Interplay between stress and osmoregulatory physiology of the anuran *Xenopus laevis* experimentally exposed to saline and pesticide-contaminated environment

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Informe de Aprobación

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Se informa a la Escuela de Postgrado de la Facultad de Ciencias que la Tesis de Magíster presentada por el candidato:

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Dedicado a Felipe, Ana María,
María Jesús, Sofía y Catalina.

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Nació en Santiago, RM, Chile, el 19 de Diciembre de 1995. Desde muy pequeño, Felipe presentó un particular interés por los animales y la naturaleza, el cual con los años se potenció en compañía de sus hermanas y amigos más cercanos. Fue este interés por los animales y la naturaleza que en el año 2015 ingresa a estudiar Licenciatura en Ciencias con mención en Biología en la Universidad de Chile, egresando el año 2018. En los últimos años del pregrado, Felipe se integra al Laboratorio de Ecofisiología Animal, donde más tarde realizaría un Magister en Ciencias biológicas bajo la tutoría del Dr. Pablo Sabat y co-tutoría del Dr. Juan Carlos Sanchez-Hernandez.

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Índice de Materias

Lista de tablas

Lista de figuras

Lista de Abreviaturas

SMR: Tasa metabólica estándar

CS: Citrato sintasa

COX: Citocromo C oxidasa

AChE: Acetilcolinesterasa

BChE: Butirilcolinesterasa

CbE: Carboxilesterasa

OP: Pesticida Organofosforado

CPF: Clorpirifos

CPFoxon: Clorpirifos-oxon

TAC: Total antioxidant capacity

SW: Agua Salada

FW: Agua Fresca

NP: No-Pesticida

P: Pesticida

ABSTRACT

Salinization and pollution (e.g., agrochemicals) are two main environmental stressors leading to degradation of aquatic ecosystems and consequently alteration in their biological communities. Amphibians are a highly sensitive group of vertebrates to environmental disturbance of aquatic ecosystems, however, studies on the combined effect of both stressors on the physiology of anurans are limited. In the present study, the energetic and biochemical response was evaluated in the invasive anurus *Xenopus laevis* acclimatized for 45 days to a high osmotic load (SW), the presence of chlorpyrifos (P) and the joint exposure to both stressors (SW/P). The results revealed a decrease in the standard metabolic rate (SMR) in both groups of animals exposed to pesticides. In addition, we found an increase in the activity of liver citrate synthase (CS) in response to salt stress. In parallel, the liver acetylcholinesterase (AChE) activity decreased by 50% in animals in the doubly stressed group, which explained the differences in the detoxification capacity of the xenobiotic. Along the same lines, we observed an increase in plasma AChE activity in the doubly stressed group, a result that we attributed to the physiological effects of salt in conjunction with the agrochemical. Finally, oxidative stress showed a synergistic effect exhibited by an increase in both lipid peroxidation and concentration of aqueous peroxides found in the experimental group simultaneously exposed to stressors.

Keywords

Amphibian ecotoxicology; Oxidative stress; Biomarkers; Organophosphorus pesticides; Climate change

RESUMEN

La salinización y la contaminación (*e.g.*, agroquímicos) son dos de los principales factores de estrés ambiental que conducen a la degradación de los ecosistemas acuáticos y, en consecuencia, a la alteración de sus comunidades biológicas. Los anfibios son un grupo de vertebrados muy sensible a la alteración ambiental de los ecosistemas acuáticos, sin embargo, los estudios sobre el efecto combinado de ambos factores de estrés en la fisiología de los anuros son limitados. En el presente estudio se evaluó la respuesta energética y bioquímica en el anuro invasor *Xenopus laevis* aclimatado durante 45 días a una alta carga osmótica (SW), la presencia de clorpirifós (P) y la exposición simultánea a ambos estresores (SW / P). Los resultados revelaron una disminución en la tasa metabólica estándar (SMR) en ambos grupos de animales expuestos a pesticidas. Además, encontramos un aumento en la actividad de la citrato sintasa (CS) hepática en respuesta al estrés salino. Paralelamente, la actividad de la acetilcolinesterasa hepática (AChE) disminuyó en un 50% en los animales del grupo doblemente estresado, lo que explicó las diferencias en la capacidad de desintoxicación del xenobiótico. En la misma línea, observamos un aumento de la actividad de la AChE plasmática en el grupo doblemente estresado, resultado que atribuimos a los efectos fisiológicos de la sal en conjunto con el agroquímico. Finalmente, el estrés oxidativo mostró un efecto sinérgico exhibido por un aumento tanto en la peroxidación lipídica como en la concentración de peróxidos acuosos encontrados en el grupo experimental simultáneamente expuestos a factores los dos factores estresantes.

Palabras clave

Ecotoxicología de anfibios; Estrés oxidativo; Biomarcadores; Plaguicidas organofosforados; Cambio climático

1. INTRODUCTION

There is general consensus on environmental factors that threat amphibian populations. Among them, global climate change (*e.g.*, droughts, excessive precipitation events, and environment salinization), habitat fragmentation, introduction of exotic species and pollution are the major environmental stressors involved in population decline of amphibians (Hayes et al., 2010; Becker et al., 2007; Collins & Storfer, 2003). However, little is known about how exotic amphibian species such as *Xenopus laevis* are able to copy with these environmental factors that dramatically change the structure and function of habitats. One of the most significant changes derived from climate change is salinization of wetlands, which represent an acute process with adverse consequences to organismal fitness (Mack et al. 2000, Heine-Fuster et al. 2010).

In freshwater ecosystems, increases of salinity affects the abundance (Amsinck et al., 2005; Sarma et al., 2006) and diversity of organisms (Jeppesen et al., 1994; Shallemberg et al., 2003). Indeed, many studies have evidenced that salinity has a significant impact on growth, reproduction, behavior and survival of aquatic invertebrates (Dana & Lenz, 1986; Achuthankutty et al., 2000; Charmantier et al., 2001; Martínez-Alvarez et al., 2002; Montory et al., 2014; Johnson et al., 2015; Rivera-Ingraham & Lignot, 2017; Boeuf & Payan, 2001; Gonzalez, 2012; Weaver et al., 2016). To acclimate to fluctuating salinity, aquatic organisms need to adjust their osmoregulation physiology to maintain the homeostasis (Martinez-Alvarez 2002; McCormick & Bradshaw, 2006; Haramura et al., 2019). Consequently, osmoregulatory changes often increase metabolic rates as a result of energetic costs involved in ionic transport, synthesis of osmo-active compounds and blood pumping (Peña-Villalobos et al. 2016; Sutton et al., 2018; Jones et al., 2019; Williard et al., 2019).

Salinity may induce oxidative stress in organisms (Hidalgo et al, 2020). Cellular oxidative

metabolism generates reactive oxygen species (ROS), which can interact with many biomolecules such as lipids, proteins, and nucleic acids (Lushchak, 2011; Cecarini et al, 2007). However, molecular (*e.g.*, glutathione) and enzymatic mechanisms (*e.g.*, superoxide dismutase, glutathione reductase, catalase, and many others) contribute to keep cellular oxidative homeostasis by inactivating ROS (Paital & Chainy, 2010; Rodríguez et al, 2003). Nevertheless, environmental factors such as pollution, salinity, and temperature, among many others, may cause an excessive production of ROS that cell is not able to mitigate because of saturation and/or inactivation of antioxidant mechanisms (Vakifahmetoglu-Norberg et al., 2017). These conditions are known as oxidative stress and cause alterations in multiple cellular processes (Dowling and Simmons 2009; Monaghan et al. 2009; van de Crommenacker et al. 2010; Selman et al. 2012), ultimately leading to cell death. Oxidative stress and induction of antioxidant systems have been described in invertebrates, fishes and birds exposed to salt stress (Tremblay and Abele, 2016; Velez et al., 2016; Rivera-Ingraham and Lignot, 2017; Sabat et al., 2017). However, little is known about oxidative processes induced by salinity in amphibians (Pinya et al., 2016; Demori et al., 2019)

Some studies suggest that global climate change has a significant impact on agroecosystems. For example, an increased pesticide input is expected because of a higher incidence of pests and new species of pests in crops, and a greater dissipation of pesticides due to greater biodegradation and abiotic metabolism (Noyes et al., 2009). Organophosphate (OP) pesticides and specifically chlorpyrifos (O,O-diethyl O-3,5,6-trichloro-2-pyridylphosphorothioate, CPF) is one of the most used pesticide in Chile (SAG, 2012). The mechanism of toxic action of CPF is the irreversible inhibition of acetylcholinesterase (AChE, EC. 3.1.1.7) activity; a hydrolase enzyme that breakdowns the neurotransmitter acetylcholine, which participates in the regulation of nerve transmission in the neuromuscular plate and the cholinergic and muscarinic synapses of the central, peripheral and autonomic nervous system (Pope et al., 2005; Lionetto et al., 2013). Likewise, CPF is actively detoxifies by oxidative desulfurization; a reaction catalyzed by cytochrome P450-dependent monooxygenases, which leads to the formation of the highly toxic metabolite chlorpyrifos-oxon

(CPFoxon). The metabolite displays a higher affinity for the active site of AChE than its parent compound CPF (Chambers et al., 2010; Dzul-Caamal et al., 2012; Narvaez et al., 2016). It has been documented that the presence of pesticides in organisms promotes the REDOX imbalance (Silvestre, 2020), as well as modifications in metabolic rates and in the enzyme activities responsible for xenobiotic detoxification (Narvaez et al, 2016; Rosenbaum et al., 2012; Blevin et al., 2017; Sandoval-Herrera et al., 2019). Because of conservation measures and the endangered status of many amphibian species, exotic species with a wide distribution range, such as *X. laevis* (36,000 Km^2) (Mora et al., 2019), emerge as suitable candidates to examine the impact of multiple environmental stressors on this vertebrate group, and to unravelling how exotic species may tolerate highly changing environments.

The aim of this study was, therefore, to explore the interactive effects of salinity and pesticide contamination on metabolism, osmoregulation, neurotoxicity and oxidative stress of adult *X. laevis.* Results from this study may provide insights on the potential adaptation capacity of this invasive species in Chilean wetlands.

Hypothesis

We hypothesize that frogs acclimatized to a hyperosmotic environment contaminated with CPF will decrease their detoxification capacity, thus increasing their sensitivity to xenobiotics. Likewise, animals also depress their metabolic capacity, displaying a lower activity of mitochondrial enzymes and an increased oxidative potential.

