthesis is catalyzed by cytochrome P450 aromatase from androgens (Diotel et al. 2010, Coumailleau et al. 2015). In the other hand, in fish the vitellogenin (VTG), a precursor of the oocyte yolk is synthesized in the liver by the activation of estrogen receptor (ER) mainly by E_2 (Sumpter and Jobling 1995).

In the aquatic ecosystems, many endocrine disrupting compounds (EDCs) can be found, such as the case of 17α -ethynil estradiol (EE_2) used in oral contraceptives with high estrogenic potency (Shanle and Xu 2011). Many studies in fish have documented that EE_2 possesses high affinity with ER and could increase the VTG expression and synthesis in both male and female fishes (Folmar et al. 2000, Rose et al. 2002, van den Belt et al. 2003).

A similar case occurs with heavy metals, which can alter the normal function of the gonads in fish (Amutha and Subramanian 2013), inducing a reduction in the size of the gonads (Łuszczek-Trojnar et al. 2014), disrupting the reproductive neuroendocrine function (Khan and Thomas 2000) and some metals are

involved in the activation of ER through “zinc fingers” at pre-transcriptomic level (Darbre 2006). In the environment, it is possible that some metals could alter the HPLG axis (Simmons et al. 2014, Olivares-Rubio et al. 2015). Divalent metals increase the expression of VTG in the western mosquitofish (*Gambusia affinis*) (Huang et al. 2014) and potentiating the estrogenic capacity of E_2 on goldfish (*Carassius auratus*) hepatocytes (Chang et al. 2011) that are essential for VTG synthesis such as Zn and Ca (Falchuk and Montorzi 2001). Several mechanisms are involved in the binding of metals to cellular components such as the metallothioneins (MT) synthesized by animal cells (Palacios et al. 2011, Sears 2013). However, a lack of information prevails about the possible toxic effects of metals alone and in mixture, and in the presence of exogenous estrogens in the performance of HPLG axis. This information is relevant because in the aquatic environment, the fish species could be exposed to the complex mixture of pollutants including metals and xeno-estrogens capable to disrupt this axis at different points of control.

The mexcalpique (*Girardinichthys viviparus*) is an endemic and critically endangered fish species belonging to family Goodeidae. This fish species inhabits only in some water bodies in the Valley of Mexico. The mexcalpique possess a matrotrophic viviparity as a reproductive strategy (Vega-López et al. 2007). Recently, our research team found that wild *G. viviparus* inhabitant of two lakes with different degree of pollution in Mexico City suffers a noticeable disruption of the sexual endocrine axis, mainly in male fish influenced by *cyp* 1A1 activity and by EE_2 exposure (Olivares-Rubio et al. 2015). In addition, negative effects of Pb in the HPLG axis were documented (Olivares-Rubio et al. 2015). Therefore the aim of this study was to assess a battery of biomarkers involved in the HPLG axis in mexcalpique treated with environmental relevant concentrations of mixture of metals and with the same mixture of metals spiked with 25 ng EE_2 /L at 7, 14 and 21 days

of exposure. This concentration of EE₂ was chosen because the maximum contents of hepatic VTG were found at this treatment after 7, 14 and 21 days of exposure in this fish species (data not published). In the head, the content of GtH I, GtH II, E₂ and MT were measured to evaluate the alteration on hypothalamus-pituitary control point. In the liver, the content of VTG and MT as biomarker of estrogenic response and binding of metals, respectively, were quantified. In the gonads the content of GtH I, GtH II, E₂, and of VTG were assessed since the gonads are a target tissue of gonadotropins and are a source of E₂ production and VTG storage in female fish.

MATERIAL AND METHODS

Fish species and experimental design

Specimens of *Girardinichthys viviparus* born in laboratory within the age of 8-10 months (24.4-25.3 and 42.3-45.1 mm of standard length for males and females, respectively) were used. These differences in the size among sexes of this fish species are due to its sexual dimorphism (Vega-López et al. 2007, Sedeño-Díaz and López-López 2009). Fishes were separated by sex, one month before the experiments to avoid the courtship and activation of the endocrine sexual axis. The exposure conditions were the following: *i*) Treatment 1 (T1), a mixture of metals at environmentally relevant concentrations found in the lakes of 2nd Section of Chapultepec Park (Cu = 0.4 mg/L, Fe = 0.9 mg/L, Mn = 0.3 mg/L, Pb = 0.13 mg/L and Zn = 0.15 mg/L) (Olivares-Rubio et al. 2015), *ii*) Treatment 2 (T2), the same mixture of metals spiked with 25 ng EE₂/L. For the experiments, 12 males and 12 females of mexcalpique were placed in each glass tank (7 L of test volume) using semi-hard synthetic water (120 mg/L as CaCO₃) as dilutant in three independent experiments. Standard solutions of zinc sulphate, chloride iron, lead nitrate, manganese sulphate and copper sulphate dissolved in ultra-pure water at 10.0 g/L were prepared just before the beginning of the experiments. Working solutions of metals were prepared and stored at 4 °C using semi-hard synthetic water as dilutant. On the other hand, standard solution of EE₂ at 1.0 mg/mL dissolved in ethanol HPLC (Sigma-Aldrich™) was done, dissolution was performed to reach 1.0 mg/L. Total ethanol content by tank was 25 µL/L in fish exposed to T2. During the exposure, controls and treated fishes were not fed and were maintained by constant aeration and temperature (23 ± 2 °C), to a natural light cycle. Fishes were not fed because

undigested food, dregs and detritus can modify the concentration and bioavailability of EE₂ and metals in the water. Each day for seven days, four males and four females were euthanized by rapid freezing (15 min/-80 °C) according to the Mexican protocol for the production, protection and welfare of experimental animals (SAGARPA 2001) and necropsy was done. A total change of water was done after each sampling, and sufficient amount of standard solutions of metals and EE₂ was added until the nominal concentrations of the test were reached. Control fish (12 males and 12 females) were maintained under the same conditions, without EE₂, and metals and were sacrificed at days 7, 14 and 21.

