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Opposite and synergistic physiological responses to water acidity and predator cues in spadefoot toad tadpoles

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ABSTRACT

Organisms are exposed to multiple environmental factors simultaneously to which they often respond behaviorally, morphologically and/or physiologically. Amphibian larvae are quite plastic and efficiently adjust their phenotype and physiology to the reigning local conditions. Here we tested whether the combination of predator presence and low water pH induces alterations in the morphology and physiology of spadefoot toad tadpoles. We raised *Pelobates cultripes* tadpoles in the laboratory in water at either pH 4 or 7, and in the presence or absence of caged dragonfly nymphs, and determined their changes in shape through geometric morphometrics to assess whether predator recognition was impaired or not at low pH. We also measured levels of plasma corticosterone, activity of four antioxidant enzymes, as well as markers of oxidative damage and redox status. We found that tadpoles altered their body shape in response to predator cues even at low pH, indicating that predator recognition was not interfered by water acidity and developmental responses were robust even under abiotic stress. Water acidity was associated with increased corticosterone levels in tadpoles, whereas predator presence consistently reduced corticosterone levels. Predator presence was linked to reduced antioxidant enzyme activity, whereas the combination of both factors resulted in negative synergistic effects on lipid peroxidation and the antioxidant capacity of tadpoles. Here we show that tadpoles detect predators even at low pH but that the development of adaptive anti-predatory morphology can magnify physiological imbalances when other stressors co-occur. These results emphasize the need to understand how multiple environmental perturbations can affect animal homeostasis.

1. Introduction

Antipredator responses are a widespread form of adaptive phenotypic plasticity that allows prey to alter their phenotype upon detection of predator cues (Agrawal, 2001; Tollrian and Harvell, 1998). These responses can be fine-tuned to the level of predation risk experienced (McCoy et al., 2012; Van Buskirk and Arioli, 2002) indicating the existence of accurate and reliable mechanisms of cue detection. Predator cue recognition, however, is critically dependent upon the joint evolutionary history between prey and predator (Ferrari et al., 2010b; Relyea, 2004). Because organisms live in rather complex environments, multiple factors can compromise the efficiency of inducible defenses even in the case of predator-prey systems with ample joint evolutionary history.

Stressful abiotic conditions (e.g. salinity, extreme pH or temperature) in particular have a two-fold potential for disruption of inducible defenses: they can interfere with cue quality by denaturalizing the cue itself or inhibiting its receptors (Troyer and Turner, 2015), or they can hamper antipredator responses by causing additional physiological stress (Hawlena and Schmitz, 2010). Exposure to multiple sources of stress could impair the organism's ability to develop adaptive antipredator phenotypes. For instance, altered water chemistry or pollution can hinder cue detection and activation of inducible defenses (Burraco et al., 2018; Ferrari et al., 2010a; Lürling and Scheffer, 2007; Polo-Cavia et al., 2016; Gabor et al., 2019), and it can also increase the costs of producing inducible defenses (Pestana et al., 2010; Teplitsky et al., 2005). Therefore the efficiency of inducible defenses depends on accurate and unobstructed predator cue recognition, but a sufficient body

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condition to overcome potential production costs of inducible defenses is also needed (Milot et al., 2014; Murren et al., 2015). From that perspective, additional stressors may prevent an appropriate expression of inducible defenses as a consequence of allocating resources to the maintenance of metabolic pathways linked to an induced stressful state, and away from the development of adaptive antipredator responses (Killen et al., 2013). Indeed, poorer body condition often entails increased vulnerability to predation (Hoey and McCormick, 2004; Murray, 2002). Conversely, antipredator responses can make prey more vulnerable to additional stressors, such as pesticides or viruses (Kerby et al., 2011; Relyea, 2003).

