

Physiological Stress Responses in Amphibian Larvae to Multiple Stressors Reveal Marked Anthropogenic Effects even below Lethal Levels

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ABSTRACT

Natural and anthropogenic disturbances cause profound alterations in organisms, inducing physiological adjustments to avoid, reduce, or remedy the impact of disturbances. In vertebrates, the stress response is regulated via neuroendocrine pathways, including the hypothalamic-pituitary-interrenal axis that regulates the secretion of glucocorticoids. Glucocorticoids have cascading effects on multiple physiological pathways, affecting the metabolic rate, reactive oxygen species production, or immune system. Determining the extent to which natural and anthropogenic environmental factors induce stress responses in vertebrates is of great importance in ecology and conservation biology. Here we study the physiological stress response in spadefoot toad tadpoles (*Pelobates cultripes*) against three levels of a series of natural and anthropogenic stressors common to many aquatic systems: salinity (0, 6, and 9 ppt), herbicide (0, 1, and 2 mg/L acid equivalent of glyphosate), water acidity (pH 4.5, 7.0, and 9.5), predators (absent, native, and invasive), and temperature (21°, 25°, and 29°C). The physiological stress response was assessed examining corticosterone levels, standard metabolic rate, activity of antioxidant enzymes, oxidative cellular damage in lipids, and immunological status. We found that common stressors substantially altered the physiological state of tadpoles. In particular, salinity and herbicides cause dramatic physiological changes in tadpoles. Moreover, tadpoles reduced corticosterone levels in the presence of natural predators but did not do so against invasive predators, indicating a lack of innate recognition. Corticosterone and the antioxidant enzyme glutathione reductase were the most sensitive parameters to stress in this study. Anthropogenic perturbations of aquatic systems pose serious threats to larval amphibians even at nonlethal concentrations, judging from the marked physiological stress responses generated, and reveal the

importance of incorporating physiological information onto conservation, ecological, and evolutionary studies.

Keywords: amphibians, corticosterone, immune system, metabolic rate, oxidative stress, stress physiology.

Introduction

Environmental disturbances, whether natural or anthropogenic, cause physiological alterations of individual organisms that allow them to reduce or avoid the impact of the stressors (Romero 2004; McCue 2010). Such physiological responses, however, may come at a cost and result in fitness trade-offs associated with reduced immune competence, delayed growth and maturity, and a shorter life span (Bonier et al. 2009; Shalev et al. 2013). In some cases, such disturbances impose entirely novel challenges to which organisms need to adapt, as is the case with many pollutants or the introduction of invasive predators. Moreover, humans are also causing faster and more acute modifications of factors to which organisms may be naturally exposed within a narrower range, as in water acidification, salinization, or global warming (Kaushal et al. 2005; Lafferty 2009). Stressors can profoundly alter the physiology of organisms well before reaching lethal levels, conditioning key aspects of their behavior, growth, or reproductive performance.

In vertebrates, the stress response is regulated by a set of neuroendocrine pathways, of which the hypothalamic-pituitary-interrenal (HPI) axis is the most studied. The HPI axis modulates a hormonal cascade resulting in the activation of the interrenal gland and glucocorticoid (GC) production: corticosterone (CORT) in amphibians, reptiles, and birds and cortisol in most mammals and fish (Romero 2004). GCs elicit the mobilization of energetic metabolic substrates (e.g., lipids; Peckett et al. 2011), which affect essential functions of the organism such as reproduction, behavior, and growth (Denver et al. 2002; Schoech et al. 2009; Kindermann et al. 2013), thus conditioning the transition between life-history stages (Crespi et al. 2013). Prolonged secretion of CORT has been associated with mobilization of energetic substrates and increased metabolic demands in multiple tissues (Peckett et al. 2011; Lattin and Romero 2015) while resulting in reduced long-term survival (Bonier et al. 2009). GCs have cascading effects on multiple physiological pathways. Elevated GCs cause an increased metabolic rate involving overproduction of reactive oxygen species (ROS; Peckett et al. 2011) that often results in cellular damage (Circu and Aw 2010). Such cellular damage, however, can be buffered by increasing the activity of antioxidant enzymes (Costantini et al. 2011; Gomez-

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Mestre et al. 2013). Additionally, GCs are immunomodulators that can exert both negative and positive effects on the immune status of individuals (Franchimont 2004), depending on the duration and intensity of the exposure to stress (Rich and Romero 2005), although chronic exposure to high GC levels seems associated with immune deregulation (Padgett and Glaser 2003).

Many amphibian species have complex life cycles with aquatic larvae, which are often exposed to a suite of biotic and abiotic natural stressors. Amphibians are also deeply affected by human disturbances and indeed constitute the most threatened group of vertebrates (Hoffman et al. 2010). CORT regulation is a common stress response in anuran tadpoles to pond drying, pollutants, predators, acidification, or UV-B radiation (Glennemeier and Denver 2001; Chambers and Belden 2009; Burraco et al. 2013; Chambers et al. 2013; Maher et al. 2013). However, CORT regulation has many potential cascading effects on other aspects of amphibian biology, and these are seldom studied. Here we analyze the physiological stress response in spadefoot toad tadpoles (*Pelobates cultripes*) against a series of natural and anthropogenic stressors common to many aquatic systems. We measure physiological parameters relevant for evaluating the stress response: CORT levels, standard metabolic rate (SMR), antioxidant enzyme activity, oxidative cellular damage, and immune status. We tested the physiological response against three levels of salinity, herbicide (glyphosate), pH, and temperature, as well as against natural and invasive predators. All factors included in this study are considered potentially stressful for tadpoles, and some of them can be magnified by human activities at either global or local scales. High salinity results in reduced tadpole survival and delayed metamorphosis (Hopkins and Brodie 2015). Herbicide exposure reduces amphibian diversity and alters the outcome of competition interactions (Relyea and Mills 2001). Water acidity also reduces survival and slows down development in embryos and larvae, particularly at pH 4.5 or lower (Merilä et al. 2004). The introduction of novel predators may have a deep impact on local populations (Siesa et al. 2011), in part because native prey are very likely to fail to recognize novel predators and hence fail to produce antipredator defenses, whether behavioral or morphological. Last, increased water temperature causes developmental acceleration, causing larvae to metamorphose smaller and with reduced hind limbs (Gomez-Mestre et al. 2010; Duarte et al. 2012).