Physicochemical analysis

Water samples (1 L) were collected every week (7, 14 and 21 days) just after the euthanization of fishes and the replacement of totality of water for chemical analysis. Physicochemical parameters, including conductivity, total dissolved solids, salinity, dissolved oxygen saturation, pH, and redox potential were evaluated each day using a YSI Mod 556 MPS multiparametric probe. Concentrations of metals (Cu, Fe, Mn, Pb and Zn) were evaluated by acid digestion followed by flame atomic absorption spectrometry using the direct air-acetylene flame method 7000B published by Environmental Protection Agency of The United States of America with a GBC 932 Plus spectrophotometer as in an earlier study (Vega-López et al. 2013). Quantification EE₂ was performed according to Wang et al. (2006) with modifications using a Shimadzu HPLC system coupled to a UV detector (Olivares-Rubio et al. 2015).

Biomarkers

Necropsy was done immediately after sacrifice to obtain the head under stereoscopic microscope and avoiding the inclusion of opercula, gills, eyes and jaws. Also, the liver and the gonads were obtained. Tissues were weighed to within 0.1 mg and homogenized in 500 µL with phosphate buffer solution (1X PBS) using Teflon micropestles. The homogenates were centrifuged at 4980 X g (9000 rpm) and 4 °C for 15 min in a Hermle Labnet Z216MK centrifuge to obtain the cytosolic fraction and stored at -70 °C until the biomarker assay (less than one week).

Gonadotropins and estradiol

Measuring of gonadotropins I and II (GtH I and GtH II) and estradiol (E₂) in the head and gonads was performed using specific ELISA kits for fish from CUSABIO™ Hubei Province 430206, P.R. China.

The ELISA kits employed were Fish Follicle-Stimulating Hormone ELISA Kit (CSB-E15790Fh), Fish Luteinizing Hormone ELISA Kit (CSB-E15791Fh) and Fish Estradiol ELISA kit (CSB-E1301Fh). CUSABIO™ guaranteed specificity for fish species GtH I, GtH II and E₂ and declares no significant cross-reactivity and interferences. No reactivity analysis with GtH I, GtH II and E₂ specific of *G. viviparus* were done since manufacturer statement and because specific standards for this species do not exist. In addition, no procedures of purification for these gonadotropins and E₂ were employed since the CUSABIO™ ensures that this quantification (GtH I and GtH II) could be done directly in serum and plasma of fish species. However, the small size of this fish species is a real impediment to obtain the blood in enough quantities for these assessments. As a consequence, the centrifuged head homogenate was employed for gonadotropins evaluation. In the case of E₂ measurement, it could be performed in tissue homogenates. According to CUSABIO™ ELISA Kits, method detection limits for GtH I was less to 0.4 mIU/mL, less to 2.5 mIU/mL in the case of GtH II, and 25 pg/mL for E₂. The intra and inter assay variance for GtH I, GtH II and E₂ were lower than 8 % expressed as variance coefficient (% CV). The results were shown as International Units (IU) of GtH I and GtH II /g tissue (wet wt), meanwhile, the estradiol concentration was presented as ng/g tissue.

Vitellogenin and metallothionein

The content of vitellogenin (VTG) was determined in the liver because in this tissue their synthesis occurs and in the gonads because in females they are the immediate target organs, meanwhile in males does not have target organ (Vega-López et al. 2007). However, VTG evaluation in the testis was performed. The quantification was made by enzyme-linked immunosorbent assay (ELISA) in an inhibition format using rabbit polyclonal anti-VTG serum as described earlier (Vega-López et al. 2006). The method detection limit (MDL) of hybrid ELISA was calculated to be 0.005 ng of *G. viviparus* VTG. MT induction and purification was performed by the method of Pedersen et al. (1994) with some modifications. Specimens of *G. viviparus* (10 males and 10 females) were exposed to 0.1 ppm of CdCl₂ (JT Baker®) for 25 days. The MT fraction of the livers was concentrated on an Amicon 202 ultra-filtration system of molecular mass cut-off 5 kDa. The purified protein was used to obtain polyclonal antibodies in New Zealand rabbits in accordance with earlier reports (Vega-López et al. 2006). MT

was measured in the liver, in the head and in the gonads of the mexcalpique, and quantification was performed with six replicates in three independent experiments by ELISA in an inhibition format using rabbit polyclonal anti-MT serum diluted at 1:7000. The MDL was of 0.012 ng of *G. viviparus* MT. Both, VTG and MT content were expressed as ng/g tissue (wet wt) or pg/g tissue as convenient. Total protein content was determined according to Bradford (1976) using bovine serum albumin (Merck) as a standard.

Statistical analysis and Integrated Biomarker Response Index version 2

With the aim to find statistical significant differences of the biomarker responses measured in *G. viviparus* with regards to control fish and among sexes at the same treatment, we used a one-way ANOVA with Bonferroni's post-test using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA. Correlations between biomarkers were estimated using the Pearson's correlation coefficient as linear correlation was performed using SPSS v.12.0 software. Integrated Biomarker Response version 2 (IBRv2) developed by Sanchez et al. (2012) to evaluate the integrated biological response by effects of treatments, exposure time and sex was used. This index assesses the deviation between biomarkers of specimens collected from a polluted, stressed site or exposed under controlled conditions compared to those from a reference site, unexposed, less polluted or not stressed animals.

RESULTS

Physicochemical analysis

Physicochemical variables evaluated with multiparametric probe were stable during both experiments (and its replicate) showed the following mean \pm standard deviation values: conductivity, 2.25 ± 0.035 mS/cm; total dissolved solids, 1615.6 ± 25.86 mg/L; salinity, 1.264 ± 0.022 ppt; dissolved oxygen saturation presented in Mexico City (68-75 %) within 6.85 ± 0.0512 mg O₂/L; pH, 8.72 ± 0.056 and redox potential, -52.63 ± 8.39 . Metals and EE₂ concentrations in water samples was lower than the nominal concentration (**Table I**).