From the previous hypothesis, the following predictions arises:

I. Individuals exposed to hypersaline treatment (400 mOsm NaCl) are expected to have the highest concentration of hepatic and plasma urea, as well as higher plasma osmolality. This response, in turn, will generate an osmoregulatory cost that translates into an increase in

SMR.

II. Individuals exposed to the presence of both salt and pesticide will present a lower metabolism and lower catalytic activity of citrate synthase and cytochrome oxidase, accounting for the synergistic and negative effect of the environmental stressors.

2. OBJETIVES

2.1 General objective

To evaluate the interaction of natural and anthropogenic stressors on the physiology and response capacity of the invasive frog *X. laevis.*

2.2 Specific objectives

a) To determine the standard metabolic rate (SMR) in animals acclimated to two levels of pesticides (chlorpyrifos) and salinity for 4 weeks.

b) Determination of biochemical variables, in particular i) activities of metabolic enzymes (citrate synthase and cytochrome c oxidase) and enzymes related to the detoxification of xenobiotics (acetylcholinesterase, carboxylesterase and butyrylcholinesterase) in addition to ii) evaluating osmoregulatory parameters such as plasma osmolality and the concentration of urea in plasma and liver. Finally, iii) markers of oxidative stress will also be evaluated.

3. MATERIALS AND METHODS

3.1. Experimental design

Impact of saline stress and pesticide exposure was assessed by a factorial experiment that involved two levels of salinity (150 mOsm NaCl or fresh water [FW] and 400 mOsm or saltwater [SW]) and two concentrations of CPF (pesticide-free [NP] or control and 1.0 µg active CPF/L [P]) (Katz & Hanke, 1993; Richards & Kendall, 2011). Nineteen male *X. laevis* adults were obtained from a feral population in San Antonio (33° 34′ S, 71° 36′ W), a mesic coastal locality of central Chile and acclimatized in the laboratory using plastic containers with dechlorinated tap water (150 mOsm NaCl). Animals were fed weekly with ground meat. The osmolality was progressively modified until reach the experimental conditions. Considering that the plasmatic osmolality described for the species is ~ 250 mOsmol/kg (Inoda & Morisawa, 1987), in the hyperosmotic group the concentration of salt in the water was increased for ten days until reaching 400 mOsm following the protocol of Peña-Villalobos., 2016), while in the group hypoosmotic was acclimatized up to 150 mOsm. After that, both groups were equally separated in two additional treatments which received the two CPF concentrations. Accordingly, an aqueous solution of CPF was prepared from a commercial emulsifiable formulation (48CE, 48% w/w CPF, Point International Ltd, Bromley, UK) to yield a stock solution of 10.0 µg/L. Chlorpyrifos was then diluted in the incubation water up to 1 µg/L. Water was replaced by fresh control (pesticide free) and CPF-spiked water was replaced by spiked fresh water every 48 h to maintain the pesticide concentration constant during the 45 day of exposure. Animals were kept in these experimental conditions at 25ºC and LD cycle of 12L:12D in starvation conditions to restrict the input of water and ions through food (Peña-Villalobos et al., 2016; Hidalgo

et al., 2020).

3.2. Standard metabolic rate

Standard metabolic rate (SMR) was estimated as the rate of oxygen consumption (VO_2) using standard flow-through respirometry methods following Peña-Villalobos et al. (2016). Briefly, individuals were gently dried with a paper towel, weighed and placed in transparent acrylic chambers of 1.5 L. This chamber was provided with a humid paper towel at the bottom and then located in a temperature controlled and illuminated cabinet (Sable Systems, Henderson, Nevada) at a constant ambient temperature (Ta=25±0.5°C).

The metabolic chamber received air at 200 mL min−1 from a mass flow controller and through Bev-A-Line tubing (Thermoplastic Processes Inc., Georgetown, Delaware). The excurrent air passed through columns of Drierite, CO_2-absorbent granules of Baralyme and Drierite and then through and Fox Box O_2-analyzer equipped with a flow meter (Sable Systems, Henderson, Nevada) calibrated with a mix of oxygen (20%) and nitrogen (80%), which was certified by chromatography (BOC, Chile). The mass flow meter of the Fox Box was calibrated monthly with a volumetric (bubble) flow meter.

Because water steam and CO_2 were scrubbed before entering the O_2 analyzer, oxygen consumption was calculated as: $VO_2 = [FR \times 60 \times (Fi\ O_2 - Fe\ O_2)]/(1 - Fi\ O2)$ (Withers, 1977), where FR is the flow rate in ml min^{-1}, and the Fi and Fe are the fractional concentrations of O_2 entering and leaving the metabolic chamber, respectively. Ten min of baseline O_2 concentrations were recorded before and after each measurement period in order to correct for drift in the O_2 analyzer. Output from the oxygen analyzer (% O_2) and flow meter was digitalized using a Universal Interface II (Sable Systems) and recorded on a personal computer using EXPEDATA data acquisition software (Sable Systems). Our sampling interval was 1 s. Frogs remained in the chamber for 4 h, long enough to reach steady-state conditions, which typically occurs after 1-2 h. We averaged O_2 concentration of the excurrent

airstream over a 20 min period after steady state was reached.

3.3. Tissue sample collection and homogenization

After metabolic determinations, animals were sacrificed by decapitation (Katz & Hanke, 1993), heart, liver, ventral pelvic patch (skin), testicular mass, kidney, and leg muscle were dissected, weighed and frozen at -80°C for further biochemical analyses. analysis. Just after sacrifice, blood was collected from the heart using capillary tubes, centrifuged at 12,000 g for 5 min, and plasma was obtained for osmolality measurement. The remaining amount of plasma was stored (-80°C) for further biochemical analysis.

3.4. Metabolic enzyme assays

Mitochondrial cytochrome *c* oxidase (COX, E.C. 1.9.3.1) and citrate synthase (CS, E.C. 4.1.3.7) activities were measured in liver, heart, leg muscle and skin. COX is an enzyme involved in the last step of the mitochondrial respiratory chain, thus being a biomarker of the energy capacity of the mitochondrial system, whereas CS activity participates in the Krebs cycle. It has been widely reported that an increase in the activity of both enzymes reflects changes in both the functional properties and the density of mitochondria (Spinazzi et al., 2012). COX activity was measured in a reaction mixture containing 10 mM Tris-HCl (pH 7), 120 mM KCl, 250 mM sucrose, and cytochrome *c* reduced with dithiothreitol in a final volume of 0.2 ml. The enzyme activity was calculated using an extinction coefficient of 21.84 mM^{-1} cm^{-1} at 550 nm (Moyes et al., 1997). CS activity was measured in an assay medium composed of 10 mM Tris-HCl (pH 8.0), 10 mM 5,5′dithiobis-(2-nitrobenzoic acid), 30 mM acetyl coenzyme A and 10 mM oxaloacetic acid (omitted in the controls) in a final volume of 0.2 mL at 25ºC (Sidell et al., 1987). Enzyme activity was calculated using an extinction coefficient of 13.6 mM^{-1} cm^{-1} at 412 nm.

3.5. Esterase enzyme assays

We measured three esterase enzymes involved in the acute toxicity of CPF ([AChE]) and its detoxification (butyrylcholinesterase [BChE], EC 3.1.1.8 and carboxylesterase [CbE] EC 3.1.1.1). The inhibition of BChE and CbE is considered a non-catalytic mechanism of pesticide detoxification whereby the organophosphorus molecule is inactivated by irreversible binding to the active site of these esterases (Lockidge & Masson, 2000; Liu et al., 2011; Cui et al., 2015).

Both AChE and BChE activities were measured according to the method by Ellman et al. (1961), adapted to the 96-well microplate format by Wheelock et al. (2005). Reaction medium (200 µL) was composed of 0.1 M Na-phosphate buffer (pH = 8.0), 320 µM 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), 3 mM acetylthiocholine iodide (AChE) or butyrylthiocholine iodide (BChE), and the sample. Kinetics were read for 2 min (10-sec intervals) at 412 nm and 25°C. A calibration curve produced with DTNB and serial concentrations of reduced glutathione allowed quantification of the product formed during hydrolysis of substrates (Eyer et al. 2003). Protein concentrations were determined using the Bradford method (Bradford, 1976) with bovine serum albumin as the standard. Carboxylesterase activity was determined using the ester substrate 1-naphthyl butyrate (1-NB) and following the discontinuous assay by Thompson (1999). The reaction mixture contained 0.1 M Tris–HCl (pH = 7.4), 2 mM 1-NB and the sample, and was incubated for 10 min at 25 °C. The hydrolysis of 1-NB was stopped by adding 50 µL of a solution made dissolving 0.1% Fast Red ITR in 2.5% (w/v) SDS and 2.5% Triton X-100. Microplates were left in the dark for 30 min for colour development. The specific carboxylesterase activity was calculated using an external curve made with the product of the hydrolytic reaction 1-naphthol (Sigma-Aldrich, Santiago de Chile, Chile). Kinetic assays were run in triplicate and read using a Thermo Scientific Multiskan GO UV/VIS spectrophotometer. Blanks (i.e., sample-free reaction media) were included in the assays to discount non-enzymatic generation of the reaction products. All enzyme activities are expressed as specific activity per milligram of protein (µmol min^{-1} mg^{-1}).

3.6. Biomarkers of oxidative stress

Oxidate stress was assessed by measuring the total antioxidant capacity (TAC), lipid peroxidation (thiobarbituric acid reactive species or TBARs assay) and water-soluble peroxides. TAC was determined by measuring the capacity of sample to quench a standardized free radical solution in a colorimetric reaction using a commercial assay kit (Sigma Aldrich, San Diego, CA, #CS0790). Decrease in absorbances was monitored in a Thermo Scientific Multiskan GO UV/VIS spectrophotometer at 25°C. TBARs were quantified using a commercial kit (Oxiselect, STA-330 Cell biolabs). This assay evaluates the adduct formed between malondialdehyde, which is a product of lipid peroxidation, and thiobarbituric acid. We determined hydrogen peroxides in plasma as a measure of ROS production using the commercial kit MAK311 (Sigma Aldrich). The assay evaluated colorimetrically (585 nm) the oxidation of Fe^{+2} to Fe^{+3} by the peroxides present in the sample.

3.7. Osmolality measurement

Plasma was thawed and 8 µL was used for osmolality measurement by vapor pressure osmometry (Wescor 5130B) (Christensen et al., 2017). The urea concentration was measured in both plasma and tissue homogenates (mg dL^{-1}) by the urease/Berthelot method using a commercial kit (Valtek, Chile).

