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Effect of salinity on locomotor performance and thermal extremes of metamorphic Andean Toads (*Rhinella spinulosa*) from Monte Desert, Argentina

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Abstract

Rhinella spinulosa is distributed from Peru to Argentina (from 1200 to 5000 m elevation), inhabiting arid mountain valleys of the Andes, characterized by salty soils. The variations in soil salinity, caused by high evapotranspiration of water, can create an osmotic constraint and high thermal oscillations for metamorphsed Andean toad (*R. spinulosa*), affecting their thermoregulation and extreme thermal tolerances. We investigated the changes in thermal tolerance parameters (critical thermal maximum and crystallization temperature) of a population of metamorphosed *R. spinulosa* from the Monte Desert of San Juan, Argentina, under different substrate salinity conditions. Our results suggest that the locomotor performance of metamorphs of *R. spinulosa* is affected by increasing salinity concentrations in the environment where they develop. On the other hand, the thermal extremes of metamorphs of *R. spinulosa* also showed changes associated with different salinity conditions. According to other studies on different organisms, the increase of the osmolarity of the internal medium may increase the thermal tolerance of this species. More studies are needed to understand the thermo-osmolar adjustments of the metamorphs of toads to environmental variability.

Key Words: Critical thermal maximum; Crystallization temperature; Locomotor performance; Monte Desert; Salinity; Osmotic stress; Thermal tolerances.

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1. Introduction

Desert anurans have a biphasic life cycle, with an aquatic phase for tadpoles and a terrestrial phase for juveniles and adult metamorphosed toads (Duellman and Trueb, 1994). Amphibians, although able to occupy brackish water during embryonic and larval development (Balinsky, 1981), cannot tolerate high salinity values because they have a poor osmoregulation. Therefore, during tadpole development, high concentrations of salinity in the aquatic environment have adverse effects on growth and development. These include delayed time to metamorphosis, malformations on body, and weight loss among others (Gosner and Black, 1957; Gómez-Mestre and Tejedo, 2003, Chinathamby et al., 2006; Squires et al., 2010). Once metamorphosis is completed, metamorphic toads colonize the terrestrial environment. The soils from Andean valleys often contain high salt concentrations, which can induce additional osmotic stress. This may affect locomotor performance due to lost in the muscle mass of limbs (Denoël et al., 2010; Alexander et al., 2012), alterations in skin physiology (Hillyard et al., 2007), decreases the survival of tadpoles (Dougherty and Smith, 2006), or restricting potential places for foraging and feeding behavior (Rose et al., 1986). In addition, a negative correlation of abundance of larvae and metamorphosed toads have been reported with an increase in salinity of environments, influencing the distribution of toads (Smith et al., 2007).

Osmotic stress is compensated by different adaptations in terrestrial amphibians: selection of less stressful microhabitats; morphological adaptations

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such as a pelvic patch (ventral section below the femur present in typical desert anuran with high vascularization that facilitates the absorption of water from the soil (Baldwin, 1974)); and the physiological response of an increase in plasma osmolality. Increased urea in response to water stress has been widely reported in different groups of amphibians, through this mechanism the toads can retain the body water, maintaining the positive osmotic gradient (Scheer and Markel, 1962; Katz, 1989; Jørgensen, 1997).

Saline soils are generated in landscapes with high evapotranspiration induced by elevated environmental temperatures that evaporate soil's water and deposits the dissolved salts. The NaCl is the most common salt dissolved in water (Sposito, 2008). Central-western Argentina area has high daily evapotranspiration that induces daily fluctuations in soil water and stream level (Sanabria and Quiroga, 2011), producing a saline soil (salt crust) where metamorphosed *R. spinulosa* feed and disperse during the day. Metamorphic *Rhinella* is active during the day (unusual in other amphibians), which is common in this genus (Navas et al., 2007; Pizzatto et al., 2008).

Rhinella spinulosa is found throughout a wide latitudinal range from Andean Peru to Argentina, Bolivia, and Chile (Cei, 1979; Veloso and Navarro, 1988; Frost, 2017). This species also has an extensive elevational distribution, up to 5000 m (Gallardo, 1987; Veloso and Navarro, 1988; Lavilla and Cei, 2001). The thermal physiology of post-metamorphic Andean toads under different environmental conditions has been poorly studied. We studied the thermal extremes (critical thermal maximum and crystallization temperature) and locomotor performance of

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R. spinulosa exposed to different salt soil concentrations. Also, we analyzed locomotor performance, as total distance covered during five minutes at four different temperatures in a population of metamorphosed Andean toad from the Monte Desert of San Juan, Argentina. We hypothesized that these post-metamorphic Andean toads would exhibit a moderate change in critical thermal maximum, crystallization temperature, and locomotor performance associated with increasing salt soil concentration. We expect an increase in thermal tolerances (CT_{max}, CT, and locomotor performance) of studied toads associated with the accumulation of solutes in the plasma, as it has been found in other groups (fish, crustacean, see below).