We expected most experimental factors to affect CORT levels, since the HPI axis is known to play a central role in amphibian stress responses (Denver 2013). We also expected changes in metabolic rate to be affected by the experimental factors chosen. Moreover, hormonal and metabolic changes may alter the production of ROS, which may cause oxidative damage unless dealt with, and therefore we also expected the activity of antioxidant enzymes to increase when metabolism itself was elevated. Also, increased GC secretion is tightly associated with the immune system, as it results in an increased neutrophils:lymphocytes ratio (Davis et al. 2008). Likewise, enhanced immune responses under stress have been shown to incur increased metabolic costs (Räberg et al. 2002).

This study will allow us to compare the magnitude of the stress responses across multiple factors and assess the asso-

ciation among physiological alterations. Comprehensive physiological studies are needed that compare the intensity and amplitude of physiological responses to both novel environmental challenges and the intensification of natural stressors to which organisms may already be adapted. Assessment of various aspects of physiological stress responses to multiple factors will identify interdependence among such responses and possibly unveil mechanisms underlying life-history trade-offs. Physiological analyses of responses to multiple factors are therefore key to both conservation and eco-evolutionary studies.

Material and Methods

Animal Collection and Experimental Setup

We collected spadefoot toad tadpoles (*Pelobates cultripes*) from three temporary ponds (80 from each location) within the biological reserve of Doñana National Park (March 2011) and from two temporary ponds within the Sierra Norte Natural Park (April 2012), both in southwestern Spain, to run five experiments (see below). All tadpoles were collected between Gosner stage 34 and 35 (Gosner 1960), and their weight was 2.50 ± 0.3 g (SE). We also collected water beetle larvae (*Dytiscus circumflexus*) and red swamp crayfish (*Procambarus clarkii*) in several ponds within the Biological Reserve. Both species are relevant tadpole predators, but *D. circumflexus* larvae are native predators, whereas the red swamp crayfish was introduced in the 1970s and has since become a common invasive predator (Diaz-Paniagua et al. 2014). Nevertheless, we still consider *P. clarkii* a novel predator since amphibians in the park show a lack of innate recognition of *P. clarkii*, as indicated by the inability of both *Pelophylax perezi* and *P. cultripes* to induce behavioral or morphological defenses against crayfish, whereas they readily deploy such defenses against native predators (Gomez-Mestre and Díaz-Paniagua 2011). We found neither crayfish nor water beetle larvae at the ponds where the tadpoles were collected. This suggests (but does not grant) that the larvae included in the study were naïve to either kind of predator. Previous exposure to native predators could have partially induced phenotypic responses, whereas exposure to invasive predators could have given tadpoles the chance to learn to recognize their cues if paired with alarm cues from attacked conspecific tadpoles (Polo-Cavia and Gomez-Mestre 2014). Tadpoles collected from natural ponds thus represent a conservative test regarding naïveté toward invasive predators. All tadpoles were acclimated for 1 wk in 4-L buckets (four individuals per bucket) filled with dechlorinated tap water in climate chambers set at 21°C and a 12L:12D cycle according to natural conditions in the field. Tadpoles were fed ad lib. with rabbit chow. Predators were maintained individually in 4-L buckets.

We conducted five independent experiments, each one testing for physiological responses to exposure to different levels of each of five factors separately: salinity, herbicide, pH, predators, and temperature. Experiments were conducted in two consecutive breeding seasons. We pooled tadpoles from all clutches collected within each season. Tadpoles collected from the Biological Reserve of Doñana in 2011 were used for salinity, pH, and predator-

exposure experiments, whereas those from the Sierra Norte Natural Park in 2012 were used for herbicide and temperature experiments. Groups of four tadpoles were kept in 3-L buckets filled with carbon-filtered dechlorinated tap water. Experimental units were randomized within each experiment across shelves in a walk-in chamber set at a constant 21°C and a 12L:12D cycle. Water was renewed twice a week, and tadpoles were fed ad lib. with rabbit chow. Each experiment had its own set of control replicates, which all had the same conditions: 21°C, pH 7, and herbicide-free freshwater without predator cues. We randomly assigned containers to experimental treatments. Treatments lasted for 10 d and were initiated after a 1-wk acclimation period in the climatic chambers under control conditions.

Stress Factors

We selected three nonlethal levels for each stress factor: salinity (0, 6, and 9 ppt NaCl), herbicide (0, 1, and 2 mg/L of glyphosate), pH (4.5, 7.0, and 9.5), predators (absent, native, and invasive), and temperature (21°, 25°, and 29°C). The levels applied for the different factors were chosen based on previous knowledge of ranges commonly experienced by amphibians in either natural systems or areas affected by human activities (Alvarez and Guerrero 2000; Gomez-Mestre et al. 2003; Solomon and Thompson 2003; Serrano et al. 2006; Diaz-Paniagua et al. 2014). We replicated each treatment 10 times, for a total of 150 experimental units and 600 tadpoles. Almost all individuals survived throughout the experimental procedure (94.67%), confirming the nonlethality of the treatments chosen during the 10 d of exposure. Tadpoles allocated to the highest levels of salinity, temperature, and herbicide were previously acclimated for 3 d at the intermediate levels, so they were exposed to the highest level for only 7 d. Even short acclimation periods seem to be critical to allow enough time to mount an effective physiological response to acute stressors (Wu et al. 2014).

After 10 d, we randomly collected one tadpole per container, and we measured their standard metabolic rate as described below. We also extracted blood for leukocyte determination from another tadpole randomly chosen from each container. We then collected the remaining tadpoles, euthanized them individually by immersion in a lethal concentration of anesthetic (MS-222), and randomly allocated one tadpole per experimental unit to CORT assay and oxidative stress assays.

