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Effect of salinity acclimation on osmoregulation, oxidative stress, and metabolic enzymes in the invasive *Xenopus laevis*

Jaime Hidalgo[1] | **Felipe Álvarez-Vergara**[1] | **Isaac Peña-Villalobos**[1,2] | **Carolina Contreras-Ramos**[1] | **Juan C. Sanchez-Hernandez**[3] | **Pablo Sabat**[1,4]

[1]Departamento de Ciencias Ecológicas, Facultad de Ciencias, Universidad de Chile, Casilla 653, Santiago, Chile

[2]Laboratorio de Células troncales y Biología del Desarrollo, Departamento de Biología, Facultad de Ciencias, Universidad de Chile, Santiago, Chile

[3]Laboratory of Ecotoxicology, Faculty of Environmental Sciences and Biochemistry, University of Castilla-La Mancha, Toledo, Spain

[4]Departamento de Ecología, Center of Applied Ecology & Sustainability (CAPES-UC), Pontificia Universidad Católica de Chile, Santiago, Chile

Correspondence
Pablo Sabat, Departamento de Ciencias Ecológicas, Facultad de Ciencias, Universidad de Chile, Casilla 653, Santiago, Chile.
Email: psabat@uchile.cl

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Abstract
Aquatic animals often display physiological adjustments to improve their biological performance and hydrosaline balance in saline environments. In addition to energetic costs associated with osmoregulation, oxidative stress, and the activation of the antioxidant system are common cellular responses to salt stress in many species, but the knowledge of osmoregulation-linked oxidative homeostasis in amphibians is scarce. Here we studied the biochemical responses and oxidative responses of *Xenopus laevis* females exposed for 40 days to two contrasting salinities: hypo-osmotic (150 mOsm·kg^{-1}·H_2O NaCl, HYPO group) and hyper-osmotic environments (340 mOsm·kg^{-1}·H_2O NaCl, HYPER group). We found an increase of plasma osmolality and plasma urea concentration in the animals incubated in the HYPER treatment. Increases in electrolyte concentration were paralleled with an increase of both citrate synthase and cytochrome *c* oxidase activities in liver and heart. Interestingly, HYPO group had higher catabolic activity of the skin and liver total antioxidant capacity (TAC), compared with animals from the HYPER group. Moreover, there was an inverse relationship between liver TAC and plasma osmolality; and with the metabolic enzymes from liver. These findings suggest that salinity induces changes in urea metabolism and specific activity of metabolic enzymes, which appears to be tissue-dependent in *X. laevis*. Contrary to our expectations, we also found a moderate change in the oxidative status as revealed by the increase in TAC activity in the animals acclimated to low salinity medium, but constancy in the lipid peroxidation of membranes.

KEYWORDS
antioxidant capacity, metabolic enzymes, oxidative status, ROS, salinization

1 | INTRODUCTION

Our planet is undergoing rapid environmental changes and there is broadly recognized evidence of a sustained rise in ambient temperatures, increased frequency of climatic extremes (e.g., droughts, excessive precipitation events, and heat waves), and substantial habitat alteration, wildlife over-exploitation, introduction of exotic species and pollution (IPCC, 2014). These phenomena often disrupt fundamental ecological processes, which in turn can have irreversible effects on wildlife, and particularly impacting animal performance (Barnosky et al., 2011; Vitousek, 1994). One of the most significant alterations derived from climate change is wetland salinization (Albecker & McCoy, 2017; Vineis, Chan, & Khan, 2011), which represents an acute process with adverse consequences to organismal fitness (Heine-Fuster, Vega-Retter, Sabat, & Ramos-Jiliberto, 2010; Mack et al., 2000). A deeper knowledge of the physiology of both native and exotic species can provide

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understanding on its adaptive potential to fluctuating environments, so predictions on species distribution and abundance can be accurately performed (Heine-Fuster et al., 2010; Sarma, Nandini, Morales-Ventura, Delgado-Martínez, & González-Valverde, 2006; Schallenberg, Hall, & Burns, 2003).

