



Biomarker and hematological fieldwork with amphibians: is it necessary to sample all night?

Julie Céline Brodeur^{1,3} · María Florencia Bahl^{2,3} · Guillermo Sebastián Natale^{2,3} · María Belén Poliserpi¹

Received: 12 November 2019 / Accepted: 2 March 2020 / Published online: 7 March 2020
© Springer-Verlag GmbH Germany, part of Springer Nature 2020

Abstract

In the context of the global amphibian crisis, biomonitoring constitutes a valuable assessment tool to provide critical up to date information on the status and health of amphibians worldwide. The aim of the current study was to evaluate the possible confounding effects of sex, size, and time since capture on enzymatic biomarkers and hematologic parameters of the South American frog *Leptodactylus latrans*. Frogs were collected by hand between 9 pm and 12 am on two consecutive nights. On the first night, captured frogs were transported for 2 h by car to laboratory installations, maintained overnight in plastic containers, and blood and tissue sampled on the next morning. In contrast, frogs collected on the second night were blood and tissue sampled in the field, immediately after the capture period. Hematological parameters were analyzed, and enzymatic activities of catalase, cholinesterase (ChE), and glutathione S-transferase (GST) were determined in the plasma, liver, kidney, and muscle. A sex difference was observed only for total white blood cell counts (WBC), females exhibiting significantly greater values than males. The packed cell volume (PCV), mean corpuscular hemoglobin concentration (MCHC), WBC, and muscle ChE activity were significantly correlated with snout-vent length (SVL). The correlation was inversed in the case of MCHC, WBC, and muscle ChE, while the correlation was positive between PCV and SVL. Most examined parameters presented similar values when frogs were sampled at night following capture or the next morning. Total red blood cells (RBCs) count, and plasma enzymatic activities of ChE and GST were the only parameters that presented significantly increased values in morning samplings compared with night samplings. Overall, the current study indicates that it is best to sample the frogs as soon as possible after capture if hematologic or plasmatic biomarkers are examined. Nevertheless, it is possible to sample on the next morning if tissular biomarkers are employed.

Keywords Amphibians · Contamination · Biomarkers · Stress

Introduction

Human-induced changes have caused a major biodiversity crisis and driven the earth into its 6th mass extinction (Wake

and Vredenburg 2008; Barnosky et al. 2011; Pimm et al. 2014; Williams et al. 2015). Among vertebrates, amphibians are the most rapidly declining group, with more than 40% of the species being threatened globally (Stuart et al. 2004; Pounds et al. 2006; Roelants et al. 2007, IPBES 2019). Six major threats have been traditionally linked to amphibian declines: habitat loss and fragmentation, commercial overexploitation, introduced species, environmental contaminants, global climate change, and emerging infectious diseases (Bishop et al. 2012). Recent evidence however suggests that the causes of the amphibian declines are probably more variable and locally driven than previously assumed (Campbell Grant et al. 2016). Amphibian declines may ultimately cause secondary impacts on ecosystems as amphibians are both predator and prey and therefore play a key role in energy flow and nutrient cycling (Crump 2010).

In the context of the global amphibian crisis, it is necessary to develop, extend, and improve monitoring programs to

Responsible Editor: Philippe Garrigues

✉ Julie Céline Brodeur
julbrodeur@hotmail.com

¹ Instituto de Recursos Biológicos, Centro de Investigaciones de Recursos Naturales (CIRN), Centro Nacional de Investigaciones Agropecuarias (CNIA), Instituto Nacional de Tecnología Agropecuaria (INTA), 1686, Hurlingham, Buenos Aires, Argentina

² Centro Investigaciones del Medio Ambiente, Departamento de Química, Facultad de Ciencias Exactas, Universidad Nacional de La Plata, La Plata, Buenos Aires, Argentina

³ Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Buenos Aires, Argentina

provide critical up to date information on the status and health of amphibian populations worldwide. In this sense, biomonitoring constitutes a valuable assessment tool as it uses field studies with biomonitors and biomarkers to understand the temporal and spatial extent of environmental contamination and its effects (Huggett et al. 2018). Biomonitors are organisms that provide quantitative information on the level of environmental contamination through measurable physiological or biochemical changes called biomarkers. Biomarkers complement and enhance the reliability of chemical monitoring data by offering an integrated evaluation of the effects of pollutants on the health of the organisms (Van der Oost et al. 2003, Hansen 2003, Cazenave et al. 2009). Traditionally, biomarkers have included a whole range of biochemical, physiological, or histological indicators of either exposure to, or effects of, xenobiotic chemicals at the suborganismal or organismal level.

Although the use of biomarkers presents many potential benefits, their improper application or interpretation may greatly reduce their utility and value. For instance, confounding factors such as temperature, pH, salinity, sex, size, and age often influence biomarker values (Amiard-Triquet and Berthet 2015). The unavoidable general stress response to capture, handling, and temporary holding may also alter biomarkers. Examples of biomarkers that respond to stress include the total oxyradical scavenging capacity and DNA damage in mussels (Wilson et al. 1998; Camus et al. 2004), enzymes in shrimps (ChE, GST, lactate dehydrogenase) (Menezes et al. 2006), and white blood cell counts and immune function (Davies et al. 2008; Brousseau et al. 2013).

As in most vertebrates, the capture or handling stress of an amphibian induces an immediate physiological response in which the sympathetic nervous system triggers an almost instantaneous release of stored catecholamines. This activates the so-called “fight or flight” reaction, which enables the animal to alter its physiology and take rapid action. Within minutes, the hypothalamus releases corticotrophin-releasing factor (CRF), which initiates the release of adrenocorticotrophin (ACTH) from the pituitary. ACTH then stimulates the synthesis and release of corticosterone from the adrenal cortex (Monaghan and Spencer 2014; Santymire et al. 2018). Corticosteroid levels of amphibians have been shown to remain elevated for various days when animals are held in captivity after being caught in the field (Narayan and Hero 2011; Narayan et al. 2013). It is therefore impossible to allow time for plasma corticosterone to return to basal levels before sampling for biomarkers, as the physiological status of the animal might be modified over such a long period.

In biomonitoring studies, adult anurans are generally captured within the first 2 to 4 h following sunset (Falfushinska et al. 2008; Attademo et al. 2011; Brodeur et al. 2011, 2012). Afterwards, researchers are faced with the decision of sampling for tissues throughout the night or to let the frogs rest in a

temporary holding tank until the next morning. This decision sometimes depends on whether or not it is possible to create a makeshift laboratory near the sampling site. Sampling on the next morning is normally more convenient, but biomarker values may be affected by the sustained stress response experienced by the animal during this holding period.