Here we study whether antipredator responses in larval amphibians are affected by simultaneous exposure to water acidity, using spadefoot toad tadpoles (*Pelobates cultripes*). Amphibians are sensitive to multiple stressors, often experiencing the activation of the hypothalamic-pituitary-interrenal (HPI) axis in response to them, which involves a physiological cascade that can induce oxidative stress and immunological imbalances (Burraco and Gomez-Mestre, 2016; Gervasi and Foufopoulos, 2008; Groner et al., 2013). However, the activation of the HPI-axis may be context dependent and result in unexpected dynamics (e.g. Gabor et al., 2018). The fact that multiple stressors may coincide, and the consequences of exposure to various concurrent stressors may be additive or even synergistic, combining to pose a much greater threat to amphibians than each factor individually (Blaustein et al., 2011; Boone et al., 2007; Davidson and Knapp, 2007). Water acidity and predator presence are common stressors to amphibians (Egea-Serrano et al., 2014), and they are the focus of this study, since their joint effect on amphibians has not been frequently studied. Low pH commonly poses a grave risk to embryonic and larval amphibians, causing an imbalance in their ionic regulation (Freda, 1986; Rasanen et al., 2002, 2003; Sadinski and Dunson, 1992). Water acidity is known to alter intraguild predator-prey interactions between amphibian species (Kiesecker et al., 1996), and to have very different impacts on swimming performance, growth and development across species (Freda and Dunson, 1985; Kutka, 1994; Rowe et al., 1992). Few studies have directly assessed the physiological stress responses of amphibian larvae to low pH, but they indicate that water acidity tends to increase their corticosterone levels (Burraco and Gomez-Mestre, 2016; Chambers et al., 2013). Likewise, the physiological stress responses to predator presence in amphibian larvae still require further research to confirm whether patterns found to date are dependent upon species identity, exposure duration, or a specific developmental stage. Wood frog (*Rana sylvatica*) tadpoles increase their corticosterone levels in the non-lethal presence of predators (Middlemis Maher et al., 2013), although this response may be dependent on the amount of time that tadpoles spend exposed to predator cues (Bennett et al., 2016). In contrast, green frog (*Rana clamitans*) and spadefoot toad tadpoles lower their corticosterone levels in response to predator cues (Burraco et al., 2013; Burraco and Gomez-Mestre, 2016; Fraker et al., 2009). In addition, the common frog (*Rana temporaria*) shows geographic and temporal variation in whether predators induce increased or decreased corticosterone levels in its tadpoles (Dahl et al., 2012). Our experiment was designed to test for additive or synergistic effects of low pH and predator presence on stress physiology of spadefoot toad tadpoles after chronic exposure to both sources of stress, quantifying the phenotypic responses of tadpoles to predators in both neutral and acidic water, their corticosterone levels and their oxidative status. We expected acidic water to interfere with the ability of tadpoles to detect predator cues. We also expected exposure to predator cues and acidic water to be associated with lower and higher corticosterone levels, respectively, and that both factors would involve redox imbalances in amphibian larvae. Finally, we predicted that the combination of acidic water and predator cues would magnify the individual effect of each factor, either in an additive or a synergistic way.

2. Methods and materials

This study was conducted at the Doñana Biological Reserve, located within the Doñana National Park, on the right bank of the Guadalquivir river mouth in southwestern Spain. The study area has a Mediterranean climate with an Atlantic influence, having hot and dry summers and rainfall occurring mostly in autumn or winter, from November to March (mean annual precipitation of 544.6 mm ± 211.3 mm; Diaz-Paniagua et al., 2010). The park comprises extensive marshes and a sandy area with shrubland, pinewoods and dunes that holds an extensive pond network of over 3000 temporary ponds (Diaz-Paniagua et al., 1997, 2010). These ponds usually fill up in the fall or in winter and last until June or July depending on the amount and timing of rainfall, showing large interannual variation in hydroperiod and physic-chemical properties (Gómez-Rodríguez et al., 2009). These ponds are common breeding habitats for eight amphibian species (Diaz-Paniagua et al., 2005), of which spadefoot toads are among the first to breed each season. Early breeding following the first strong rainfalls is important for this species given that it has a very long larval period (Gomez-Mestre et al., 2013; Kulkarni et al., 2011) and ponds dry up at the beginning of the summer. Breeding early and having a high growth rate grant spadefoot toad tadpoles a priority effect over the rest of the local anuran guild and makes them very strong competitors (Arribas et al., 2014). However, early breeding also puts them at risk of experiencing low water pH during their early ontogenetic stages. When some ponds in the area flood again in the fall after the summer drought, they experience a process of acidification due to pyrite oxidation (Serrano et al., 2016). Such acidification can be locally quite pronounced, at times reaching pH values as low as 3.3, which can become buffered over time if the pond is connected to groundwater (Serrano et al., 2016). Spadefoot toad tadpoles are also prey to multiple types of invertebrate predators, from dragonfly nymphs, to water beetle larvae or crayfish (Diaz-Paniagua et al., 2005; Gomez-Mestre and Diaz-Paniagua, 2011).

2.1. Experimental setup

We collected between 30 and 40 eggs from each of four clutches of *P. cultripes* from a pond at Doñana National Park (37°N, 6°20′W). The clutches were newly laid and the surrounding water was at pH 6.8. We brought the eggs into the laboratory at Doñana Biological Reserve and placed them in shallow trays with filtered water from their ponds of origin until they hatched. Once they reached the free-feeding stage (Gosner 25; Gosner, 1960), we pooled all larvae together and haphazardly selected 200 of them for the experiment.

