

# Predator-induced physiological responses in tadpoles challenged with herbicide pollution

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**Abstract** Predators induce plastic responses in multiple prey taxa, ranging from morphological to behavioral or physiological changes. In amphibians, tadpoles activate plastic responses to reduce predation risk by reducing their activity rate and altering their morphology, specifically tail depth and pigmentation. Furthermore, there is now evidence that tadpoles' defenses are modified when predators combine with other stressful factors such as pollutants or competitors, but our knowledge on the physiological responses underlying these responses is still scarce. Here we study physiological responses in *Pelobates cultripes* tadpoles exposed to a natural predator (larvae of the aquatic beetle *Dytiscus circumflexus*), non-lethal concentrations of herbicide (glyphosate, 0.5 mg/L and 1.0 mg/L) or both factors combined. We measured corticosterone levels, standard metabolic rate, oxidative damage (TBARS) and activity of antioxidant enzymes, and immune response (via leukocyte count). Tadpoles reduced their corticosterone concentration by ca. 24% in the presence of predator cues, whereas corticosterone did not change in the presence of glyphosate. Two enzymes involved in antioxidant response also decreased in the presence of predators (14.7% and 13.2% respectively) but not to glyphosate. Herbicide, however, increased the number of neutrophils and reduced that of lymphocytes, and had an interaction effect with predator presence. Standard metabolic rate did not vary across treatments in our experiment. Thus we show a marked physiological response to the presence of predators but little evidence for interaction between predators and low levels of herbicide. Multiple assessment of the physiological state of animals is important to understand the basis and consequences of phenotypic plasticity [*Current Zoology* 59 (4): 475–484, 2013].

**Keywords** Inducible defenses, Physiological responses, Corticosterone, Glyphosate, Spadefoot toads, Oxidative stress

Many organisms respond to the presence of predators or herbivores by producing plastic phenotypic alterations, whether morphological, behavioral or physiological, hence reducing predation or herbivory risk (Karban et al., 1997; Tollrian and Harvell, 1999). Thus, plants respond to herbivores by modifying leaf morphology and/or chemical composition and release (Agrawal et al., 2002; Mithöfer and Boland, 2012) and many animals develop spines, thicker shells or other defensive structures in response to predators (Spitze 1992; Freeman and Byers, 2006; Brönmark et al., 2011). Many organisms do also alter the timing of ontogenetic switch points between life history stages to reduce predation risk (Susarczyk, 1995; Benard, 2004; Gomez-Mestre et al., 2008; Warkentin, 2011). A trade-off between predation risk and resource acquisition is a central tenet of life-history theory (Sih, 1982; Skelly, 1992), and many theoretical models and experimental approaches evaluate the consequences of such trade-offs to individual fitness and community structure (Lima and Dill, 1990; Peckarky and McIntosh, 1998; Downes, 2001; Hebble-

white and Merrill, 2009).

Amphibian species with aquatic larvae commonly encounter aquatic predators, and often activate plastic responses against them: tadpoles exposed to predator chemical cues will likely reduce activity rate, increase relative tail depth, and even modify the coloration of the tail (Anholt and Werner, 1995; Van Buskirk and McCollum, 2000; Touchon and Warkentin, 2008; Gomez-Mestre and Diaz-Paniagua, 2011). Moreover, the presence of predators may also modify tadpole development, extending the time to metamorphosis (Benard, 2004), reducing growth rate (Van Buskirk and Saxer, 2001), or shortening the tail resorption phase (Vonesh and Warkentin, 2006). However, the ability to detect and respond to predator cues may be altered or dampened by other biotic or abiotic factors, which may inhibit tadpoles' ability to detect cues or simply condition the tadpoles' stress response.

Water pollution by herbicides is a common amphibian source of anthropogenic stress because it affects a large fraction of the aquatic systems worldwide. Herbi-

cides have the potential for incapacitating tadpoles to detect natural predators, or even impair the activation of their plastic defenses. Previous studies have found a synergic interaction when herbicide and predator cues were combined, which decreased survival (Relyea 2003; Relyea, 2003b), reduced biomass (Relyea, 2005), or else decreased activity rate (Rohr and Crumrine, 2005). Furthermore, interference of a herbicide with predator recognition has been described in common toad tadpoles (Mandrillon and Saglio, 2007).

Stress responses in amphibian larvae are strongly dependent upon the activation of the hypothalamic-pituitary-adrenal (HPA) axis, which regulates the production of glucocorticoids such as corticosterone (Denver, 2009). Increased corticosterone levels in amphibian larvae can accelerate metamorphosis (Hayes and Wu, 1995; Denver, 1997) although its effects may be stage-specific (Hayes et al., 1993). Additionally, increased glucocorticoid production can increase oxidative stress in vertebrates, although it may depend on treatment duration and its effects would be tissue-dependent (Costantini et al., 2011). Some pesticides seem to mimic the corticosterone-induced changes in tadpoles (Hayes et al., 1997; Hayes et al., 2006). Corticosterone levels are also associated with activity rate (Astheimer et al., 1992; Cash and Holberton, 1999) and with standard metabolic rate (Buttemer et al., 1991; Miles et al., 2007) in many organisms. Moreover, increases in glucocorticoids are often linked to the immune response by increasing the neutrophils/lymphocytes ratio (Davis et al., 2008).

Environmental stress factors also frequently result in the production of reactive oxygen species (ROS) at a cellular level (Metcalf and Alonso-Alvarez, 2010). Increased ROS production may constrain life-histories by acting as a physiological cost in reproduction, immune function, or activity of individuals (Costantini, 2008). The antioxidant enzymes maintain cell homeostasis by detoxifying ROS. Hence, we expect to find a higher activity of these enzymes in individuals exposed to stress, although it can be tissue-dependent (Noeman et al., 2011; Costa et al., 2008).

Here we study the physiological stress response in western spadefoot toad tadpoles *Pelobates cultripes* against a native predator (*Dytiscus* beetle larvae), but also their response to herbicide (glyphosate), and ‘predator x herbicide’ interaction. Glyphosate is a broad-spectrum herbicide that is very commonly used across the world. Its effects on tadpoles are being increasingly unveiled, but data is still scarce about the physiological mechanisms underlying the expression of the behavioral or

developmental changes it induces, and how it affects their inducible defenses against predators. Here we analyze changes in four key indicators of physiological stress: corticosterone concentration, standard metabolic rate, immune response (via leukocyte counts), oxidative damage (in the form of accumulated thiobarbituric acid reactive substances, TBARS), and activity of antioxidant enzymes (superoxide dismutase, glutathione reductase, glutathione peroxidase and catalase). These responses are likely to be somewhat entangled because predator-induced changes in activity rate may require changes in corticosterone levels that in turn affect metabolic rate, the immune response, and the production of ROS. We expected that exposure to herbicide would generally have opposite effects to those induced by predators, causing an increase in corticosterone, metabolic rate, and production of ROS.