Although a number of studies have focused on biomonitoring with adult anurans (Attademo et al. 2007, 2011; Brodeur et al. 2011, 2012; Christin et al. 2013; Hedge and Krishnamurthy 2014; Pollo et al. 2017), few studies have examined the influence of confounding factors and capture stress on anuran biomarker determinations. Falfushinska et al. (2008) reported seasonal variations in oxidative stress biomarkers. Orton et al. (2014) examined the influence of body size on biomarkers of reproductive health, while a few studies examined the sex and season dependence of hematologic parameters, although with varying results (Mahapatra et al. 2012; Young et al. 2012; Meesawat et al. 2016; Zhelev et al. 2017; Xiong et al. 2018). In this context, the objective of the current study was to evaluate the possible confounding effects of the time since capture and the sex and size of the animal on enzymatic biomarkers and hematologic parameters of the South American frog *Leptodactylus latrans*.

Methods

Sampling site and test species

The semiaquatic frog *Leptodactylus latrans* was selected as the test species because its wide abundance over a range of latitudes and its large size, which facilitates blood sampling, makes it increasingly popular as a biomarker/bioindicator species of contaminated environments (Brodeur et al. 2011; Brodeur and Vera Candiotti 2017). Widely distributed over South America, east of the Andes from Venezuela to Argentina, *L. latrans* is a large long-legged anuran species that can reach up to 120–140 mm in snout-vent length (SVL) (Ceï 1980). An active and vigorous frog, it is frequently found resting at the margin of shallow waterbodies and jumps into the water if disturbed. Classified as a generalist consumer, it feeds on insects and their larvae and, occasionally, on other smaller anurans (Maneyro et al. 2004). Reproductive activity and calling is typically observed after heavy rainfalls in the spring and summer (September to February). The conservation status of *L. latrans* is listed as “Least Concern” in view of its wide distribution, tolerance to a broad range of habitats, and presumed large population (Heyer et al. 2010).

Frogs were captured in the natural reserve “El Destino”, Department of Magdalena, Buenos Aires Province, Argentina (35°08'15.87" S; 57°23'35.21" O). Created in 1979, El Destino Reserve encompasses a surface of 1879 ha and is located within the “Parque Costero del sur,” a

UNESCO Biosphere Reserve of 265 km² created in 1997. Frogs were captured at night on the shores of the Primera Estancia River, within the natural reserve. All animal captures were realized under a permit from “Dirección de Áreas Naturales Protegidas” of the “Organismo Provincial para el Desarrollo Sostenible” (OPDS) of Buenos Aires Province, Argentina.

Sampling designs

Frog captures occurred between 9 pm and 12 am on the nights of October 24–25, 2016. Frogs were captured by hand and placed in plastic containers fitted with air holes and containing river water to a depth of approximately 5 cm. On the night of October 24, captured frogs were transported for 2 h by car to our laboratories in Buenos Aires, and maintained overnight at ambient temperature in the plastic containers. These frogs were blood and tissue sampled on the next morning, approximately 12 to 16 h after capture. In contrast, frogs collected on October 25 were blood and tissue sampled immediately after the capture period (i.e., approximately 1 to 4 h after capture) in a makeshift laboratory setup at approximately 400 m from the sampling site.

When sampling for blood and tissues, frogs were anesthetized individually in well water containing 100 mg/L of tricaine methanesulfonate, and their body mass and SVL were measured (Mitchell 2009; Brodeur et al. 2011, 2012). Blood was collected from the ventral abdominal vein in a heparinized 1-mL syringe fitted with a sterile of 25-g needle. Blood sampling took no more than 2 min, and 0.15 mL of blood was collected on average for each frog. Collected blood was used for hematology determinations as described below. The remaining blood was centrifuged at 10,000 *g* for 5 min, and plasma was removed and stored at –80 °C for biochemical analyses. Next, the animals were sacrificed by cutting the neural cord behind the brain, and frogs were sexed by examination of the gonads. The liver and kidneys were excised, and a 1-cm³ piece of white muscle was sampled from the left thigh. It took on average of 5 to 8 min to process each frogs. All sampled tissues were immediately frozen in liquid nitrogen and stored at –80 °C until biochemical analyses were performed within the following 6 months. All animal manipulations were performed under approval of the local institutional ethics committee for the care and use of laboratory animals (University of La Plata CICUAL Protocol 023-22-15).

Body condition

Body condition was calculated according to the scaled mass index (SMI) method described by Peig and Green (2009). The SMI method consists in first quantifying the scaling exponent *b* from the function $Mass = aLength^b$ for the studied species

and then calculating the predicted body mass of studied individuals at a given length. In the present study, a value of 3.11 was used for the exponent *b* based on a previous study realized by our group with *L. latrans* (Brodeur et al. 2020). SMI was calculated for the SVL corresponding to the average SVL of all frogs sampled in this study.

Hematology

Red blood cells (RBCs) were counted in duplicate in an improved Neubauer chamber, after a 1:200 dilution of entire blood in Natt and Herrick’s Solution (Natt and Herrick 1952). Hemoglobin (Hb) concentrations were determined by the cyanmethemoglobin method using a 1:200 dilution of entire blood in Drabkin’s solution (Drabkin and Austin 1935). To estimate packed cell volume (PCV), blood was collected in heparinized capillary tubes that were centrifuged at 10,000 *g* for 5 min. Mean corpuscular volume (MCV), which represents an estimate of RBCs’ volume, was calculated as:

$$MCV(fL) = (PCV (L/L)/RBC(10^{12}cells/L))$$

Mean corpuscular hemoglobin (MCH), the average concentration of Hb per RBC, was calculated as:

$$MCH(pg) = [Hb (g/dL)*10]/RBC (10^{12}cells/L)$$

Finally, mean corpuscular hemoglobin concentration (MCHC), a measure of the concentration of Hb in a given volume of packed red blood cells, was calculated using the following equation:

$$MCHC(g/dL) = [Hb (g/dL)*100]/PCV (%)$$

Two blood smears were prepared for each frog in order to perform total thrombocyte counts and total and differential white blood cell (WBC) counts. Smears were air-dried, fixed with methanol, and stained with 5% Wright-Giemsa solution. Total numbers of platelets and WBCs were estimated, per 1000 RBCs, based on the numbers of cells present in a monocellular layer examined at 1000× and counting a total of 1000 erythrocytes per individual frog. For differential WBC counts, 100 cells were counted at 1000× on a monocellular layer of a stained blood smear, and the relative percentages of lymphocytes, neutrophils, monocytes, basophils, and eosinophils were calculated.