Biomarkers

Gonadotropins and estradiol

The content of GtH I in the head and gonads in the mexcalpique exposed to T1 and T2 was greater than in control fish, particularly in the head of males ($p < 0.05$;

TABLE I. CHEMICAL ANALYSIS OF WATER SAMPLES OBTAINED FROM THE TWO TREATMENTS BY DUPLICATED

Control			
Cu (mg/L)	0.098 ± 0.042		
Fe (mg/L)	0.062 ± 0.022		
Mn (mg/L)	0.041 ± 0.029		
Pb (mg/L)	ND		
Zn (mg/L)	0.106 ± 0.073		
EE ₂ (ng/L)	ND		
T1			
	7 days	14 days	21 days
Cu (mg/L)	0.308 ± 0.102	0.239 ± 0.069	0.236 ± 0.098
Fe (mg/L)	0.725 ± 0.122	0.684 ± 0.186	0.689 ± 0.206
Mn (mg/L)	0.029 ± 0.013	0.037 ± 0.026	0.067 ± 0.041
Pb (mg/L)	0.103 ± 0.041	0.099 ± 0.058	0.078 ± 0.049
Zn (mg/L)	0.097 ± 0.022	0.021 ± 0.015	0.026 ± 0.016
EE ₂ (ng/L)	ND	ND	ND
T2			
	7 days	14 days	21 days
Cu (mg/L)	0.207 ± 0.058	0.189 ± 0.075	0.250 ± 0.105
Fe (mg/L)	0.793 ± 0.233	0.788 ± 0.216	0.803 ± 0.103
Mn (mg/L)	0.103 ± 0.07	0.178 ± 0.08	0.109 ± 0.06
Pb (mg/L)	0.086 ± 0.075	0.091 ± 0.064	0.076 ± 0.062
Zn (mg/L)	0.024 ± 0.017	0.023 ± 0.019	0.024 ± 0.025
EE ₂ (ng/L)	12.25 ± 5.98	13.32 ± 6.41	14.65 ± 7.54

Mean ± standard deviation, ND = no detected. T1 = treatment 1, mixture of metals at environmentally relevant concentrations (Cu = 0.4 mg/L, Fe = 0.9 mg/L, Mn = 0.3 mg/L, Pb = 0.13 mg/L and Zn = 0.15 mg/L). T2 = treatment 2, the same mixture of metals spiked with 25 ng EE₂/L.

Fig. 1A and B). The amount of this hormone was higher in male fishes than in female fishes. By exposure to metals (T1), GtH I in males presented an inverse time-dependent response in both head and gonads (**Fig. 1A and 1B**). The effect of metals in addition to EE₂ (T2) about GtH I content induced an irregular response in the time in the males and females (**Fig. 1A and B**). In contrast, GtH II in both tissues (head and gonads) was lower than in the control group. In the head of males exposed to T1, GtH II levels were inversely related to the exposure time ($p < 0.05$; **Fig. 1C**); meanwhile, in the fish exposed to T2, a noticeable diminution in GtH II was found (**Fig. 1D**). In the gonads of males and females, an inverse time-dependent response about GtH II levels was detected, undetectable at the end of the exposure ($p < 0.001$; **Fig. 1D**).

On the other hand, the content of estradiol showed differences between sexes and tissues. In the head of male *G. viviparus* treated with T1 and T2, concentration of E₂ increased almost in a

time-dependent manner, particularly in those exposed to T2 that showed significant differences regarding to controls ($p < 0.05$ and 0.01) (**Fig. 2A and 2B**). In contrast, in the head of female fishes exposed to T1, a peak of E₂ was detected at day 14, posterior a decline occurs. In the gonads of females exposed to T1 and T2, a decay of E₂ levels was noted particularly at day 7 ($p < 0.05$). However, a slight recovery of E₂ levels at day 14 and 21 was detected; but in no case the basal level was reached.

Vitellogenin and metallothionein

In general, metals did not stimulate the VTG content, neither in the liver nor in gonads of the mexcalpique with regard to control group. In contrast, the effect of T2, VTG concentration in liver and gonads of male fishes were higher than in the control group ($p < 0.05$ and 0.01 ; **Fig. 2C and 2D**). However, this induction was not sustained over the time as observed also in the liver of female fish