Salinity Experiment. To obtain the target salinity levels (0, 6, and 9 ppt), we added commercial sea salt (Instant Ocean) as required for each treatment. To prevent possible osmotic shocks derived from direct transfer to 9 ppt (Wu et al. 2012), we acclimated tadpoles assigned to the 9-ppt treatment in a solution at 6 ppt for 3 d before onset of the experiment. We monitored salinity twice a week using an osmometer (Multi 340i; WTW) and a refractometer (RHS-10; LABOLAN). Salinity varied ± 0.2 ppt for 6- and 9-ppt treatments and did not vary for 0 ppt.

Herbicide Experiment. We tested tadpoles' response to glyphosate, which is one of the herbicides most widely used in crop

fields worldwide (Solomon and Thompson 2003). We used a stock solution containing 360 g/L of isopropylamine salt of glyphosate (Fortin; Industrial Química Key). We used two different glyphosate concentrations: 1 and 2 mg/L acid equivalent. Glyphosate was made fresh before each water change to avoid possible glyphosate degradation since its half-life in water is 7–14 d (Giesy et al. 2000).

pH Experiment. We obtained the target pH levels (4.5, 7.0, and 9.5) by adding either sodium carbonate (PQS) or sodium bisulphate (PQS) from concentrated stock solutions. We checked water pH daily with a pH meter (Multi 340i; WTW), adjusting it as necessary. pH values varied by ± 0.4 regardless of the pH level.

Predator Experiment. Predators were introduced in cages made of plastic cups (250 mL) with a mesh screen bottom that allowed water flow and cue diffusion. Buckets in the predator-absent treatment contained empty cages. We surveyed the experiment daily and replaced any dead predators. We fed predators in external housing tanks to avoid confounding the detection of predator kairomones with detection of alarm cues from injured tadpoles in the experimental buckets.

Temperature Experiment. We used individual aquarium heaters (25 W) in each bucket to regulate temperature. Experimental units in the 21°C treatment also contained heaters but were switched off. We increased temperature in a two-step process so that tadpoles assigned to the 29°C treatment were first maintained at 25°C for 3 d to allow tadpoles to acclimate. We verified the water temperature daily with a thermometer (RTD; Delta Ohm) and found it to be very stable. The temperature was constant and varied only ± 0.3 °C in each level (21°, 25°, and 29°C).

Corticosterone Assay

CORT levels were determined from whole-body homogenates by performing enzyme immunoassay (EIA; Burraco et al. 2015) using commercial kits (Cayman Chemical). This procedure is a conservative test of CORT differences across treatments, as it has lower sensitivity than radioimmunoassay (RIA) or EIA on plasma samples (Burraco et al. 2015). However, it does not require the use of radioactive isotopes and allowed us to keep the number of animals used in the study at a minimum given the high combination of factors and levels required (Burraco et al. 2015).

Tadpole homogenates were centrifuged at 4,000 rpm at 4°C for 15 min. We took 50 μ L from the resulting supernatant for EIAs. EIAs are quantitative assays based on competitive binding between the target hormone and a conjugated CORT tracer (CORT-acetylcholinesterase) for a limited number of CORT-specific sheep antiserum binding sites that bind to the rabbit polyclonal antiserum IgG previously attached to the well. Quantitative estimates were obtained by reading absorbance at 412 nm, and CORT concentrations were determined based on standard curves run in duplicate on each plate. Each sample was run by duplicate. The detection limit (80% B/B₀) for

this kit is 30 pg/mL as indicated by the manufacturer, and cross reactivity with other steroids is below 1%.

Standard Metabolic Rate

We used an aquatic respirometer to measure standard metabolic rate (SMR) consisting of a set of 10 flow-through cells (plexiglass cylinders, 44 m in diameter \times 163 mm in length) with 20 optical sensors. Two sensors flanked each chamber to simultaneously measure the oxygen concentration (mg/L) coming in and out of the chamber. We connected the optical sensors to an oxymeter (Oxy 10-PreSens), and we programmed it to record oxygen partial pressure every 15 s. The optical sensors used (optodes) do not consume oxygen during measurements and have long-term stability, and their signal does not depend on the flow rate of the sample. We calibrated the respirometer at the same temperature as the experimental units experienced: 25° and 29°C for the experimental temperature treatments and 21°C for all the rest. Calibration took place at least once daily using a sodium sulphite solution and oxygen-saturated water to reach 0% and 100% concentrations. From each bucket, we introduced one random tadpole individually in each plexiglass chamber. All tadpoles were at Gosner stage 35 (Gosner 1960). We recorded oxygen consumption for 25 min but discarded the first 5 min of the data series, considered as an acclimation period of the animals to the chambers, and SMR values were calculated following Álvarez et al. (2006). Based on our previous experience, after 5 min, tadpoles seem to behave normally, and respirometer output is quite stable. In our experience, 20 min of effective recording time provides reliable measurements compared to longer recordings on the same species. This procedure allowed us to compact the overall number of days required to collect all the data, hence avoiding the potentially confounding factor of having later-assayed tadpoles being more advanced in development than earlier-assayed tadpoles. All trials were conducted between 0900 and 1400 hours to avoid circadian effects. On release from the chambers, tadpoles were blotted dry and weighed to the nearest 0.1 mg on a high-precision balance (CP324S; Sartorius).

Oxidative Stress

We quantified the activity of four antioxidant enzymes: catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), and glutathione reductase (GR). We also quantified the cellular damage by measuring thiobarbituric acid substances (TBARS) formed during lipid peroxidation.

Upon completion of the experiment, we euthanized tadpoles in benzocaine 0.01%, snap froze them in liquid nitrogen, and stored them at -80°C until the assays were conducted. We thawed the samples and dissected the specimens to remove the gut in order to avoid possible interferences with the assays. Samples were then individually homogenized in a buffered solution (100 mM Tris-HCL with 0.1 mM EDTA, 0.1% triton X-100, pH 7.8 and 0.1 mM PMSF, for the inhibition of proteolysis) using a homogenizer at 35,000 rpm (Micra D-1). We mixed 1 g of tissue

in 4 mL of homogenization buffer (1:4, w:v). The homogenated tissues were centrifuged at 14,000 rpm for 30 min at 4°C . We aliquoted the resulting supernatant into several 0.6-mL tubes, and we cryopreserved it at -80°C . We determined the total protein content assessed to calculate the antioxidant enzymes activity by the standard Bradford's method (Bradford 1976).