Enzymatic biomarkers

Enzymatic activities of ChE, CAT, and GST were measured in the liver, kidney, muscle, and plasma as previously described (Brodeur et al. 2011; Brodeur et al. 2017). Briefly, tissues were homogenized in ice-cold 50 mM tris (hydroxymethyl) aminomethane buffer (pH 7.4) containing 1 mM of

ethylenediaminetetraacetic acid and 0.25 M of sucrose using a Teflon-glass Potter-Elvehjem homogenizer. Homogenates were centrifuged at 10,000 *g* and 4 °C for 5 min to remove debris, and the resulting supernatant was used for enzymatic determinations.

ChE activity was determined by Ellman's method (Ellman et al. 1961). The reaction mixture consisted of 200 μL of phosphate-buffered saline (PBS) (100 mM, pH 8), 10 μL of acetylthiocholine iodide (1 mM), 10 μL of 5,5'-dithiobis-(2-nitrobenzoic acid) (0.5 mM), and 50 μL of diluted sample. The change in absorbance was recorded at 25 °C and 412 nm. Enzymatic activity was calculated using a molar extinction coefficient of $14,150 \text{ M}^{-1} \text{ cm}^{-1}$. CAT activity was determined by measuring the decrease in absorbance resulting from hydrogen peroxide (H_2O_2) consumption using a molar extinction coefficient of $43.6 \text{ M}^{-1} \text{ cm}^{-1}$. The reaction mixture consisted of 300 μL of PBS (100 mM, pH 7), 10 μL of 10% H_2O_2 , and 10 μL of diluted sample. The change in absorbance was recorded at 240 nm and 25 °C. Finally, GST activity was measured in a reaction mixture containing 300 μL of PBS (100 mM, pH 7) with added reduced glutathione, 10 μL of 1-chloro-2,4-dinitrobenzene (0.1 M), and 10 μL of diluted sample. The change in absorbance was recorded at 340 nm and 25 °C. Enzymatic activity was calculated using a molar extinction coefficient of $9.6 \text{ mM}^{-1} \text{ cm}^{-1}$. Protein concentrations were measured by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

Statistical analysis

For all parameters evaluated, the presence of an association with SVL was first tested using a Spearman correlation analysis. For parameters exhibiting a significant correlation with SVL, a generalized linear model (GLM) was used to compare sexes and sampling times (night or morning). Logarithmic transformations of both SVL and the examined parameter were used in the models to ensure linearity. The full model was first fitted as $\text{Parameter} = \text{B}_0 + \text{Length} + \text{Sex} + \text{Time} + \text{Sex} * \text{Length} + \text{Time} * \text{Length} + \text{Sex} * \text{Time} + \text{Length} * \text{Sex} * \text{Time}$, and the significance of the covariate, factors, and interactions were tested with an *F*-test. Next, nonsignificant interactions were removed from the model, and significance of the remaining terms was examined using the *F*-test after fitting the new model. This sequential removal of nonsignificant terms was continued until only significant terms were left in the final model. GLM analysis was performed using Systat 11 software package, and model validation was realized by controlling through the Durbin-Watson D Statistic, which the dispersion of the residuals was normal.

Parameters that were not correlated with SVL were compared among sexes and sampling time as follows: (1) For hematological parameters, the data were first compared among sexes at each sampling time (night or morning) using a Mann-Whitney

Rank Sum Test because the data rarely presented the normality and equal variance necessary to allow the execution of a two-way ANOVA. Data from both sexes were then grouped to compare the influence of sampling time with a Mann-Whitney Rank Sum Test, as sex differences were never observed. (2) Enzymatic parameters were compared among sexes and sampling time using a two-way ANOVA when normality and equal variance of the data could be achieved. Enzymatic activities were compared through a Mann-Whitney Rank Sum Test as described above for hematological parameters in case where normality and equal variance could not be obtained.

Results

Thirty-two frogs were captured on October 24 (sampled next morning), and 30 frogs were captured on October 25 (sampled at night). The body weight of captured frogs ranged between 9.4 and 62.6 g, for an average body weight of $33.1 \pm 14.7 \text{ g}$ (mean \pm S.D.). The SVL of sampled frogs ranged between 45.2 and 82.0 mm for an average SVL of $64.7 \pm 9.47 \text{ mm}$ (mean \pm S.D.). Body weight and SVL of frogs captured on both dates were not significantly different.

Among the different tissues sampled, the activity of all three enzymes examined, catalase, GST, and ChE, was greatest in the liver (Table 1). In the case of both catalase and GST, the second largest level of enzymatic activity was found in the kidneys with activity values equivalent to 16.8% and 22.7% of the activity level determined in the liver, respectively. Catalase activity was too low to be detected in muscle and plasma, while GST exhibited very low levels of activity in muscle and plasma (0.34 and 0.17% liver activity levels) (Table 1). The case of ChE was different from the other two enzymes, the activity in muscle and plasma being respectively equivalent to 50.4 and 55.6% of the activity detected in the liver (Table 1).

A sex difference was observed only in the case of WBC, females exhibiting significantly greater WBC counts than males ($p = 0.040$). All other parameters examined were not significantly different between males and females, so data from both sexes were pooled to compare the influence of sampling time on the measurements. Of all the parameters measured, only PCV ($R^2 = 0.418$, $p = 0.001$), MCHC ($R^2 = -0.408$, $p = 0.002$), WBC ($R^2 = -0.281$, $p = 0.03$) and muscle ChE activity ($R^2 = -0.341$, $p = 0.005$) presented a significant correlation with SVL. The correlation was inverse in the cases of MCHC, WBC, and muscle ChE (i.e., parameters decreased as animals grew larger), whereas the correlation between PCV and SVL was positive (i.e., they increased together).

The great majority of examined parameters presented similar values when frogs were sampled at night following capture or the next morning (Table 1, Table 2, and Fig. 1). Total RBC counts (Table 2) and enzymatic activities of ChE and

Table 1 Enzymatic biomarker values (mean \pm S.E.) of *Leptodactylus latrans* frogs when sampled the night of their capture or the next morning

	Night sampling (30)	Morning sampling (32)	All animals (62)
Catalase			
Liver	1664 \pm 96	1557 \pm 92	<i>1610 \pm 66</i>
Kidney	280 \pm 40	338 \pm 42	<i>309 \pm 29</i>
Cholinesterases			
Liver	13.5 \pm 0.8	13.8 \pm 1.2	<i>13.7 \pm 0.7</i>
Muscle	6.8 \pm 0.7	6.3 \pm 0.8	<i>6.5 \pm 0.5</i>
Plasma	7.5 \pm 0.8	10.6 \pm 1.2*	
Glutathione-S-transferase			
Liver	8.8 \pm 0.6	8.3 \pm 0.6	<i>8.5 \pm 0.4</i>
Kidney	2.0 \pm 0.1	2.2 \pm 0.2	<i>2.1 \pm 0.1</i>
Muscle	0.029 \pm 0.003	0.0028 \pm 0.003	<i>0.029 \pm 0.002</i>
Plasma	0.015 \pm 0.004	0.021 \pm 0.004*	

The average of values obtained for all sampled animals is indicated in italics in cases where morning and night samples are not significantly different. Enzyme activity is expressed in $\mu\text{mol}/\text{min}/\text{mg}$ protein in all cases. The number of animals sampled is indicated in brackets.