2. Methods

2.1 Environment and experimental animals

The Monte Desert is characterized by an arid climate with a mean annual temperature of 17.3 ºC, a mean maximum temperature of 25.7 ºC, a mean minimum temperature of 10.4 ºC, and a mean annual rainfall of 89 mm (most of which falls during the wet season in austral summer months, November-March) (Cabrera, 1976). We collected toads after metamorphosis during three consecutive days May 23th to 25th 2013 (beginning of the austral winter), at a study area located 100 km south of the city of San Juan (31.9044 S; 68.7098 W; elevation 1200 m; Fig. 1).

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2.2 Experimental setting and salinity concentration

The metamorphosed Andean toads are small toads (mean of body mass 0.43 ± 0.02 g; range: 0.25 – 0.5 g). Our experimental design consisted of three groups of metamorphosed Andean toads (55 individuals per group). The groups were incubated in the laboratory for five days at 16 ºC and a photoperiod 12:12, provided with food *ad libitum*. Salt concentration treatments were chosen based on field estimates obtained from field substrate salinity concentrations. We measured 10 sites where the toads typically feed and bask (temperature: 15.8 ºC ± 0.13 ºC and salinity 630 ± 1.06 ppm) with a handheld conductivity meter (Adwa Instruments Corp., Mauritius). In the laboratory, we recreated three substrates with different NaCl concentrations on moistened paper towels: 1) Similar salinity concentration found in the field at 16 °C (630 ppm), and two concentrations of salinity (above and below) from the average concentration found in the typical habitat of *R. spinulosa* metamorphs. 2) 150 ppm at 16 ºC, 76% below mean field concentration; and 3) 1800 ppm at 16 ºC, 285 % upper mean field concentration. These solutions of known salinity were placed on the bottom of containers of different groups of toads. We dissolved sodium chloride (Sigma-Aldrich, USA) in distilled water at 16 °C until reaching the desired concentration in ppm. The salt concentration selection will assess the constraints imposed by soil salinity under field conditions.

2.3 Determination of locomotor performance

For each salinity concentration group, we measured the locomotor performance as the total distance covered for 5 min of forced locomotion at

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different temperatures (5 ºC, 15 ºC, 25 ºC, and 35 ºC). We used 20 toads for each salinity concentration (*n*= 60), and test 5 individual for each selected temperature. We divided the total distance covered (cm) by the SVL of the toad (cm) and used this value as a representation of locomotor performance (Titon et al., 2010). Toads were kept in plastic containers at target experimental temperatures (5 ºC, 15 ºC, 25 ºC and 35 ºC) for 20 min before testing. The arena consisted of a circular aluminum tank (79 cm diameter with 10 cm high walls) with controlled temperature by running water through metal tubes below the floor of the circular tank. The appropriate temperature for each test was maintained by pumping water through the metal tubes using an electrical chiller (Thermo NESLAB RTE 7, Newington, NH). The temperature at the floor of the arena was monitored using a digital thermometer (TES 1312, TES Electrical Electronic Corp., Taipei, Taiwan, ± 0.1 ºC). Toads were induced to jump by gently tapping them with a small brush during the 5 min duration trial, all toads were tested one time.

2.3 Determination of thermal tolerances

We measured the critical thermal maximum (CT_{max}) and crystallization temperature (*CT*) of 30 toads that were acclimated for five days at 16 °C with a photoperiod of 12:12 L:D. CT_{max} was determined using the dynamic method of Hutchison (1961). Individual toads were placed in a glass container (15 x 20 cm) with 150 ml of water, 15 mm column water height, at acclimation temperature (16 ± 2 ºC). An electric heating mantle raised the temperature of the water bath at a rate of 1 ºC min^{-1}. The studied toads were small (~ 0.78 cm^3, and mean body mass of

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0.43 ± 0.02 g). When the animals showed abrupt spasms, we measured the water temperature as an indicator of body temperature of the toads (following Luttersschmidt and Hutchison, 1997).

On the other hand, we considered the thermal minimum of this species as the crystallization temperature (*CT*) (Claussen and Costanzo, 1990). We used this thermal parameter as an estimate of the minimum temperature that the animals endure without dying. The determination of *CT* is unequivocal, it is the minimum temperature value recorded before the abrupt increase in the corporal temperature associated with the freezing of the body fluids occurs. This parameter has a higher repeatability than the thermal critical minimum, avoiding mistakes in the estimation of the thermal parameter (Sanabria, unpublished data).