## 1 Material and Methods

We collected *Pelobates cultripes* tadpoles from three different ponds within the Biological Reserve of Doñana National Park (Huelva, Spain). This species is distributed throughout most of the Iberian Peninsula and in southeastern France. Adults occupy habitats with soft or sandy soils as they spend extended periods of time buried, and breed in a variety of water bodies, from temporary to more permanent ponds. Tadpoles are larger than any other sympatric tadpoles and have a long larval period (4–6 months). Their extended larval period may render them more vulnerable to predators and pond drying (IUCN - <http://www.iucnredlist.org/details/58052/0>), but they are also developmentally plastic and hence capable of accelerating metamorphosis to evade deteriorating conditions (Gomez-Mestre and Buchholz 2006, Kulkarni et al 2011).

At the time of collection all tadpoles were between developmental stages 35–36 Gosner (Gosner, 1960). We also collected dytiscid beetle larvae *Dytiscus circumflexus* as predators from various ponds within the National Park. We kept tadpoles in 3L plastic buckets, placing four tadpoles per bucket filled with carbon-filtered dechlorinated tapwater in a walk-in chamber at Doñana Biological Station in Seville. The chamber was set at constant 21°C and a 12:12 light-dark cycle. We completely renewed the water twice a week and fed the tadpoles 30 mg of ground rabbit chow twice weekly. We maintained the animals for two weeks under such standardized conditions prior to initiation of the experiment. Experimental treatments were initiated after the two-week acclimation period, and lasted for 20 days. To

test for synergistic physiological responses to combined natural and anthropogenic stress, we crossed the presence of herbicide (glyphosate, absent, 0.5 mg/L, and 1 mg/L) with presence/absence of dytiscid larvae in a 3×2 factorial design. The glyphosate concentrations used were expected to be non-lethal, were similar to those used in previous studies (Relyea, 2005b; Jayawardena et al., 2010), and below those considered as chronically toxic for aquatic vertebrates (EC directive 67/548/EEC). Glyphosate solution was made fresh at each water change, hence ensuring that the glyphosate would not degrade in between water changes since its half-life in aquatic environments is 7–14 days (Giesy et al., 2000). Each treatment was replicated 10 times, distributed in 3 random blocks grouped by shelves within the experimental chamber for a total of 60 experimental units (each containing four tadpoles).

To obtain the target herbicide concentrations we used a stock solution of 360 g/L isopropylamine glyphosate salt (Fortin, Industrial química key, S.A.). A single *Dytiscus* larva was added in each replicate assigned to predator treatment, caged inside a suspended plastic cup (250 mL) with a mesh screen bottom that allowed water flow and cue diffusion. Empty cages were placed in buckets in treatments with absence of predator. We monitored the experiment daily and replaced any dead predators. To avoid confounding the detection of predator kairomones with detection of alarm cues from injured tadpoles, we did not feed predators directly in the experimental buckets, but in external housing tanks instead.

After 20 days we randomly collected one tadpole per bucket and measured their standard metabolic rate as described below. We then collected the remaining tadpoles, euthanized them individually by immersion in a lethal concentration of MS-222, and randomly assigned one tadpole per replicate to corticosterone assay, antioxidant enzymes determination, or immune response quantification.

### 1.1 Corticosterone assay

We determined corticosterone levels from whole body homogenates of tadpoles using an enzyme-linked immunosorbent assay (ELISA). ELISAs are quantitative biochemical assays based on competitive binding between corticosterone and a corticosterone-acetylcholinesterase conjugate (corticosterone tracer) for a limited number of corticosterone-specific sheep antiserum binding sites. The corticosterone concentration varies but the corticosterone tracer concentration is constant, so the concentration of corticosterone is inversely proportional

to the amount of tracer left to bind to the antiserum during incubation (90 min). The plate was then washed twice with buffer to remove all the unbound material and the bound hormone was detected by the addition of a substrate that generated a coloured reaction after 30 min incubation. Quantitative estimates were obtained reading absorbance at 405 nm. We took 50 µL from tadpole homogenates previously centrifuged to use on an EIA Kit (Cayman Chemical Company). Corticosterone concentration in homogenate samples was calculated by interpolation from standard curves. Each plate contained two blanks, two non-specific binding wells, two maximum binding wells and an eight-point standard curve run in duplicate. All samples were run in duplicate. We read absorbance at 410 nm.

### 1.2 Standard metabolic rate

To measure standard metabolic rate (SMR) we used an aquatic respirometer consisting of a set of ten optical sensors, mounted at the entrance and exit of five plexi-glass chambers (44 mm in diameter × 163 mm long cylinders). This way we simultaneously obtained five independent measures of oxygen differential (mg/L). The optical sensors were connected to an oxymeter (Oxy 10-PreSens, Germany), and were programmed to record oxygen concentration every 15 seconds. The optical sensors used (optodes; Oxy 10-PreSens, Germany), unlike common electrodes, do not consume oxygen during measurements, have long-term stability and their signal does not depend on the flow rate of the sample. The respirometer was calibrated at least once daily using a sodium sulphite solution and oxygen saturated water to achieve 0 and 100% oxygen concentrations. We calibrated the oxymeter at 21°C and all measurements were taken at this temperature. Tadpoles were introduced in the chambers individually. We discarded the first five minutes of data recording, considered as acclimation period of the animals to the chambers. SMR was calculated following Alvarez et al (2006) as:

$$VO_2 = V_w \cdot \Delta C_w$$

where  $VO_2$  ( $\mu\text{g h}^{-1}$ ) is the SMR measured as rate of oxygen consumption,  $V_w$  is the flow rate through the chamber ( $l \text{ h}^{-1}$ ), and  $\Delta C_w$  is the instantaneous difference in  $O_2$  concentration between the inflow and outflow. We blotted dry and weighed the tadpoles to the nearest 0.1 mg on a high precision balance (CP324S, Sartorius) after the SMR measurement, for inclusion of body mass as a covariate in the models.