We quantified CAT activity in terms of catalytic activity with an indirect method, according to Cohen and Somerson (1969). We used potassium permanganate (KMnO_4), which is an oxidizing agent and colored compound that acts on the H_2O_2 (reducing agent), producing H_2O and O_2 . KMnO_4 is reduced, producing a red product (absorbance read at a wavelength at 480 nm). We performed standard curves of commercial catalase (SIGMA-60634), and we determined absorbance at a wavelength of 480 nm 5 min after adding KMnO_4 . We expressed the catalase activity as units per milligram of total proteins. Following Cord and Fridovich (1969) we obtained the SOD activity levels by measuring the cytochrome C inhibition rate, produced by SOD: superoxide free radicals (O_2^-) reduce the ferrocycytochrome C (xantine-xantine oxidase enzymatic system), but in SOD presence this reaction is inhibited because of superoxide radicals producing hydrogen peroxide and molecular oxygen. One unit of SOD is defined as the amount of enzyme that inhibits the rate of reduction of ferrocycytochrome C by 50% at 25°C at 550 nm (Cord and Fridovich 1969). We determined GPx activity as described by Paglia and Valentine (1967). GPx converts hydrogen peroxide into water but requires reduced glutathione that is produced by GR through oxidized glutathione reduction. To quantify the GPx activity, we measured NADPH oxidation by reading absorbance at a wavelength of 340 nm. We quantified GR activity following Cribb et al. (1989). We measured the change in absorbance at 340 nm due to NADPH oxidation, as described in the GPx assay. The formation of TBARS is due to lipid peroxidation and is increased during cellular damage processes. One product of lipid peroxidation is malondialdehyde (MDA), which reacts with thiobarbituric acid and produces a red product absorbing at 535 nm. We measured TBARS concentration according to Buege and Aust (1978). We measured the optical density values for the blank and the calibration curve. We then calculated the TBARS concentration (nmol MDA/mL) from the absorbance of each sample, subtracting the blank values and comparing with the calibration values.

Immune Status

We counted leukocytes to assess stress condition through direct cell observation from blood smears. Although a single observer recorded leukocyte proportion, we estimated the variation between observers (coefficient of variation [CV] = 8.71%). The CV intrasample was 6.99%. The blood was obtained via cardiac venipuncture with a 29G syringe (BD Micro-Fine Insuline U-100, 0.5 mL) in tadpoles anesthetized with MS-222. The resulting blood smears were stained using the Pappenheim method (May-Grünwald-Giemsa staining) and were fixed onto the glass slides with DPX (Eukitt mounting medium). We identified and counted the proportion of lymphocytes and granulocytes (ba-

sophils, neutrophils, and eosinophils) out of 100 white cells in each sample using a $\times 100$ ocular Zeiss microscope (total magnification $\times 1,000$) with the aid of immersion oil DC.

Statistical Analyses

We conducted all statistical analyses in R, version 2.14-1 (R Development Core Team 2007). We tested for normality by means of Kolmogorov-Smirnov tests (`lillie.test`, package `nortest`, ver. 1.0-3) and for homogeneity of variances with Bartlett's tests (`bartlett.test`), as well as through visual inspection of residuals. Otherwise, we used the Akaike information criterion to assess the goodness of fit of each model and chose the appropriate error distribution. When parametric assumptions were met, we used linear models with a Gaussian distribution and an identity link function. We used gamma distributions where appropriate in generalized linear models with the `glm` function included in the MASS package (ver. 7.3-40). For SMR analysis, we used body weight as a covariate to control for the effect of body mass in oxygen consumption. CORT, GR, and GPx were also significantly affected by body mass, although its explanatory power was very low ($R^2 < 0.05$) except for GR ($R^2 = 0.156$, $P < 0.001$). Therefore, we included only body mass as a covariate in SMR and GR analyses. CORT and SMR data were log transformed to meet parametric assumptions. We tested for differences in the proportion of leukocytes by fitting generalized linear models with a binomial distribution. We conducted post hoc tests (Tukey tests) using the `TukeyHSD` function (`multcomp` package, ver. 1.2-13) when overall tests were significant to test for differences among treatments.

Results

Pelobates cultripes tadpoles exposed to nonlethal levels of salinity and glyphosate experienced changes in most of the physiological parameters measured, modifying CORT levels, metabolic rate, antioxidant enzymatic activity, and leukocyte counts (see table 1). Furthermore, changes in pH, temperature, and predators also produced hormonal, enzymatic, or immune alterations in spadefoot toad tadpoles. Because experiments were run in two consecutive breeding seasons including animals from two different locations, the absolute values were not directly comparable across all experiments. Consequently, and since each experiment had its independent control treatment at 21°C and neutral water, we plotted the results for each experimental treatment as the relative change of each variable with respect to the control.