Salinity is a critical environmental driver in the acclimatization capacity of many aquatic animals, thus modulating many traits of their biology such as growth rate, reproductive success, behaviour and survival (Montory, Pechenik, Diederich, & Chaparro, 2014; Rivera-Ingraham & Lignot, 2017; Weaver et al., 2016). Changes in salinity generally induces an increase in the metabolic cost because of an enhanced energetic demand for transmembrane ion transport, synthesis of osmoactive molecules and blood pumping. These cellular and physiological responses have been documented in several taxa including aquatic invertebrates (Fiess et al., 2007; Ramaglia, de Castro, & Augusto, 2018) and vertebrates (Jones, Johnson, & Kelly, 2019; Peña-Villalobos, Narváez, & Sabat, 2016; Sutton, Turko, McLaughlin, & Wright, 2018; Williard, Harden, Jones, & Midway, 2019), and recently in terrestrial vertebrates (Gutiérrez et al., 2011; Peña-Villalobos, Valdés-Ferranty, & Sabat, 2013; Sabat et al., 2017). In the mollusk *Mya arenaria*, for instance, the increase of salinity affects the respiratory rate (Lasota, Sokolowski, Smolarz, Sromek, & Dublinowska, 2018) and triggers changes in mitochondrial enzyme activities that lead to alterations in energy supply (Sokolova, 2018).

There is a consensus that amphibians are extremely sensitive organisms to changes in the physicochemical properties of their environments (Becker, Fonseca, Haddad, Batista, & Prado, 2007; Collins & Storfer, 2003; Hopkins & Brodie, 2015; Relyea, 2003). In amphibians, a negative effect of salinity has been reported elsewhere, such as, changes in behavior, development and growth rate, among others (Katz & Hanke, 1993; Kearney, Byrne, & Reina, 2012). Particularly, physiological adjustments of amphibians to saline stress includes enhanced urea synthesis and plasma concentration (Jørgensen, 1997). Recently, we found that the anuran *Xenopus laevis* acclimatized to contrasting regimes of water salinity, responded with a significant increase of metabolic rate (ca., 80%) in hyperosmotic conditions respect to animals kept in isosmotic conditions (Peña-Villalobos et al., 2016). These metabolic changes were accompanied by an increase in plasma osmolality and urea concentration as well as higher activity of metabolic enzymes in liver and heart of hyperosmotic-exposed anurans.

In addition to the energetic costs associated with osmoregulatory responses, oxidative metabolism is also a costly biological process (Jimenez, 2018). The cellular oxidative metabolism generates reactive oxygen species (ROS), which can interact with macromolecules (e.g., lipids, proteins, and nucleic acids) ultimately leading to disruption of multiple cellular processes when ROS are produced in excess (oxidative stress status; Giraud-Billoud et al., 2019; Monaghan, Metcalfe, & Torres, 2009, 2010; Selman, Blount, Nussey, & Speakman, 2012). Several studies with aquatic invertebrates have revealed that oxidative disturbance caused by hypersalinity could be an indirect effect of tissue hypoxia (An, Kim, Shin, Kil, & Choi, 2010; Freire, Togni, & Hermes-Lima, 2011; Rivera-Ingraham & Lignot, 2017) or changes in respiration rates, which in turn increase the ROS formation (Rivera-Ingraham et al., 2016). To protect against ROS-generated oxidative stress, aerobic animals often activate both enzymatic and molecular antioxidant systems in response to elevated environmental salinity; a physiological response that can be observed in invertebrates, fishes, turtles, and birds (Ding et al., 2019; Rivera-Ingraham & Lignot, 2017; Sabat et al., 2017; Velez, Figueira, Soares, & Freitas, 2016). However, knowledge of how amphibians use these antioxidant mechanisms to compensate oxidative stress under salinity fluctuations is still limited. To fill this gap, we explore the responses of selected biomarkers of oxidative stress, plasma osmolality, urea concentration, and mitochondrial metabolic enzymes of *X. laevis* during chronic exposure to saltwater and freshwater.

We hypothesized that toads acclimated to the hypersaline environment will trigger antioxidant mechanisms to maintain oxidative homeostasis, and we predict that they will exhibit higher levels of mitochondrial enzyme activities in the internal organs than animals exposed to an hypoosmotic medium.