3.8. Data analysis

Variations in the mean activity of metabolic enzymes were detected using a two-way analysis of variance (ANOVA) with pesticide concentration (0 and 1.0 µg/L) and water salinity (SW and FW) as the independent factors. Similarly, the effect of the interplay between pesticides and salinity on metabolic rates, osmoregulatory parameters and oxidative stress parameters of each tissue was

analyzed using a two-way ANOVA or ANCOVA when necessary (*e.g.,* SMR, organ mass) with the values of each parameters as the response variable. The *post hoc* Tukey test was used to test for specific differences among means in all measured traits. In the particular case of body mass, a repeated measures ANOVA (RM ANOVA) was performed to evaluate the mass change over time. To assess whether there were potential relationships between the morphological, physiological and metabolic variables in the treatments, a correlation matrix was constructed which was analyzed by means of a Pearson's correlation coefficient (r). Before performing each statistical analysis, the Shapiro-Wilk and Levene tests were run to determine the normality and homoscedasticity of the data, respectively. Data are presented as mean ± SD.

The statistical analyzes performed in this thesis were performed by using the statistical package IBM SPSS Statistics software (SPSS) (version 15.0; SPSS Inc., Chicago, IL, USA) and the statistical package STATISTICA® (2004) for Windows.

4. RESULTS

4.1 Morphology

After the acclimation all experimental groups decreased body mass (RM ANOVA, $F_{(1,15)} = 8.192$; $p = 0.012$), but at the end of acclimatization, body mass was not affected by the level of salinity ($F_{(1, 15)} = 0.03$, $p = 0.868$), pesticide concentration ($F_{(1,15)} = 0.99$, $p = 0.337$) or by the interaction between factors ($F_{(1,15)} = 0.001$, $p = 0.96$) (Table 1). Nevertheless, ANCOVA analysis showed that liver mass covaried with body mass ($F_{(1,14)} = 18.91$, $p = 0.001$), and the liver was larger in animals acclimatized to 400 mOsm NaCl ($F_{(1, 14)} = 20.52$, $p = 0.0001$). Neither pesticide ($F_{(1,14)} = 0.19$, $p = 0.673$) nor the interaction between salinity and pesticide ($F_{(1.14)} = 0.93$, $p = 0.351$) affected liver mass. The a *posteriori* test revealed that SW-1.0 µg/L group had larger livers than both FW-1.0 µg/L ($p = 0.025$) and FW-pesticide free ($p = 0.009$) groups (Table 1). Likewise, kidney size covaried with body mass ($F_{(1,14)} = 24.57$, $p = 0.0001$), and they were higher in animals acclimatized to 400 mOsm NaCl (ANCOVA, $F_{(1,14)} = 13.80$, $p = 0.002$) and to the 2 stressors simultaneously ($F_{(1,14)} = 9.77$, $p = 0.007$), but not due to the individual effect of the pesticide ($F_{(1,14)} = 2.93$, $p = 0.109$). The *posteriori* test revealed the largest kidneys in the SW / P group (Table 1).

Heart mass was affected by body mass (ANCOVA, $F_{(1,14)} = 42.69$, $p = 0.0001$) and were larger in SW than in FW treatments ($F_{(1,14)} = 19.23$, $p = 0.001$). We found no effect of pesticide ($F_{(1,14)} = 0.02$, $p = 0.886$), nor by the interaction salt x pesticide ($F_{(1,14)} = 0.80$, $p = 0.387$). The *a posteriori* test showed a 1.5 fold increase in SW/NP groups than in the FW/NP group (Table 1). Finally, the testicular mass was not affected by body mass (ANCOVA, $F_{(1,14)} = 1.560$, $p = 0.232$), but SW animals exhibited smaller testis than FW groups. Finally, animals acclimated in the presence of CPF had smaller testis

($F_{(1,14)} = 5.993$, $p = 0.028$) than the NP groups. In particular, the Tukey test showed that the testicular mass of the SW/P group was 40% smaller than in the FW/NP group ($p = 0.01$) (Table 1). We did not find an effect of the interaction of both factors on testicular size ($F_{(1,14)} = 0.090$, $p = 0.769$).

4.2. Metabolic Rate

Standard metabolic rate was significantly affected by body mass ($F_{(1,14)} = 11.70$, $p = 0.004$) and the ANCOVAs showed that when body mass was accounted for, SMR was lower in OP acclimated animals ($F_{(1,14)} = 14.21$, $p = 0.002$). We found no effect of the salt factor ($F_{(1,14)} = 0.34$ $p = 0.570$), nor of the interaction between both variables ($F_{(1,14)} = 0.28$, $p = 0.604$) on SMR (Table 1).

Table 1. Metabolic rate, body mass and organ masses, after 45 days of acclimatization to 400 mOsm NaCl (SW/NP), 50 mOsm NaCl (FW/NP), 1 µg/L CPF (FW/P) and 400 mOsm NaCl and 1 µg/L CPF (SW/P). Letters represent statistical differences and ± SD.

Treatments	**SW/NP**	**FW/P**	**SW/P**	**FW/NP**
Mb initial (g)	44.34 ± 8.97	46.82 ± 9.53	48.57 ± 7.15	44.6 ±11.40
Mb final (g)	33.24 ± 7.31	41.16 ± 8.98	38.12 ± 7.73	36.38 ± 11.12
SMR (mL/h)	34.48 ± 4.55	29.68 ±7.53	27.62 ± 3.70	39.69 ±12.91
LSM	36.92 ± 2.90^{ab}	27.21 ± 2.90^{a}	27.04 ± 3.15^{a}	40.18 ± 2.81^{b}
Liver (g)	1.38 ± 0.63^{ab}	1.06 ± 0.3^{b}	1.81 ± 0.53^{a}	0.94 ± 0.5^{ab}
Kidney (g)	0.16 ± 0.03^{b}	0.16 ± 0.05^{b}	$0.27 \pm .,08^{a}$	0.17 ± 0.06^{b}
Heart (g)	0.24 ± 0.04^{a}	0.22 ± 0.05^{ab}	0.26 ± 0.08^{a}	0.18 ± 0.07^{b}
Fatty bodies (g)	0.87 ± 0.6	0.82 ± 0.47	1.64 ± 0.66	1.10 ± 0.57
Testicles (g)	0.12 ± 0.03^{ab}	0.12 ± 1.38^{ab}	0.10 ± 0.02^{a}	0.31 ± 0.29^{b}

4.3 Metabolic enzymes

Liver COX activity was affected by the presence of salt ($F_{(1,15)}$ = 11,32, p = 0.004); in particular, acclimatization to the FW/NP treatment increases the enzymatic activity; but it was not affected by pesticide ($F_{(1,15)}$ =3.53, p=0.08), nor by the interaction of both factors ($F_{(1,15)}$ = 0.07, p=0.795). Nevertheless, the Tukey test indicated a decrease four times of COX hepatic activity in the SW/NP group, in contrast to FW/P group (p=0.008) (Figure 1b). Regarding the hepatic activity of CS, we observed an ca. 200 % increase in the SW/NP group compared to that found in the FW/P (p = 0.0024) and FW/NP (p = 0.0019) treatments (Figure1b). On the other hand, COX activity in the skin was affected of environment salinity (ANOVA $F_{(1,15)}$ =13.64, p=0.002), with an increase in the activity in the SW/NP group (see Figure 1), but we found no effect of the OP ($F_{(1,15)}$ =0.12, p=0.731) nor of the interaction between both factors ($F_{(1,15)}$=0.95, p=0.346). The Tukey test indicated an increase 1.5 times the SW/NP group compared with that found in the FW treatments (Figure 1b). Finally, the CS activity of the skin was only affected by the interaction of the factors (ANOVA $F_{(1,15)}$ =21.03, p<0.01). In particular, the CS activity of skin was significantly higher in the SW/NP than in the FW/NP group, being the values intermediate for both groups with pesticide (Figure 1a).

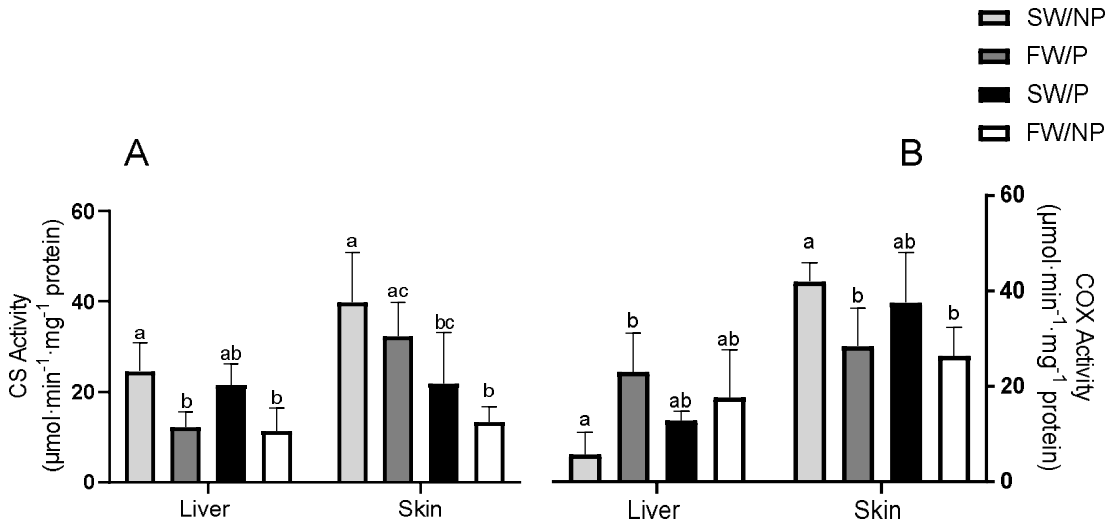

Figure 1. Activity of the mitochondrial enzymes Citrate synthase (CS) and Cytochrome c oxidase (COX) measured in the liver and ventral pelvic patch of *Xenopus laevis* acclimated to contrasting regimens of Salt and Chlorpyrifos. Different letters indicate statistically significant differences between treatments.