### 1.3 Oxidative stress

We quantified five parameters related to oxidative stress: activity of four enzymes (catalase, superoxide

dismutase, glutathione peroxidase and glutathione reductase), and thiobarbituric acid reactive substances (TBARS) formed during lipid peroxidation (Kappus, 1985). Euthanized tadpoles were dissected to remove the gut and avoid possible interference of its content, snap frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until assayed. Samples were immersed 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 individually homogenized at 35,000 rpm with a Micra homogenizer (Micra D-1). We used a proportion of 1 g tissue in 4 ml of homogenization buffer (1:4; w:v). The homogenates were centrifuged at 14,000 rpm for 30 min at  $4^{\circ}\text{C}$ . We further aliquoted the resulting supernatant into several 0.6 mL tubes and cryopreserved it at  $-80^{\circ}\text{C}$ . Total protein content was assessed by standard Bradford's procedure (Bradford, 1976).

### 1.3.1 Catalase assay (CAT)

We determined catalase activity indirectly by measuring catalytic activity, following Cohen and Somerson (1969). The procedure used potassium permanganate ( $\text{KMnO}_4$ ) as an oxidizing and coloured agent that reacts with hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), the catalase substrate.  $\text{KMnO}_4$  is hence reduced, a red product is produced and absorbance read at a wavelength of 480 nm five minutes after  $\text{KMnO}_4$  is added. We prepared standard curves of commercial catalase (SIGMA-60634) and expressed the catalase activity as U / mg of total proteins.

### 1.3.2 Superoxide dismutase assay (SOD)

According to Cord and Fridovich (1969) we obtained estimates of SOD activity measuring spectrophotometrically the ferrocytochrome c using xanthine, xanthine oxidase as the source of superoxide radicals ( $\text{O}_2^-$ ). One unit of SOD was defined as the amount of enzyme that inhibits the rate of reduction of cytochrome C by 50% at  $25^{\circ}\text{C}$  at 550 nm (Cord and Fridovich, 1969):

$$\text{Inhibition \%} = \left[ \frac{\Delta E_{550} / \text{min (control)} - \Delta E_{550} / \text{min (problem)}}{\Delta E_{550} / \text{min (control)}} \right] \times 100$$

$$\text{U SOD / ml} = \frac{\text{inhibition \%}}{50\%} \times \frac{1}{\text{Sample Volume}} \times \text{dilution factor}$$

### 1.3.3 Glutathione reductase assay (GR)

Following Cribb et al. (1989) we measured the decrease in absorbance at 340 nm, due to NADPH oxidation. To obtain the glutathione peroxidase reductase and glutathione activity we applied the formula

$$\text{mU / ml} = \text{nmol / min / Mg prot} = \frac{(\Delta \text{O.D.} / \Delta t) \times V_t}{E_{340} \times d \times 10^{-6} \times V_e \times P}$$

where D.O. is the optical density increment per minute,

$V_t$  is the total reaction volume (in mL),  $E_{340}$  is NADH's molar extinction coefficient at 340 nm ( $6.22 \times 10^{-3} \text{ M}^{-1} \times \text{cm}^{-1}$ ),  $V_e$  is the volume of added extract (in mL) and  $d$  is the light path length (0.6 cm for microplates).  $P$  is the soluble protein in mg/ml.

### 1.3.4 Glutathione peroxidase assay (GPX)

We determined glutathione peroxidase activity following an assay described by Paglia and Valentine (1967). The oxidized glutathione (GSSG) takes part in the GPX enzymatic reaction. It is continually reduced due to an excess of glutathione reductase (GR) and produces a constant level of reduced glutathione (GSH). We measured NADPH oxidation by reading absorbance at a wavelength of 340 nm.

### 1.3.5 Thiobarbituric acid reactive substance (TBARS) assay

We measured thiobarbituric acid reactive substance (TBARS) as a generic measure of oxidative damage, according to Buege and Aust (1978). One product of lipid peroxidation is malondialdehyde (MDA), which reacts with acid to give a thiobarbituric acid reactive substance (TBARS), a red product absorbing at 535 nm. To obtain the TBARS concentrations we measured the optical density values for the blank and for the calibration curve. We calculated the TBARS concentration (in nmol TBARS/ml) from the absorbance of each sample, subtracting the blank values and comparing with the calibration values.

## 1.4 Immune response

Immune response was assessed by counting white blood cells (leukocytes) in blood smears. The blood was obtained via cardiac puncture with a non-heparinized insulin syringe (BS Micro-Fine Insulin Y-100 0.5 ml) in tadpoles anesthetized with MS-222. Blood smears were prepared and stained with a May-Grunwald-Giemsa technique, according to Pappenheim's stain (May-Grunwald-Giemsa). We made two or three blood smears per individual to ensure sufficient leukocyte count. The slides were mounted and fixed with DPX (EUKITT). To determine the leukocyte fraction we made a differential count of white blood cells by using oil immersion in a ZEISS microscope, model Zi, with a 100X ocular for a total magnification of 1000 $\times$ . We counted 100 white blood cells from each replicate and recorded the abundance of the various white cell types: lymphocytes, neutrophils, eosinophils and basophils (heterophils), and monocytes. Neutrophils and, in general, heterophils, are the principal phagocytic leukocyte and proliferate in response to infections, stress, or inflammation (Jain, 1993; Campbell, 1995; Harmon, 1998; Thrall, 2004).

Lymphocytes have multiple functions, such as infected cell lysis, antibody secretion, or immunoregulation (Janeway et al., 2001; Campbell, 1996)

### 1.5 Statistical analyses

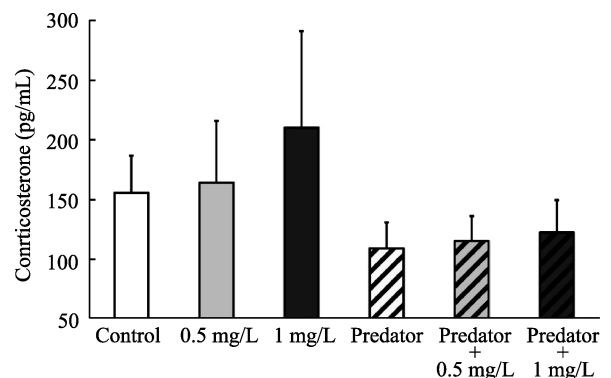
We fitted generalized linear models to test the effect of the different treatments on standard metabolic rate, corticosterone concentration, antioxidant enzymes activity, and immune response. We used Gaussian or gamma distributions (with identity or log link functions, respectively) to model the underlying error distribution of all variables except cell counts, using Akaike Information Criterion (AIC) to assess the goodness of fit of each model and choose the appropriate distribution. We included weight as a covariate in analyses of the standard metabolic rate to control for the effect of body mass. We tested for differences in the proportion of cell types by fitting generalized linear models with a binomial distribution and a logit link function. When overall tests of main factors or their interaction were significant we conducted Tukey-Kramer *post-hoc* tests. All analyses were run in SAS v. 9.1 (SAS Institute Inc., USA).