2 | METHODS

Adults female *X. laevis* (*n* = 10) were obtained from a feral population in San Antonio, a mesic coastal locality of central Chile (for a detailed description of the capture site, see Peña-Villalobos et al., 2016). Animals were trapped in summer 2017, transported to the laboratory and randomly assigned to two salinity conditions (hypoosmotic and hyper-osmotic, five individuals per treatment), which were simulated by altering NaCl concentration. Animals were maintained in water with salinity at 109 $mOsm \cdot kg^{-1} \cdot H_2O$ using individual plastic containers (61 L) at 25°C, and light:dark cycle of 12 hr:12 hr. In the hyperosmotic group (HYPER), the salt concentration was increased at a rate of 23.1 $mOsm \cdot kg^{-1} \cdot H_2O$ per day for 10 days until it reach 340 $mOsm \cdot kg^{-1} \cdot H_2O$. The hypo-osmotic (HYPO) group was acclimated up to 150 $mOsm \cdot kg^{-1} \cdot H_2O$ (at a rate of 4.1 $mOsm \cdot kg^{-1} \cdot H_2O$ per day); a value considered as hypo-osmotic because *X. laevis* inhabiting freshwater environments exhibit a plasma osmolarity of 235 $mOsm \cdot kg^{-1} \cdot H_2O$ (McBean & Goldstein, 1967). Animals were kept in these saline conditions for 40 days in starvation conditions, to restrict the input of water and ions through food (Peña-Villalobos et al., 2016).

2.1 | Biochemical determinations

At the end of the acclimation, animals were euthanized by decapitation (Katz & Hanke, 1993), the organs were weighted and tissues (liver, heart, ventral skin, and leg muscle) were frozen at −80°C for further biochemical analysis. Blood was collected from heart using capillary tubes, then centrifuged at 12,000*g* for 5 min, and the plasma (8 μL) was used for osmolality measurement by vapor pressure osmometry (Wescor 5130B). The remained plasma was stored (−80°C) for further analyses. The urea concentration (mg/dl) was determined by the urease/Berthelot method using a commercial kit

(Valtek, Chile). Liver, kidney, skin, muscle, and heart samples were homogenized in Na–phosphate buffer (0.1 M, pH 7.3), and protein concentrations were determined using the method by Bradford (1976) with bovine serum albumin as the standard.

The mitochondrial enzymes cytochrome *c* oxidase (COX; E.C. 1.9.3.1) and citrate synthase (CS; E.C. 4.1.3.7) were measured in liver, heart, leg muscle, and skin. The former is an enzyme involved in the last step of the mitochondrial respiratory chain, and it is indicative of the energy capacity of the mitochondrial system, whereas the latter enzyme activity participates in Krebs cycle. An increase in the activity of these enzymes reflects changes in both the functional properties and the density of mitochondria. COX activity was measured using a spectrophotometric method. Briefly, enzyme 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. Enzyme activity was calculated using an extinction coefficient of 21.84 mM/cm at 550 nm for cytochrome *c*. CS activity was measured as follow. The enzyme assay medium contained 10 mM Tris-HCl (pH 8.0), 10 mM 5,5′-dithiobis(2-nitrobenzoic acid), 30 mM acetyl coenzyme A (acetyl CoA) and 10 mM oxaloacetic acid (OAA; omitted in the controls) in a final volume of 0.2 ml. Enzyme activity was calculated using an extinction coefficient of 13.6 mM/cm at 412 nm. For both enzyme activities, changes in absorbance were monitored using a Thermo Scientific Multiscan monochromator-based UV/VIS spectrophotometer at 25°C. All enzyme activities are expressed as specific activity per milligram of protein ($\mu mol \cdot min^{-1} \cdot mg^{-1}$).

2.2 | Oxidative stress biomarkers

2.2.1 | Total antioxidant capacity (TAC)

Tissues were diluted at 10× and homogenized with phosphate-buffered saline (PBS), then centrifuged at 10,000*g* for 10 min at 4°C. The supernatant was immediately stored at −80°C for future analyses. We evaluated the total antioxidant capacity. The assay kit is produced by Cell Biolabs OxiSelect™ (San Diego, CA; # STA-360) is based on the reduction of copper (II) to copper (I) by antioxidants such as uric acid, the copper reduced react with a coupling chromogenic reagent that produces a color with a maximum absorbance at 490 nm. Antioxidant capacity was determined by comparison with the uric acid standards optical density lectures (0-1 mM).