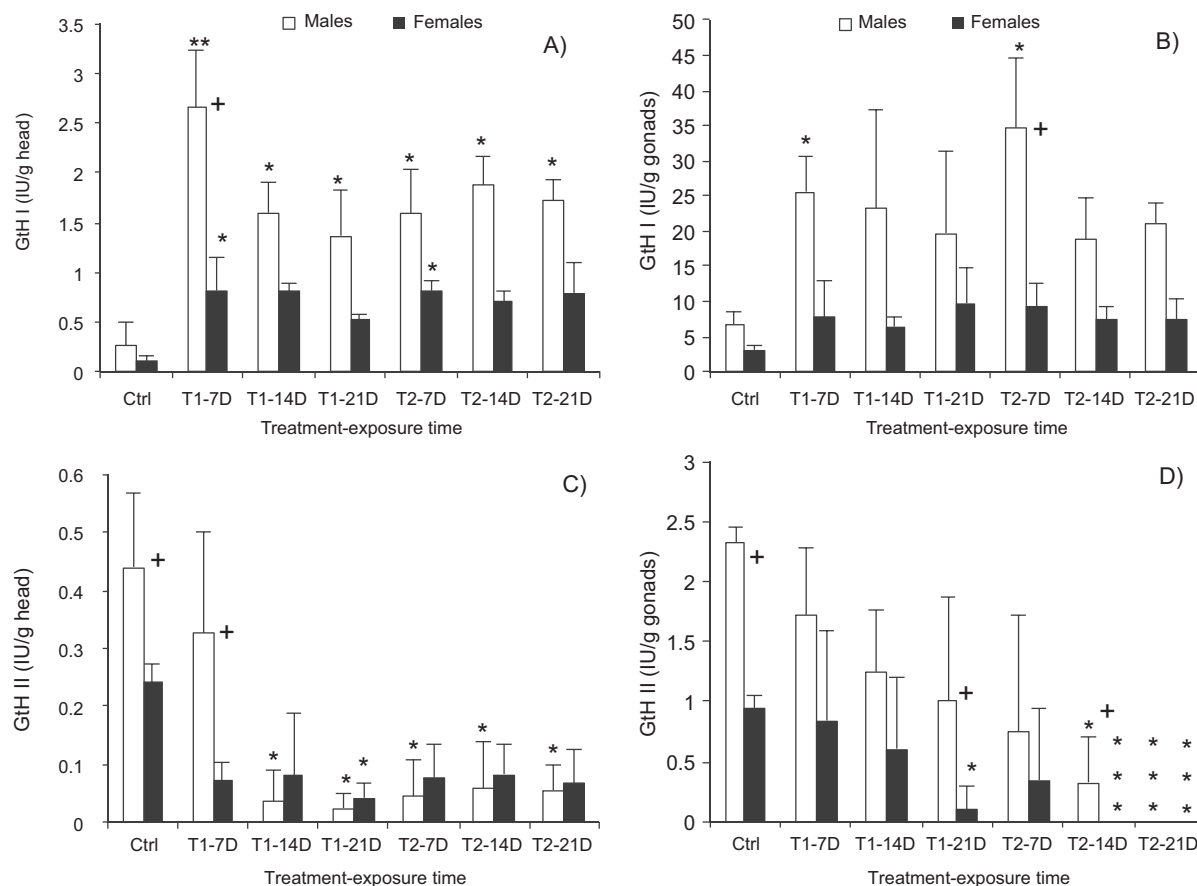


Fig. 1. Content of gonadotropins I (GtH I) and II (GtH II) in the head and gonads of *Girardinichthys viviparus* exposed under controlled conditions to mixture of metals (T1) and mixture of metals spiked with 25 ng/L of ethinyl estradiol (T2). 7D = 7 days, 14D = 14 days and 21D = 21 days. A) content of GtH I in the head, B) content of GtH I in gonads, C) content of GtH II in head and D) content GtH II in gonads. Bars represent the mean and standard deviation of data set. Asterisks denote significance difference regarding to their corresponding control group (sex) * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$; + denote significance difference at $p < 0.05$ between sexes in the same treatment

(Fig. 2C). Meanwhile, in the gonads of female fish exposed to T2, VTG induction in a time-dependent manner was observed (Fig. 2D).

The concentration of MT was higher in males than in females in the tissues under study in control fish as in fish exposed with the exception of hepatic MT female controls. In the head of fish of both sexes exposed to T1 and T2 induction of MT, an inverse time-dependent manner was detected (Fig. 3A). However, in the liver and in the gonads in fish exposed to T1, an induction of MT with a peak at 14 days was found mainly in male fish ($p < 0.01$), but MT levels were not sustained at 21 days. In fishes exposed to T2, initial induction of MT prevails particularly in male fish than in female fish (Fig. 3B and 3C).

In the *G. viviparus* exposed with T1, a number of relations between biomarkers were found, particularly in male fish. In contrast, in fish exposed to T2,

the relations were lower than in fish exposed to T1 (Table II).

Integrated Biomarker Response Index version 2 (IBRv2)

In an interesting way, the greater values of IBRv2 were found at 14 days in male and female fish exposed to both treatments (T1 and T2), except for female fish treated with T2 where the greater IBRv2 was found at 7 days (Table III). The star plot area values showed a sex-linked response. In male fish exposed to T1, an induction of MT in the head, liver and gonads and elevation of GtH I and E_2 in the head was noted with regard to T2; in contrast, in male fish treated with T2, an increase of VTG in the liver was found (Fig. 4). Compared with male fish, in female fish, the integration of the response was lower in specimens treated with both treatments (Fig. 5).

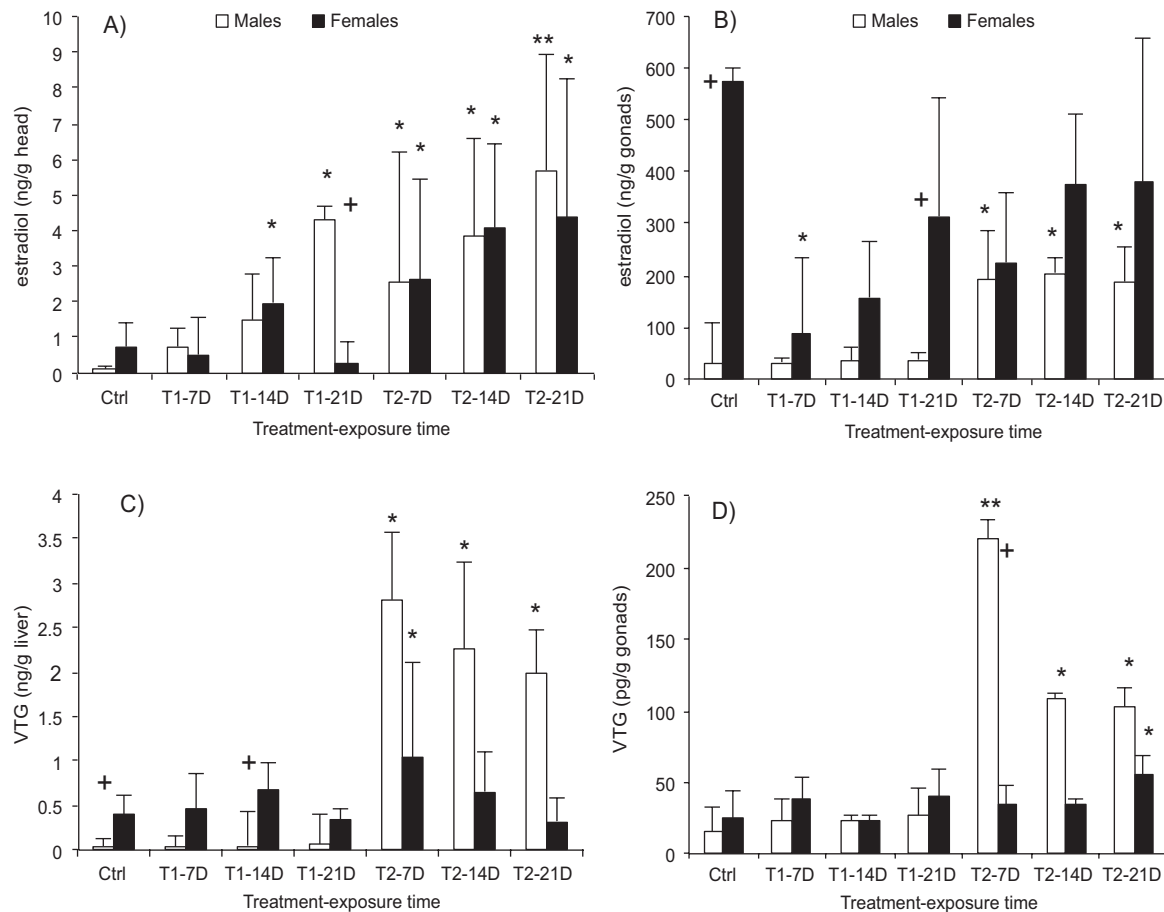


Fig. 2. Content of estradiol (E_2) in the head and gonads and vitellogenin (VTG) in the liver and gonads of *Girardinichthys viviparus* exposed under controlled conditions to mixture of metals (T1) and mixture of metals spiked with 25 ng/L of ethynil estradiol (T2). 7D = 7 days, 14D = 14 days and 21D = 21 days. A) content of E_2 in the head, B) content of E_2 in the gonads, C) content of VTG in the liver, D) content of VTG in the gonads. Bars represent the mean and standard deviation of data set. Asterisks denote significance difference regarding to their corresponding control group (sex) * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$; + denote significance difference at $p < 0.05$ between sex in the same treatment