*Significantly different from night samplings ($p < 0.005$)

GST in plasma (Table 1) were the only parameters that presented significantly different values depending on when the frogs were sampled. In all cases, measured values were increased in morning samplings compared with night samplings. Interestingly, plasma protein concentrations were not significantly different among the two groups of frogs (data not shown).

Discussion

Hematologic parameters measured in *L. latrans* were, overall, within the ranges of values previously reported for anuran species, although PCV and the proportion of neutrophils were among the lowest values reported in anurans and the proportion of lymphocyte among the largest (Cathers et al. 1997; Cabagna

Table 2 Hematological parameters (mean \pm S.E.) of *Leptodactylus latrans* frogs when sampled the night of their capture or the next morning

	Night sampling (30)	Morning sampling (32)	All animals (62)
RBC (10^{12} cells/L)	0.73 \pm 0.03	0.90 \pm 0.06*	
PCV (%)	21.0 \pm 1.2	20.1 \pm 1.3	<i>20.6 \pm 0.9</i>
Hb (g/dL)	6.5 \pm 0.2	6.9 \pm 0.3	<i>6.7 \pm 0.2</i>
MCV (fL)	314.6 \pm 34.3	246.0 \pm 21.9	<i>281.5 \pm 21.0</i>
MCH (pg)	93.7 \pm 5.3	86.5 \pm 6.8	<i>90.1 \pm 4.3</i>
MCHC (g/dL)	34.5 \pm 2.5	39.5 \pm 3.1	<i>36.9 \pm 2.0</i>
Total WBCs ^a	39.3 \pm 2.5	38.3 \pm 3.3	<i>38.8 \pm 2.0</i>
Total Thrombocytes ^b	18.7 \pm 1.6	16.1 \pm 1.2	<i>17.4 \pm 1.0</i>
Neutrophils (%)	5.1 \pm 0.8	6.9 \pm 1.1	<i>6.1 \pm 0.7</i>
Lymphocytes (%)	87.8 \pm 1.5	86.1 \pm 1.5	<i>86.9 \pm 1.0</i>
Monocytes (%)	2.2 \pm 0.3	2.9 \pm 0.4	<i>2.6 \pm 0.3</i>
Eosinophils (%)	1.4 \pm 0.5	1.1 \pm 0.3	<i>1.2 \pm 0.3</i>
Basophils (%)	3.2 \pm 0.4	2.8 \pm 0.4	<i>3.0 \pm 0.3</i>
N/L Ratio	0.06 \pm 0.01	0.09 \pm 0.02	<i>0.08 \pm 0.01</i>

The average of values obtained for all sampled animals is indicated in italics in cases where morning and night samples are not significantly different. The number of animals sampled is indicated in brackets

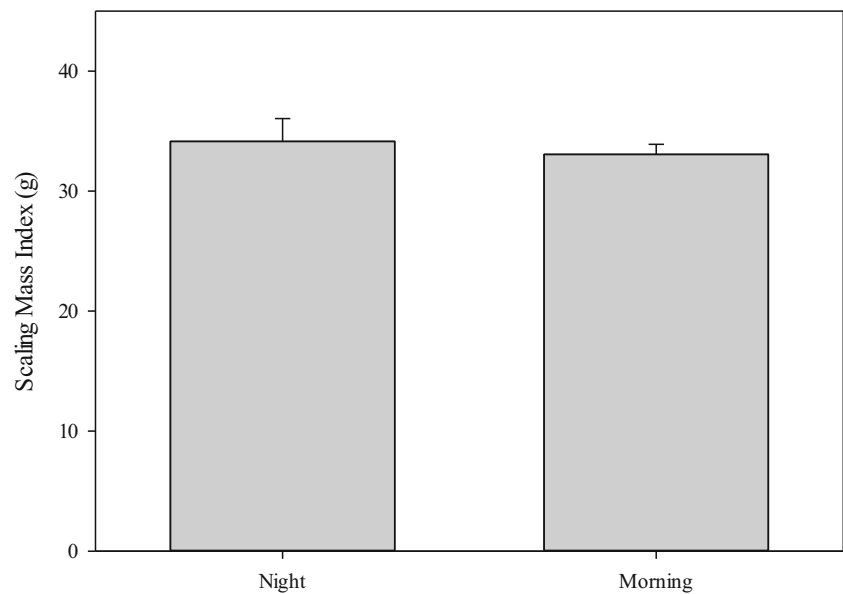
*Significantly different from night samplings ($p < 0.005$)

RBC red blood cells, PCV packed cell volume, Hb hemoglobin, MCV mean corpuscular volume, MCH mean corpuscular hemoglobin, MCHC mean corpuscular hemoglobin concentration, WBCs white blood cells, N/L Ratio Neutrophils/Lymphocytes

^a Estimated number of WBCs per 1000 RBCs

^b Estimated number of thrombocytes per 1000 RBCs

Fig. 1 Body condition (mean \pm S.E.) of *Leptodactylus latrans* frogs when sampled the night of their capture or the next morning



et al. 2005; Forbes et al. 2006; Davies and Durso 2009; Forzán et al. 2017). Of all the hematologic parameters examined, only total WBC counts differed significantly among sexes; females presenting greater WBC counts than males. This result is different from previous reports available in amphibian species where, when present, sex differences in leukocytes were in terms of cell type proportions and not at the levels of total WBC counts (Mahapatra et al. 2012; Meesawat et al. 2016; Xiong et al. 2018). However, similar sex-related differences in total WBC counts have been reported in fish (Motlagh et al. 2012; George and Akinrotimi 2017; Omeje et al. 2019). With respect to erythrocyte-related parameters such as PCV, HB, MCV, and MCH, the current study on *L. latrans* did not find any sex-related differences in contrast to previous studies with other amphibian species which, in most cases, identified one or two sex-dependent parameters. (Mahapatra et al. 2012; Meesawat et al. 2016; Zhelev et al. 2017). Altogether, it would seem that in adult anurans, the influence of sex on hematological parameters is either species specific or that more data is needed in order to obtain a clearer understanding of the topic. For their part, catalase, GST, and ChE enzyme activities measured in the various tissues were not influenced by gender in *L. latrans*, a result consistent with previous observations in both amphibians (Lajmanovich et al. 2004; Brodeur et al. 2011, 2012; Prokic et al. 2018) and fish (Beauvais et al. 2002; Pathiratne et al. 2008; Moura Costa et al. 2010; Brodeur et al. 2017).