## 2 Results

Corticosterone levels, activity of antioxidant enzymes and leukocyte count varied significantly in response to at least one of the experimental treatments, whereas standard metabolic rate did not, although it showed a tendency towards increased SMR in tadpoles exposed to 1 mg/L herbicide (Table 1). Corticosterone levels decreased by 23.7% in tadpoles exposed to

chemical cues from predators ( $F_{1,46} = 5.19$ ,  $P = 0.030$ ; Fig. 1) but did not change in tadpoles exposed to herbicide ( $F_{2,46} = 0.19$ ,  $P = 0.821$ ), and neither did we find a significant ‘predator x herbicide’ interaction ( $F_{2,46} = 0.09$ ,  $P = 0.916$ ) in corticosterone levels. The CV between corticosterone assay plates was 15.46% (15.3 and 15.61 in each plate).

As for the activity of the antioxidant enzymes, we found that superoxide dismutase activity also decreased ( $F_{1,52} = 7.42$ ,  $P = 0.009$ ) in tadpoles exposed to predator cues, with an average reduction of 14.7%. The SOD activity decreased by 13.3% when tadpoles were exposed to herbicide but the effect was only marginally significant ( $F_{2,52} = 2.85$ ,  $P = 0.057$ ), and we found no



**Fig. 1** Whole-body corticosterone concentration after a 20-day exposure to different herbicide concentrations (glyphosate), presence/absence of cues from predatory aquatic beetle larvae, or to a combination of both. Error bars indicate  $\pm$  SE.

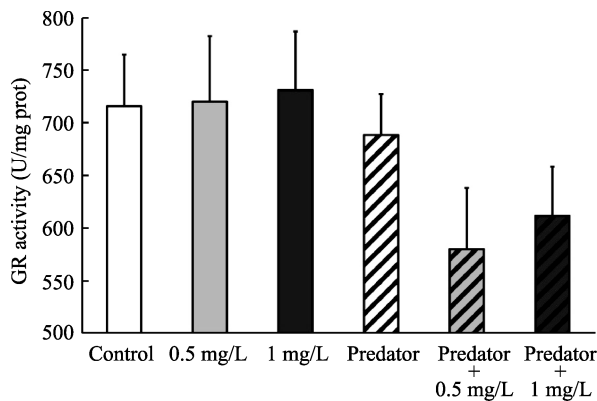
**Table 1** Physiological parameter averages  $\pm$  standard error on tadpole responses to experimental exposure to herbicide and predator cues

	<i>n</i>	Control	0.5 mg/L	1 mg/L	Predator	Predator+0.5 mg/L	Predator+1 mg/L
CORT	52	155.59 $\pm$ 31.04	164.09 $\pm$ 51.68	135.51 $\pm$ 34.62	109.27 $\pm$ 21.87	115.52 $\pm$ 20.75	122.71 $\pm$ 107.30
SMR	58	0.038 $\pm$ 0.002	0.028 $\pm$ 0.004	0.067 $\pm$ 0.005	0.030 $\pm$ 0.008	0.027 $\pm$ 0.007	0.038 $\pm$ 0.003
GPX	58	25.85 $\pm$ 1.54	26.75 $\pm$ 2.01	25.21 $\pm$ 1.22	33.43 $\pm$ 1.03	26.99 $\pm$ 1.43	36.25 $\pm$ 1.55
SOD	58	137.6 $\pm$ 11.91	131.57 $\pm$ 11.86	140.34 $\pm$ 14.88	139.32 $\pm$ 8.46	101.96 $\pm$ 4.10	108.31 $\pm$ 10.49
GR	57	715.65 $\pm$ 49.04	720.06 $\pm$ 62.04	731.02 $\pm$ 55.56	688.30 $\pm$ 38.96	580.11 $\pm$ 58.14	611.88 $\pm$ 46.35
CAT	57	235.64 $\pm$ 24.98	230.69 $\pm$ 22.41	197.30 $\pm$ 15.46	232.94 $\pm$ 26.16	249.96 $\pm$ 25.01	228.68 $\pm$ 11.76
TBARs	57	6.56 $\pm$ 1.06	6.98 $\pm$ 1.13	5.38 $\pm$ 0.81	7.78 $\pm$ 1.09	7.68 $\pm$ 1.77	8.33 $\pm$ 1.26
Neutr	53	8.33 $\pm$ 2.62	7.00 $\pm$ 1.83	11.56 $\pm$ 2.55	10.00 $\pm$ 1.92	9.56 $\pm$ 1.72	9.71 $\pm$ 3.56
Lymph	53	95.89 $\pm$ 2.60	96.43 $\pm$ 1.80	89.70 $\pm$ 1.97	90.50 $\pm$ 2.65	92.10 $\pm$ 2.19	92.86 $\pm$ 3.80
Basoph	53	0.22 $\pm$ 0.15	0.86 $\pm$ 0.34	0.44 $\pm$ 0.17	0.60 $\pm$ 0.21	0.80 $\pm$ 0.25	0.57 $\pm$ 0.30
Monoc	53	0.33 $\pm$ 0.17	0.14 $\pm$ 0.143	0.30 $\pm$ 0.15	0.10 $\pm$ 0.10	0.20 $\pm$ 0.13	0.29 $\pm$ 0.18
Eosin	53	0.78 $\pm$ 0.32	0.57 $\pm$ 0.30	1.00 $\pm$ 0.30	1.20 $\pm$ 0.49	1.00 $\pm$ 0.33	1.00 $\pm$ 0.31

The parameters reported are: corticosterone (CORT, in pg/ml), standard metabolic rate (SMR, in ml O<sub>2</sub>/h/g), glutathione peroxidase (GPX, in mU/mg prot), superoxide dismutase (SOD, in U/mg prot), glutathione reductase (GR, in U/mg prot), catalase (CAT, in U/mg/prot), thibarbituric acid responsive substances (TBARs, in nmol/ml), and neutrophil (Neutr), lymphocyte (Lymph), basophil (Basoph), monocyte (Monoc), and eosine (Eosin) counts from a total of 100 white blood cells observed per replicate.