4.4. Esterase enzymes

The presence of OP in water decreased the activity of liver AChE (ANOVA $F_{(1,15)} = 12.97$, $p = 0.003$), while the salt factor has no effect on this variable ($F_{(1,15)} = 0.06$, $p = 0.809$). The interaction between factors had no effect on liver AChE activity ($F_{(1,15)} = 1.26$, $p = 0.279$) (Table 2). On the other hand, the plasma AChE activity was significantly lower in animals acclimatized to 50 mOsm NaCl (ANOVA $F_{(1,15)} = 26.64$, $p < 0.01$), while the presence of pesticides did not affect the AChE activity ($F_{(1.15)} = 4.47$, $p = 0.52$). We found that the interaction between factors affected the activity of plasma AChE ($F_{(1,15)} = 25.49$, $p < 0.01$). The *a posteriori* test revealed that animals in acclimated to the SW/P treatment, had the higher values (more than two fold) for plasma AChE than the other groups (Table 2). Likewise, the liver activity of BChE was lower in presence of pesticide (ANOVA $F_{(1,15)} = 13.41$, $p = 0.002$). However, BChE activity in liver did not change in the presence of salt ($F_{(1,15)} = 2.10$ $p = 0.168$), nor was affected by the interaction between factors ($F_{(1,15)} = 0.09$, $p = 0.772$). Although there was a trend towards to having e higher activity in both groups without pesticide, only the FW/NP group differentiate from FW/P and SW/ groups.

The pesticide also inhibited plasma BChE activity (ANOVA $F_{(1,15)} = 8.372$, $p = 0.011$), but the salinity factor did not ($F_{(1,15)} = 2.757$, $p = 0.118$). The interaction between the factors was significant ($F_{(1,15)} = 4.829$, $p = 0.044$) and the *a posteriori* test showed that the FW/P group had four times lower activities than SW/NP and FW/NP groups (Table 2). Finally, the pesticide treatment affected the liver CbE activity (ANOVA $F_{(1,15)} = 10.14$, $p = 0.006$), being lower in the CPF-treated groups respect to controls. Neither salinity treatment nor the interaction between factors had a significant effect on liver CbE activity ($F_{(1,15)} = 2.41$, $p = 0.142$ and $F_{(1,15)} = 0.23$, $p = 0.635$, respectively, see Table 2 for the *a posteriori* analyses). Conversely, plasma CbE activity was significantly altered by salinity ($F_{(1,15)} = 8.593$, $p = 0.01$), but not by the pesticide ($F_{(1,15)} = 2.471$, $p = 0.137$), nor by the interaction of both factors ($F_{(1,15)} = 0.743$, $p = 0.402$). The strong and significant difference was found between the SW/NP and FW/P groups, where a lower CbE activity was found in plasma of the FW/P group. (Table 2).

Table 2. Average values of the enzymatic activities of acetylcholinesterase (AChE), butyrylcholinesterase (BChE) and carboxylesterase (CbE), measured in plasma and liver after 45 days in the respective acclimatization regimes. Enzyme activities are reported in μmol · min^{-1} · mg $protein^{-1}$ and are tabulated as the group mean ± SD. Letters represent statistical differences.

Treatments	**SW/NP**	**FW/P**	**SW/P**	**FW/NP**
Plasma AChE	8.86 ± 1.98^{a}	3.66 ± 0.92^{a}	21.93 ± 7.83^{b}	9.02 ± 2.82^{a}
Liver AChE	7.37 ± 2.68^{a}	4.15 ± 2.45^{ab}	2.83 ± 0.14^{b}	6.53 ± 1.72^{ab}
Plasma BChE	2.27 ± 0.71^{a}	0.59 ± 0.60^{b}	2.02 ± 1.12^{ab}	2.47 ± 0.76^{a}
Liver BChE	5.43 ± 3.47^{ab}	3.61 ± 3.20^{a}	1.44 ± 0.13^{a}	5.58 ± 2.23^{b}
Plasma CbE	1957.48 ± 937.18^{a}	888.50 ± 275.83^{b}	1600.05 ± 1051.01^{ab}	1485.18 ± 879.39^{ab}
Liver CbE	560.38 ± 266.01^{a}	210.02 ± 97.64^{b}	289.01 ± 73.56^{ab}	409.77 ± 110.47^{ab}

4.5. Oxidative stress

ANOVA revealed that neither pesticide treatment nor environmental salinity affected the concentration of aqueous peroxides in plasma, muscle and skin, but a significant effect of the interaction between both factors was found ($F_{(1,15)}$=6.181, p=0.025; $F_{(1,15)}$=5.027, p=0.04 and $F_{(1,15)}$ =7.757, p=0.014 respectively). Specifically, SW/P group had ca. 200% higher values than the SW/NP and FW/P groups in the plasma; a 30% increment H_2O_2 concentrations in the FW/NP group compared with SW groups in skeletal muscle; and an increase of 25% in SW/P group compared to SW/NP group in the skin (Figure2b). Lipid peroxidation of the plasma was affected by the pesticide concentration (ANOVA $F_{(1,14)}$ = 4.816, p = 0.046), being higher in OP groups. Salinity did not affect lipid peroxidation in plasma ($F_{(1,14)}$ = 0.799, p = 0.386), and the interaction between factors was non-significant ($F_{(1,14)}$ = 0.124, p = 0.730), Figure 2a. Lipid peroxidation of the liver was not affected by salinity (ANOVA $F_{(1,14)}$ = 3.235, p=0.098), pesticide presence ($F_{(1,14)}$ =2.432, p=0.141), nor by the interaction ($F_{(1,14)}$ =2.382, p=0.145) (Figure2a). The TAC in plasma was higher in the pesticide treated groups ($F_{(1,15)}$ = 5.18, p = 0.038), but was unaffected by salinity ($F_{(1,15)}$ = 3.63, p = 0.078). Nevertheless, we found that the TAC was affected by the interaction between both factors (ANOVA $F_{(1,15)}$ = 92.14, p <0.01, see Figure 2c for details). Finally, TAC levels in liver was not affected by salt (ANOVA $F_{(1,14)}$ = 2.614, p=0.128) nor by pesticide ($F_{(1,14)}$ = 2.510, p=0.135) treatment. Finally, the interaction between both factors affected TAC in liver ($F_{(1,14)}$ = 6.335, p=0.025) and the *a posteriori* test showed higher 15% values in the FW/NP group than in SW/NP and FW/P groups (Figure 2c).

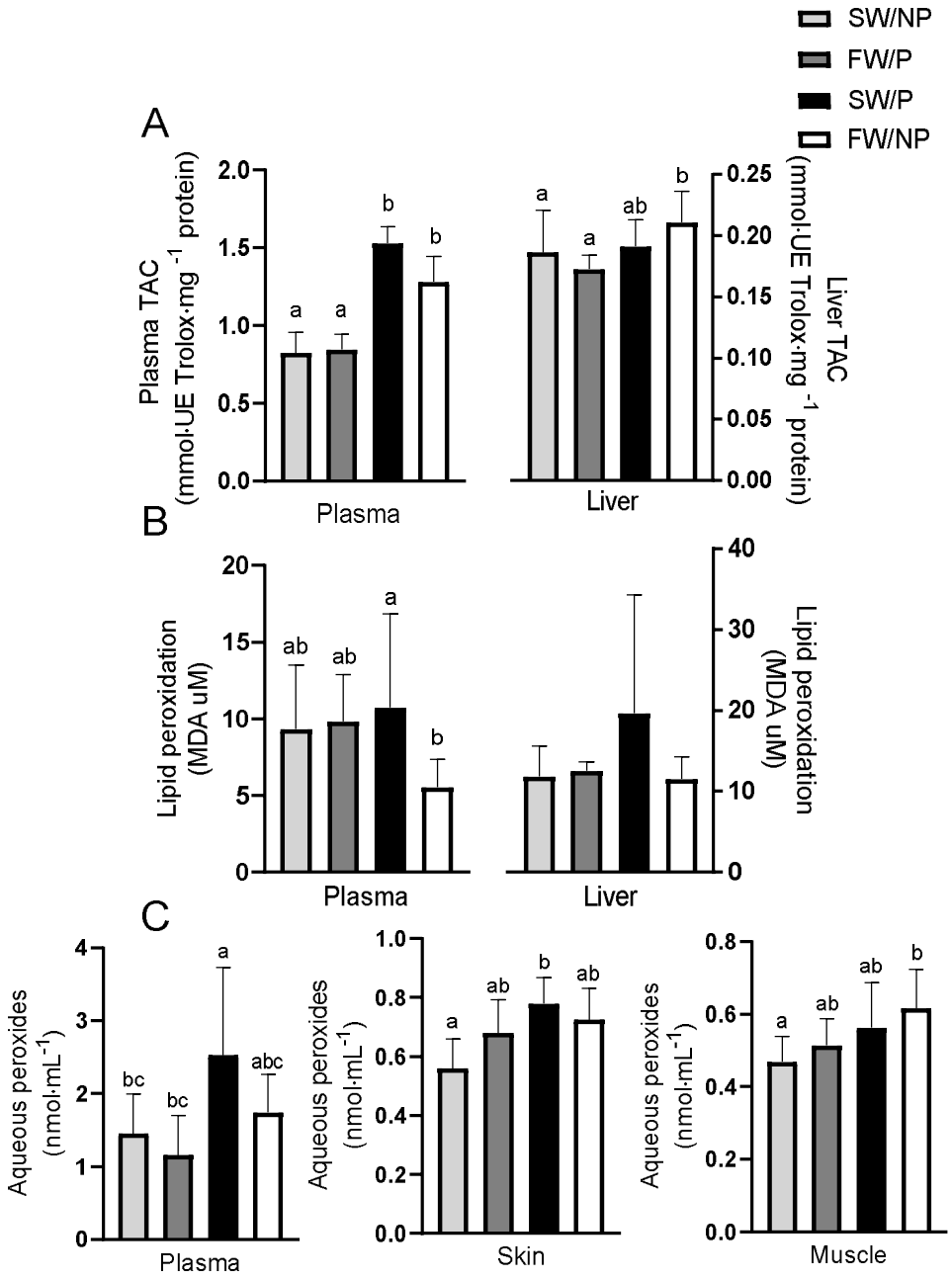

Figure 2. Levels of oxidative stress markers in *Xenopus laevis* acclimated to contrasting regimes of salinity and chlorpyrifos (A-C). (A) Total antioxidant capacity (TAC) liver and plasma of acclimatized individuals. (B) Lipid peroxidation in plasma. (C) Aqueous peroxides measured in plasma, ventral pelvic patch and skeletal muscle. Data are means ± S.D. Different letters indicate differences between groups.