Three hematologic parameters, WBC, PCV, and MCHC, and one enzyme activity, muscle ChE, were significantly correlated with the size of the frogs. This observation is of interest as hematological parameters are not traditionally examined in terms of body size, and few reports exist regarding the dependence of these parameters on body length. In goldfish, HCT and HB were inversely correlated with total length (Burton and Murray 1979). However, in anurans, Xiong et al. (2018)

found no relationship between blood parameters and SVL in the toad *Oreolalax rugosus*. Our results highlight the importance of considering the possibility of a dependence of blood parameters on SVL before undertaking the monitoring of a new animal species. Indeed, whenever the monitored parameters are dependent on body length, it is necessary to consider SVL as a covariate during data analysis to avoid introducing a bias to the results.

For its part, the inverse correlation observed between muscle ChE and SVL in *L. latrans* is not unexpected as a similar phenomenon has repeatedly been observed in fish for both brain (Beauvais et al. 2002; Phillips et al. 2002; Pathiratne et al. 2008) and muscle (Flammarion et al. 2002; Pathiratne et al. 2008; Koenig and Sole 2013) ChE activity. To our knowledge, this is, however, the first time that a relationship between muscle ChE activity and SVL is reported in an anuran species. Interestingly, in *L. latrans*, the dependence of ChE activity on SVL was detected only in the muscle tissue, ChE activity in liver and plasma being independent of SVL (Table 1). Lajmanovich et al. 2004 previously obtained a similar result with the toad, *Bufo paracnemis*, in which they failed to detect a relationship between serum ChE and SVL. The apparent difference between muscle, liver, and plasma ChE activity and their relationship to SVL may be linked to a difference in the type and regulation of ChE expressed in each tissue. In fish, for example, the brain and muscle tissue express mainly acetylcholinesterase, whereas butyrylcholinesterase is found mainly in the liver and plasma (Habig and Di Giulio 1991).

The influence of the stress of capturing, handling, and holding on enzymatic biomarkers and hematologic parameters was evaluated by comparing frog sampled either at night following capture or the next morning. Most parameters examined presented similar values at both sampling times, RBC counts and plasma activities of ChE and GST being the only parameters

significantly increased in morning compared with night samplings. The exact mechanistic processes leading to these responses can, at best, be hypothesized from information available in mammals given the paucity of detailed mechanistic studies performed on amphibians. For example, the observed increase in RBC counts is possibly a direct result of the stress-induced elevation of plasma corticosterone levels, as glucocorticoids have been shown to increase erythropoiesis in mammals (Voorhees et al. 2013). With respect to the increase in plasma activities of ChE and GST, dehydration is apparently not at cause since plasma protein concentrations did not differ among the two sampling times. In the case of plasma GST, in mammals, this parameter is used as a marker of hepatocellular injury because plasma GST originates mainly from the liver and has a short half-life in the blood (Beckett et al. 1985; Parida et al. 2018). This information, combined to the fact that acute stress has been shown to provoke tissue injuries at the hepatic level in mice (Fernandez et al. 2000), make it possible to suggest that increased plasma GST activity observed in frogs sampled on the next morning might suggest the presence of a slight stress-induced damage to the liver tissue. This hypothesis is also coherent with the fact that GST activity was unchanged in liver, kidney, and muscle tissues. Finally, for plasma ChE, a direct induction by the stress hormones is plausible, considering that increased ChE activity has been linked to acute stress in mammals. Indeed, in mice, acute stress induces a phase of enhanced neuronal excitability coupled with a transient increase in acetylcholine in some regions of the brain (Imperato et al. 1991). ChE activity is then overexpressed to reduce the acetylcholine excess (Tsakiris 1993; Kaufer et al. 1998; Meshorer et al. 2002). Similarly, salivary ChE is significantly increased after restraint and transport stress in pigs (Tecles et al. 2016). However, if the increase in plasma ChE observed in *L. latrans* sampled on the next morning is due to a direct action of stress, the question remains as to why liver and muscle ChE were not affected.

It is interesting to note that, although the variation of some blood parameters seems to point out to the presence of an underlying stress response in frogs sampled on the next morning, these observations were not accompanied by an increase in neutrophils to lymphocytes ratio (N/L ratio), as could have been expected if the animals were under stress. Indeed, in amphibians, as in any other vertebrate, glucocorticoid hormones act to increase the number and percentage of neutrophils while decreasing the number and percentage of lymphocytes, i.e., they increase the N/L ratio (Davies et al. 2008). This is why an elevation of the N/L ratio is commonly used as an indicator of stress and high glucocorticoid levels. However, in ectothermic animals, because of their temperature-dependent metabolism, the leukocyte response can sometimes take longer to develop. For example, the leukocyte differential took from 12 h to 3 days to occur following exogenous administration of stress hormones in amphibian species (Bennett and Harbottle 1968; Bennett et al. 1972). It is therefore possible that, in the current study,

L. latrans N/L ratio was still in the process of increasing when frogs were sampled on the next morning, as a nonsignificant tendency towards an elevation was observed (Table 2). Narayan and Hero (2011), for their part, observed a faster response in the Fijian ground frog (*Platymantis vitiana*), the increase in N/L ratio being present 6 h after transportation.

Finally, from an applied and operative point of view, the current study shows that it is best to sample the frogs as soon as possible after capture when considering hematologic or plasma biomarkers, as these values tend to rapidly change over time. Alternatively, if only tissular biomarkers are used, it is possible to wait until reaching more adequate installations and sample on the next morning. The need to promptly conduct blood samplings demonstrated here is of particular importance in the present context of amphibian declines given that nondestructive monitoring scheme should be increasingly favored in the future. Indeed, for the sake of amphibian conservation, nondestructive monitoring designs based on the capture, quick blood sampling, and release of the animal should be prioritized over destructive samplings. When conducting nondestructive samplings, rules for minimal blood sampling should be applied as described by Heatley and Johnson (2009). In the context of current amphibian declines, it is essential to intensify research efforts into the technical aspects of biomarker sampling in order to provide better and more complete assessments of amphibian health in the wild. In this sense, it would be important to repeat the evaluation performed in the current study in a range of other species to verify whether the findings obtained with *L. latrans* also apply to other amphibian species.

Funding information Funding for this work was provided by the “Agencia Nacional de Promoción Científica y Técnica” and the “Instituto Nacional de Tecnología Agropecuaria”.