Individual toads were placed in a glass container (15 x 20 cm) at acclimation temperature (16 ºC ± 2 ºC) inside chiller's bath (Thermo NESLAB RTE 7, Newington, NH) with ethylenglycol. The temperature was decreased at a rate of 0.5 ºC min^{-1}. A 40 AWG thermocouple positioned against the abdomen provided a continuous recording of body temperature on a computing data logger (PP 222; Pico logic, UK). When the toads' skin showed an exothermic reaction, we measured the minimal abdominal temperature as an estimator of the *CT*. All toads recovered from the experiment in a few minutes. Toads used to determine the *CT* ware tested once and used only for this experiment. All toads used for the determination of extreme thermo-physiological parameters (*CT* and CT_{max}) survived the experiment.

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2.5 Data Analysis

We calculated the mean and standard error for all thermal data. We applied the non-parametric Kruskal-Wallis test to evaluate differences in CT_{max}, CT, and locomotor performance among salinity substrate concentrations. In addition, we used the generalized linear model, two fixed model ANOVA for the test to evaluate differences in locomotor performance among salinity substrate concentrations and temperatures. The data didn't have a normal distribution and variances among treatments were not homogeneous. The PAST version 9.4 statistical package was used for analysis.

3. Results

3.1 Variations in locomotor performance

The locomotor performance of metamorphosed Andean toads was affected significantly by temperature and varied among salinity concentrations (Fig. 2). The lowest performance were observed at 1800 ppm for 5 ºC (Kruskal-Wallis test: $H_{(2, N= 15)}$ =10.6; p= 0.004), 15 ºC (Kruskal-Wallis test: $H_{(2, N= 15)}$= 5.41; p= 0.05), 25 ºC (Kruskal-Wallis test: $H_{(2, N= 15)}$= 10.11; p= 0.006), and 30 ºC (Kruskal-Wallis test: $H_{(2, N= 15)}$= 7.07; p= 0.02). On the other hand, the locomotor performance was affected by the increasing concentration of NaCl (Fig. 3). Especially, the performance curves at 5 and 25 ºC (Fig. 3 A and C) that describe the typical dose-response inverse U-shape curve of hormesis compensation. In contrast at 15 and 35 ºC the locomotor performance decreased more linearly in relation to the amount

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of NaCl concentration (Fig. 3 B and D). Results of the two fixed model ANOVA, showed that the locomotor performance is affected by the temperature ($F_{(3,57)}$=128.0 p=0.0001), and salinity ($F_{(3,57)}$=33.4, p=0.0001). Furthermore, there was interaction of temperature and salinity over the locomotor performance ($F_{(6,54)}$=2.9, p=0.01).

3.2 Variation in thermal extremes

Thermal resistances of metamorphosed Andean toads were higher with increasing salinity concentrations. CT_{max} was significantly different among concentrations (Kruskal-Wallis test: $H_{(2, N= 17)}$= 9.15; p< 0.01). The CT_{max} lowest values were at 150 ppm (34.2 ± 0.69 ºC) and highest at 1800 ppm (37.67 ± 0.38 ºC) (Fig. 4). Finally, the *CT* was different among salinity concentrations (Kruskal-Wallis test: $H_{(2, N= 16)}$= 6.78; p< 0.03). The lowest *CT* values were found at 1800 ppm (-0.91 ± 0.32 ºC) (Fig. 5).

4. Discussion

The locomotor performance curve of metamorphsed Andean toad (*R. spinulosa*) shows an increase in locomotor performance with increasing temperature and tends to decrease or stabilize when the experimental temperature approaches the critical thermal maximum and the optimal temperature has been largely exceeded; this is commonly found for most ectotherms (Angilletta, 2009). Increasing salinity concentrations diminished locomotor performance suggesting that higher osmotic levels are stressful across all the temperatures. Wijethunga, et

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al. (2016) showed enhanced locomotor ability (speed) with increased salinity in *Rhinella marina* metamorphs. Our data for metamorphosed *R. spinulosa* revealed a contrasting trend with significant locomotor performance decline, especially at higher (>1000 ppm) NaCl concentrations. This reversal trend is a consequence of measuring divergent locomotor performance traits (maximum speed vs maximum resistance) which may express trade-offs (Dohm et al., 1996). Both species (*R. spinulosa* and *R. marina*) are considered walkers toads (Duellman and Trueb, 1994) and the differences found in locomotion ability are attributable to the genetically based physiological balance between speed and resistance (Dohm et al., 1996).