2.2.2 | Lipid peroxidation

A sample of each tissue was washed with PBS containing heparin and diluted 10-fold with PBS containing 5% butylated hidroxytoluene to prevent sample oxidation. Tissues were homogenized and centrifuged at 10,000*g* for 5 min at 4°C. The supernatant was removed and stored at −80°C until measurement. Lipid peroxidation was assessed via measurement of thiobartbituric acid. The assay kit test by Cell Biolabs OxiSelect™ (# STA-330) evaluates a 1:2 adduct formed between malondialdehyde (MDA) and thiobarbituric acid (TBA). The MDA-TBA adduct was determined by a colorimetric assay at 532 nm and using a calibration curve constructed with MDA (0–125 μM).

2.2.3 | Nitric oxide

A subsample of blood was collected with ethylenediaminetetraacetic acid and centrifuged at 10,000 RPM for 10 min at 4°C. Liver tissue was diluted (10×), homogenized with PBS and centrifuged at 12,000 RPM for 10 min at 4°C. After discarding the pellet, the homogenate was again centrifuged in a 10 kDa filter nitric oxide (NO) concentration was determined according to Patton and Kryskalla (2011) using the commercial kit from Biovision, Milpitas, CA (# K262). NO is rapidly oxidized to nitrite and nitrate which allows the quantification of NO production. The test consisted of two steps, involving the conversion of nitrate to nitrite using nitrate reductase, and then the use of Griess Reagents to convert nitrite into an azo component. The amount of azochromophore formed reflects the amount of NO in the sample, which was monitored at 540 nm. Nitrite concentrations were determined by comparing the absorbance values of the test samples with a standard curve generated by serial dilution of a stock solution (100 mM) of sodium nitrite.

2.3 | Statistical analysis

Variations in body mass as a function of salinity and time (before and after the acclimation period) were evaluated by repeated-measures analysis of variance, whereas comparisons of biochemical parameters and enzymatic activities were performed using the Student *t* test. We evaluated the potential associations between physiological and biochemical variables within treatments by determining the Pearson correlation coefficient. Data were examined for assumptions of normality and homogeneity of variance using Kolmogorov–Smirnov and Levene tests, before each statistical analysis. Statistical analyses were performed using the STATISTICA® (2004) statistical package for Windows. Data are reported as mean ± standard deviation.

3 | RESULTS

3.1 | Body mass and blood parameters

After the acclimation period, body mass decreased on average ca., 6% (repeated measures analysis of variance $F_{(1,8)} = 130.4$; $p < .001$) but was not affected by treatment $F_{(1,8)} = 1.98.4$; $p = .19$). Toads acclimated to HYPER condition lost ca., 4.5% of body mass, whereas toads acclimated to HYPO condition lost 7% (Table 1).

The animals from both groups had marked differences in osmoregulatory parameters. Plasma osmolality values were around 50% higher in the HYPER group than those from the HYPO group (Table 1). Accordingly, urea concentration was about two times higher in the

TABLE 1 Body mass, mass of internal organs, and urea concentration of female *Xenopus laevis* acclimated for 40 days to 150 mOsm·kg^{-1}·H_2O (hypo-osmotic treatment) and 340 mOsm·kg^{-1}·H_2O (hyper-osmotic treatment)

	Hypo-osmotic	Hyper-osmotic	T_8	*p* value
Body mass (g) initial	84.38 ± 2.51	91.25 ± 8.72	1.69	.130
Body mass (g) final	80.56 ± 2.45	84.860 ± 8.34	1.11	.301
Plasma urea (mmol/L)	10.37 ± 3.98	18.39 ± 6.54	2.34	**.047**
Liver urea (mmol/L)	2.46 ± 1.24	5.57 ± 1.52	3.55	**<.001**
Muscle urea (mmol/L)	3.59 ± 1.86	6.85 ± 1.79	2.82	**.022**
Skin urea (mmol/L)	3.17 ± 0.49	6.57 ± 2.80	2.68	**.028**
Osmolality (mOsm·kg^{-1}·H_2O)	245.60 ± 13.56	360.20 ± 19.37	10.84	**<.001**

Note: Significant *p*-values are in bold.

plasma, liver, legs muscle, and skin of animals acclimated to the saline treatment (Table 1). In addition, osmolality was positively correlated with urea concentration of plasma (Pearson's $r = .659$, $p = .038$), liver (Pearson's $r = .797$, $p = .006$), muscle (Pearson's $r = .756$, $p = .012$), and skin (Pearson's $r = .733$, $p = .016$).