References

- Amiard-Triquet C, Berthet B (2015) Individual biomarkers. In: Amiard-Triquet C, Amiard J-C, Mouneyrac C (eds) Aquatic Ecotoxicology. Advancing Tools for Dealing with Emerging Risks. Academic Press, New York, USA
- Attademo AM, Peltzer PM, Lajmanovich RC, Cabagna M, Fiorenza G (2007) Plasma B-esterase and glutathione S-transferase activity in the toad *Chaurus schneideri* (Amphibia, Anura) inhabiting rice agroecosystems of Argentina. *Ecotoxicology* 16:533–539
- Attademo AM, Cabagna-Zenkhusen M, Lajmanovich RC, Peltzer PM, Junges C, Basso A (2011) B-esterase activities and blood cell morphology in the frog *Leptodactylus chaquensis* (Amphibia: Leptodactylidae) on rice agroecosystems from Santa Fe Province (Argentina). *Ecotoxicology* 20:274–282
- Barnosky AD, Matzke N, Tomiya S, Wogan GOU, Swartz B, Quental TB, Marshall C, McGuire JL, Lindsey EL, Maguire KC, Mersey B, Ferrer EA (2011) Has the Earth’s sixth mass extinction already arrived? *Nature* 471:51–57
- Beauvais SL, Cole KJ, Atchison GJ, Coffey M (2002) Factors affecting brain cholinesterase activity in bluegill (*Lepomis macrochirus*). *Water Air Soil Pollut* 135:249–264

- Beckett GJ, Chapman BJ, Dyson EH, Hayes JD (1985) Plasma glutathione S-transferase measurements after paracetamol overdose: evidence for early hepatocellular damage. *Gut* 26:26–31
- Bennett MF, Harbottle JA (1968) The effects of hydrocortisone on the blood of tadpoles and frogs, *Rana catesbeiana*. *Biol Bull* 135:92–95
- Bennett MF, Gaudio CA, Johnson AO, Spisso JH (1972) Changes in the blood of newts, *Notophthalmus viridescens*, following administration of hydrocortisone. *J Comp Physiol* 80:233–237
- Bishop, P.J., Angulo, A., Lewis, J.P., Moore, R.D., Rabb, G.B., Garcia Moreno, J. 2012. The amphibian extinction crisis - what will it take to put the action into the amphibian conservation action plan? *S.A.P.I.E.N.S* [online], 5.2. <http://sapiens.revues.org/140>
- Brodeur JC, Suarez RS, Natale GS, Ronco AE, Zaccagnini ME (2011) Reduced body condition and enzymatic alterations in frogs inhabiting intensive crop production areas. *Ecotoxicol Environ Saf* 74:1370–1380
- Brodeur, J.C., Vera Candiotti, J. Soloneski, S. Larramendy, M.L., Ronco, A.E. 2012. Evidence of reduced feeding and oxidative stress in common tree frogs (*Hypsiboas pulchellus*) from an agroecosystem experiencing severe drought. *J Herpetol* 46:72–78
- Brodeur JC, Vera Candiotti J (2017) Impacts of agriculture and pesticides on amphibian terrestrial life stages: potential biomonitor/bioindicator species for the pampa region of Argentina. In: Larramendy ML (ed) *Ecotoxicology and Genotoxicology - Non-traditional Terrestrial Models*. Royal Society of Chemistry, London
- Brodeur JC, Sanchez M, Castro L, Rojas DE, Cristos D, Damonte MJ, Poliserpi MB, D'Andrea MF, Andriulo AE (2017) Accumulation of current-use pesticides, cholinesterase inhibition and reduced body condition in juvenile one-sided livebearer fish (*Jenynsia multidentata*) from the agricultural Pampa region of Argentina. *Chemosphere* 185:36–46
- Brodeur JC, Damonte MJ, Vera Candiotti J, Poliserpi MB, D'Andrea MF, Bahl MF (2020) Frog body condition: basic assumptions, comparison of methods, and characterization of natural variability with field data from *Leptodactylus latrans*. *Ecol Indic* 112:106098
- Brousseau P, Pillet S, Frouin H, Auffret M, Gagne F, Fournier M (2013) Linking Immunotoxicity and Ecotoxicological effects at higher biological levels. In: Amiard-Triquet C, Amiard J-C, Rainbow PS (eds) *Ecological Biomarkers. Indicators of Ecotoxicological Effects*. CRC Press, New York
- Burton CB, Murray SA (1979) Effects of density on goldfish blood. *J Hematology Comp Biochem Physiol* 62A:555–558
- Cabagna MC, Lajmanovich RC, Stringhini G, Sanchez-Hernandez JC, Peltzer PM (2005) Hematological parameters of health status in the common toad *Bufo arenarum* in agroecosystems of Santa Fe Province. *Argentina Applied Herpet* 2:373–380
- Campbell Grant EH, Miller DAW, Schmidt BR, Adams MJ, Amburgey SM, Chambert T, Cruickshank SS, Fisher RN, Green DM, Hossack BR, Johnson PTJ, Joseph MB, Rittenhouse TAG, Ryan ME, Waddle JH, Walls SC, Bailey LL, Fellers GM, Gorman TA, Ray AM, Pilliod DS, Price SJ, Saenz D, Sadinski W, Muths E (2016) Quantitative evidence for the effects of multiple drivers on continental-scale amphibian declines. *Sci Rep*. <https://doi.org/10.1038/srep25625>