To test the effect of both water acidity and predator presence on the phenotype and physiology of spadefoot toad tadpoles we designed a 2 × 2 experiment where we crossed presence/absence of caged dragonfly nymphs (*Anax imperator*) with two levels of water pH, acid (pH 4) and neutral (pH 7). Each treatment combination was replicated ten times using 4 L round plastic containers (23 cm upper diameter, 17 cm high), where each container held five tadpoles. Inside each container, we placed a 250 mL plastic cup with a mesh bottom from the rim with a metal wire so that the bottom of the cup hung approximately half way deep into the container. In each cup of the containers assigned to 'predator presence' we placed one dragonfly nymph, leaving the rest empty. Dragonflies were fed one spadefoot toad tadpole every other day inside the cup throughout the duration of the experiment. Water in the neutral pH treatments was untreated well water, whose pH ranged between 7.31 and 7.79, as measured with a benchtop pH meter. For the low pH treatments, we used the same well water but acidified it to pH 4 with sulfuric acid. We renewed the water of all containers and treatments every third day to ensure that pH in the acidic treatments did not drift away from target (pH range 3.96–4.45). Tadpoles were fed rabbit chow (Complete, Versele-Laga) ad libitum after each water renewal.

We ran the experiment for twenty days. After that period, we took

pictures of each tadpole in an ad hoc plexiglass photo booth using the respective experimental water type but without predator cues for morphometric analysis. Tadpoles were returned to their experimental containers, and six days after taking the photographs we extracted blood from them to measure corticosterone. We immersed tadpoles in a buffered solution of MS-222 (Ethyl 3-aminobenzoate methanesulfonate, Sigma) until they were deeply anesthetized. We then extracted blood from four individuals through direct heart puncture with non-heparinized insulin syringes (BD Micro-Fine Insuline U-100 0.5 mL) under a dissecting scope at 20× magnification. Blood extraction took under 3 min per individual, and blood from all four individuals from each container (ca. 30 μL per tadpole) was pooled together in heparinized tubes to ensure sufficient plasma volume was collected for the hormonal assay (Burraco et al., 2015). The blood was then centrifuged at 4000 rpm, at 4 °C for 20 min to obtain plasma (Gomez-Mestre et al., 2013). Plasma samples were stored at −80 °C until assayed. The remaining tadpole per container was eviscerated to avoid possible interference of gut content on biochemical determinations and snap-frozen for oxidative status determination.

2.2. Morphometric analyses

We used geometric morphometrics to describe shape variation in spadefoot toad tadpoles across treatments. We photographed all tadpoles laterally and scaled the resulting photographs using a grid. We delimited the shape of each tadpole digitizing 18 landmarks (Fig. 1) with tpsDig2 software (Rohlf, 2008). We chose these landmarks according to their ability to capture the overall body shape of tadpoles while satisfying statistical restrictions associated with geometric morphometrics (Rufino et al., 2006). We then performed generalized procrustes analysis on the landmark dataset (Rohlf and Slice, 1990) with the package *geomorph*, version 3.1.2 (Adams and Otárola-Castillo, 2013) in R (version 3.6.1). We tested for a potential allometric relationship between shape and body size testing the effect of log centroid size on shape across all individuals regardless of their allocation to experimental container or treatment. We found no such allometric component ($F_{1,\ 197} = 2.54$, $P = .667$). We therefore excluded centroid size from subsequent analyses. To avoid pseudoreplication, we calculated average procrustes coordinates values per container and used these average values to test for the effect of experimental treatments on tadpole shape. We conducted principal components analysis (i.e. relative warps, abbreviated RWs) to determine average body shape variation among containers. The first two relative warps, explained 45% and 21% of the total morphometric variance, respectively. These warps explained variation in common morphological features previously described for amphibian larvae (Hossie et al., 2010; Orizaola et al., 2013), especially associated with tail depth and relative length, body roundness and the angle of the anterior dorsal insertion of the tail. We conducted a procrustes ANOVA (*procD.lm* function of *geomorph*) on the resulting shape variables to test whether water pH, predator presence or their interaction affected the shape of tadpoles. Statistical significance of shape variation was based on 1,000 random permutations, using randomized residual permutation procedure (RRPP; Collyer et al., 2015).