3.2 | Enzymatic activities and oxidative status

Heart and liver CS activities were higher in the HYPER group than in the HYPO group (Table 2). We also found an increase of liver COX activity of HYPER-acclimated animals compared with that of the HYPO group, but such difference was in the limit of statistical significance. Likewise, liver CS activity increased as concentration of urea and osmolality of plasma increased ($r^2 = .522$, $p = .018$; $r^2 = 0.853$, $p < .001$). Interestingly, catabolic activity in toad skin was markedly different between treatments (Table 2); both CS and COX activities were significantly higher in the HYPO group than in the HYPER group. We also found a 25% increase in liver TAC of toads from the HYPO group respect to that of the HYPER group ($t_8 = 5.71$, $p = .0004$, Figure 1). This enhanced antioxidant capacity remained unchanged in plasma ($t_8 = 0.61$, $p = .55$). Plasma and liver NO concentrations ($t_8 = 1.26$, $p = .24$ and $t_8 = 0.01$, $p = .99$, respectively) and lipid peroxidation ($t_8 = 0.40$, $p = .702$ and $t_8 = 0.20$, $p = .849$) did not change between treatments (Figure 1). Moreover, we found a significant and inverse relationship between liver TAC and plasma osmolality, as well as an inverse trend with the urea concentration and mitochondrial enzyme activities in liver (Figure 2).

4 | DISCUSSION

Current results together with previous studies (Peña-Villalobos et al., 2016) confirmed that *X. laevis* tolerated chronic exposure to moderate levels of saltwater. The body weight of hyperosmotic-exposed animals did not change to those acclimatized to field-simulated salinity. However, we found significant changes in the biochemical and physiological responses examined in this study, in particular metabolic enzymes and, at a lesser extent, biomarkers of oxidative stress.

One of the initial and acute responses of *Xenopus* to an increase in water salinity is the synthesis of urea and its accumulation in the tissues—including the blood—likely to decrease the osmotic potential with the environment (Schmidt-Nielson, Borut, Lee, & Crawford, 1963). Accordingly, we found an increase of urea concentration in HYPER group than that in the HYPO group, which was coupled with higher plasma osmolality (see also Jørgensen, 1997; Katz & Hanke, 1993; Romspert, 1976). The urea accumulation is probably due to enhanced urea-ornithine cycle in the liver (McBean & Goldstein, 1970). This assumption is consistent with the higher CS and COX activities measured in the liver of toads from the HYPER group. All changes in the biochemical capacities of heart and liver are used by anurans, probably because of maintenance the water influx to the body, increasing internal osmolality and hence avoiding dehydration and osmotic pressure losses (Hoffmann, Lambert, & Pedersen, 2009; Wehner, Olsen, Tinel, Kinne-Saffran, & Kinne, 2003).

Osmoregulation is an integrative process of molecular, physiological and even behavioral mechanisms to maintain water balance with the surrounding environment, involving organs such as kidneys, gut, or avian salt gland (Rivera-Ingraham et al., 2016). This is true of the amphibian

TABLE 2 Cytochrome *c* oxidase and citrate synthase activities in selected organs of female *Xenopus laevis* acclimated to 150 mOsm·kg^{-1}·H_2O (Hypo-osmotic treatment) and 340 mOsm·kg^{-1}·H_2O (Hyper-osmotic treatment) for 40 days

Enzyme activities	Hypo-osmotic	Hyper-osmotic	t_8	*p*
Cox (UI/mg protein)				
Heart	7.26 ± 2.34	10.42 ± 3.66	0.73	.48
Leg muscle	0.17 ± 0.03	0.15 ± 0.014	0.59	.57
Liver	4.01 ± 0.82	7.36 ± 1.29	2.18	.06
Ventral skin	0.123 ± 0.018	0.061 ± 0.01	3.00	**.017**
CS (UI/mg protein)				
Heart	7.53 ± 1.83	14.06 ± 1.75	2.57	**.03**
Leg muscle	0.07 ± 0.02	0.07 ± 0.01	0.27	.79
Liver	2.45 ± 0.29	5.41 ± 0.40	6.01	**.0003**
Ventral skin	0.03 ± 0.001	0.02 ± 0.001	2.90	**.02**

Note: Significant *p*-values are in bold.
CS, citrate synthase; COX, cytochrome *c* oxidase.