‘predator x herbicide’ interaction ( $F_{2,52} = 2.12$ ,  $P = 0.130$ ). Glutathione reductase also showed a significant decrease in activity in tadpoles exposed to predator cues ( $F_{1,51} = 5.64$ ,  $P = 0.021$ ) but not when exposed to herbicide ( $F_{2,51} = 0.62$ ,  $P = 0.5424$ ) or their interaction ( $F_{2,51} = 0.78$ ,  $P = 0.462$ ). Catalase and glutathione peroxidase showed no differences across treatments, and neither did TBARS. SOD, GPX, and GR activity was measured in eight different plates, with an average within-plate CV of 9.74%, 6.90%, and 7.00% respectively, and among-plate CV of 16.99%, 10.79%, and 11.37%. Only two plates were used for CAT and TBARS, and the average within plate CV was 1.53% and 3.83%, respectively, with among-plate CV of 8.89% and 16.74%, respectively.

We observed changes in the immune response (leu-



**Fig. 2** Glutathione reductase activity was reduced in the presence of cues from predatory dytiscid larvae but not affected by the herbicide glyphosate at the indicated concentrations

Error bars indicate +SE.

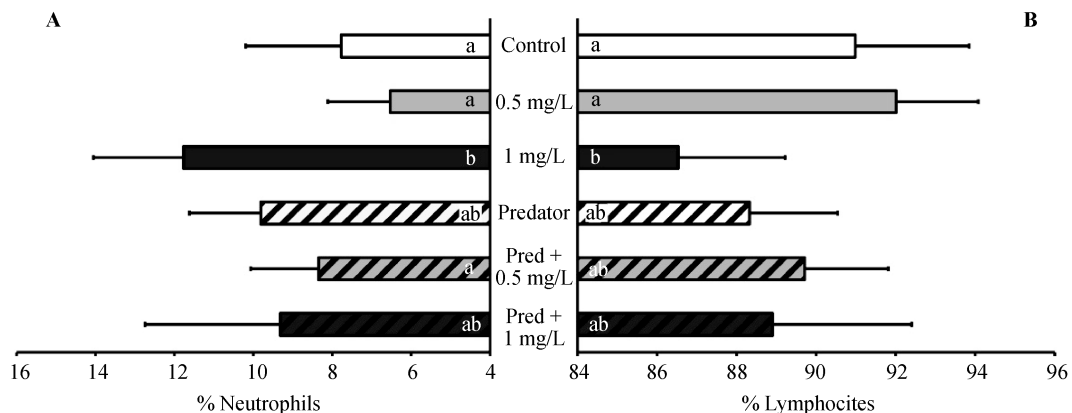
kocyte count) of tadpoles to the experimental treatments so that neutrophil increased in tadpoles exposed to herbicide ( $F_{2,47} = 4.97$ ,  $P = 0.011$ ; Fig. 3). The increase in neutrophils was on average 38.7% between control tadpoles and those exposed to highest herbicide concentration. We also observed a significant ‘predator x herbicide’ interaction ( $F_{2,47} = 3.45$ ,  $p = 0.040$ ). Herbicide also decreased the number of lymphocytes ( $F_{2,47} = 4.97$ ,  $P = 0.015$ ), resulting in a 6.5% reduction on average between the control tadpoles and those exposed to 1 mg/L of herbicide. The interaction also decreased the lymphocyte production ( $F_{2,47} = 3.75$ ,  $P = 0.031$ ). We did not find significant variation in eosinophil, basophil, and monocyte abundance with any experimental treatment (all  $P$ -values > 0.25)

At the end of the experiment all tadpoles were between 38–40 Gosner stages and did not differ across treatments.

### 3 Discussion

#### 3.1 Physiological responses to predators

Predator chemical cues induced hormonal and enzymatic responses in *Pelobates cultripes* tadpoles, whereas we found no detectable immune response. Several previous studies show activation of the HPA axis and increased corticosterone levels in tadpoles in response to stressful factors like radiation, intraspecific competition, or infectious diseases (Glennemeier and Denver, 2002; Belden et al., 2003; Warne et al., 2011). Increased glucocorticoid levels accelerate developmental rates in the later stages of tadpole development (Hayes et al, 1993; Denver 2009) and increase activity



**Fig. 3** Neutrophil and lymphocyte abundance in *Pelobates cultripes* tadpoles changed in response to predator presence and herbicide

A. 1 mg/L of glyphosate increased the neutrophil number compared to the control and the 0.5 mg/L treatment. B. The number of lymphocytes decreased when added 1 mg/L of herbicide compared to the control and the 0.5 mg/L treatments. Different letters on the bars indicate statistical significance of *post-hoc* tests. In all cases error bars indicate +SE.

rate (Glennemeier and Denver, 2002). That way, tadpoles can accelerate development and evade adverse aquatic conditions and thus increase odds of survival (Denver, 1998; Boorse and Denver, 2004; Warne et al., 2011).

In the context of predators, however, the role of corticosterone is not that straightforward. Middlemis Maher et al. (2013) have recently shown that wood frog tadpoles increase their corticosterone levels in the presence of predators, whereas our results indicate a 23.7% reduction in corticosterone levels in Western spadefoot toad tadpoles in response to the presence of dytiscid larvae (Fig. 1). Numerous studies show that tadpoles increase survival against predators by decreasing their activity rate (Van Buskirk and Yurewicz, 1998; Anholt et al., 2000; Gomez-Mestre and Diaz-Paniagua, 2011). Consequently, our observation of reduced corticosterone levels in the presence of a predator is congruent with hormonally driven adaptive reduction in activity rate. Moreover, it has been shown that tadpoles also respond to conspecific alarm cues by reducing corticosterone levels (Fraker et al., 2009). However, we did not find significant differences in standard metabolic rate between tadpoles exposed and not exposed to predators, despite it being well established that corticosterone levels affect the standard metabolic rate (Buttemer et al., 1991; Miles et al., 2007) suggesting that we may have lacked sufficient statistical power to detect differences. Nonetheless, we did not find differences in leukocyte count either despite the relationship between corticosterone and immune function (Davis et al., 2008), so it could also be that, while detectable, differences in corticosterone levels were not large enough to have cascading effects on metabolism or immune function. The effects of corticosterone on metabolism, growth and development may be strongly conditioned by the developmental stage and the nutritional state of the animal, and hence more studies will be needed to explain the apparent current discrepancies. Also, further analyses on physiological and hormonal responses to different predator types in the presence or absence of conspecific alarm cues is needed.