Responses to Salinity

Increased salinity significantly increased CORT levels ($F_{2,22} = 3.856$, $P = 0.040$; fig. 1), with tadpoles in 9 ppt showing a 2.78-fold increase on average compared to tadpoles in 0 and 6 ppt. High salinity also increased SMR ($F_{2,28} = 3.86$, $P = 0.035$; fig. 2). Tadpoles in 9 ppt increased their SMR on average by 2.41-fold ($P = 0.026$) compared to the ones in 0 ppt. Salinity altered the activity of antioxidant enzymes, particularly for GR ($F_{2,29} = 4.09$,

$P = 0.029$; fig. 3) and SOD ($F_{2,30} = 10.43$, $P \leq 0.001$). High salinity (9 ppt) resulted in an average reduction of 26.8% in GR activity ($P = 0.025$) compared to control tadpoles, although tadpoles in 6 ppt did not vary GR activity. Tadpoles in 6 ppt reduced their SOD activity by 28.8% ($P = 0.005$) compared with tadpoles in freshwater, whereas the reduction in SOD activity at 9 ppt reached on average 35.9%. We found no significant variation in CAT and GPx activity in response to salinity (all $P > 0.686$) and no evidence for oxidative cellular damage (TBARS; $F_{2,30} = 0.03$, $P = 0.962$). Furthermore, salinity increased the proportion of neutrophils 41.3% and 41.6% on average in 6 and 9 ppt, respectively ($F_{2,28} = 20.19$, $P < 0.001$), whereas lymphocytes decreased by 10.1% and 8.5% in 6 and 9 ppt, respectively ($F_{2,28} = 13.43$, $P < 0.001$). The proportion of basophils or eosinophils did not vary among treatments ($P > 0.286$).

Responses to Herbicide

Herbicide increased CORT levels ($F_{2,28} = 3.94$, $P = 0.033$; fig. 1) at both concentrations used, ranging from a 65% to a 91.4% increase, although in the case of the 2-mg/L glyphosate treatment, the difference with the control was marginally non-significant ($P = 0.060$). Exposure to herbicide also increased SMR ($F_{2,22} = 5.69$, $P = 0.0122$; fig. 2). SMR increased on average by 2.6- and 2.7-fold in 1 and 2 mg/L, respectively, both differing from the control treatment ($P < 0.029$). Herbicide exposure also altered antioxidant activity. GR activity decreased with herbicide ($F_{2,29} = 5.15$, $P = 0.012$; fig. 3) at both concentrations (37.6% at 1 mg/L and 30.72% at 2 mg/L) compared to tadpoles in nonherbicide treatment. GPx activity tended to decrease in tadpoles exposed to 1 mg/L of glyphosate, but this change was marginally nonsignificant ($F_{2,30} = 2.74$, $P = 0.082$). We found no changes in SOD or CAT activity ($P > 0.372$) and no evidence of oxidative damage (TBARS; $F_{2,29} = 0.615$, $P = 0.548$). Herbicide exposure did not significantly affect leukocyte proportion ($P > 0.369$).

Responses to Changes in pH

Exposure to acid or basic pH did not cause tadpoles to vary their CORT levels ($F_{2,23} = 1.37$, $P = 0.278$; fig. 1) or SMR ($F_{2,30} = 1.45$, $P = 0.448$; $P = 0.252$; fig. 2). We found, however, a marginally nonsignificant decrease in GR activity ($F_{2,30} = 2.94$, $P = 0.070$; fig. 3) so that tadpoles exposed to pH 4.5 showed on average 20.5% lower GR activity than tadpoles in neutral water. We found no significant changes in GPx, SOD, or CAT activities (all $P > 0.205$) and no sign of oxidative damage (TBARS; $F_{2,30} = 0.26$, $P = 0.774$). Leukocyte count was similarly unaffected by exposure to acidic and basic pH ($P > 0.267$) except for the proportion of basophils, which increased by 1.46-fold in tadpoles exposed to basic pH ($F_{2,24} = 4.76$, $P = 0.009$).

Responses to Predator Exposure

Exposure to predators altered CORT levels ($F_{2,23} = 4.11$, $P = 0.032$; fig. 1). Tadpoles raised in the presence of native beetle

Table 1: Mean values of the main parameters analyzed in spadefoot toad tadpoles (*Pelobates cultripes*) exposed to five environmental factors