4.6. Osmoregulation

Plasma osmolality was higher in SW groups than in FW groups (ANOVA $F_{(1,15)} = 12.12$, $p < 0.01$), while the pesticide had no effect ($F_{(1,15)} = 0.96$, $p = 0.34$). On the other hand, the combined effect of stressors also modified plasma osmolality ($F_{(1,15)} = 139.92$, $p < 0.01$) and the *a posteriori* test *showed* that all treatments differed. Interestingly, the osmolality of SW/P group was 10% lower that SW/NP group (Figure 3a). The concentration of urea in liver was only affected by the salinity of water (ANOVA $F_{(1,15)} = 21.10$, $p < 0.01$), being urea levels highest in the SW/NP group (see Figure 3b for detailed comparisons). Plasma urea concentration, meanwhile, was also affected only by the presence of salt (ANOVA $F_{(1,15)} = 56.50$, $p < 0.01$), specifically, we found an increase in plasma urea in individuals acclimatized to SW treatments (Figure 3b).

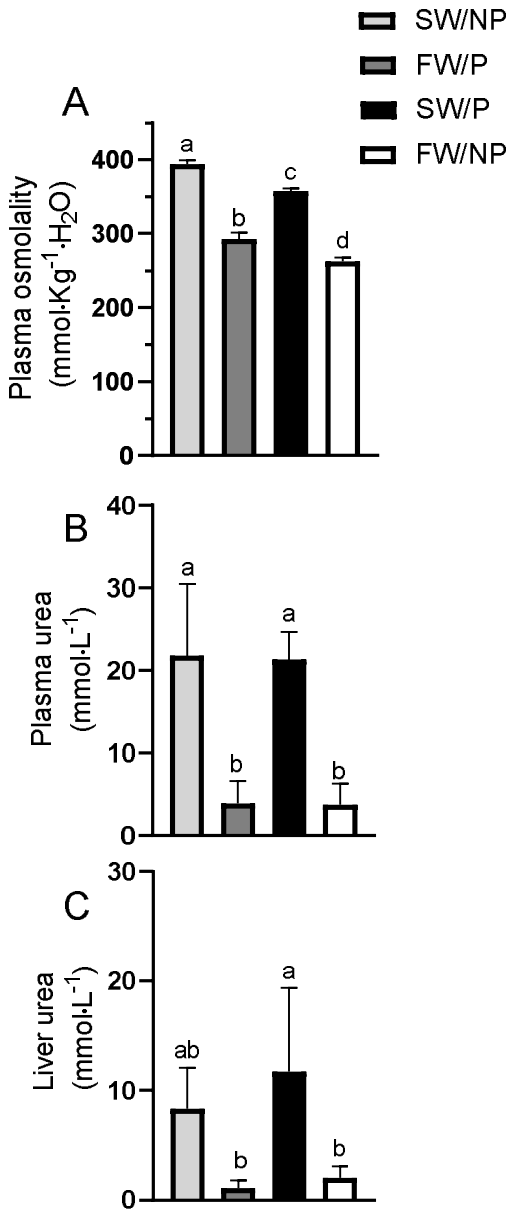

Figure 3. Osmoregulatory parameters (A, B, C). (A) Plasma osmolality after 45 days of acclimatization to 400 mOsm NaCl, 50 mOsm NaCl, 1 µg / L and 0 µg / L. (B) Urea concentration in plasma and liver (C). On the bars are letters that indicate significant differences between treatments.

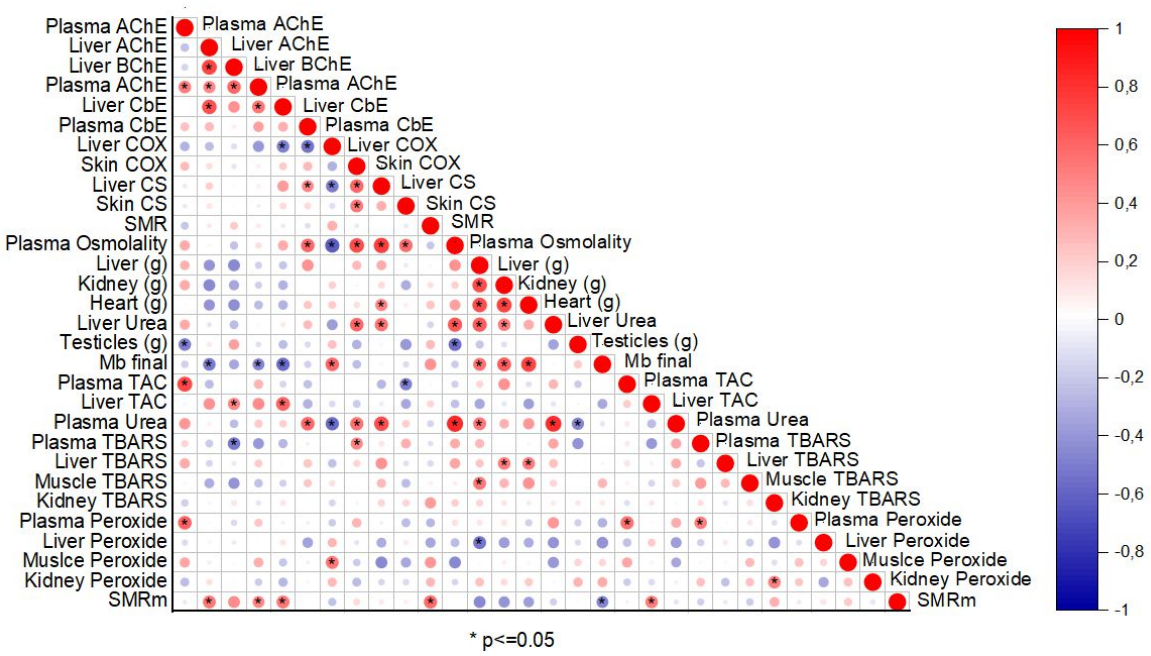

Figure 4. Graphic representation of the correlation matrix. The bar on the side indicates the pearson correlation coefficient (*r*) (-1 to 1) and the graph indicates significant correlations with an asterist ($p<0.05$).

5. DISCUSSION

5.1 Osmoregulation and energy expenditure

The main objective of this study was to evaluate the combined effect of the salinity and the exposure to CPF on several physiological and biochemical traits of *X. laevis*. At the level of osmoregulatory response, the increase in plasma and urea concentration we found in *X. laevis* (Figure 3a) is in line with what was described as a common response of amphibian species to the increase of environmental water concentration (Peña-Villalobos et al., 2016; Hidalgo et al., 2020). It is noteworthy that plasma osmolality -but not urea concentration- observed in both SW groups differed and appears to be lower in the group treated with pesticide (Figure 3a). This fact suggests that the presence of pesticides could exert an adverse or inhibitory effect on the osmoregulatory response in aquatic vertebrates, or at least in this model species. Previous studies have shown that increase plasma osmolality would be explained by increase of density and activity of Na^+/K^+ -ATPase, (Dunson & Dunson, 1975; Marshall, 2002; Lin et al., 2004; Shui et al., 2018). For example, Persian sturgeons (*Acipenser persicus*) acclimatized from freshwater to brackishwater environment, do increase the branchial Na^+ /K^+-ATPase activity, but when the environmental salinity was changed in the presence of diazinon (0.54 and 0.9 mg / mL), the enzymatic activity remain stable, accounting for the toxic effect of the pesticide on the enzyme (Hajirezaee et al., 2016). We hypothesize that differences in plasma concentration in frogs between pesticide treatments could be attributed to a decrease in the activity and concentration of Na^+ / K^+ -ATPase (Eastin et al., 1982; Lavado et al., 2009; Ajilore et al., 2018).

Changes in the biochemical capacities mentioned above could also be manifested in changes at the organism level, such as the SMR. Interestingly, experimental exposure to OP has produced

contrasting results in SMR. For example, the fish (*e.g*. *Fundulus heteroclitus* and *Astyanax aeneus*) exposed to OP pesticides depressed its metabolic rate, mainly due to muscle fatigue, which led to a reduction in locomotor and respiratory capacities (Agrahari et al., 2007; Weis et al., 2012; Sandoval-Herrera et al., 2019). In contrast, fenitrothion-exposed, starved lizards did not produce changes in SMR or feeding rate (Bain et al., 2004). Our results showed that SMR exhibited lower values in the animals treated with pesticides, reached an intermediate value in the group maintained in hypersaline medium without pesticide, while the group maintained in fresh water and without pesticide had the highest values (Table 1). Along with this, we found an increase in liver mass, as well as in CS activity in response to saline stress (Figure 1), which added to a positive correlation between hepatic COX activity and the concentration of urea in the liver ($r = 0.56$, $p = 0.013$, Figure 4), supports the idea that mounting an osmoregulatory response is an energy-demanding process, which accompanies the increase in the activities of the enzymes of the urea cycle (Shambaugh, 1977; Yiamouyiannis et al., 1992; Hoffman et al., 2009). Our results of the metabolic rate are in the same line of previous studies carried out in *X. laevis*, where an increase in the enzymatic activity of COX and CS was found in the skin of animals acclimatized to a hypoosmotic environment (Larsen, 2020; Hidalgo et al., 2020). It is likely that the increase of enzymatic activities can be explained by the active influx of Na^+ ions through the skin which in some cases can explains up to 30% of the energy budget in anuran (Zerahn, 1956).

5.2 Detoxification and stress response

Exposure to CPF produced a decrease in liver AChE activity, although only a significant difference close to 50% was found between the animals acclimated to salt water and those acclimated to both stressors (Table 2). It is interesting to note that the enzymatic activity in individuals acclimatized only to the pesticide does not alter its catabolic activity, which can be attributed to the observed hepatic inhibitions of BChE and CbE. This fact suggeststhat the activation of the enzyme protection system

is consistent with previous results found in birds and amphibians (Liendro et al., 2015; Narvaez et al., 2016) (Table 2). However, even though CPF is responsible for the inhibition of AChE (Lionetto et al., 2013; John and Shaike, 2015), the simultaneous presence of stressors would increase the inhibitory effects on hepatic AChE. This effect of salt on animal toxicity has previously been attributed to the direct effect of salt *per se* on the physiology of the species (Heugens et al., 2001, Fortin et al., 2007, Noyes et al., 2009).

AChE activity in plasma did not vary between individual treatments. However, contrary to expectations, the simultaneous presence of CPF and 400 mOsm of NaCl increases the activity of AChE (Table 2). Previous studies have reported that AChE activity decreased in fish and mollusks kept in hyperosmotic environments (Pfeifer et al., 2005; Kim et al., 2021), although under physical or chemical stress there is evidence of overexpression of the enzyme in response to inhibition of its activity (Grisaru et al., 2001; Kwom et al., 2012). Thus, we hypothesize that the increased activity of plasma AChE in animals subjected to both salinity and OP is due to the rapid and long-lasting overexpression of the AChE-R isoform, which increases its concentration and activity under stress physical or chemical precisely in response to the uninterrupted cholinergic synapse in the presence of CPF (Meshorer et al., 2005; Jameson et al., 2007; Kehat et al., 2007).