- Camus L, Pampanin DM, Volpato E, Delaney E, Sanni S, Nasci C (2004) Total oxyradical scavenging capacity responses in *Mytilus galloprovincialis* transplanted into the Venice lagoon (Italy) to measure the biological impact of anthropogenic activities. *Mar Pollut Bull* 49:801–808
- Cathers T, Lewbart GA, Correa M, Stevens JB (1997) Serum chemistry and hematology values for anesthetized American bullfrogs (*Rana catesbeiana*). *J Zoo Wildlife Med* 28:171–174
- Cazenave J, Bacchetta C, Parma MJ, Scarabotti PA, Wunderlin DA (2009) Multiple biomarkers responses in *Prochilodus lineatus* allowed assessing changes in the water quality of Salado River basin (Santa Fe, Argentina). *Environ Pollut* 157:3025–3033
- Cei JM (1980) Amphibians of Argentina. *Monitore zool ital Monogr* 2: 609 p
- Christin SM, Ménard L, Giroux I, Marcogliese DJ, Ruby S, Cyr D, Fournier M, Brousseau P (2013) Effects of agricultural pesticides on the health of *Rana pipiens* frogs sampled from the field. *Environ Sci Pollut Res* 20:601–611
- Crump ML (2010) Amphibian diversity and life history. In: Dodd CK Jr (ed) *Amphibian ecology and conservation. A handbook of techniques, Techniques in Ecology and Conservation Series*. Oxford University Press, Oxford, U.K., pp 3–35
- Davies AK, Maney DL, Maerz JC (2008) The use of leukocyte profiles to measure stress in vertebrates: a review for ecologists. *Funct Ecol* 22: 760–772
- Davies AK, Durso AM (2009) White blood cell differentials of northern cricket frogs (*Acris c. crepitans*) with a compilation of published values from other amphibians. *Herpetologica* 65(3):260–267
- Drabkin DL, Austin JH (1935) Spectrophotometric studies. II. Preparations from washed blood cells; nitric oxide hemoglobin and sulfhemoglobin. *J Biol Chem* 112:51–65
- Ellman GL, Courtney KD, Valentino AJ, Featherstone RM (1961) A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem Pharmacol* 7:88–95
- Falfushinska H, Loumbourdis N, Romanchuk L, Stolyar O (2008) Validation of oxidative stress responses in two populations of frogs from Western Ukraine. *Chemosphere* 73(7):1096–1101
- Fernandez G, Mena MP, Arnau A, Sanchez O, Soley M, Ramirez I (2000) Immobilization stress induces c-Fos accumulation in liver. *Cell Stress Chaperones* 5(4):306–312
- Flammarion P, Noury P, Garric J (2002) The measurement of cholinesterase activities as a biomarker in chub (*Leuciscus cephalus*): the fish length should not be ignored. *Environ Pollut* 120(2):325–330
- Forbes MR, McRuer DL, Shutler D (2006) White blood cell profiles of breeding American toads (*Bufo americanus*) relative to sex and body size. *Comp Clin Pathol* 15:155–159
- Forzán MJ, Heatley J, Russell KE, Horney B (2017) Clinical pathology of amphibians: a review. *Vet Clin Pathol* 46(1):11–33
- George ADI, Akinrotimi OA (2017) Influence of sex on haematological response of *Clarias gariepinus* juveniles treated with atrazine and metalochlor. *Trends Green Chem* 3:1–6
- Habig C, Di Giulio RT (1991) Biochemical characteristics of cholinesterases in aquatic organisms. In: Mineau P (ed) *Cholinesterase-inhibiting insecticides*. Elsevier, Amsterdam, pp 20–33
- Hansen P-D (2003) Biomarkers. In: Markert BA, Breure AM, Zechmeister HG (eds) *Bioindicators and biomonitors*. Elsevier Science Ltd, Oxford
- Heatley JJ, Johnson M (2009) Clinical technique: amphibian hematology: a practitioner's guide. *J Exotic Pet Med* 18:14–19
- Hegde G, Krishnamurthy SV (2014) Analysis of health status of the frog *Fejervarya limnocharis* (Anura: Ranidae) living in rice paddy fields of Western Ghats, using body condition factor and AChE content. *Ecotoxicol Environ Contam* 9:69–76
- Heyer, R., Langone, J., La Marca, E., Acevedo-Ramos, C., di Tada, I., Baldo, D., Lavilla, E., Scott, N., Aquino, L., Hardy, J. 2010. *Leptodactylus latrans*. The IUCN red list of threatened species: e.T57151A11592655
- Huggett RJ, Kimerle RA, Mehrle PM, Bergman HL (2018) Biomarkers: biochemical, physiological, and histological markers of anthropogenic stress. CRC Press, Boca Raton
- Imperato A, Puglisi-Allegra S, Casolini P, Angellucci L (1991) Changes in brain dopamine and acetylcholine release during and following stress are independent of the pituitary-adrenocortical axis. *Brain Res* 538:111–117
- IPBES. 2019. Global assessment report on biodiversity and ecosystem services of the intergovernmental science-policy platform on biodiversity and ecosystem services. E.S. Brondizio, J. Settele, S. Díaz, and H.T. Ngo (editors). IPBES secretariat, Bonn, Germany