DISCUSSION

It was documented for the first time that mixture of metals induces changes in gonadotropins levels in the head and gonads of the mexcalpique. The GtH II in the head and gonads of *G. viviparus* were lower than in control group in both treatments (T1 and T2). It has been documented that some metals could potentially act on the male axis GnRH system impairing the GtH II secretion (Klein et al. 1994, Khan and Thomas 2000, Szczerbik et al. 2006) as in female fish (Crump and Trudeau 2009). Previous report and current results suggested that the lead present in the mixture of metals could disrupt the system GnRH-GtH II in the mexcalpique of both sexes. This effect possibly occurs because depletion of GtH II in the head as a decay in the gonads. However, it is

not possible to distinguish the possible toxic effects of the other metals in or not in addition to estrogenic compounds on the disruption of GtH II release.

In an interesting way, the content of GtH I showed an induction in the head and gonads particularly in male fish exposed to T1 and T2. It was found by Pearson moment correlation analysis that the induction of MT in the head of male fish exposed to T1 (Fig. 3A) was related to GtH I in the gonads ($p < 0.05$, Table II) suggesting that some metals in the mixture could enhance GtH I. In agreement with these results, in male *G. viviparus* inhabitant of a highly polluted lake, a positive relationship of zinc with VTG in vivo using multivariate approach was documented (Olivares-Rubio et al. 2015). Current results suggest that certain metals at threshold endogenous levels would modify the mRNA expression

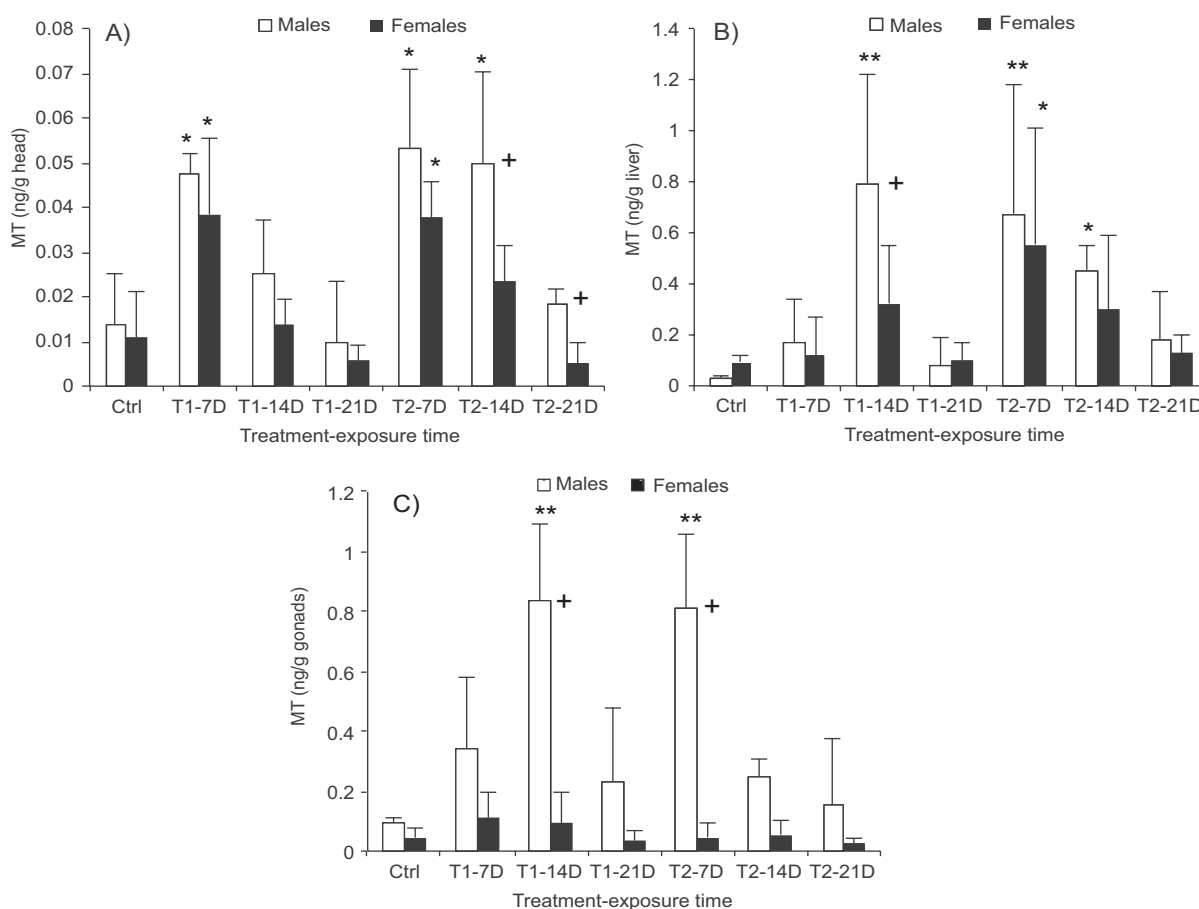


Fig. 3. Content of metallothioneins (MT) in the head, liver and gonads of *Girardinichthys viviparus* exposed under controlled conditions to mixture of metals (T1) and mixture of metals spiked with 25 ng/L of ethinyl estradiol (T2). 7D = 7 days, 14D = 14 days and 21D = 21 days. A) content of MT in the head, B) content of MT in the liver, and C) content of MT in gonads. Bars represent the mean and standard deviation of data set. Asterisks denote significance difference regarding to their corresponding control group (sex) * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$; + denote significance difference at $p < 0.05$ between sexes in the same treatment