Predator presence also induced a decrease in the activity of two antioxidant enzymes: SOD and GR. The activity of these enzymes is related to the reactive oxygen species production, which correlates with the degree of individual stress (Di Giulio et al., 1989; Costa et al., 2008). SOD activity was reduced by 14.7%, which was congruent with the observed reduction in corticosterone because decreased corticosterone reduces food intake (De Pedro et al., 1993; Crespi and Denver, 2005) and

activity, so it can involve a reduction in the production of superoxide anions. Food intake decreases mitochondrial metabolism, and hence diminishes ROS production (Sohal et al., 1994). Similarly, the observed 13.3% reduction in GR activity in the presence of predators was congruent with a lower production of NADPH due to reduced food intake (Viganò, et al., 1993). NADPH molecules provide the reduction power needed for the GR reaction in which glutathione disulfide (GSSG) is reduced to the sulfhydryl form (GSH).

The reduction in antioxidant enzymes activity observed when predator cues were present may be due to a general reduction in activity and concomitant reduction of the catabolic pathways. However, it is important to note that although we detected reduced antioxidant activity, it did not seem to come at the cost of increased oxidative damage, as TBARS, used here as a proxy for general measure of oxidative damage, did not vary in the presence/absence of predator cues. Our results are thus in agreement with observed reduction in growth rate in damselflies in response to predators associated with reduced catalase activity (Slos and Stocks, 2008). Similarly, slowly growing garter snakes with long lifespan showed more efficient mitochondria and reduced ROS production than faster paced snakes (Robert and Bronikowski, 2010).

### 3.2 Physiological responses to herbicide

Herbicide was applied in sublethal doses and did not cause tadpole mortality. Such low dosage did not affect corticosterone levels or the activity of enzymes involved in oxidative stress response (Figs. 1–3). We observed a slight and non-significant increase in metabolic rate in tadpoles exposed to 1 mg/L glyphosate. Herbicide, however, had a deeper effect on the immune system of tadpoles. Tadpoles exposed to 1 mg/L of glyphosate increased the proportion of neutrophils and decreased that of lymphocytes in comparison to tadpoles in clean water or exposed to 0.5 mg/L glyphosate. The activation of the immune system depends on the intensity and duration of the stress (Dhabhar et al., 1995; Koolhaas et al., 1997; Tilders et al., 1999; Davis et al., 2008). Previous experiments described an increase of the neutrophils: lymphocytes ratio in response to stressful factors in multiple taxa (Bennet, 1986; Jain, 1993; Rupley, 1997; Thrall 2004), which are in part related to the levels of glucocorticoids (Davis et al., 2008). The observed increase in neutrophils and decrease in lymphocytes may indicate a superior ability of tadpoles to respond (e.g. phagocytosing pathogens) to potential infections associated with stress (Davis et al., 2008) although our leuko-

cyte profile data does not give any information about inflammation or diseases associated with the stress exposure.

### 3.3 Physiological responses to the interaction between predator and herbicide

In this experiment we have found no interaction between predator presence and herbicide, indicating that at these concentrations, the physiological responses observed in spadefoot toads were mostly driven by either factor alone, with no synergistic effects between them. However, the multiple approach we have taken outlines the importance of assessing multiple physiological parameters simultaneously because they differ in sensitivity to different stressors, and depend to different extent on the state of the animal, providing complementary information on the physiological state.

In summary, our results show that *Pelobates cultripes* tadpoles show different responses to exposure to dystiscid larvae than to non-lethal herbicide levels. Tadpoles modified their corticosterone levels and antioxidant enzymes activity in response to predators, and their leukocyte count when exposed to herbicide, but no synergistic effects were detected. Further studies are needed to evaluate the impact of common stressful factors on tadpole physiology, and a combined analysis of various physiological parameters will reveal a more complete view of the physiological alterations experienced.

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## References

- Agrawal AA, Conner JK, Johnson MTJ, Wallsgrrove R, 2002. Ecological genetics of an induced plant defense against herbivores: Additive genetic variance and costs of phenotypic plasticity. *Evolution* 56: 2206–2213.
- Álvarez D, Cano JM, Nicieza AG, 2006. Microgeographic variation in metabolic rate and energy storage of brown trout: Counter-gradient selection or thermal sensitivity? *Evolutionary Ecology* 20: 345–363.
- Anholt BR, Werner EE, 1995. Interaction between food availability and predation mortality mediated by adaptive behavior. *Ecology* 76: 2230–2234.
- Anholt BR, Werner E, Skelly DK, 2000. Effect of food and predators on the activity of four larval Ranid frogs. *Ecology* 81: 3509–3521.
- Astheimer LB, Buttemer WA, Wingfield JC, 1992. Interactions of corticosterone with feeding, activity and metabolism in passerine birds. *Ornis Scandinavica* 23: 355–365.
- Belden LK, Moore IT, Mason RT, Wingfield JC, Blaustein AR, 2003. Survival, the hormonal stress response and UV-B avoidance in Cascades frog tadpoles *Rana cascadae* exposed to UV-B radiation. *Functional Ecology* 17: 409–416.
- Benard MF, 2004. Predator-induced phenotypic plasticity in organisms with life histories. *Annual review of Ecology, Evolution and Systematics* 35: 651–673.
- Bennett MF, 1986. Stress and changes in the blood of newts *Notophthalmus viridescens* during early regeneration. *Journal of Comparative Physiology, Part A* 6: 823–826.
- Boorse G, Denver R, 2004. Endocrine mechanisms underlying plasticity in metamorphic timing in spadefoot toads. *Integrative and Comparative Biology* 43: 646–657.
- Bradford M, 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* 72: 248–254.
- Brönmark C, Lakowitz T, Hollander J, 2011. Predator-induced morphological plasticity across local populations of a freshwater snail. *PLoS One* 6: e21773.
- Buege JA, Aust SD, 1978. Microsomal lipid peroxidation. *Methods in Enzymology* 52: 302–310.
- Buttemer WA, Astheimer LB, Wingfield, JC, 1991. The effect of corticosterone on standard metabolic rates of small passerine birds. *Journal of comparative physiology, Part B. Biochemical, Systemic, and Environmental Physiology* 161: 427–431.
- Campbell TW, 1995. *Avian Hematology and Cytology*. Ames, Iowa: Iowa State University Press.
- Campbell TW, 1996. *Clinical pathology*. In: Mader DR ed. *Reptile Medicine and Surgery* Philadelphia: W.B. Saunders Company, 248–257.
- Cash WB, Holberton RL, 1999. Effects of exogenous corticosterone on locomotor activity in the red-eared slider turtle *Trachemys scripta elegans*. *The Journal of Experimental Zoology* 284: 637–644.
- Cohen G, Somerson NL, 1969. Catalase-aminotriazole method for measuring secretion of hydrogen peroxide by microorganisms. *Microbiology* 98: 543–546.
- Cord JM, Fridovich I, 1969. An enzymic function for Erythrocyte (Hemocypreïn). *The Journal of Biological Chemistry* 244: 6049–6055.
- Costa, MJ, Monteiro DA, Oliveira-Neto AL, Rantin FT, Kalinin AL, 2008. Oxidative stress biomarkers and heart function in bullfrog tadpoles exposed to Roundup Original. *Ecotoxicology* 17: 153–163.
- Costantini, D, 2008. Oxidative stress in ecology and evolution: Lessons from avian studies. *Ecology Letters* 11: 1238–1251.
- Costantini D, Marasco V, Møller AP, 2011. A meta-analysis of glucocorticoids as modulators of oxidative stress in vertebrates. *Journal of Comparative Physiology, Part B*. 181: 447–456.
- Crespi EJ, Denver RJ, 2005. Roles of stress hormones in food intake regulation in anuran amphibians throughout the life cycle. *Comparative Biochemistry and Physiology, Part A*. 141: 381–390.
- Cribb AE, Leeder JS, Spielberg SP, 1989. Use of a microplate reader in an assay of glutathione reductase using 5,5'-dithiobis (2-nitrobenzoic acid). *Analytical Biochemistry* 183: 195–196.
- Davis AK, Maney DL, Maerz JC, 2008. The use of leukocyte