On the other hand, we observed an increase of lipid peroxidation in animals treated only with salt or OP (SW and P), which agrees with previous studies in other ectothermic animals (Amaral et al., 2012; Sanchez-Hernandez 2018; Hidalgo et al., 2020; Rivera-Ingraham et al., 2016). While, in animals acclimated to both salinity and the OP, a 200% increase in membrane damage was found (Figure 2b), suggesting that oxidative damage would be synergistically affected by high osmotic load and the pesticide, as previously reported for other environmental stressors (Sharma et al., 2010; Freitas et al., 2017; Peluso et al; 2021). The higher oxidative damage evidenced by lipid peroxidation is consistent with the higher concentration of aqueous peroxides in the skin and plasma in response to simultaneous acclimatization to both pesticide and salty water (Figure 2c). It is noteworthy to note that in skeletal muscle there is no difference between animals acclimated to salt, pesticide or both

stressors, which we hypothesize is due to the increase in urea concentration which could play a non-enzymatic antioxidant role in the animals acclimatized to a high osmotic load (Bhaskara Reddy et al., 2015; Hidalgo et al., 2020).

Regarding the antioxidant capacity, animals acclimated to low salinity water (150 mOsm NaCl) exhibited higher levels of plasma and liver TAC than the animals acclimated to a brackish environment, which could also be due to the presence of urea acting as an antioxidant, and thus prevent the synthesis of endogenous antioxidants (Figure 2a). Interestingly, if we analyze the effect of the pesticide on TAC levels, we find a decrease in the response to oxidative stress, which suggests an inhibition of antioxidant enzymatic defenses, as seen previously (Oruc et al., 2004; Janssens & Stoks, 2017; Özaslan et al., 2018). Unfortunately, our experimental design did not consider the analysis of antioxidant enzymes (*e.g.* superoxide dismutase, catalase), which due to its exclusively biological nature could have a different response. Finally, there is a tendency to increase the TAC in the plasma of the animals exposed to both stressors, which is consistent with the increase in the levels of MDA and aqueous peroxides (Figure 2a and Figure 2b). This fact, added to the positive correlation between TAC and aqueous peroxides ($r = 0.55$, $p = 0.013$, Figure 4), allows us to hypothesize that animals produce an organism-level response to oxidative damage caused by the simultaneous presence of both stressors (Costantini and Verhulst, 2009; Singh et al., 2017; Sabat et al., 2017).

6. CONCLUSION

Although the effect of salt and CPF on the physiology of anurans is relatively well described in the literature, this study focused on evaluating the physiological, biochemical, energy and oxidative responses to the simultaneous exposure of these stressors in a model species. To our knowledge, this is the first study to evaluate the factorial effect of CPF and NaCl on integrated physiological responses to osmoregulation and detoxification, as well as energy and oxidative damage in an anuro species. In summary, we found a decrease in the ability to concentrate plasma in animals acclimated simultaneously to both stressors, a condition that we attribute to the presence of CPF. Along with that, the simultaneous presence of 400 mOsm NaCl and 1 µg/L CPF has a synergistic effect on oxidative damage in frogs. Finally, despite the evident damage that the CPF develops on the physiology of the animal, we were able to identify that at the plasma level the activity of AChE increased its activity about 8 times, which we postulate is due to the presence of AChE-R isoform. While liver AChE activity remained unaltered because of the existence of an enzymatic protection system. Thus, our results suggest that the invasive *X. laevis* can tolerate moderate levels of salinity and CPF and even resisting the various physiological and biochemical adjustments that this species displays, including changes in the energy balance.

7. REFERENCES

Achuthankutty, C. T., Shrivastava, Y., Mahambre, G. G., Goswami, S. C., Madhupratap, M., 2000. Parthenogenetic reproduction of *Diaphanosoma celebensis* (Crustacea: Cladocera): influence of salinity on feeding, survival, growth and neonate production. Mar. Biol. 137, 19-22.

Agrahari, S., Pandey, K. C., Gopal, K., 2007. Biochemical alteration induced by monocrotophos in the blood plasma of fish, *Channa punctatus* (Bloch). Pesticide Biochemistry and Physiology. 88(3), 268-272.

Ajilore, B. S., Alli, A. A., Oluwadairo, T. O., 2018. Effects of magnesium chloride on in vitro cholinesterase and ATP ase poisoning by organophosphate (chlorpyrifos). Pharmacology research & perspectives. 6(3), e00401.

Amaral, M. J., Bicho, R. C., Carretero, M. A., Sanchez-Hernandez, J. C., Faustino, A. M., Soares, A. M., Mann, R. M., 2012. The use of a lacertid lizard as a model for reptile ecotoxicology studies: Part 2–Biomarkers of exposure and toxicity among pesticide exposed lizards. Chemosphere. 87(7), 765-774.

Amsinck, S. L., Jeppesen, E., Landkildehus, F., 2005. Relationships between environmental variables and zooplankton subfossils in the surface sediments of 36 shallow coastal brackish lakes with special emphasis on the role of fish. J. Paleolimnol. 33, 39-51.

Bain, D., Buttemer, W. A., Astheimer, L., Fildes, K., Hooper, M. J., 2004. Effects of sublethal fenitrothion ingestion on cholinesterase inhibition, standard metabolism, thermal preference, and prey-capture ability in the Australian central bearded dragon (*Pogona vitticeps*, agamidae). Environmental Toxicology and Chemistry: An International Journal. 23(1), 109-116.

Becker, C. G., Fonseca, C. R., Haddad, C. F. B., Batista, R. F., Prado, P. I., 2007. Habitat split and the global decline of amphibians. Science. 318(5857), 1775-1777.

Blévin, P., Tartu, S., Ellis, H. I., Chastel, O., Bustamante, P., Parenteau, C., Herzke, D., Angelier, F., Gabrielsen, G. W., 2017. Contaminants and energy expenditure in an Arctic seabird: Organochlorine pesticides and perfluoroalkyl substances are associated with metabolic rate in a contrasted manner. Environmental research. 157, 118-126.

Bhaskara Reddy, M. V., Srinivasulu, D., Peddanna, K., Apparao, C., Ramesh, P. ,2015. Synthesis and antioxidant activity of new thiazole analogues possessing urea, thiourea, and selenourea functionality. Synthetic Communications. 45(22), 2592-2600.

Bradford, M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248-254.

Boeuf, G., Payan, P., 2001. How should salinity influence fish growth? Comp. Biochem. Physiol. C. 130, 411–423.

Cecarini, V., Gee, J., Fioretti, E., Amici, M., Angeletti, M., Eleuteri, A. M., Keller, J. N., 2007. Protein oxidation and cellular homeostasis: emphasis on metabolism. Biochimica et Biophysica Acta (BBA)-Molecular Cell Research. 1773(2), 93-104.

Charmantier, G., Haond, C., Lignot, J., Charmantier-Daures, M., 2001. Ecophysiological adaptation to salinity throughout a life cycle: a review in homarid lobsters. Journal of Experimental Biology. 204(5), 967-977.

Christensen, E. A. F., Svendsen, M. B. S., & Steffensen, J. F. (2017). Plasma osmolality and oxygen consumption of perch *Perca fluviatilis* in response to different salinities and temperatures. Journal of fish biology. 90(3), 819-833.

Collins, J. P., Storfer, A., 2003) Global amphibian declines: sorting the hypotheses. Diversity and distributions. 9(2), 89-98.

Costantini, D., Verhulst, S., 2009. Does high antioxidant capacity indicate low oxidative stress?. Functional Ecology. 23(3), 506-509.

Cui, F., Li, M. X., Chang, H. J., Mao, Y., Zhang, H. Y., Lu, L. X., Yag, S.G., Lang, M.L., Qiao, C. L., 2015. Carboxylesterase-mediated insecticide resistance: Quantitative increase induces broader metabolic resistance than qualitative change. Pesticide biochemistry and physiology. 121, 88-96.

Dana, G., Lenz, P., 1986. Effects of Increasing Salinity on an Artemia Population from Mono Lake, California. Oecologia. 68(3), 428-436.

Demori, I., Rashed, Z. E., Corradino, V., Catalano, A., Rovegno, L., Queirolo, L., Salvidio, S., Biggi, E., Zanotti-Russo, M., Canesi, L., Catenazzi, A., Grasselli, E. 2019. Peptides for Skin Protection and Healing in Amphibians. Molecules (Basel, Switzerland). 24(2), 347.

Dowling, D. K., Simmons, L. W., 2009. Reactive oxygen species as universal constraints in life-history evolution. Proceedings of the Royal Society B: Biological Sciences. 276(1663), 1737-1745.

Dunson, M. K., Dunson, W. A., 1975. The relation between plasma Na concentration and salt gland Na− K ATPase content in the diamondback terrapin and the yellow-bellied sea snake. Journal of comparative physiology. 101(2), 89-97.

Dzul-Caamal, R., Domínguez-López, M. L., García-Latorre, E., Vega-López, A., 2012. Implications of cytochrome 450 isoenzymes, aryl-esterase and oxonase activity in the inhibition of the acetylcholinesterase of *Chirostoma jordani* treated with phosphorothionate pesticides. Ecotoxicology and environmental safety. 84, 199-206.

Eastin Jr, W. C., Fleming, W. J., Murray, H. C., 1982. Organophosphate inhibition of avian salt gland Na, K-ATPase activity. Comparative biochemistry and physiology. C: Comparative pharmacology. 73(1), 101-107.

Ellman, G. L., Courtney, K. D., Andres Jr, V., Featherstone, R. M., 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochemical pharmacology. *7*(2), 88-95.

Eyer, P., Worek, F., Kiderlen, D., Sinko, G., Stuglin, A., Simeon-Rudolf, V., Reiner, E., 2003. Molar absorption coefficients for the reduced Ellman reagent: reassessment. Analytical biochemistry. 312(2), 224-227.

Freitas, J. S., Felício, A. A., Teresa, F. B., de Almeida, E. A., 2017. Combined effects of temperature and clomazone (Gamit®) on oxidative stress responses and B-esterase activity of *Physalaemus nattereri* (Leiuperidae) and *Rhinella schneideri* (Bufonidae) tadpoles. Chemosphere. 185, 548-562.