at DNA element response of GnRH-gonadotropins system. In addition, some metals could disrupt the GnRH-gonadotropins system by alteration of calcium homeostasis (Yuan et al. 2013). In the brain of rainbow trout (*Oncorhynchus mykiss*) exposed to Cd (5 and 10 $\mu\text{g/L}$) mRNA levels of GnRH1 and GnRH2 was greatly enhanced in a concentration-dependant manner; probably because Cd alters E_2 signalling pathways and could affect the reproductive axis by non-estrogenic mechanisms (Vetillard and Bailhache 2005). However, if exogenous estrogens are present, the response elicited by metals could be modified. Vetillard and Bailhache (2005) showed that E_2 treatments did not modify GnRH1 and GnRH2 mRNA levels in the brain of rainbow trout. However, Cd in combination with E_2 stimulated these mRNAs. In wild male *G. viviparus*, it was found that Zn and Pb could (up and down) regulate hypothalamus-pituitary

axis (Olivares-Rubio et al. 2015). More studies are needed to clarify interactions of exogenous estrogens with metals at GnRH-gonadotropins system.

GtH II levels in the head and gonads of male fish exposed to T2 significantly decreased with regard to control fish, particularly in the gonads. However, levels of GtH I in the head of male and female fish exposed to T2 with a peak at days 14 and 7, respectively coincides with the greater values of IBRV2 for this treatment (Table III).

Different pattern of response was observed by Integrated Biomarker Response index version 2 (IBRV2) in fish exposed to T1. Gonadal GtH I of fish exposed to T2 showed a more pronounced response in male fish with regard to their own controls; additionally in both sexes a peak at day 7 was observed (females higher IBRV2). No apparent relation among MT in the head and GtH I in fish of both sexes

TABLE II. SIGNIFICANT CORRELATIONS OF THE BIOMARKERS EVALUATED IN *Girardinichthys viviparus* EXPOSED TO T1 AND T2 USING PEARSON MOMENT CORRELATION

Biomarker 1	Biomarker 2	r ²	p value
Male exposed to T1			
GtH II G	E ₂ G	-0.998	0.040
GtH I G	MT H	0.997	0.050
E ₂ H	VTG G	0.998	0.046
E ₂ G	VTG L	0.997	0.048
E ₂ G	MT H	-1.000	0.012
VTG L	MT H	-0.998	0.036
MT L	MT G	0.998	0.039
Female exposed to T1			
GtH II G	E ₂ G	-1.000	0.000
GtH II H	VTG G	0.998	0.040
E ₂ H	VTG G	-1.000	0.003
E ₂ H	MT L	0.999	0.028
Male exposed to T2			
GtH I H	GtH II G	-0.999	0.024
E ₂ H	MT L	-0.999	0.028
Female exposed to T2			
GtH I H	GtH I G	1.000	0.018
GtH II H	E ₂ G	-0.999	0.026
VTG L	MT L	0.997	0.048

GtH = gonadotropins, E₂ = estradiol, VTG = vitellogenin, MT = metallothioneins, H = head, L = liver, G = gonads. T1 = treatment 1, mixture of metals at environmentally relevant concentrations (Cu = 0.4 mg/L, Fe = 0.9 mg/L, Mn = 0.3 mg/L, Pb = 0.13 mg/L and Zn = 0.15 mg/L). T2 = treatment 2, the same mixture of metals spiked with 25 ng EE₂/L.

exposed to T2 was observed, suggesting a main role of EE₂ in the disruption of GnRH-gonadotropin system (**Fig. 4** and **5**) despite toxic effects elicited by the mixture of metals previously detailed.

Two findings suggest a feedback mechanism of exogenous estrogens involved in the disruption of metals on the GnRH-gonadotropin system: *i*) The depletion of GtH II at the head of male fish exposed to T2 showed an inverse response with enhancement of GtH I in the testis ($p < 0.05$). *ii*) In female fish, the stimulation GtH I in the head and gonads presented a similar induction during the exposure time, and their levels were above that of the control fish ($p < 0.05$). The probable consequence of these toxic effects in male *G. viviparus* is the disruption of 11-keto testosterone production by an imbalance in the activity of *cyp* 450 aromatase (Cheshenko et al. 2008) favouring the production of E₂ mediated by

gonadotropins. The induction of GtH I in the head and gonads and depletion of GtH II could also be hazardous to adult female mexcalpique because GtH II plays a main role in E₂ synthesis. Previous reports and current findings corroborate that this feedback mechanism is also regulated by exogenous estrogen. However, other interactions involved in up-regulation and down-regulation of release of gonadotropins are not discernable (Yaron et al. 2003, Aroua et al. 2012, Karigo et al. 2014).

In females, GtH I and GtH II induced the release of female steroid hormones as is the case of E₂ (Rempel and Schlenk 2008). Meanwhile in males stimulates testicular androgen secreted by Sertoli and Leydig cell proliferation (Swanson et al. 2003). The gonads of female fishes are the main source of E₂. However, in the brain, the expression of aromatase (*cyp*19), which convert androgens to estrogens has been reported in teleost fish (Diotel et al. 2010). In this regard, it was documented that the estrogens can up-regulate aromatase activity leading an elevated serum E₂ concentration in both, male and female fish (Filby et al. 2007). In male *G. viviparus*, it is possible that the same phenomena occur by exposure to metals and by EE₂. In teleost fish, GtH I stimulated vitellogenesis through the production of E₂ (Janz and Weber 2000). The potential consequence of induction of E₂-mediated by metals exposure in male *G. viviparus* could favour their feminization. This suggestion could be supported through the high levels of E₂ in the head of male mexcalpique and high VTG levels in their liver were found ($p < 0.05$). Similar feminization elicited by E₂ was documented in male *C. auratus* exposed in situ to exogenous estrogens, among other compounds (Yan et al. 2012).

However, in female fish, induction of E₂ in the head by exposure to T1 was not sustained during the exposure suggesting a feedback mechanism. This finding is possible since the basal level of E₂ was overcome and because there is a possible inverse relation between head E₂ levels with ovarian VTG observed ($p < 0.001$). In contrast, a peak of E₂ was accompanied by noticeable decay of GtH II in a time-dependant manner in the head of male *G. viviparus* treated with T1. These responses suggest the main role of GtH I in the synthesis of E₂ because both biomarkers showed an inverse response in the male mexcalpique. Similar results were documented in the hypophysectomized walking catfish (*Clarias batrachus*) treated with semi-purified GtH I and GtH II at the dose level of 5 µg/fish/day for 7 days (Sarkar et al. 2014).