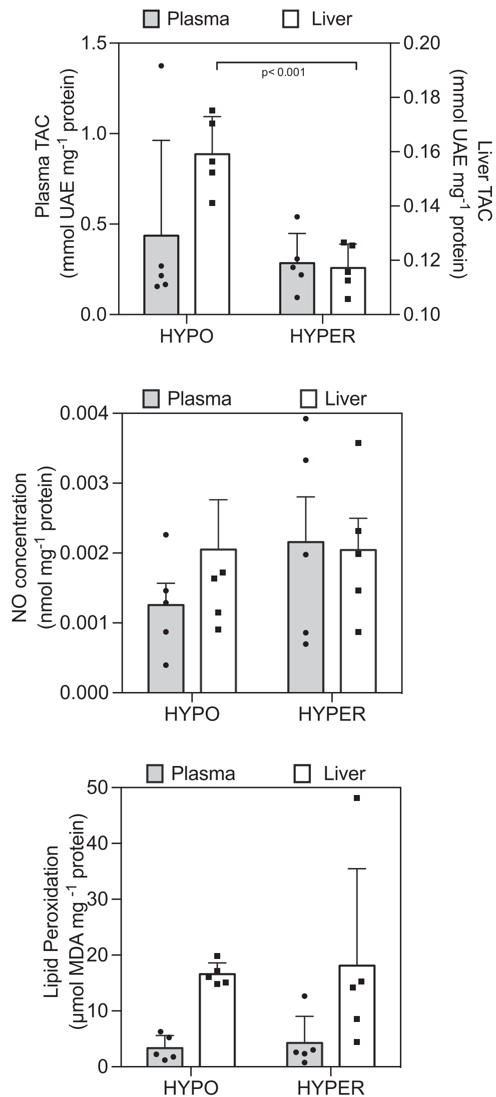

FIGURE 1 Effects of salt acclimation on total antioxidant activity, nitric oxide (NO) concentration an lipid peroxidation of liver and plasma. The total antioxidant activity in the liver of *Xenopus laevis* increased when frogs were acclimated for 40 days to a hypo-osmotic medium (HYPO, 150 mOsm·kg^{-1}·H_2O) compared with frogs acclimated to a hyper-osmotic environment (HYPER, 340 mOsm·kg^{-1}·H_2O). NO concentration and lipid peroxidation in the liver and plasma did not significantly differ between experimental treatments. Data represented as mean ±1 standard deviation

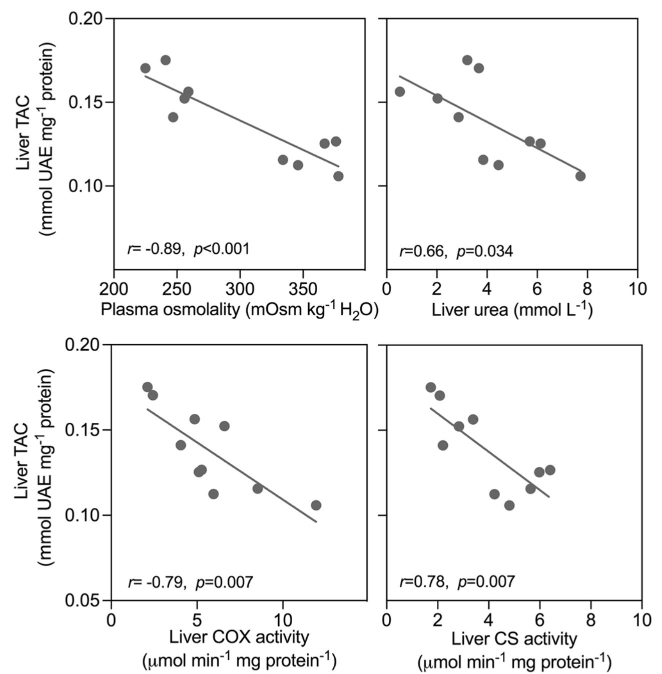

FIGURE 2 Relationship between total antioxidant activity of liver and selected biochemical parameters in *Xenopus laevis* acclimated contrasting salinities. Data points represent one individual. Each plot also shows the best-fit line and the associated Pearson's correlation coefficient, *r*. CS, citrate synthase; COX, cytochrome *c* oxidase; TAC, total antioxidant capacity