Salinity is an important osmotic stressor for permeable skinned amphibians that have poor osmoregulatory capacities, dispersion pattern, growth rate, mortality, and skin physiology (Rose et al., ´86; Dougherty and Smith, 2006; Hillyard et al., 2007; Smith et al., 2007; Denoël et al., 2010). We observed changes in locomotor performance under different experimental salinity conditions, being significantly lower at higher NaCl concentrations (1800 ppm). The restriction imposed by the salinity over locomotor ability probably limits the metamorphs of *R. spinulosa* in their ability for escaping from predators, dispersal, and foraging abilities. This physiological constraint may limit the toads to use microhabitats where salinity is suitable for efficient locomotor performance. Alexander et al. (2012) observed changes in muscle mass and limbs length of *Incilius nebulifer* (Anura: Bufonidae) related to an increase in salinity concentration. Both the length

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and muscle mass of limbs are important parameters related to locomotion in toads (Tejedo et al., 2000; Choi et al., 2003).

On the other hand, we observed a hormesis response for locomotion of *R. spinulosa*. The hormesis is a dose–response phenomenon characterized by either a U-shaped or an inverted U-shaped dose-response depending on the end-point (Calabrese and Baldwin, 2002; 2003). This response has been observed at two different temperatures (5 and 25 °C), where field salinity (630 ppm) improved the locomotor performance. This U-shaped curves might occur as a response to a disruption in homeostasis. That is, at low levels of salinity *Rhinella spinulosa* displayed an overcompensation response (increasing of locomotor performance), which results in the apparent low-dose stimulation component of the response curve. At higher doses with greater initial salinity, the system often displays a more limited capacity for a compensatory response (decreasing of locomotive capacity), usually insufficient to return to control values (Calabrese and Baldwin, 2001). Probably, this effect is determined by coadaptation in this population, where individuals typically experience ambient temperatures of ~5 °C at the beginning and end of their activity period. Indeed under field conditions, *R. spinulosa* has a maximum locomotor performance at ~25 °C (Sanabria et al., 2015). Also, the reaction norm between salinity and temperature was not observed because the effect of increased salinity was not linear (Angilletta, 2009).

The thermal extremes of metamorphs of *R. spinulosa* also showed changes associated with different salinity conditions. The critical thermal maximum gradually increased with salinity concentration. Similar observations for other groups of

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animals have reported, where the salinity of the environment where they develop has effects on their thermal tolerances. The *Daphnia pulex* (Crustacean) shows an increase in tolerance to high temperatures with an increase of environment salinity where organism develop (Chen and Stillman, 2012). On the other hand, *Limia melanonotata* (fish) shows high critical thermal maximum associated with the increase of the salinity of the medium similar to that described in this study. Haney and Walsh (2003) observe an increase in thermal tolerance in a hypersaline medium associated with a reduction in the metabolism. This phenomenon due to the decrease in oxygen permeability related to changes in the internal osmolality.

Probably the toad improves the thermal resistance due to the increase of osmolytes in the internal medium, although we do not have data of the variation of the internal osmolarity of *R. spinulosa*. In amphibians, it is well known that changes in the osmolarity of the external environment have associated changes in the internal osmolarity of toads. This increases the urea molarity of the toads' internal environment, avoiding water loss in consequence. This water balance mechanism has been described and studied mainly in species with prolonged periods of aestivation (Jones, 1980; Katz, 1989). Further investigations should be directed to understand how changes in plasma osmolarity affect the thermal physiology of amphibian.

Furthermore, the crystallization temperature of the metamorphs of *R. spinulosa* shows a significant reduction at intermediate and highest experimental salinity concentrations. This increase in cold tolerance could be associated with an increase of the internal medium osmolytes of metamorphs *R. spinulosa* acclimated

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to high and intermediate salt concentration. The crystallization temperature decreases inversely to the osmolarity of the internal medium of the organisms (Storey and Storey, 2004). Amphibians accumulate urea when exposed to low water potential, a response that aids in maintaining proper hydration during saline adaptation, estivation, and transient exposure to arid environments (Shpun et al., 1992). Urea accumulation is a universal amphibian response to osmotic challenge (Jørgensen, 1997) and low temperatures promote urea retention through diminished renal function (Nielsen and Jørgensen, 1990). *Acanthopagrus latus* (Fish) showed an increase in the tolerance to the low temperatures when they were acclimatized in high concentrations of salinity, increasing the survival at low temperatures (Jian et al., 2003).

The variations in soil salinity caused by the evapotranspiration of water in the desert can create a constraint for metamorphs of *R. spinulosa* affecting their locomotion capacity and thermal tolerances. Restrictions on locomotion can have direct effects on dispersal, prey capture and escape from predators, decreasing the survival of metamorphs of Andean toads. On the other hand, the increase of thermal tolerance associated with an increase of soil salinity would be beneficial to the salinity concentrations found in the field (~ 630 ppm), conferring to the toads thermal plasticity for avoiding the rapid changes of environmental temperature. The deserts regions are characterized by rapid fluctuations of environmental temperatures (Warner, 2004). Further studies are needed to understand the thermo-osmolar adjustments of metamorphs of Andean toad (*R. spinulosa)* to environmental variability.