On the other hand, an inverse relationship of MT in head of male *G. viviparus* exposed to T1 with testis

TABLE III. AREA VALUES OF INTEGRATED BIOLOGICAL RESPONSE VERSION 2 FOR THE BIOMARKERS IN THE TISSUES UNDER STUDY OF *Girardinichthys viviparus* EXPOSED TO T1 AND T2

	T1						T2					
	Male			Female			Male			Female		
	7 D	14 D	21 D	7 D	14 D	21 D	7 D	14 D	21 D	7 D	14 D	21 D
GtH I H	2.146	1.617	1.453	2.578	2.556	2.153	0.617	0.738	0.675	2.130	2.006	2.109
GtH I G	-0.319	-0.418	-0.591	0.135	-0.032	0.343	-0.935	-0.935	-1.371	0.606	-0.259	-0.254
GtH II H	-0.835	-3.118	-3.525	-0.676	-0.545	-1.135	-0.894	-1.199	-1.405	-0.836	-0.750	-0.945
GtH II G	-1.555	-1.892	-2.113	-0.265	-0.557	-2.192	-2.425	-3.013	-1.624	-1.291	-0.457	-0.457
E ₂ H	1.907	2.634	3.735	-0.379	0.821	-0.928	0.969	0.969	0.969	0.694	1.056	1.108
E ₂ G	-2.212	-2.118	-2.062	-4.657	-4.109	-3.486	-1.377	-1.321	-1.383	-4.018	-3.597	-3.581
VTG L	0.079	0.547	0.822	0.326	0.692	0.074	3.008	2.849	2.757	0.714	0.335	-0.249
VTG G	-1.626	-1.596	-1.440	-1.211	-1.665	-1.156	-0.520	-1.022	-1.063	-1.620	-1.612	-1.227
MT H	2.304	1.629	0.628	2.913	1.973	1.159	1.584	1.535	0.810	2.507	2.116	2.116
MT L	2.294	3.929	1.514	1.048	1.958	0.864	2.364	2.082	1.435	2.022	1.504	0.782
MT G	1.544	2.488	1.123	1.979	1.7936	0.804	1.335	0.492	0.1552	0.883	1.027	0.371
IBRv2	16.821	21.986	19.008	16.167	16.703	14.294	16.027	16.159	13.649	17.321	14.719	13.200

GtH I = gonadotropin I, GtH II = gonadotropin II, E₂ = estradiol, VTG = vitellogenin, MT = metallothioneins, H = head, L = liver, G = gonads, D = days after exposure. T1 = treatment 1, mixture of metals at environmentally relevant concentrations (Cu = 0.4 mg/L, Fe = 0.9 mg/L, Mn = 0.3 mg/L, Pb = 0.13 mg/L and Zn = 0.15 mg/L). T2 = treatment 2, the same mixture of metals spiked with 25 ng EE₂/L. EE₂ = 17 α -ethynil estradiol

E₂ levels was found. In female mexcalpique, a similar response between hepatic MT with E₂ in the head was observed ($p < 0.05$). Probably, the binding of metals in the head of male fish and in the liver of female fish allows the deregulation of the GnRH-gonadotropins system. Homeostatic regulation of metals are in part by MT which permits their transportation to specific cellular compartments where its function is required or altering its toxicity (Sears 2013).

EE₂ could increase the toxic effects of metals in *G. viviparus*. The estrogenic effects elicited by xeno-estrogens by binding to ER is well documented (Sumpter and Jobling 1995, Shanle and Xu 2011). However, the mechanism of EE₂ in addition to metals on the disruption of HPLG axis is not clear. In this regard, increases of P450aromB transcript abundance have been reported in zebrafish (*Danio rerio*) juveniles exposed to EE₂ (Kazeto et al. 2004, Vosges et al. 2010) besides in the head of roach (*Rutilus rutilus*) at 28 and 56 days after spawning (Lange et al. 2008). These findings indicate that metals and EE₂ could disrupt the HPLG axis by non specific mechanism. Increases in the level of E₂ were more evident in mexcalpique exposed to T2 than in T1. Additionally, an inverse response between hepatic MT with E₂ in the head of male mexcalpique exposed to T2 was detected ($p < 0.05$), suggesting a negative regulation of hepatic MT on the disruption of GnRH-gonadotropins system. However, only in the gonads of female

G. viviparus exposed to T2, the content of E₂ was lower than in their own controls. This particular finding could be explained probably by early increase of VTG synthesis in the liver and by late increases of VTG in the gonads. Although, EE₂ is likely responsible for this protein induction, it is not possible to rule out that endogenous E₂ could participate in VTG synthesis since this steroid is the natural agonist of ER (Sumpter and Jobling 1995).

No significant changes in VTG content were observed in the liver and gonads of male and female *G. viviparus* exposed T1. However, the increase of MT in the head was apparently related to the lack of induction of VTG in male fish ($p < 0.05$). Current results indicate that disruption of HPLG axis by exposure of metals occurs at hypothalamus-pituitary control point.

In contrast, in the liver of fish exposed T2, levels of VTG showed an early induction, particularly in male mexcalpique explained by the high estrogenic effects elicited by EE₂ via ER in fishes. The opposite, in female mexcalpique, a late capture of VTG in the gonads matched with E₂ content in the ovary and with total depletion of GtH II was detected. These findings suggest that EE₂ in presence of metals elicited a sex-linked response. In male fish, it is probable that the deregulating of the function of Sertoli cell allows an up-regulating of aromatase activity leading a female hormonal profile (Yan et al.