2.3. Corticosterone assay

We estimated the level of circulating corticosterone from plasma through enzyme immunoassay (EIA). We took 50 μL of plasma from each sample and conducted EIA with a commercial kit following manufacturer specifications (Cayman Chemical Company – # 500655). Samples and the standard curve were run in duplicate. The plate was incubated for two hours at room temperature on an orbital shaker and then developed with Ellman's reagent. We read absorbance in each well at a wavelength of 412 nm using a spectrophotometer (Victor 31,420, Perkin-Elmer, MA, USA). According to the manufacturer, the detection limit (80% B/B0) is approximately 40 pg/mL, and cross reactivity with other tested steroids is below 1%. Corticosterone concentrations were estimated from interpolation to the standard curves using a four-parameter fit. The average coefficient of variation for sample duplicates was 17.68%.

2.4. Oxidative stress analyses

To assess the level of oxidative stress experienced by either low pH or predator presence on our experimental tadpoles we determined the activity of four antioxidant enzymes: catalase, superoxide dismutase, glutathione peroxidase, and glutathione reductase. We also measured malondialdehyde, formed during lipid peroxidation, as a marker of oxidative damage, and the reduced-to-oxidized glutathione ratio as an indicator of redox status. We thawed the individual samples in a buffered solution to inhibit proteolysis (100 mM Tris-HCl with 0.1 mM EDTA, 0.1% triton X-100, pH 7.8 and 0.1 mM PMSF) and homogenized individuals at 35,000 rpm using a Miccra homogenizer (Miccra D-1) at a ratio of 1 g of homogenized tadpole per 4 mL of buffer (1:4, w:v). We centrifuged the homogenates at 20817 *g* for 30 min at 4 °C and aliquoted supernatants into several 0.6 mL tubes and stored at −80 °C. We determined total protein content using standard Bradford's method (Bradford, 1976). We indirectly quantified catalase activity by measuring catalysis of hydrogen peroxide (H_2O_2, i.e. catalase substrate), in the presence of potassium permanganate ($KMnO_4$) as an oxidizing and coloring agent (Cohen and Somerson, 1969). Reduced $KMnO_4$ gives a red color that can be read at 480 nm five minutes after $KMnO_4$ is added. Standard curves were prepared using commercial catalase (SIGMA – 60,634). Catalase activity is expressed as U/mg of total protein. Superoxide dismutase activity was indirectly determined by measuring the inhibition rate of cytochrome C reduction (Cord and Fridovich, 1969). Cytochrome C is oxidized by superoxide radicals (O_2^-) except in the presence of superoxide dismutase, which competes for O_2^- generated by xanthine and hypoxanthine and reduces cytochrome C producing hydrogen peroxide (H_2O_2) and oxygen. One unit of superoxide dismutase is defined as the amount of enzyme that inhibits the rate of reduction of cytochrome C by 50% at 25 °C at 550 nm. The quantification of glutathione peroxidase was conducted according to (Paglia and Valentine, 1967). The oxidized glutathione is continually reduced due to an excess of glutathione reductase producing a constant level of reduced glutathione, which requires NADPH. We spectrophotometrically quantified NADPH oxidation at 340 nm. For glutathione reductase determination we measured the decrease in absorbance at 340 nm due to NADPH oxidation (Cribb et al., 1989). We also measured lipid peroxidation according to (Buege and Aust, 1978). Malondialdehyde results from lipid peroxidation and reacts with thiobarbituric acid, reporting a red product that absorbs at 535 nm. For the determination of total glutathione level we used the protocol developed by (Galván et al., 2010). We diluted the tadpole homogenates 1:10 (*w/v*) and homogenized again in a stock buffer (0.01 M PBS and 0.02 M EDTA). We prepared three working solutions as follows: (A) 0.03 mM of NADPH, (B) 6 mM 5,5′-Dithiobis (2-nitrobenzoic acid) (DNTB), and (C) 50 units of GR/mL. Solution A and B were mixed at a ratio of 7:1 respectively and 160 μL of this mixture was added to 40 μL of supernatant. After 15 s, we added 20 μL of the solution C and we read absorbance at 405 nm after 30 and 60 s. We determined the total concentration of glutathione comparing the changes in absorbance between consecutive readings, according to a standard curve generated by serial dilution of glutathione from 1 mM to 0.031 mM.