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Figure legends

Figure 1: A) Collection site, the white layer on the ground is salt produced by evaporation. B) Location of the study area (star); C) A close-up view of typical soil condition during field work and metamorphosed Andean toad *Rhinella spinulosa* (Anura: Bufonidae).

Figure 2: Variation in the locomotor performance (total distance covered during 5 min) of metamorphosed Andean toads among different salinity concentrations (ppm at 16ºC) and temperatures (ºC). Different letters indicate significant differences ($p < 0.05$).

Figure 3: Variations in locomotor performance as a function of the concentration of NaCl (ppm at 16 ºC) at different temperatures. The locomotor performances at 5 and 25 ºC show a U shape curve typical of hormesis. At 15 and 35 ºC the locomotor performances are decremented in relation to the amount of concentration of NaCl (ppm). The dotted lines show the performance at a minimal concentration of NaCl (reference level).

Figure 4: Variations in the critical thermal maximum (CT_{max}) of metamorphosed Andean toads (*Rhinella spinulosa*) among different salinity concentrations.

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Figure 5: Variations in the crystallization temperature (CT) of metamorphosed Andean toads (*Rhinella spinulosa*) between different salinity concentrations.

Highlights

- Exposure to high salt concentrations decreases the locomotor performance of Andean toads.
- We observed changes in thermal extremes (maximum and minimum) in different salt concentrations.
- The Andean toad have a hormesis response for the locomotor performance at different temperatures and salt concentration