- Kaufer D, Friedman A, Seidman S, Soreq H (1998) Acute stress facilitates long-lasting changes in cholinergic gene expression. *Nature* 393:373–377
- Koenig S, Sole M (2013) Muscular cholinesterase and lactate dehydrogenase activities in deep-sea fish from the NW Mediterranean. *Mar Environ Res* 94:16–23
- Lajmanovich RC, Sanchez-Hernandez JC, Stringhini G, Peltzer PM (2004) Levels of serum cholinesterase activity in the rococo toad (*Bufo paracnemis*) in agrosystems of Argentina. *Bull Environ Contam Toxicol* 72(3):586–591
- Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the folin phenol reagent. *J Biol Chem* 193:265–75
- Mahapatra BB, Das M, Dutta SK, Mahapatra PK (2012) Hematology of Indian rhacophorid tree frog *Polypedates maculatus* gray, 1833 (Anura: Rhacophoridae). *Comp Clin Pathol* 21:453–460
- Maneyro R, Naya DE, da Rosa I, Canavero A, Camargo A (2004) Diet of the south American frog *Leptodactylus ocellatus* (Anura, Leptodactylidae) in Uruguay, Iheringia, Sér. Zool. Porto Alegre 94:57–61
- Menezes S, Soares AMVM, Guilhermino L, Peck MR (2006) Biomarker responses of the estuarine brown shrimp *Crangon crangon* L. to non-toxic stressors: temperature, salinity and handling stress effects. *J Exp Mar Biol Ecol* 335:114–122
- Meshorer E, Erb C, Gazit R, Pavlovsky L, Kaufer D, Friedman A, Glick D, Ben-Arie N, Soreq H (2002) Alternative splicing and neuritic mRNA translocation under long-term neuronal hypersensitivity. *Science* 295:508–512
- Meesawat, S., Kitana, N., Kitana, J. 2016. Hematology of wild caught *Hoplobatrachus rugulosus* in northern Thailand. *Asian Herpetol. Res.* 7(2):131–138
- Mitchell MA (2009) Anesthetic considerations for amphibians. *J Exotic Pet Med* 18:40–49
- Monaghan P, Spencer KA (2014) Stress and life history. *Curr Biol* 24(10):R408–R412
- Motlagh SP, Zarejabad AM, Nasrabadi RG, Ahmadifar E, Molaei M (2012) Haematology, morphology and blood cells characteristics of male and female Siamese fighting fish (*Betta splendens*). *Comp Clin Pathol* 21(1):15–21
- Moura Costa DD, Filipak, Neto F, Costa MDM, Morais RN, Garcia JRE, Esquivel BM, Ribeiro O, C.A. (2010) Vitellogenesis and other physiological responses induced by 17- β -estradiol in males of freshwater fish *Rhamdia quelen*. *Comp Biochem Physiol* 151C:248–257
- Narayan E, Hero JM (2011) Urinary corticosterone responses and haematological stress indicators in the endangered Fijian ground frog (*Platymantis vitiana*) during transportation and captivity. *Aust J Zool* 59:79–85
- Narayan EJ, Cockrem JF, Hero JM (2013) Sight of a predator induces a corticosterone stress response and generates fear in an amphibian. *PLoS One* 8(8):e73564. <https://doi.org/10.1371/journal.pone.0073564>
- Natt MP, Herrick CA (1952) A new blood diluent for counting the erythrocytes and leucocytes of the chicken. *Poult Sci* 31:735–738
- Omeje VO, Lambrechts H, Brink D (2019) Hepatic and haematobiochemical alterations in juvenile *Mozambique tilapia* (*Oreochromis mossambicus*) on pawpaw (*Carica papaya*) seed meal. *J Agric Sci* 11(8):20–30
- Orton F, Baynes A, Clare F, Duffus ALJ, Larroze S, Scholze M, Garner TWJ (2014) Body size, nuptial pad size and hormone levels: potential non-destructive biomarkers of reproductive health in wild toads (*Bufo bufo*). *Ecotoxicology* 23(7):1359–1365
- Pathiratne A, Chandrasekera WHU, De Seram PKC (2008) Effects of biological and technical factors on brain and muscle cholinesterases in Nile tilapia, *Oreochromis niloticus*: implications for biomonitoring neurotoxic contaminations. *Arch Environ Contam Toxicol* 54:309–3017
- Parida S, Panga R, Rajappa M, Kundra P (2018) Study of glutathione S-transferase levels in patients receiving intravenous paracetamol perioperatively: a randomized controlled trial. *Indian J Gastroenterol* 37(6):511–519
- Pimm SL, Jenkins CN, Abell R, Brooks TM, Gittleman JL, Joppa LN, Raven PH, Roberts CM, Sexton JO (2014) The biodiversity of species and their rates of extinction, distribution, and production. *Science* 344:987–998
- Peig J, Green AJ (2009) New perspectives for estimating body condition from mass/length data: the scaled mass index as an alternative. *Oikos* 118:1883–1891
- Phillips TA, Summerfelt RC, Atchison GJ (2002) Environmental, biological, and methodological factors affecting cholinesterase activity in walleye (*Stizostedion vitreum*). *Arch Environ Contam Toxicol* 43(1):75–80
- Pollo FE, Grenat PR, Salinas ZA, Otero MA, Salas NE, Martino AL (2017) Evaluation in situ of genotoxicity and stress in South American common toad *Rhinella arenarum* in environments related to fluorite mine. *Environ Sci Pollut Res* 24(22):18179–18187.
- Pounds, J.A., Bustamante, M.R., Coloma, L.A., Consuegra, J.A., Fogden, M.P.L., Foster, P.N., La Marca, E., Masters, K.L., Merino-Viteri, A., Puschendorf, R., et al. 2006. Widespread amphibian extinctions from epidemic disease driven by global warming. *Nature* 439: 161–167
- Prokic MD, Petrovic TG, Gavric JP, Despotovic SG, Gavrilovic BR, Radovanovic TB, Faggio C, Saicic ZS (2018) Comparative assessment of the antioxidative defense system in subadult and adult anurans: a lesson from the *Bufo viridis* toad. *Zoology* 130:30–37
- Roelants K, Gower DJ, Wilkinson M, Loader SP, Biju SD, Guillaume K, Moriau L, Bossuyt F (2007) Global patterns of diversification in the history of modern amphibians. *Proc Natl Acad Sci U S A* 104:887–892
- Santymire RM, Manjerovic MB, Sacertote-Velat A (2018) A novel method for the measurement of glucocorticoids in dermal secretions of amphibians. *Conserv Physiol* 6(1):coy008. <https://doi.org/10.1093/conphys/coy008>
- Stuart SN, Chanson JS, Cox NA, Young BE, Rodrigues ASL, Fischman DL, Waller RW (2004) Status and trends of amphibian declines and extinctions worldwide. *Science* 306:1783–1785
- Tecles F, Escribano D, Martinez-Miro S, Hernandez F, Contreras MD, Ceron JJ (2016) Cholinesterase in porcine saliva: analytical characterization and behavior after experimental stress. *Res Vet Sci* 106: 23–28
- Tsakiris S, Kontopoulos AN (1993) Time changes in Na⁺, K⁺-ATPase, mg⁺-ATPase, and acetylcholinesterase activities in the rat cerebrum and cerebellum caused by stress. *Pharmacol Biochem Behav* 44: 339–342
- Van der Oost R, Beyer J, Vermeulen NP (2003) Fish bioaccumulation and biomarkers in environmental risk assessment: a review. *Environ Toxicol Pharmacol* 13:57–149
- Voorhees JL, Powell ND, Moldovan L, Mo X, Eubank TD, Clay BM (2013) Chronic restraint stress upregulates erythropoiesis through glucocorticoid stimulation. *PLoS One* 8(10):e77935. <https://doi.org/10.1371/journal.pone.0077935>
- Wake DB, Vredenburg VT (2008) Are we in the midst of the sixth mass extinction? A view from the world of amphibians. *Proc Natl Acad Sci U S A* 105:11466–11473
- Williams M, Zalasiewicz J, Haff PK, Schwägerl C, Barnosky AD, Ellis EC (2015) The Anthropocene biosphere. *The Anthropocene Rev* 2: 196–219
- Wilson JT, Pascoe PL, Parry JM, Dixon DR (1998) Evaluation of the comet assay as a method for the detection of DNA damage in the cells of a marine invertebrate, *Mytilus edulis* L. (Mollusca: Pelecypoda). *Mutat Res* 399:87–95

- Xiong J, Zhang Y, Chen W, Min Y, Gou J (2018) Some haematological parameters of wild caught warty toothed toad *Oreolalax rugosus* (Liu, 1943) (Anura: Megophryidae). *Acta Zool Bulg* 70(1):69–74
- Young S, Warner J, Speare R, Berger L, Skerratt LF, Muller R (2012) Hematologic and plasma biochemical reference intervals for health monitoring of wild Australian tree frogs. *Vet Clin Pathol* 41(4):478–492
- Zhelev ZM, Georgieva KN, Todorov OB, Peeva KG (2017) Haematological parameters of *Bufo viridis* (Laurenti, 1768) (Anura: Bufonidae) from southern Bulgaria. *Acta Zool Bulg* 69(3):335–343

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.