2.5. Statistical analyses

Geometric morphometric analyses were conducted as described above. We tested differences in corticosterone levels across experimental treatments through a linear model using logarithmic-transformed data to meet parametric assumptions. Collinearity between the

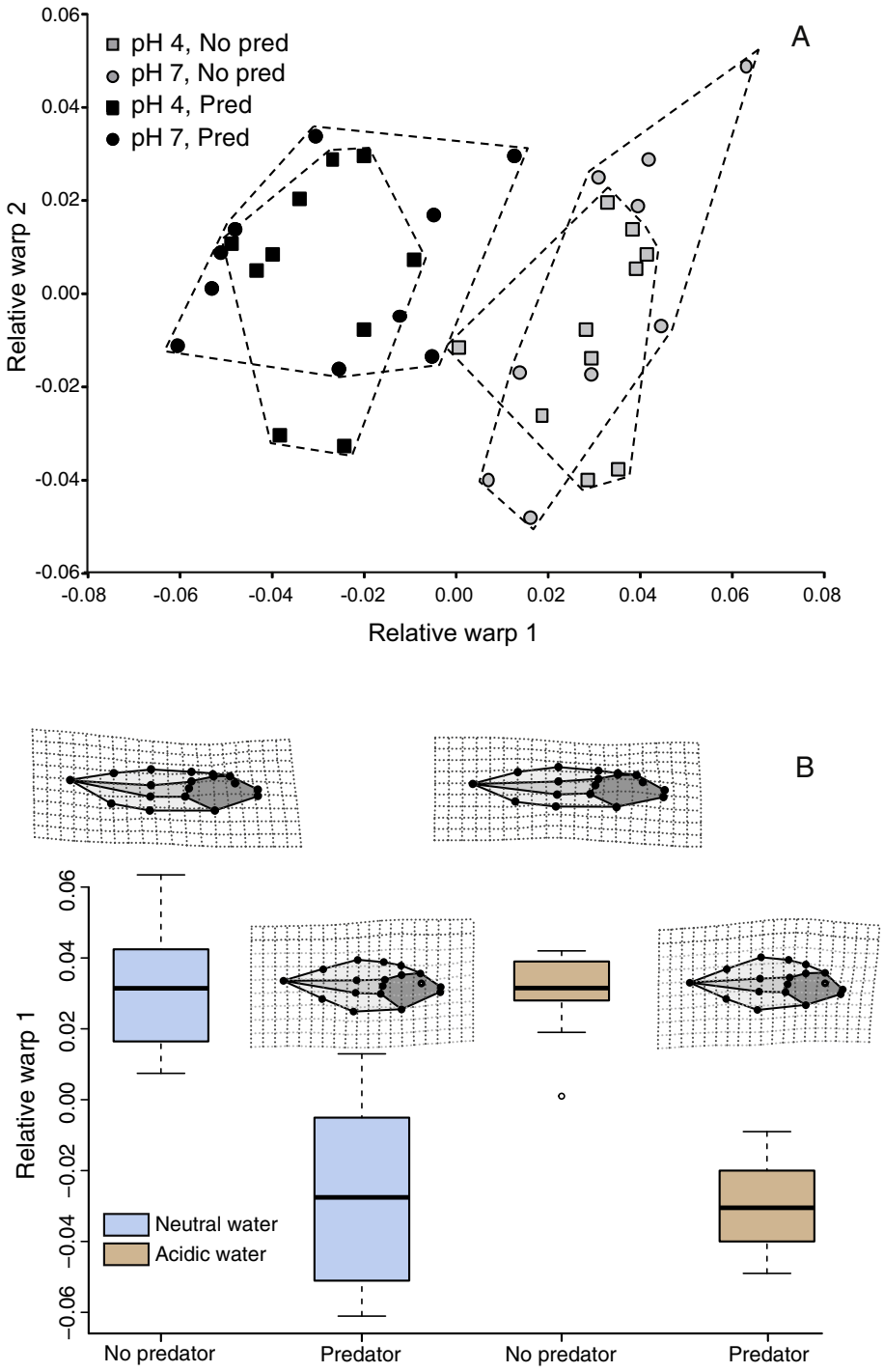

Fig. 1. A) Changes in the shape of spadefoot toad tadpoles when exposed to the non-lethal presence of a predator (dragonfly nymph) either in neutral or acidic water. Relative warps obtained from a principal components analysis on shape indicated that RW1 was strongly associated with our experimental treatments whereas RW2 was not. B) Subsequent analyses on RW1 showed that tadpoles in the presence of predator cues grew a deeper and shorter tail and a rounder body, as shown by the thin-plate spline deformation grids. Boxplots show the median, range and first and third quartiles in RW1 for each of the experimental treatments; dots indicate outliers. Low pH did not interfere with predator recognition or with the activation of inducible defenses.

redox-based markers was very low and we therefore fitted linear models for each parameter separately. The effect of experimental factors on antioxidant enzymes activity, malondialdehyde levels and the reduced-to-oxidized glutathione ratio were tested on untransformed data as they met parametric assumptions. All analyses were conducted in R (version 3.6.1, R Core Team, 2018).