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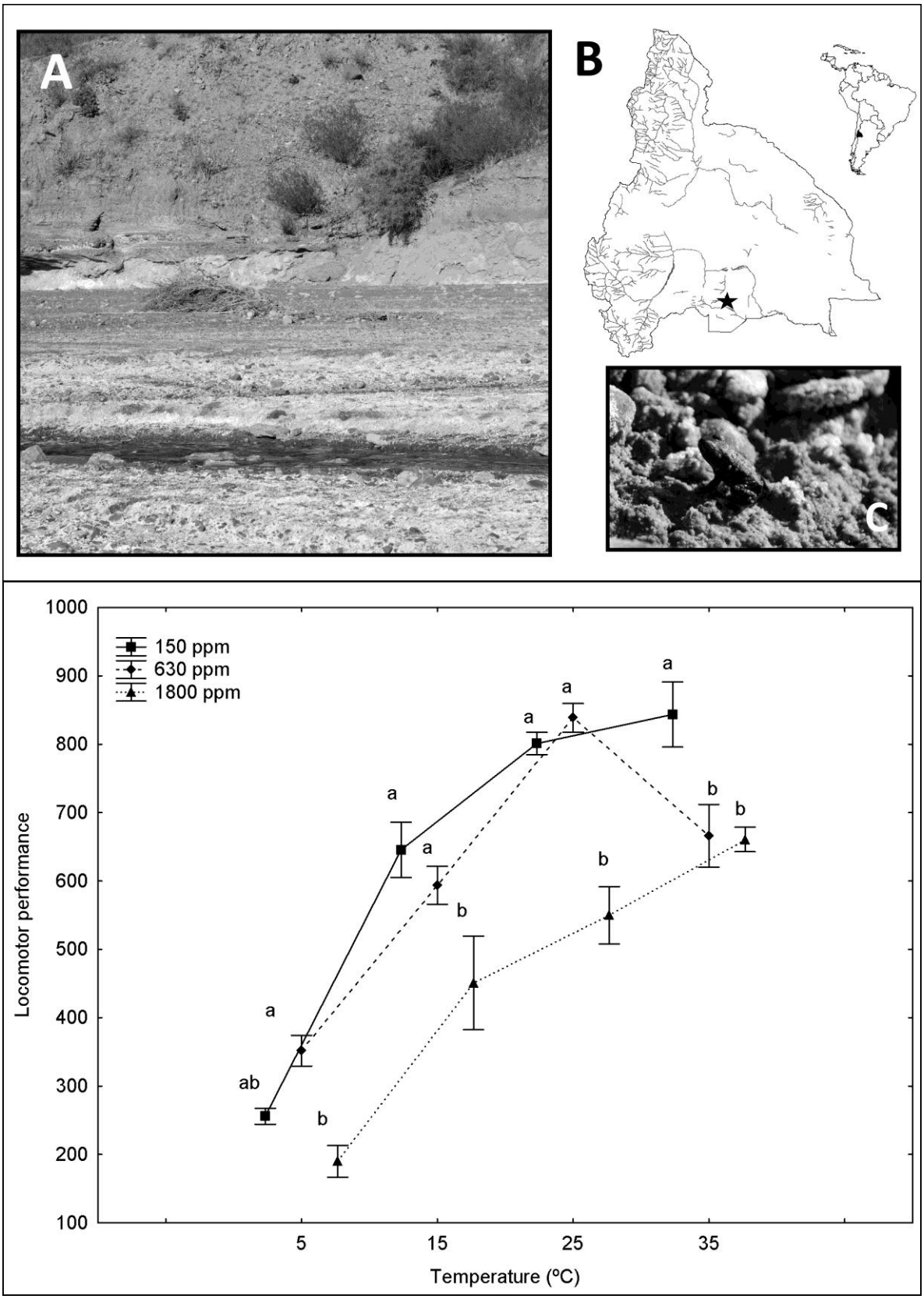
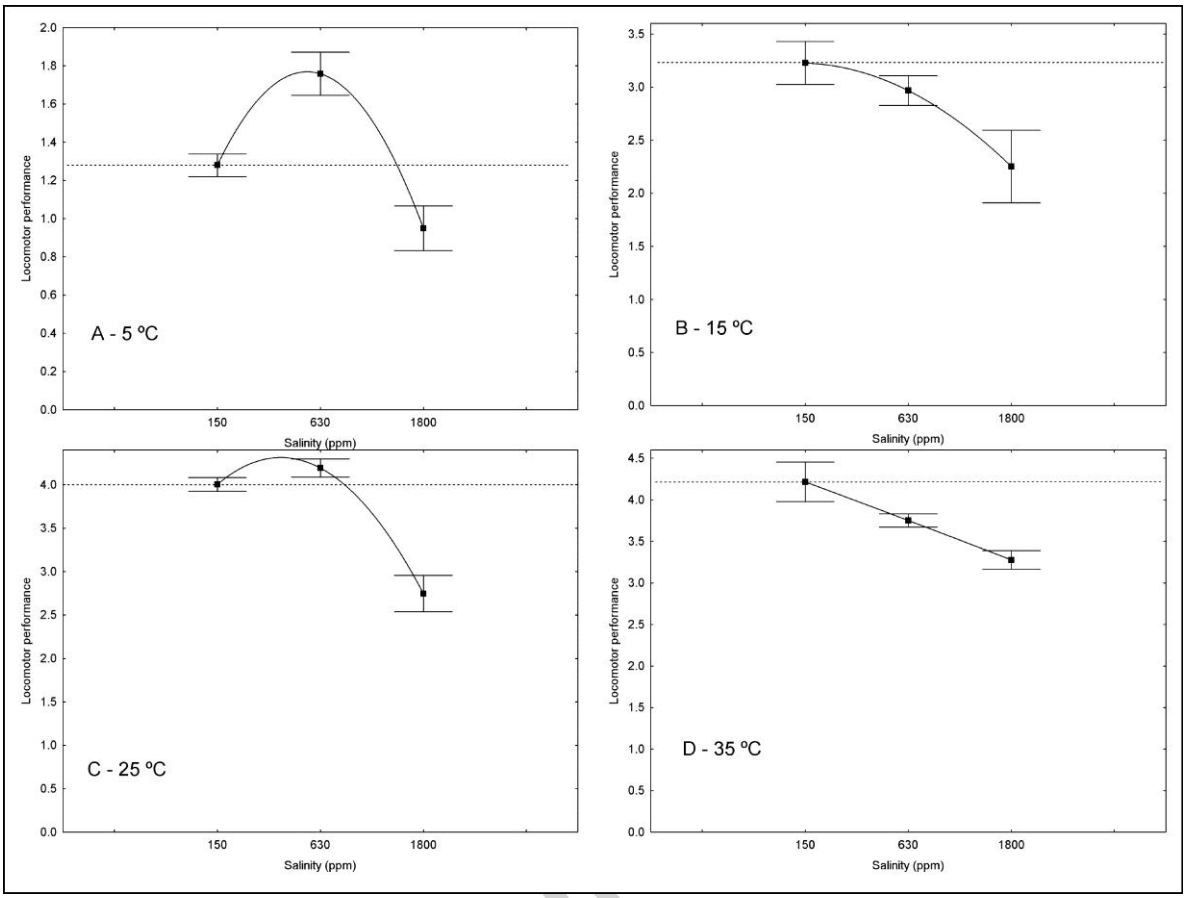

A
B
C
150 ppm
630 ppm
1800 ppm
Locomotor performance
Temperature (ºC)
1000
900
800
700
600
500
400
300
200
100
5
15
25
35
a
a
a
a
a
b
b
b
b
b
ab


A - 5 ºC
B - 15 ºC
C - 25 ºC
D - 35 ºC
Locomotor performance
Salinity (ppm)
150
630
1800