| | CORT | SMR | CAT | SOD | GPx | GR | TBARS | % Neutrophils | % Basophils | % Eosinophils | % Lymphocytes |
|------------------|---------------------|-----------------|--------------------|--------------------|------------------|------------------|-----------------|------------------|----------------|----------------|------------------|
| Salinity 0 ppt | 125.60; 27.51 (8) | .040; .013 (9) | 247.90; 14.70 (10) | 142.90; 12.01 (10) | 20.75; 1.03 (10) | 7.18; .62 (9) | 8.97; 1.34 (10) | 18.66; 2.25 (10) | 3.79; .40 (10) | 1.21; .40 (10) | 76.33; 2.42 (10) |
| Salinity 6 ppt | 64.27; 9.74 (7) | .047; .012 (10) | 259.23; 16.79 (10) | 106.14; 5.41 (10) | 22.30; 1.56 (10) | 6.53; .43 (10) | 8.96; 1.08 (10) | 26.36; 3.34 (9) | 3.42; .53 (9) | 1.54; .41 (9) | 68.67; 3.83 (9) |
| Salinity 9 ppt | 269.86; 118.38 (6) | .095; .013 (9) | 266.73; 14.88 (10) | 91.58; 5.25 (10) | 24.36; 2.13 (10) | 5.26; .40 (10) | 9.42; 1.81 (10) | 26.41; 3.05 (9) | 2.85; .59 (9) | 2.86; .59 (9) | 69.85; 2.65 (9) |
| Herbicide 0 mg/L | 493.54; 144.73 (9) | .010; .008 (9) | 202.24; 9.70 (8) | 107.50; 5.04 (8) | 17.05; 2.66 (9) | 13.61; 1.84 (9) | 3.50; 1.11 (8) | 15.85; 2.57 (8) | .48; .18 (8) | 1.08; .46 (8) | 82.59; 2.88 (8) |
| Herbicide 1 mg/L | 944.87; 104.78 (10) | .032; .008 (10) | 195.44; 7.54 (10) | 120.91; 7.69 (10) | 12.88; 1.50 (10) | 8.49; .645 (10) | 3.94; .84 (10) | 17.10; 4.93 (5) | .97; .20 (5) | .63; .39 (5) | 81.30; 4.81 (5) |
| Herbicide 2 mg/L | 860.20; 147.53 (10) | .028; .007 (10) | 205.57; 7.64 (10) | 119.18; 6.63 (10) | 19.30; 1.83 (10) | 9.44; .71 (10) | 2.67; .56 (10) | 16.67; 3.59 (8) | .81; .40 (8) | .19; .36 (8) | 81.32; 3.94 (8) |
| pH 7.0 | 91.77; 10.78 (7) | .061; .008 (10) | 335.13; 34.79 (10) | 153.62; 8.41 (10) | 23.97; 2.25 (10) | 8.27; .60 (10) | 8.10; .79 (10) | 12.14; 2.25 (8) | 1.44; .65 (8) | 1.32; .52 (8) | 92.54; 6.56 (8) |
| pH 4.5 | 153.06; 35.95 (8) | .052; .012 (10) | 282.99; 13.18 (10) | 134.70; 8.99 (10) | 28.99; 3.23 (10) | 6.57; .40 (10) | 7.15; .74 (10) | 10.16; 2.77 (7) | 1.37; .35 (7) | 3.30; 1.16 (7) | 84.07; 4.25 (7) |
| pH 9.5 | 164.97; 40.89 (10) | .040; .011 (10) | 283.97; 20.19 (10) | 132.70; 9.47 (10) | 24.32; 2.09 (10) | 7.08; .51 (10) | 8.05; 1.48 (10) | 12.62; 2.16 (8) | 3.37; 1.55 (8) | 2.04; .61 (8) | 90.50; 9.92 (8) |
| No predator | 168.84; 41.14 (7) | .076; .019 (9) | 217.40; 17.07 (9) | 131.00; 5.80 (9) | 22.84; 1.86 (9) | 7.57; .45 (9) | 8.02; .69 (10) | 15.44; 2.48 (10) | .49; .22 (10) | 2.51; .68 (10) | 81.56; 2.96 (10) |
| Native | 83.78; 24.42 (7) | .048; .017 (10) | 307.14; 40.65 (7) | 117.16; 14.08 (8) | 23.62; 1.90 (8) | 7.21; .59 (8) | 7.57; 1.28 (7) | 13.3; 2.40 (9) | .86; .16 (9) | 1.73; .36 (9) | 84.10; 2.79 (9) |
| Invasive | 131.92; 13.43 (10) | .064; .017 (10) | 294.03; 40.49 (9) | 146.41; 16.76 (9) | 23.82; 1.49 (9) | 8.42; .64 (9) | 6.76; 1.03 (10) | 13.76; 2.07 (9) | .96; .84 (9) | 1.62; .67 (9) | 83.66; 2.36 (9) |
| 21°C | 444.51; 69.29 (9) | .024; .006 (9) | 171.20; 4.04 (10) | 105.06; 2.14 (10) | 16.62; 1.66 (10) | 8.42; .69 (10) | 1.64; .49 (10) | 13.98; 3.11 (9) | .67; .31 (9) | 1.34; .39 (9) | 83.71; 3.24 (9) |
| 25°C | 672.06; 93.88 (8) | .015; .007 (9) | 173.46; 8.03 (10) | 94.75; 6.45 (10) | 17.95; 1.75 (10) | 12.12; 1.24 (10) | 3.66; .72 (10) | 7.29; 3.72 (4) | .00; .00 (4) | .49; .28 (4) | 91.98; 3.93 (4) |
| 29°C | 460.33; 102.10 (9) | .018; .006 (9) | 170.45; 7.18 (10) | 110.88; 3.08 (10) | 14.54; 2.66 (10) | 8.33; .61 (10) | 1.52; .35 (10) | 8.15; 3.72 (5) | .68; .30 (5) | .19; .19 (5) | 90.77; 3.77 (5) |

Note. Italicized numbers indicate standard error of the mean, and numbers in parentheses indicate the sample size for each treatment. CAT = catalase activity (U/mg protein); CORT = corticosterone (pg/mL); GPx = glutathione peroxidase activity (mU/mg protein); GR = glutathione reductase activity (mU/mg protein); SMR = standard metabolic rate (mL O₂/h/g); SOD = superoxide dismutase activity (U/mg protein); TBARS = thiobarbituric acid reactive substances (nmol MDA/mL). The last four columns show the percentage of neutrophils, basophils, eosinophils, and lymphocytes out of 100 white cell counts.

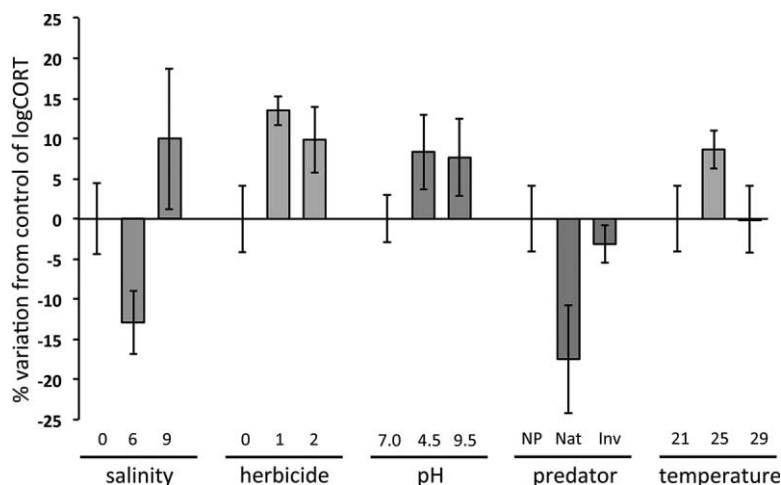


Figure 1. Effect of experimental stress factors on corticosterone concentration in spadefoot toad tadpoles, expressed as percent variation from the average concentration (\pm SE) in control tadpoles. Corticosterone (CORT) was measured in picograms per milliliter of sample after 10 d of exposure to each factor: salinity (0, 6, and 9 ppt), herbicide (0, 1, and 2 mg/L of glyphosate), pH (4.5, 7.0, and 9.5), predators (absence, native, and invasive), and temperature (21°, 25°, and 29°C). A color version of this figure is available online.

larvae decreased CORT by an average of 50.1% ($P = 0.046$). CORT levels, however, did not change when exposed to invasive crayfish ($P = 0.618$). Neither native nor alien predators altered SMR ($F_{2,29} = 0.37$, $P = 0.693$; fig. 2). Likewise, the activity of antioxidant enzymes was unaffected by the presence of either type of predator ($P > 0.081$), and no cellular damage was detected ($F_{2,27} = 0.42$, $P = 0.66$). The presence of predators had no effect on leukocyte count ($P > 0.275$).