3. Results

3.1. Induced morphological responses despite water acidity

Using geometric morphometrics, we assessed the degree to which spadefoot toad tadpoles detected and responded to predator cues under either neutral water or acidic water conditions. Variation in the first relative warp (RW1) was strongly influenced by presence/absence of predators, regardless of water pH, whereas RW2 captured variation in tadpole shape was not associated with any of the experimental treatments (Fig. 1A). A linear model using RW1 as dependent variable indicated that tadpoles significantly altered their shape in response to predators ($F_{1,38}$ = 3.82, P = .001), whether in neutral or acidic water (Fig. 1B). Under both acidic and neutral water conditions tadpoles exposed to predator cues developed a more elliptical body, a shorter and deeper tail that showed a more anterior insertion with the body, which occurred at a greater angle (Fig. 1B). Water pH had no detectable effect on RW1 ($F_{1,38}$ = 0.94, P = .173), and the interaction term 'water pH x predator presence' was not significant either ($F_{1,38}$ = 0.12, P = .45).

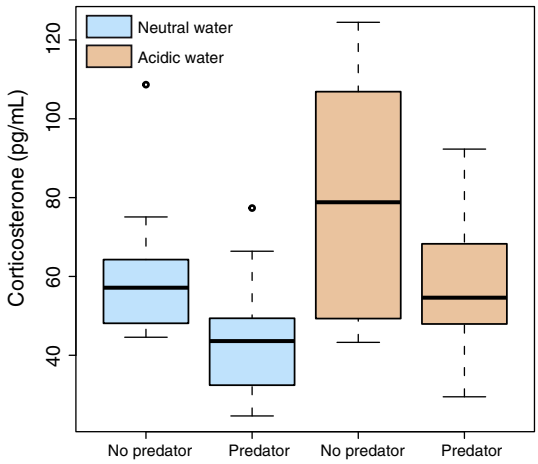

Fig. 2. Changes in corticosterone levels in the plasma of spadefoot toad tadpoles in the presence or absence of predator chemical cues in either neutral or acidic water. Water acidity (pH = 4) was associated with increased corticosterone levels whereas predator presence was associated with lower corticosterone. No interaction between both factors was observed. Box plots indicate median, range and first and third quartiles; dots indicate outliers.

3.2. Opposite response of corticosterone to predator and water acidity

Tadpoles reared in acidic water showed on average 27% higher plasma corticosterone levels than tadpoles in neutral water ($F_{1,35}$ = 4.27, P = .046). Predator presence, however, was associated with significant reductions in the level of corticosterone, and did so to a similar extent in either neutral or acidic water. Thus, predator presence decreased corticosterone equally (26% on average) in both neutral and acidic water ($F_{1,35}$ = 7.68, P = .009; Fig. 2). Consequently, no 'water pH x predator presence' interaction was found ($F_{1,35}$ = 0.015, P = .903).

3.3. Oxidative stress due to water acidity and predator presence

Both water acidity and predation risk were associated with disturbed oxidative status of tadpoles. This was reflected in up/down-regulation of antioxidant enzymes with respect to tadpoles in neutral water and absence of predator cues. In addition, the combined action of both factors was linked to higher lipid peroxidation and reduced tadpoles' antioxidant ability.

Glutathione reductase activity was higher in tadpoles raised at low pH (18.98% increase on average; $F_{1,40}$ = 6.1881; P = .018, Fig. 3A). In contrast, predator presence was associated with lower glutathione reductase activity both in neutral and acidic water, causing on average a 31.95% decrease in activity ($F_{1,40}$ = 15.09; P < .001; Fig. 3A). Glutathione reductase activity showed an additive response to both experimental factors as the interaction term was not significant ($F_{1,40}$ = 2.34; P = .135). Nevertheless, we observed a significant interaction between water pH and presence of predator cues in the activity of glutathione peroxidase and superoxide dismutase. Tadpoles exposed to predation risk had lower glutathione peroxidase activity (by 19.55% on average; $F_{1,40}$ = 4.67, P = .037) although it did so only when raised in neutral water, whereas tadpoles under acidic water had intermediate glutathione peroxidase activities ($F_{1,40}$ = 7.48; P = .010; Fig. 3B). Predator cues in acidic water were associated with induced lower values of superoxide dismutase activity than in the other three treatments (26.28% on average, $F_{1,40}$ = 9.63; P = .004; Fig. 3C). Neither pH nor predator presence altered catalase activity (all P > .109; Fig. 3D).