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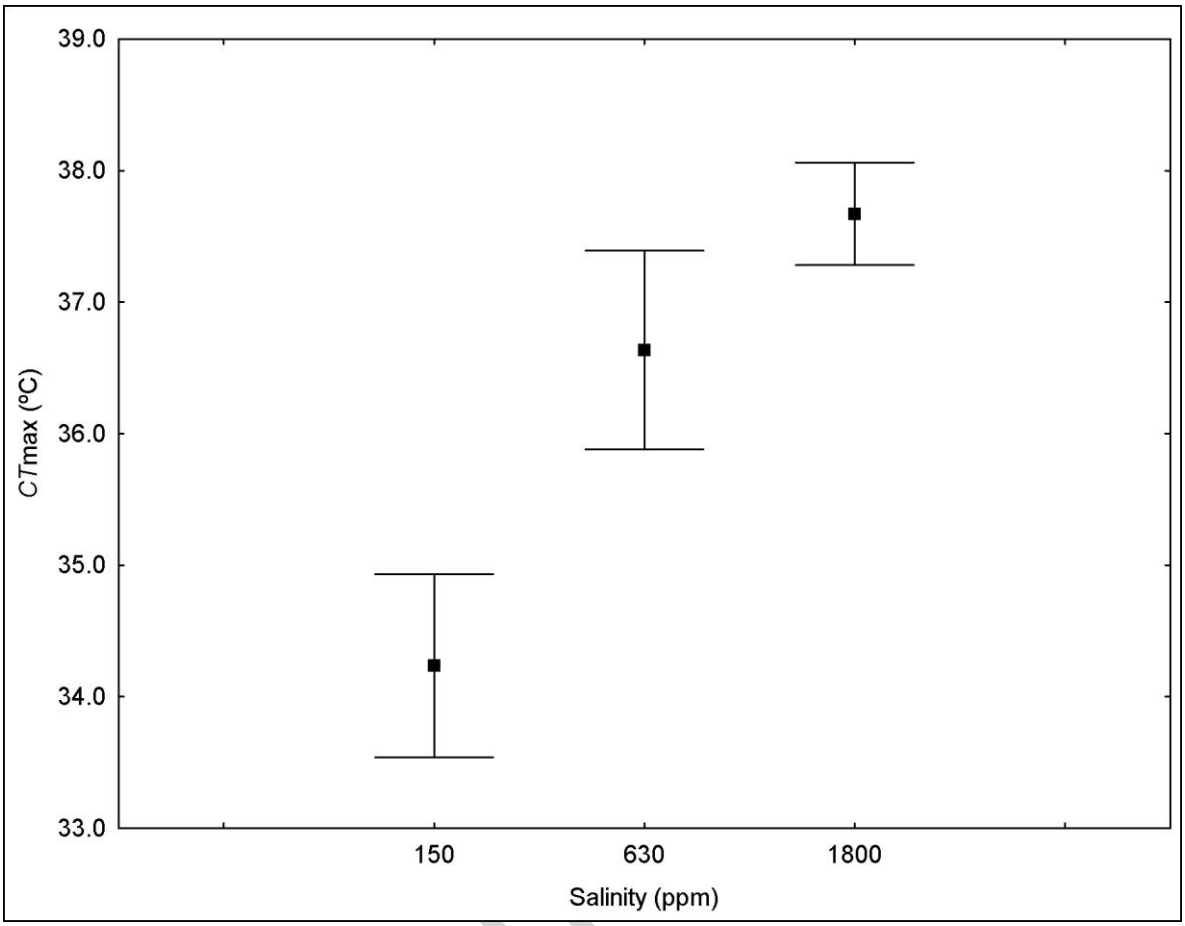