Responses to Temperature

We observed no variation in CORT levels ($F_{2,26} = 1.83$, $P = 0.183$; fig. 1) or SMR ($F_{2,27} = 0.80$, $P = 0.462$; fig. 2) across the three temperatures used in the experiment. However, tempera-

ture affected the activity of antioxidant enzymes and leukocyte count. Tadpoles reared at 25°C decreased their SOD activity with respect to the other two temperatures (21° and 29°C; $F_{2,30} = 3.59$, $P = 0.041$). GR activity also varied with temperature ($F_{2,30} = 5.80$, $P = 0.008$; fig. 3), with tadpoles exposed to 25°C showing on average 30.6% higher activity with respect to tadpoles reared at 21°C and 31.3% higher activity with respect to those raised at 29°C. We found no effects of temperature on GPx or CAT activity (all $P > 0.512$). However, tadpoles exposed to 25°C increased TBARS ($F_{2,30} = 4.98$, $P = 0.014$) by 1.23- and 1.40-fold compared to tadpoles at 21° and 29°C, respectively. Moreover, tadpoles in either 25° or 29°C showed a decrease in the neutrophil proportion by 52.17% and 58.30% and an increase in the lymphocyte proportion by 7.6% and 8.4% ($F_{2,18} = 15.12$, $P <$

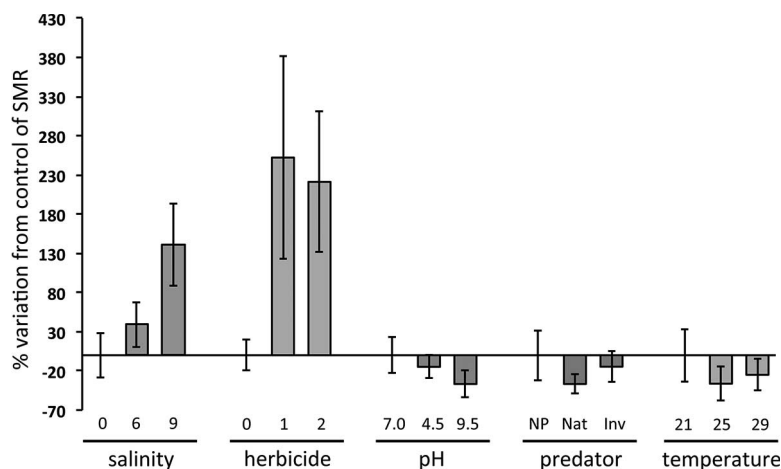


Figure 2. Percent deviation from average control values (\pm SE) of standard metabolic rate (SMR; mL O₂/h/g) after 10 d of exposure to five factors: salinity (0, 6, and 9 ppt), herbicide (0, 1, and 2 mg/L of glyphosate), pH (4.5, 7.0, and 9.5), predators (absence, native, and invasive), and temperature (21°, 25°, and 29°C). A color version of this figure is available online.

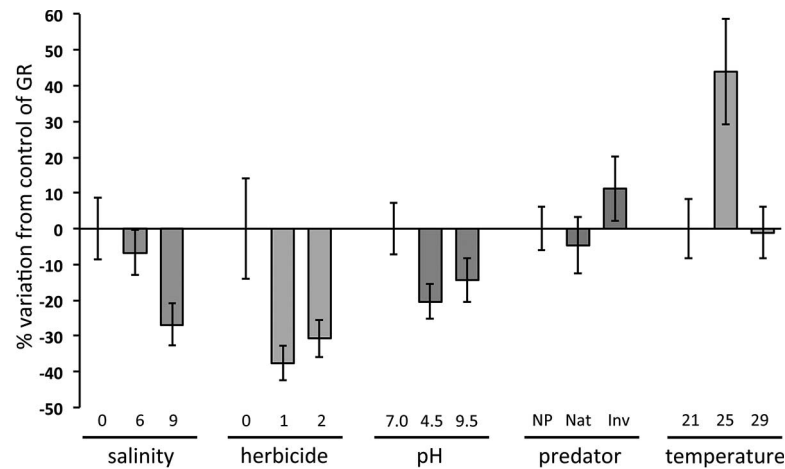


Figure 3. Percent deviation from control treatments (\pm SE) of glutathione reductase activity (GR; mU/mg protein) after 10 d of exposure against five stress factors: salinity (0, 6, and 9 ppt), herbicide (0, 1, and 2 mg/L of glyphosate), pH (4.5, 7.0, and 9.5), predators (absence, native, and invasive), and temperature (21°, 25°, and 29°C). A color version of this figure is available online.

0.001) compared to tadpoles raised at 21°C, respectively. The proportion of eosinophils decreased by 41.6% and 85.3% ($F_{2,18} = 3.63$, $P < 0.026$) in tadpoles exposed to 25° and 29°C, respectively. We did not find a significant change in basophil proportions ($F_{2,18} = 2.66$, $P = 0.070$).

Discussion

Exposure to nonlethal levels of salinity, pH, temperature, herbicide (glyphosate), and predators caused marked physiological alterations in spadefoot toad tadpoles. Of all the potential stressors studied, salinity and herbicide seemed to affect amphibian physiology the most. These two factors altered CORT levels, standard metabolic rate, and antioxidant enzymes activity in *Pelobates cultripes* tadpoles (table 1). The highest levels of salinity and herbicide (i.e., 9 ppt and 2 mg/L of glyphosate, respectively) produced comparable physiological unbalances since hormonal and metabolic rate changes occurred in the same direction and similar magnitude. These results outline that even nonlethal levels of stressors may have marked physiological effects, and it is important to take this into consideration when designing conservation policies.