Acidic water and predator presence had synergistic effects on lipid peroxidation and the antioxidant status of tadpoles regarding the variation observed in malondialdehyde levels and the reduced-to-oxidized glutathione ratio of tadpoles, respectively. Tadpoles raised in neutral water with predation cues did not significantly alter their malondialdehyde levels, but the combination of predation risk and acidic water were associated with great increases in malondialdehyde levels (by 41.65% on average with respect to tadpoles raised in acidic water without predator cues; interaction effect: $F_{1,40}$ = 4.41; P = .042; Fig. 3E). Similar to malondialdehyde results, the combination of acidic water and predator cues were associated with lower values of the reduced-to-oxidized glutathione ratio (26.64% lower on average) compared to the other three treatments (interaction effect: $F_{1,40}$ = 5.66; P = .023; Fig. 3F).

4. Discussion

Contrary to our expectations, spadefoot toad tadpoles were capable of accurately detecting predator cues and developing an anti-predatory morphology in acidic water. This suggests that the cue recognition system is quite robust and that even at pH = 4 the cue remains recognizable and the tadpoles' receptors continue to be operative. Other studies have shown that chemoreception in aquatic systems can be obstructed by acidic substances such as humic acid, both in cases of conspecific recognition (Fisher et al., 2006; Hubbard et al., 2002) and predator cue recognition (Moore et al., 2014; Ehrsam et al., 2016; Polo-Cavia et al., 2016). However, this effect may not be directly linked to water pH but to the specific pollutant effect of humic acid, as chemoreception does not seem to be prevented by low pH, at least within the pH range used in this study. Although tadpoles retained the ability to detect predator cues, low pH was associated with substantial physiological stress in spadefoot toad tadpoles, as indicated by increased corticosterone levels and redox imbalance, particularly in terms of increased glutathione reductase activity and increased lipid peroxidation. These physiological imbalances agree with our predictions, and may offer a mechanistic explanation for the decline in amphibian performance and growth often observed when larvae are raised in acidic water (Freda and Dunson, 1985; Hangartner et al., 2012; Rowe et al., 1992).

Opposite to water acidity, the non-lethal presence of predators mediated lower corticosterone levels in spadefoot toad tadpoles. The downregulation effect of predator presence on the hormone secretion occurred regardless of water pH, showing an additive, rather than synergistic, response to the combined effect of both environmental factors. Upon perceiving cues from predators or injured conspecifics, most amphibian species tend to reduce their activity rate, and consequently their foraging (Benard, 2004; Relyea, 2007; Skelly, 1994). Suppression of the HPI axis and consequently inhibition of the production of corticosterone may be a major promotor of the predator-induced behaviorally quiescent state (Fraker et al., 2009; Hossie et al., 2010). Predator-induced reduction in activity rate implies that tadpoles also spend less time feeding, but may also experience morphological alterations in their mouthparts and intestines that reduce their feeding efficiency (Relyea and Auld, 2004, 2005). Tadpoles also greatly reduce their metabolism in response to predator cues, bringing down their respiration rate by 19–56% (Barry and Syal, 2013), perhaps as a mechanism to cope with the limited food intake and less efficient digestion. Our results show that the antioxidant activity of glutathione reductase and glutathione peroxidase were indeed reduced in the presence of predators, consistent with lower metabolic activity, although this effect was masked at low pH in the case of glutathione peroxidase. In contrast, superoxide dismutase activity was only affected by the combined effect of predator presence and acidic water, which was associated with the lower enzymatic activity. Intriguingly, the combination of predator presence and low pH also had negative synergistic effects on the lipid peroxidation and redox status of tadpoles regarding the higher and lower values of malondialdehyde levels and the reduced-to-oxidized glutathione ratio, respectively. Damage to lipids of the cellular membranes and reductions in antioxidant ability may have detrimental