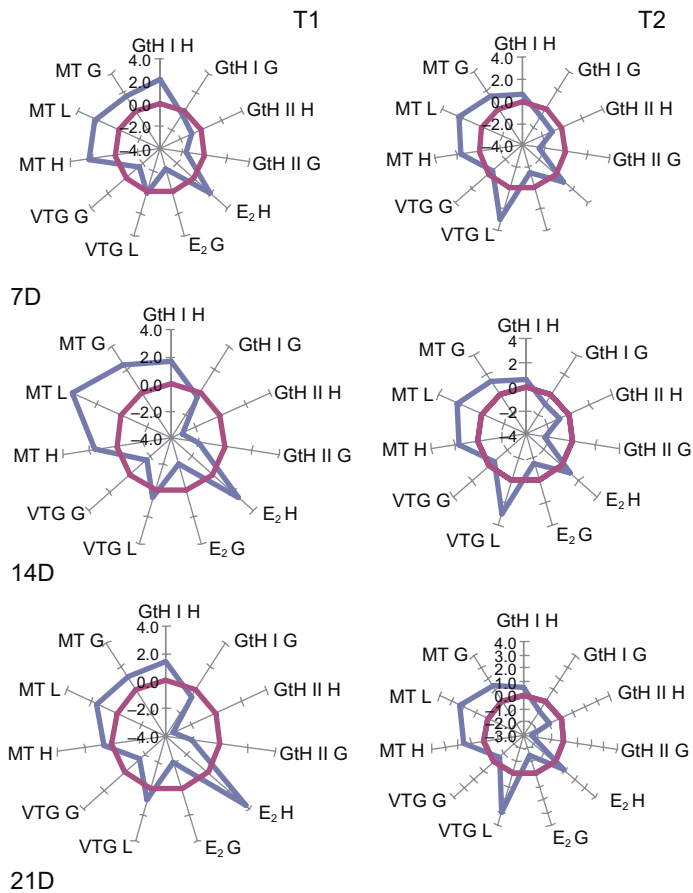


Fig. 4. Star plot areas obtained by the method of Integrated Biological Response version 2 for the biomarkers evaluated in male *Girardinichthys viviparus* exposed to T1 (Cu = 0.4 mg/L, Fe = 0.9 mg/L, Mn = 0.3 mg/L, Pb = 0.13 mg/L and Zn = 0.15 mg/L) and T2 (Cu = 0.4 mg/L, Fe = 0.9 mg/L, Mn = 0.3 mg/L, Pb = 0.13 mg/L and Zn = 0.15 mg/L plus 25 ng EE₂/L). GtH I = gonadotropin I, GtH II = gonadotropin II, E₂ = estradiol, VTG = vitellogenin, MT = metallothioneins, H = head, L = liver, G = gonads. D = days after exposure. The blue line represents the deviation with regard to basal values (red line)

2012). Meanwhile, in female mexcalpique, metals and EE₂ induce an imbalance between both gonadotropins involved in regulation of HPLG axis that in the current study favouring a late vitellogenesis mediated by EE₂.

Early induction of MT in the liver coincided with early induction of VTG. Thompson et al. (2003) has reported that changes in zinc concentration in the bloodstream of female squirrelfish (*Holocentrus adscensionis*) followed the same time course as VTG transport from the liver. In addition, hepato-ovarian translocation of zinc suggests that VTG might be a vehicle for this metal (Thompson et al. 2012). Current results and preceding studies

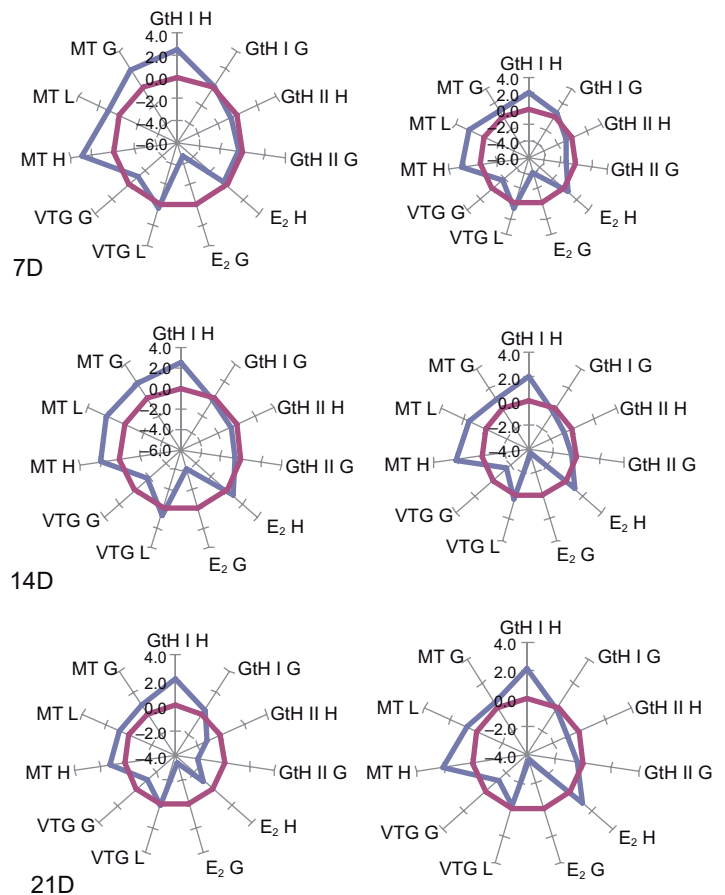


Fig. 5. Star plot areas obtained by the method of Integrated Biological Response version 2 for the biomarkers evaluated in female *G. viviparus* exposed to T1 (Cu = 0.4 mg/L, Fe = 0.9 mg/L, Mn = 0.3 mg/L, Pb = 0.13 mg/L and Zn = 0.15 mg/L) and T2 (Cu = 0.4 mg/L, Fe = 0.9 mg/L, Mn = 0.3 mg/L, Pb = 0.13 mg/L and Zn = 0.15 mg/L plus 25 ng EE₂/L). GtH I = gonadotropin I, GtH II = gonadotropin II, E₂ = estradiol, VTG = vitellogenin, MT = metallothioneins, H = head, L = liver, G = gonads. D = days after exposure. The blue line represents the deviation with regard to basal values (red line)

indicate that Zn chelated by MT is closely related to VTG induction.

CONCLUSION

The endocrine disruption of the sexual control axis in fish with matrotrophy viviparity showed clear differences in between sexes in *G. viviparus* exposed to a mixture of metals and metals spiked with EE₂. The main disruption of the HPLG axis elicited by both, metals and spiked with EE₂ occurs at hypothalamic-pituitary control point affecting basically the gonads function. By exposure to these toxicants, the male fish was the most damaged; however, female

fish could counterpart this damage apparently by feedback mechanism mediated by endogenous E₂. Despite current findings, more studies are required to clarify the disruption of HPLG axis elicited by metals and spiked with xeno-estrogens in fish with matrotrophic viviparity.

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