Fortin, M. G., Couillard, C. M., Pellerin, J., Lebeuf, M., 2008. Effects of salinity on sublethal toxicity of atrazine to mummichog (*Fundulus heteroclitus*) larvae. Marine environmental research. 65(2), 158-170.

Gonzalez J., 2012. The physiology of hype-salinity tolerance in teleost fish, a review. J. Comp. Physiol. B. 182, 321–329.

Grisaru, D., Sternfeld, M., Eldor, A., Glick, D., Soreq, H., 1999. Structural roles of acetylcholinesterase variants in biology and pathology. European Journal of Biochemistry. 264(3), 672-686.

Hajirezaee, S., Mirvaghefi, A. R., Farahmand, H., Agh, N., 2016. Effects of diazinon on adaptation to sea-water by the endangered Persian sturgeon, *Acipenser persicus*, fingerlings. Ecotoxicology and environmental safety. 133, 413-423.

Haramura, T., Ikegami, T., Wong, M. K., Takei, Y., 2019. Preparatory Mechanisms for Salinity Tolerance in Two Congeneric Anuran Species Inhabiting Distinct Osmotic Habitats. Zoological science. 36(3), 215-222.

Hayes, T. B., Falso, P., Gallipeau, S., Stice, M., 2010. The cause of global amphibian declines: a developmental endocrinologist's perspective. Journal of Experimental Biology. 213(6), 921-933.

Heine-Fuster, I., Vega-Retter, C., Sabat, P., Ramos-Jiliberto, R., 2010. Osmoregulatory and demographic responses to salinity of the exotic Cladoceran *Daphnia Exilis*. J. Plankton Res. 32, 1405-1411.

Heugens, E. H., Hendriks, A. J., Dekker, T., Straalen, N. M. V., Admiraal, W., 2001. A review of the effects of multiple stressors on aquatic organisms and analysis of uncertainty factors for use in risk assessment. Critical reviews in toxicology. 31(3), 247-284.

Hidalgo, J., Álvarez-Vergara, F., Peña-Villalobos, I., Contreras-Ramos, C., Sanchez-Hernandez, J. C., Sabat, P., 2020. Effect of salinity acclimation on osmoregulation, oxidative stress, and metabolic enzymes in the invasive *Xenopus laevis*. Journal of Experimental Zoology Part A: Ecological and Integrative Physiology. 333(5), 333-340.

Hoffmann, E. K., Lambert, I. H., Pedersen, S. F., 2009. Physiology of cell volume regulation in vertebrates. Physiological reviews. 89(1), 193-277.

Inoda, T., & Morisawa, M., 1987. Effect of osmolality on the initiation of sperm motility in *Xenopus laevis*. Comparative biochemistry and physiology. A, Comparative physiology, 88(3), 539-542.

Jameson, R. R., Seidler, F. J., Slotkin, T. A., 2007. Nonenzymatic functions of acetylcholinesterase splice variants in the developmental neurotoxicity of organophosphates: chlorpyrifos, chlorpyrifos oxon, and diazinon. Environmental health perspectives. 115(1), 65-70.

Janssens, L., Stoks, R., 2017. Chlorpyrifos-induced oxidative damage is reduced under warming and predation risk: Explaining antagonistic interactions with a pesticide. Environmental Pollution. 226, 79-88.

Jeppesen, E., Søndergaard, M., Kanstrup, E., Petersen, B., Eriksen, R. B., Hammershøj, M., Mortensen, E., Jensen, J. P., Have, A., 1994. Does the impact of nutrients on the biological structure

and function of brackish and freshwater lakes differ?. Hydrobiologia. 276, 15-30.

John, E. M., Shaike, J. M., 2015. Chlorpyrifos: pollution and remediation. Environmental Chemistry Letters. 13(3), 269-291.

Johnson, K. R., Hindmarch, C. C. T., Salinas, Y. D., Shi, Y., Greenwood, M., Hoe, S. Z., Murphy, D., Gainer, H., 2015. A RNA-Seq analysis of the rat supraoptic nucleus transcriptome: effects of salt loading on gene expression. PloS one. 10(4), e0124523.

Jones, H. R., Johnson, K. M., Kelly, M. W., 2019. Synergistic effects of temperature and salinity on the gene expression and physiology of *Crassostrea virginica*. Integrative and comparative biology. 59(2), 306-319.

Katz, U., Hanke, W., 1993. Mechanisms of hyperosmotic acclimation in *Xenopus laevis* (salt, urea or mannitol). Journal of Comparative Physiology B. 163(3), 189-195.

Kehat, R., Zemel, E., Cuenca, N., Evron, T., Toiber, D., Loewenstein, A., Soreq, H., Perlman, I., 2007. A novel isoform of acetylcholinesterase exacerbates photoreceptors death after photic stress. Investigative ophthalmology & visual science. 48(3), 1290-1297.

Kim, J. H., Jeong, E. H., Jeon, Y. H., Kim, S. K., Hur, Y. B., 2021. Salinity-mediated changes in hematological parameters, stress, antioxidant responses, and acetylcholinesterase of juvenile olive flounders (*Paralichthys olivaceus*). Environmental Toxicology and Pharmacology. 83, 103597.

Kwon, D. H., Choi, J. Y., Je, Y. H., Lee, S. H., 2012. The overexpression of acetylcholinesterase compensates for the reduced catalytic activity caused by resistance-conferring mutations in

Tetranychus urticae. Insect biochemistry and molecular biology. 42(3), 212-219.

Larsen, E. H., 2020. Dual skin functions in amphibian osmoregulation. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology. 110869.

Lavado, R., Aparicio-Fabre, R., Schlenk, D., 2013. Effects of salinity acclimation on the pesticide-metabolizing enzyme flavin-containing monooxygenase (FMO) in rainbow trout (*Oncorhynchus mykiss*). Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology. 157(1), 9-15.

Liendro, N., Ferrari, A., Mardirosian, M., Lascano, C. I., Venturino, A., 2015. Toxicity of the insecticide chlorpyrifos to the South American toad *Rhinella arenarum* at larval developmental stage. Environmental toxicology and pharmacology. 39(2), 525-535.

Lin, C. H., Tsai, R. S., Lee, T. H., 2004. Expression and distribution of Na, K-ATPase in gill and kidney of the spotted green pufferfish, *Tetraodon nigroviridis*, in response to salinity challenge. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology. 138(3), 287-295.

Lionetto, M. G., Caricato, R., Calisi, A., Giordano, M. E., Schettino, T., 2013. Acetylcholinesterase as a biomarker in environmental and occupational medicine: new insights and future perspectives. BioMed research international, 2013.

Liu, Y., Zhang, H., Qiao, C., Lu, X., Cui, F., 2011. Correlation between carboxylesterase alleles and insecticide resistance in *Culex pipiens* complex from China. Parasites & vectors. 4(1), 236.

Lockridge, O., Masson, P., 2000. Pesticides and susceptible populations: people with butyrylcholinesterase genetic variants may be at risk. Neurotoxicology. 21(1-2), 113-126.

Lowe, C. N., Butt, K. R., 2005. Culture techniques for soil dwelling earthworms: a review. Pedobiologia. 49(5), 401-413.

Lushchak, V. I., Matviishyn, T. M., Husak, V. V., Storey, J. M., Storey, K. B., 2018. Pesticide toxicity: a mechanistic approach. EXCLI journal. 17, 1101.

Mack, R. N., Simberloff, D., Mark Lonsdale, W., Evans, H., Clout, M., Bazzaz, F. A., (2000). Biotic invasions: causes, epidemiology, global consequences, and control. Ecol. Appl. 10, 689-710.

Marshall, W. S., 2002. Na^+, Cl^-, Ca^{2+} and Zn^{2+} transport by fish gills: retrospective review and prospective synthesis. Journal of experimental zoology. 293(3), 264-283.

McCormick, S. D., Bradshaw, D., 2006. Hormonal control of salt and water balance in vertebrates. General and comparative endocrinology. 147(1), 3-8.

Martinez-Alvarez, R. M., Hidalgo, M. C., Domezain, A., Morales, A. E., García-Gallego, M., Sanz, A, 2002. Physiological changes of sturgeon *Acipenser naccarii* caused by increasing environmental salinity. Journal of experimental biology. 205(23), 3699-3706.

Meshorer, E., Bryk, B., Toiber, D., Cohen, J., Podoly, E., Dori, A., Soreq, H., 2005. SC35 promotes sustainable stress-induced alternative splicing of neuronal acetylcholinesterase mRNA. Molecular psychiatry. 10(11), 985-997

Monaghan, P., Metcalfe, N. B., Torres, R., 2009. Oxidative stress as a mediator of life history trade-offs: mechanisms, measurements and interpretation. Ecology letters. 12(1), 75-92.

Montory, J. A., Pechenik, J. A., Diederich, C. M., Chaparro, O. R., 2014. Effects of low salinity on adult behavior and larval performance in the intertidal gastropod *Crepipatella peruviana* (Calyptraeidae). PloS one. 9(7), e103820.

Mora, M., Pons, D. J., Peñafiel-Ricaurte, A., Alvarado-Rybak, M., Lebuy, S., & Soto-Azat, C., 2019. High abundance of invasive African clawed frog *Xenopus laevis* in Chile: challenges for their control and updated invasive distribution. Management of Biological Invasions. 10(2), 377-388.

Moyes, C. D., Mathieu-Costello, O. A., Tsuchiya, N., Filburn, C., Hans-ford, R. G., 1997. Mitochondrial biogenesis during cellular differentiation. Am. J. Physiol. 272, C1345-C1351.

Narváez, C., Ríos, J. M., Píriz, G., Sanchez-Hernandez, J. C., Sabat, P., 2016. Subchronic exposure to chlorpyrifos affects energy expenditure and detoxification capacity in juvenile Japanese quails. Chemosphere. 144, 775-784.

Naz, H., Abdullah, S., Abbas, K., Hassan, W., Batool, M., Perveen, S., Maalik, S., Mushtaq, S. (2019). Toxic effect of insecticides mixtures on antioxidant enzymes in different organs of fish, *Labeo rohita*. Pakistan J. Zool, 51(4). 1355-1361.

Noyes, P. D., McElwee, M. K., Miller, H. D., Clark, B. W., Van Tiem, L. A., Walcott, K. C., Erwin, K.N., Levin, E. D., 2009. The toxicology of climate change: environmental contaminants in a warming world. Environment international. 35(6), 971-986.

Ohkawa, H., Ohishi, N., Yagi, K., 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Analytical biochemistry. 95(2), 351-358.