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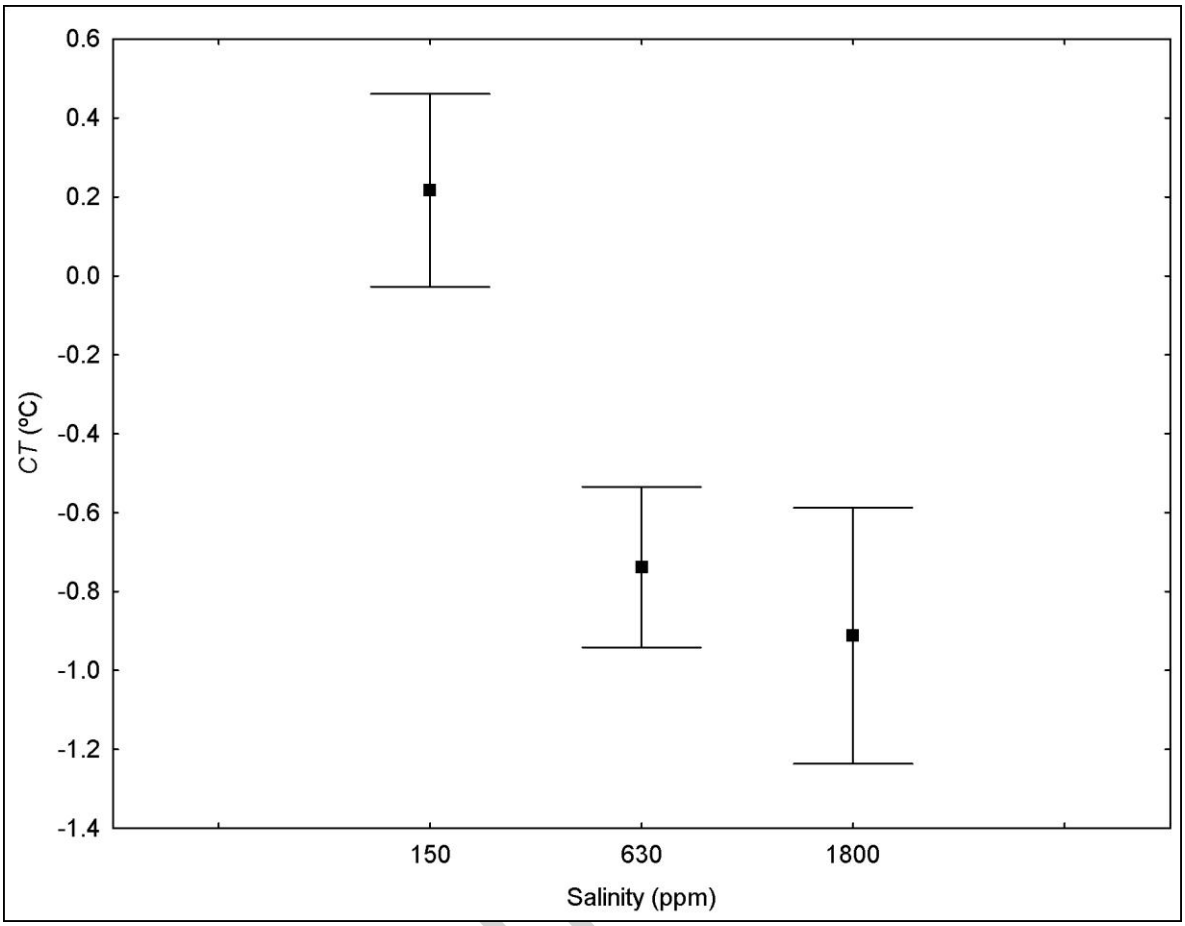
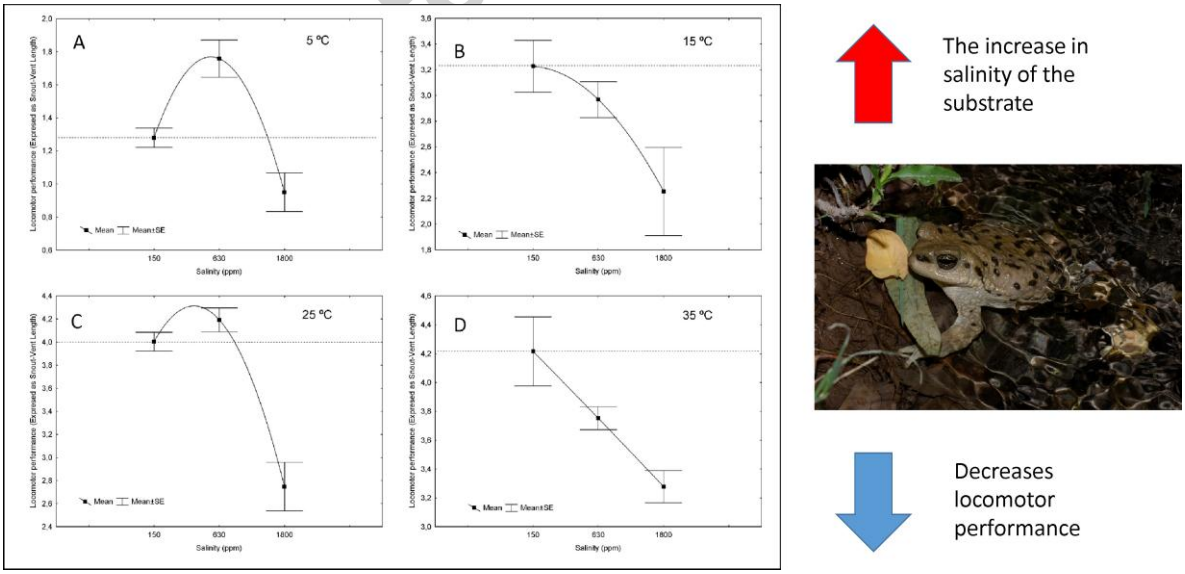
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