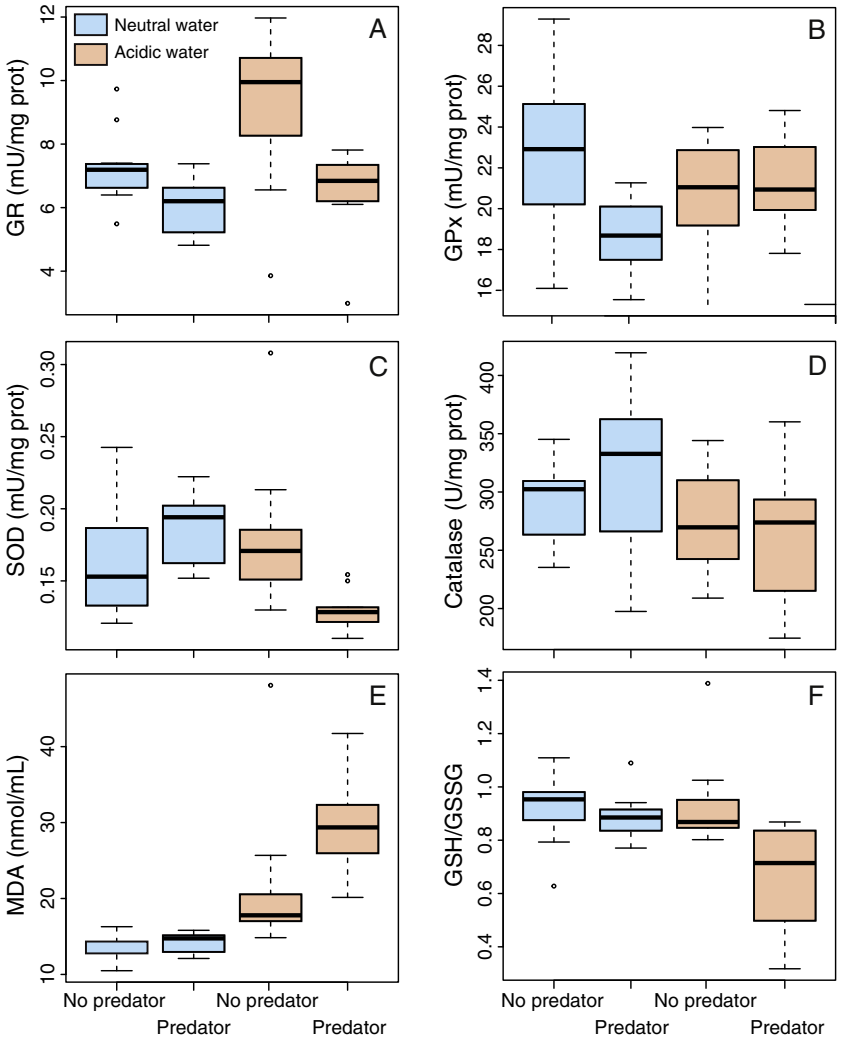

Fig. 3. Oxidative stress in spadefoot toad tadpoles from the exposure to low water pH, predator presence, or the combination of both factors. Box plots indicate median, range and first and third quartiles; dots indicate outliers. The activity of the enzymes A) glutathione reductase, B) glutathione peroxidase activity, C) superoxide dismutase and D) catalase are shown. We also show the concentration of E) malondialdehyde and F) the ratio of reduced to oxidized glutathione.

consequences for cellular stability. These signs of oxidative stress might have been provoked by energy-demanding processes linked to the development of the anti-predatory morphology as well as to the maintenance of cellular homeostasis against acidic water. Oxidative stress early in life can constrain life histories (Metcalfe and Alonso-Alvarez, 2010) and involve long-term consequences including faster senescence and reductions in fitness (Monaghan et al., 2009), although some of these damages might be reversible (Costantini, 2019). In the case of amphibians with biphasic life cycles, oxidative damage may constrain somatic development of larvae undergoing metamorphosis (Menon and Rozman, 2007; Prokić et al., 2019) and therefore compromise their survival odds during subsequent ontogenetic stages.

Stress has always been an elusive concept because it is used in different contexts to mean somewhat different things. Glucocorticoids, in particular cortisol and corticosterone, are often referred to as 'stress hormones' because they are secreted upon activation of the HPA-axis in response to external factors that often represent a threat to the well-being of the organism. However, the idea of a 'stress hormone' can be misleading if we apply circular reasoning and define 'stressful factors' those that increase the level of 'stress hormones' (MacDougall-Shackleton et al., 2019). The presence of predators is clearly perceived as a risk by amphibian larvae, which respond readily altering their morphology, reducing their activity rate, and lowering their metabolism. Achieving such quiescent state, however, seems to require a reduction in the tadpoles' corticosterone level, exemplifying how it can be misleading to directly interpret changes in corticosterone as a direct metric of physiological stress. Our results therefore highlight the complex dynamics that potential stressors can have on the phenotype and physiology of individuals. The ability of larvae to detect predators was not impaired by a harsh abiotic factor like water acidity, but the potential costs associated with the development of the anti-predator morphology and with the homeostatic responses to low pH resulted in high levels of oxidative damage and redox unbalance.

Declaration of Competing Interest

None.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbpa.2020.110654.

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