Both salinity and herbicide exposure caused tadpoles to increase their energy expenditure, possibly driven by increased corticotropin-releasing hormone that ultimately elevates thyroid hormone and CORT levels (fig. 1), causing associated increases in metabolic rate (Denver et al. 2002; Wack et al. 2012; fig. 2). Moreover, the activation of corticotropin-releasing hormone increases expression of mineralocorticoid receptors (Gesing et al. 2001). These receptors are involved in the regulation of body fluid osmolality and ion balance (Terker and Ellison 2015), which is essential for amphibian osmoregulation, especially under osmotic stress (Hopkins and Brodie 2015). As predicted, factors that increased CORT concentration and metabolic rate resulted in redox imbalance, as indicated by alterations in antioxidant enzyme activity (fig. 3; table 1). Decreased GR activity might be due

to low levels of NADPH, a secondary manifestation of cellular free radical stress (Moreno et al. 2005). Likewise, decreased SOD activity is likely related to oxidative inactivation of enzymes by free radicals (Pigeolet et al. 1990) due to reduced de novo synthesis (Kaushik and Kaur 2003). Despite possible redox imbalance, no cellular damage in cell membranes was observed, as indicated by a lack of differences among treatments in TBARS (Lin et al. 2004). In addition, salinity affected the leukocyte profile, causing increased proportions of lymphocytes and granulocytes (table 1). Common stress-induced changes in the proportion of leukocytes include neutrophilia (abnormally high number of neutrophils) and lymphopenia (abnormally low number of neutrophils) and are often associated with increased GC levels (Davis et al. 2008). Herbicide, however, had no apparent effects on the immune system in this experiment; glyphosate has been shown to affect the leukocyte proportion of tadpoles (Shutler and Marcogliese 2011; Burraco et al. 2013).

Contrary to our expectations, high temperature did not affect either CORT levels or SMR. This could be due to lack of statistical power, but other stressful factors did cause marked changes in these parameters, so at least we can conclude that the effect of temperature was milder than that of factors such as salinity or herbicide. Nevertheless, we observed increased GR activity at 25°C, as well as evidence for oxidative cellular damage (TBARS). Tadpoles at 25°C may have been closer to their optimum temperature (from a physiological point of view) and consequently experienced higher growth and developmental rates than tadpoles at either 21° or 29°C, hence increasing lipid peroxidation (by-product of fat degradation). However, tadpoles reared at 29°C may have developed too quickly to even have time to accumulate fat (Kulkarni et al. 2011), hence reducing the rate of lipid peroxidation. More detailed analysis of lipid consumption during the course of anuran development and in response to changes in developmental and growth rates is needed to clarify the observed nonlinear patterns in oxidative stress with varying temperature.

Changes in pH within the range used in this study did not cause deep physiological adjustments. A slight (and marginally nonsignificant) reduction in GR activity may reflect increased free radicals against acidic and basic pH. Chambers and Belden (2009) found effects of acid or basic pH on CORT levels in amphibian larvae, but these changes were species dependent. The range of water pH in the Doñana National Park is wide (from 4 or 5 to 7 or 8; Serrano et al. 2006); thus, *P. cultripes* tadpoles could be adapted to large fluctuations of pH.

The observed responses to predators indicate a conflict between the population-level and the organismal-level concepts of environmental stress. Predators clearly pose a threat to individual survival and could consequently dramatically reduce fitness within populations. In that respect, predators are clearly a source of environmental stress. In some species, predators also trigger physiological stress responses that would typically characterize them as a stressful factor, such as when raptors induce overexpression of heat-shock proteins in nestling passerines (Thomson et al. 2010). Similarly, Maher et al. (2013) reported CORT elevation in *Rana sylvatica* tadpoles exposed to dragonfly nymphs. However, we observed reduced CORT levels in spadefoot toad tadpoles exposed to native beetle larvae (fig. 1). Reduced CORT in the presence of predators may simply be associated with the reduction in activity rate observed in *P. cultripes*, which can be up to 57% in the presence of native predators (Polo-Cavia and Gomez-Mestre 2014). Other amphibians have been shown to also lower their metabolic rate in the presence of predators (Barry and Syal 2013), sometimes after an initial transient increase (Steiner and Van Buskirk 2009). Invasive predators, however, did not alter tadpoles' CORT levels (fig. 1). This lack of hormonal response to invasive crayfish is congruent with past observations that local tadpoles do not activate their morphological or behavioral defenses against invasive crayfish at the study site for lack of innate recognition (Gomez-Mestre and Díaz-Paniagua 2011; Polo-Cavia and Gomez-Mestre 2014).

Our data show that common stressors to aquatic systems substantially alter the physiological state of tadpoles. Herbicides constitute a major threat because they are novel to amphibians at an evolutionary scale and have marked physiological consequences. However, while most of the other factors may vary considerably in nature, they are often drastically intensified by human activities, as in salinization or acidification of aquatic systems, raises in temperature, or the introduction of alien predators. In particular, high levels of salinity and herbicide cause similarly steep physiological alterations in tadpoles. It is, however, important to understand the nature of the responses against each type of stressor, because different risks may induce physiological changes of very different magnitudes and even in opposite directions. Among the parameters used, CORT and GR were the most sensitive to environmental stress in our study, although a combined approach determining several other physiological parameters such as metabolic rate or leukocyte profile provides a more comprehensive assessment of the physiological responses. Systematic comparisons of physiological alterations against multiple factors and factor combinations will fuel larger-scale comparative

physiology, providing mechanistic insights into conservation, ecological, and evolutionary studies and contributing to explaining large geographical and temporal patterns (Chown and Gaston 2015). Moreover, stress experienced during early life stages and high levels of GCs in particular have long-lasting effects (Weaver 2009; Wu et al. 2012). Therefore, long-term studies are needed to fully understand the consequences of stress during the larval stages on the phenotype and fitness of the adults. Comparative physiological studies will also contribute to inform effective management decisions aimed at soothing the impact of anthropogenic disturbances before marked population declines are detected (Chown and Gaston 2008).

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