Oruc, E. O., Sevgiler, Y., Uner, N., 2004. Tissue-specific oxidative stress responses in fish exposed to 2, 4-D and azinphosmethyl. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology. 137(1), 43-51.

Özaslan, M. S., Demir, Y., Aksoy, M., Küfrevioğlu, Ö. I., Beydemir, Ş., 2018. Inhibition effects of pesticides on glutathione-S-transferase enzyme activity of Van Lake fish liver. Journal of biochemical and molecular toxicology. 32(9), e22196.

Paital, B., Chainy, G. B. N., 2010. Antioxidant defenses and oxidative stress parameters in tissues of mud crab (*Scylla serrata*) with reference to changing salinity. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology. 151(1), 142-151.

Peluso, J., Furió Lanuza, A., Pérez Coll, C. S., Aronzon, C. M., 2021. Synergistic effects of glyphosate-and 2, 4-D-based pesticides mixtures on *Rhinella arenarum* larvae. Environmental Science and Pollution Research. 1-10.

Peña-Villalobos, I., Narváez, C., Sabat, P., 2016. Metabolic cost of osmoregulation in a hypertonic environment in the invasive African clawed frog *Xenopus laevis*. Biol. Open. 5, 955-961.

Pettersen, A. K., Marshall, D. J., White, C. R., 2018. Understanding variation in metabolic rate. Journal of Experimental Biology. 221(1), jeb166876.

Pfeifer, S., Schiedek, D., & Dippner, J. W., 2005. Effect of temperature and salinity on

acetylcholinesterase activity, a common pollution biomarker, in *Mytilus sp.* from the south-western Baltic Sea. Journal of experimental marine biology and ecology. 320(1), 93-103.

Pinya, S., Tejada, S., Capó, X., & Sureda, A. (2016). Invasive predator snake induces oxidative stress responses in insular amphibian species. Science of the Total Environment. 566, 57-62.

Pope, C., Karanth, S., Liu, J., 2005. Pharmacology and toxicology of cholinesterase inhibitors: uses and misuses of a common mechanism of action. Environmental toxicology and pharmacology. 19(3), 433-446.

Racke, K. D., 1993. Environmental fate of chlorpyrifos. Reviews of environmental contamination and toxicology. 1-150.

Richards, S., & Kendall, R. (2003). Physical effects of chlorpyrifos on two stages of *Xenopus laevis.* Journal of Toxicology and Environmental Health Part A. 66(1), 75-91.

Rivera-Ingraham, G. A., Barri, K., Boël, M., Farcy, E., Charles, A. L., Geny, B., Lignot, J. H., 2016. Osmoregulation and salinity-induced oxidative stress: is oxidative adaptation determined by gill function?. Journal of Experimental Biology. 219(1), 80-89.

Rivera-Ingraham, G.A., Lignot, J.H., 2017. Osmoregulation, bioenergetics and oxidative stress in coastal marine invertebrates: raising the questions for future research. J. Exp. Biol. 220:1749–1760.

Rodriguez, C., Mayo, J. C., Sainz, R. M., Antolín, I., Herrera, F., Martín, V., Reiter, R. J., 2004. Regulation of antioxidant enzymes: a significant role for melatonin. Journal of pineal research. 36(1), 1-9.

Rodriguez, M C., Tarnopolsky, M. A., 2003. Patients with dystrophinopathy show evidence of increased oxidative stress. Free Radical Biology and Medicine. 34(9), 1217-1220.

Rosenbaum, E. A., Duboscq, L., Soleño, J., Montagna, C. M., Ferrari, A., Venturino, A., 2012. Response of biomarkers in amphibian larvae to in situ exposures in a fruit-producing region in North Patagonia, Argentina. Environmental toxicology and chemistry. 31(10), 2311-2317.

Sabat, P., Narváez, C., Peña-Villalobos, I., Contreras, C., Maldonado, K., Sanchez-Hernandez, J. C., Newsome, S.D., Nespolo, R., Bozinovic, F., 2017. Coping with salt water habitats: metabolic and oxidative responses to salt intake in the rufous-collared sparrow. Frontiers in physiology. 8, 654.

SAG., 2012. Declaración de Venta de Plaguicidas, año 2012. Santiago, Chile: Servicio Agrícola y Ganadero, División Protección Agrícola y Forestal.

Sanchez-Hernandez, J. C., 2018. Biochar activation with exoenzymes induced by earthworms: A novel functional strategy for soil quality promotion. Journal of hazardous materials. 350, 136-143.

Sanchez-Hernandez, J. C., Del Pino, J. N., Capowiez, Y., Mazzia, C., Rault, M., 2018. Soil enzyme dynamics in chlorpyrifos-treated soils under the influence of earthworms. Science of the Total Environment. 612, 1407-1416.

Sandoval-Herrera, N., Mena, F., Espinoza, M., Romero, A., 2019. Neurotoxicity of organophosphate pesticides could reduce the ability of fish to escape predation under low doses of exposure. Scientific reports. 9(1), 10530.

Sarma, S. S. S., Nandini, S., Morales-Ventura, J., Delgado-Martínez, I., González-Valverde, L., 2006.

Effects of NaCl salinity on the population dynamics of freshwater zooplankton (rotifers and cladocerans). Aquat. Ecol. 40, 349-360.

Seebacher, F., Brand, M. D., Else, P. L., Guderley, H., Hulbert, A. J., Moyes, C. D., 2010. Plasticity of oxidative metabolism in variable climates: molecular mechanisms. Physiological and Biochemical Zoology. 83(5), 721-732.

Selman, C., Blount, J. D., Nussey, D. H., Speakman, J. R., 2012. Oxidative damage, ageing, and life-history evolution: where now? Trends in ecology & evolution. 27(10), 570-577.

Schallenberg, M., Hall, C. J., Burns, C. W., 2003. Consequences of climate-induced salinity increases on zooplankton abundance and diversity in coastal lakes. Mar. Ecol. Prog. Ser. 251, 181-189.

Schiedek, D., Sundelin, B., Readman, J. W., Macdonald, R. W., 2007. Interactions between climate change and contaminants. Marine pollution bulletin. 54(12), 1845-1856.

Shambaugh III, G. E., 1977. Urea biosynthesis I. The urea cycle and relationships to the citric acid cycle. The American journal of clinical nutrition. 30(12), 2083-2087.

Sharma, H., Zhang, P., Barber, D. S., Liu, B., 2010. Organochlorine pesticides dieldrin and lindane induce cooperative toxicity in dopaminergic neurons: role of oxidative stress. Neurotoxicology. 31(2), 215-222.

Shui, C., Shi, Y., Hua, X., Zhang, Z., Zhang, H., Lu, G., Xie, Y., 2018. Serum osmolality and ions, and gill Na+/K+-ATPase of spottedtail goby *Synechogobius ommaturus* (R.) in response to acute salinity changes. Aquaculture and fisheries. 3(2), 79-83.

Singh, N., Gupta, V. K., Kumar, A., Sharma, B., 2017. Synergistic effects of heavy metals and pesticides in living systems. Frontiers in chemistr. 5, 70.

Sidell, B. D., Driedzic, W. R., Stowe, D. B., Johnston, I. A., 1987. Biochemical correlations of power development and metabolic fuel preferenda in fish hearts. Physiological Zoology. 60(2), 221-232.

Spinazzi, M., Casarin, A., Pertegato, V., Salviati, L., Angelini, C., 2012. Assessment of mitochondrial respiratory chain enzymatic activities on tissues and cultured cells. Nature protocols. 7(6), 1235-1246.

Southwood Williard, A., Harden, L. A., Jones, T. T., Midway, S. R., 2019. Effects of temperature and salinity on body fluid dynamics and metabolism in the estuarine diamondback terrapin (*Malaclemys terrapin*). Journal of Experimental Biology. 222(10), jeb202390.

Sutton, A. O., Turko, A. J., McLaughlin, R. L., Wright, P. A., 2018. Behavioral and physiological responses of an amphibious, euryhaline mangrove fish to acute salinity exposure. Copeia. 106(2), 305-311.

Tremblay, N., Abele, D., 2016. Response of three krill species to hypoxia and warming: an experimental approach to oxygen minimum zones expansion in coastal ecosystems. Mar. Ecol. 37, 179–199.

Thompson, H. M., 1999. Esterases as markers of exposure to organophosphates and carbamates. Ecotoxicology. 8(5), 369-384.

Vakifahmetoglu-Norberg, H., Ouchida, A.T., Norberg, E., 2017. The role of mitochondria in metabolism and cell death. Biochemical and Biophysical Research Communications.482(3):426-431.

Van de Crommenacker, J., Horrocks, N. P., Versteegh, M. A., Komdeur, J., Tieleman, B. I., Matson, K. D., 2010. Effects of immune supplementation and immune challenge on oxidative status and physiology in a model bird: implications for ecologists. Journal of Experimental Biology. 213(20), 3527-3535.

Velez, C., Figueira, E., Soares, A. M., Freitas, R., 2016. Native and introduced clams biochemical responses to salinity and pH changes. Sci. Total Environ. 566, 260–268.

Weaver, P. F., Tello, O., Krieger, J., Marmolejo, A., Weaver, K. F., Garcia, J. V., Cruz, A., 2016. Hypersalinity drives physiological and morphological changes in *Limia perugiae* (Poeciliidae). Biology Open. 5(8), 1093-1101.

Weis, J. S., Candelmo, A., 2012. Pollutants and fish predator/prey behavior: a review of laboratory and field approaches. Current Zoology. 58(1), 9-20.

Wheelock, C. E., Eder, K. J., Werner, I., Huang, H., Jones, P. D., Brammell, B. F., Elskus, A.A., Hammock, B. D. (2005). Individual variability in esterase activity and CYP1A levels in Chinook salmon (*Oncorhynchus tshawytscha*) exposed to esfenvalerate and chlorpyrifos. Aquatic Toxicology. 74(2), 172-192.

Withers, P. C., 1977. Measurement of VO_2, VCO_2, and evaporative water loss with a flow-through mask. Journal of Applied Physiology. 42(1), 120-123.

Yiamouyiannis, C. A., Sanders, R. A., Watkins III, J. B., Martin, B. J., 1992. Chronic physical activity: hepatic hypertrophy and increased total biotransformation enzyme activity. Biochemical

pharmacology. 44(1), 121-127.

Zerahn, K., 1956. Oxygen Consumption and Active Sodium Transport in the Isolated and Short-Circuited Frog Skin1. Acta physiologica Scandinavica. 36(4), 300-318.