skin, which has a key role in ion exchange with water. The influx of water through the skin is tightly associated to the adenosine triphosphate (ATP)-dependent incorporation of Na^+ (Jansen et al., 2003; Larsen, Willumsen, Møbjerg, & Sørensen, 2009). Moreover, in vitro studies have demonstrated that the active transport of Na^+ could represent around 25% of the total oxygen consumption (Zerahn, 1956), being the skin contribution to the total energy budget up to 28% in frogs (Flanigan, Withers, & Guppy, 1991). Interestingly, the enhanced skin COX and CS activities of HYPO group compared with that of HYPER group is consistent with the higher mitochondrial density (Spinazzi, Casarin, Pertegato, Salviati, & Angelini, 2012) needed to produce ATP, which is needed in the Na^+ gradient maintenance. Therefore, our findings support the hypothesis that ion gradients responsible for the passive water exchange through the skin are a costly process, allowing anurans to exhibit an efficient osmoregulatory capacity when are subjected to salinity switches.

Our results revealed that *X. laevis* acclimated to saltwater had ca. 30% lower total antioxidant capacity in the liver, than the HYPO group (Figure 1). Although in the current study we did not measured metabolic rates of individuals, in a similar study, Peña-Villalobos et al. (2016) reported that *X. laevis* chronically exposed to saltwater had higher metabolic rates than those exposed to isosmotic conditions. Thus, it would be not surprisingly that whole metabolic rates of toads could also change in our experimental groups. The fact that we found higher TAC in the HYPO group instead of in the HYPER group is intriguing. Surprisingly, TAC inversely correlated with both urea concentrations in plasma and liver metabolic enzyme activities (Figure 2). Because osmolality and urea concentration were higher in the HYPER group, together these results seem to support the idea that urea has a protective effect (i.e., antioxidant) to face ROS increases in individuals acclimated to saltwater. In this vein, an increase in circulating ROS as a result of salt-induced increase in metabolic activity could be compensated by the effect of higher urea concentrations without the needs of synthetize endogenous antioxidants which its likely associated costs (Grundy & Storey, 1998; Zhang, Yang, & Cohen, 1999). In fact, the production of antioxidants is thought to have costs because the resources required to build antioxidant defenses may have alternative roles within the body associated with growth, coloration (Monaghan et al., 2009),

reproduction (Wiersma, Selman, Speakman, & Verhulst, 2004) and immune response in vertebrates (Bertrand, Criscuolo, Faivre, & Sorci, 2006). This hypothesis is supported by the results of Wang et al. (1999) who suggested that urea and derivatives are cardioprotective agents against oxidative stress-induced myocardium damage in rats and sharks. Whether the effect of the increase in total antioxidant activity or the possible protective effect of urea, the overall result is constancy in the levels of oxidative damage (i.e., lipid peroxidation), when animals are exposed to different salinities.

Alternatively, several studies have reported that significant increases in metabolic rate can trigger ROS and therefore activate antioxidant defenses (Cohen, Hau, & Wikelski, 2008). Other studies suggest a functional relationship between mass-specific metabolic rates and oxidative state (Fletcher et al., 2013; Sabat et al., 2017, 2019), although this association continues to be a subject of active debate (Jimenez, 2018; Selman et al., 2012; Speakman & Garratt, 2014). In particular, Speakman and Garratt (2014) proposed that the notion that ROS production is linked to metabolism in a fixed proportion is incorrect. Indeed, these researchers postulated that a greater metabolism is probably linked to lesser free-radical production because of their impact on lowering the inner-membrane potential of mitochondria (Speakman & Garratt, 2014 and references therein). This is consistent with previous data on the oxidative status of avian species, which vary in the intake of hyper-osmotic vs terrestrial prey (Tapia-Monsalve et al., 2018).

In conclusion, our study shows that *X. laevis* is able to trigger molecular mechanisms to tolerate hypersaline environments, which involve alterations of plasma urea concentration and modulation of metabolic enzymes. However, our results do not support the hypothesis that salinity induces greater oxidative damage, at least in this anuran species.

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ORCID

Isaac Peña-Villalobos https://orcid.org/0000-0002-5599-5290
Carolina Contreras-Ramos https://orcid.org/0000-0001-9732-9176
Juan C. Sanchez-Hernandez https://orcid.org/0000-0002-8295-0979
Pablo Sabat http://orcid.org/0000-0002-6609-9969

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