- profiles to measure stress in vertebrates: A review for ecologists. *Functional Ecology* 22: 760–772.
- Di Giulio RT, Washburn PC, Wenning RJ, Winston GW, Jewell CS, 1989. Biochemical responses in aquatic animals: A review of determinants of oxidative stress. *Environmental Toxicology and Chemistry* 8: 1103–1123.
- De Pedro N, Alonso-Gómez AL, Gancedo B, Delgado MJ, Alonso-Bedate M, 1993. Role of corticotropin-releasing factor (CRF) as a food intake regulator in goldfish. *Physiology & Behavior* 53: 517–520.
- Denver RJ, 1997. Environmental stress as a developmental cue: Corticotropin-releasing hormone is a proximate mediator of adaptive phenotypic plasticity in amphibian metamorphosis. *Hormones and Behavior* 31: 69–79.
- Denver RJ, 1998. Hormonal correlates of environmentally induced metamorphosis in the Western spadefoot toad *Scaphiopus hammondi*. *General and Comparative Endocrinology* 110: 326–336.
- Denver RJ, 2009. Stress hormones mediate environment-genotype interactions during amphibian development. *General and Comparative Endocrinology* 164: 20–31.
- Dhabhar FS, Miller AH, McEwen BS, Spencer RL, 1995. Effects of stress on immune cell distribution - dynamics and hormonal mechanisms. *Journal of Immunology* 154: 5511–5527.
- Downes S, 2001. Trading heat and food for safety: Costs of predator avoidance in a lizard. *Ecology* 82: 2870–2881
- Fraker ME, Hu F, Cuddapah V, McCollum SA, Relyea RA et al., 2009. Characterization of an alarm pheromone secreted by amphibian tadpoles that induces behavioral inhibition and suppression of the neuroendocrine stress axis. *Hormones and behavior* 55: 520–529.
- Freeman AS, Byers JE, 2006. Divergent induced responses to an invasive predator in marine mussel populations. *Science* 313: 831–833.
- Geisy JP, Dobson S, Solomon KR, 2000. Ecotoxicological risk assessment for Roundup herbicide. *Reviews of Environmental Contamination and Toxicology*, 167: 35–120.
- Glennemeier KA, Denver RJ, 2002. Role for corticoids in mediating the response of *Rana pipiens* tadpoles to intraspecific competition. *Journal of Experimental Zoology* 292: 32–40.
- Gomez-Mestre I, Buchholz DR, 2006. Developmental plasticity mirrors differences among taxa in spadefoot toads linking plasticity and diversity. *Proceedings of the National Academy of Sciences, USA* 103: 19021–19026.
- Gomez-Mestre I, Wiens JJ, Warkentin KM, 2008. Evolution of adaptive plasticity: Risk-sensitive hatching in neotropical leaf-breeding treefrogs. *Ecological Monographs* 78: 205–224.
- Gomez-Mestre I, Díaz-Paniagua C, 2011. Invasive predatory crayfish do not trigger inducible defences in tadpoles. *Proceedings of The Royal Society, Part B* 278: 3364–3370.
- Gosner KL, 1960. A simplified table for staging anuran embryos and larvae with notes on identification. *Herpetologica* 16: 183–190.
- Harmon BG, 1998. Avian heterophils in inflammation and disease resistance. *Poultry Science* 77: 972–977.
- Hayes TB, Chan R, Licht P, 1993. Interactions of temperature and steroids in larval growth, development, and metamorphosis in a toad *Bufo boreas*. *Journal of Experimental Zoology* 266: 206–215.
- Hayes TB, Wu TH, 1995. Interdependence of corticosterone and thyroid hormones in toad larvae *Bufo boreas*. *Journal of Experimental Zoology* 271: 103–111
- Hayes TB, Wu TH, Gill TN, 1997. DDT-like effects as a result of corticosterone treatment in an anuran amphibian: Is DDT a corticoid mimic or a stressor? *Environmental Toxicology and Chemistry* 16: 1948–1953.
- Hayes TB, Case P, Chui S, Chung D, Haeffele C et al., 2006. Pesticide mixtures, endocrine disruption, and amphibian declines: Are we underestimating the impact? *Environmental Health Perspectives* 114: 40–50.
- Hebblewhite M, Merrill E, 2009. Trade-offs between predation risk and forage differ between migrant strategies in a migratory ungulate. *Ecology* 90: 3445–3454.
- Jain NC, 1993. *Essentials of Veterinary Hematology*. Philadelphia: Blackwell Publishing.
- Janeway C, Travers P, Walport M, Shlomchik M, 2001. *Immunobiology*. 5<sup>th</sup> edn. New York and London: Garland Science.
- Jayawardena UA, Rajakaruna RS, Navaratne AN, Amerashinghe PH, 2010. Toxicity of agrochemicals to common hourglass tree frog. *International Journal of Agriculture and Biology* 12: 641–648.
- Kappus H, 1985. Lipid peroxidation: Mechanisms, analysis, enzymology and biological relevance. In: Sies H ed. *Oxidative Stress*. London: Academic Press.
- Karban R, Agrawal AA, Mangel M, 1997. The benefits of induced defenses against herbivores. *Ecology* 78: 1351–1355.
- Koolhaas JM, Meerlo P, De Boer SF, Strubbe JH, Bohus B, 1997. The temporal dynamics of the stress response. *Neuroscience and Biobehavioral Reviews* 21: 775–782.
- Kulkarni S, Gomez-Mestre I, Moskalik C, Storz B, Buchholz D, 2011. Evolutionary reduction of developmental plasticity in desert spadefoot toads. *Journal of Evolutionary Biology* 24: 2445–2455
- Lima SL, Dill LM, 1990. Behavioral decisions made under the risk of predation: A review and prospectus. *Canadian Journal of Zoology* 68: 619–640.
- Mandrillon A-L, Saglio P, 2006. Herbicide exposure affects the chemical recognition of a non native predator in common toad tadpoles *Bufo bufo*. *Chemoecology* 17: 31–36.
- Metcalfe NB, Alonso-Alvarez C, 2010. Oxidative stress as a life-history constraint: The role of reactive oxygen species in shaping phenotypes from conception to death. *Functional Ecology* 24: 984–996.
- Middlemis Maher JM, Werner EE, Denver RJ, 2013. Stress hormones mediate predator-induced phenotypic plasticity in amphibian tadpoles. *Proceedings of the Royal Society, Part B*. 280: 20123075
- Miles DB, Calsbeek R, Sinervo B, 2007. Corticosterone, locomotor performance, and metabolism in side-blotched lizards *Uta stansburiana*. *Hormones and behavior* 51: 548–554.
- Mithöfer A, Boland W, 2012. Plant defense against herbivores: Chemical aspects. *Annual Review of Plant Biology* 63: 431–450.
- Noeman SA, Hamooda HE, Baalash AA, 2011. Biochemical study of oxidative stress markers in the liver, kidney and heart of high fat diet induced obesity in rats. *Diabetology and Metabolic Syndrome* 3: 17.
- Paglia DE, Valentine WN, 1967. Studies on the quantitative and

- qualitative characterization of erythrocyte glutathione peroxidase. *Journal of Laboratory and Clinical Medicine* 70: 158–169.
- Peckarsky BL, McIntosh AR, 1998. Fitness and community consequences of avoiding multiple predators. *Oecologia* 113: 565–576.
- Relyea R, 2003. How prey respond to combined predators: A review and an empirical test. *Ecology* 84: 1827–1839.
- Relyea RA, 2003b. Predator cues and pesticides: A double dose of danger for amphibians. *Ecological Applications* 13: 1515–1521.
- Relyea RA, Schoeppner NM, Hoverman JT, 2005. Pesticides and amphibians: The importance of community context. *Ecological Applications* 15: 1125–1134.
- Relyea RA, 2005. The lethal impacts of Roundup and predatory stress on six species of North American tadpoles. *Archives of Environmental Contamination and Toxicology* 48: 351–357.
- Robert KA, Bronikowski AM, 2010. Evolution of senescence in nature: Physiological evolution in populations of garter snake with divergent life histories. *American Naturalist* 175: 147–159.
- Rohr JR, Crumrine PW, 2005. Effects of a herbicide and an insecticide on pond community structure and processes. *Ecological Applications* 15: 1135–1147.
- Rupley AE, 1997. *Manual of Avian Practice*. Philadelphia: W.B. Saunders Company.
- Sih A, 1982. Foraging strategies and the avoidance of predation by an aquatic insect *Notonecta hoffmanni*. *Ecology* 63: 786–796.
- Sih, A, 1982. Foraging strategies and the avoidance of predation by an aquatic insect. *Ecology* 63: 786–796.
- Skelly DK, 1992. Field evidence for a cost of behavioral anti-predator responses in a larval amphibian. *Ecology* 73: 704–708.
- Slos S, Stoks R, 2008. Predation risk induces stress proteins and reduces antioxidant defense. *Functional Ecology* 22: 637–642.
- Slusarczyk M, 1995. Predator-induced diapause in *Daphnia*. *Ecology* 76: 1008–1013.
- Sohal RS, Ku H-H, Agarwal S, Forster MJ, Lal H, 1994. Oxidative damage, mitochondrial oxidant generation and antioxidant defenses during aging and in response to food restriction in the mouse. *Mechanisms of Ageing and Development* 74: 121–133.
- Spitze K, 1992. Predator-mediated plasticity of prey life history and morphology: *Chaoborus americanus* predation on *Daphnia pulex*. *The American Naturalist* 139: 229–247.
- Thrall MA, 2004. Hematology of amphibians. In: Thrall MA ed. *Veterinary Hematology and Clinical Chemistry: Text and Clinical Case Presentations*. Philadelphia: Lippincott Williams and Wilkins.
- Tilders FJ, Schmidt ED, Hoogendijk WJ, Swaab DF, 1999. Delayed effects of stress and immune activation. *Baillieres Clinical Endocrinology and Metabolism* 13: 523–40.
- Tollrian R, Harvell CD, 1999. *The Ecology and Evolution of Inducible Defenses*. Princeton, New Jersey: Princeton University Press.
- Touchon JC, Warkentin KM, 2008. Fish and dragonfly nymph predators induce opposite shifts in color and morphology of tadpoles. *Oikos* 117: 634–640.
- Van Buskirk J, Yurewicz KL, 1998. Effects of predators on prey growth rate: Relative contributions of thinning and reduced activity. *Oikos* 82: 20–28.
- Van Buskirk J, McCollum SA, 2000. Influence of tail shape on tadpole swimming performance. *The Journal of Experimental Biology* 203: 2149–2158.
- Van Buskirk, J, Saxer G, 2001. Delayed costs of an induced defense in tadpoles? Morphology, hopping, and development rate at metamorphosis. *Evolution* 55: 821–829.
- Viganò L, Arillo A, Bagnasco M, Bennicelli C, Melodia F, 1993. Xenobiotic metabolizing enzymes in uninduced and induced rainbow trout *Oncorhynchus mykiss*: Effects of diets and food deprivation. *Comparative Biochemistry and Physiology Part C*. 1: 51–55.
- Vonesh JR, Warkentin KM, 2006. Opposite shifts in size at metamorphosis in response to larval and metamorph predators. *Ecology* 87: 556–562
- Warkentin KM, 2011. Environmentally cued hatching across taxa: Embryos respond to risk and opportunity. *Integrative and Comparative Biology* 51: 14–25.
- Warne RW, Crespi EJ, Brunner JL, 2011. Escape from the pond: Stress and developmental responses to ranavirus infection in wood frog tadpoles. *Functional Ecology* 25: 139–146.