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RESEARCH ARTICLE

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

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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

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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 Krishnamurty 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 Candioti 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) (Cei 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 km^2 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-cm^3 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:

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

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

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

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:

$$\mathrm{MCHC}(g/dL) = [\mathrm{Hb}\ (g/dL)*100]/\mathrm{PCV}\ (\%)$$

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 M^{-1} 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 M^{-1} 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 mM^{-1} 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 Parameter = B_o + Length + Sex+Time + Sex*Length + Time*Length + Sex*time + Length*Sex*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 ± 14.7 g (mean ± S.D.). The SVL of sampled frogs ranged between 45.2 and 82.0 mm for an average SVL of 64.7 ± 9.47 mm (mean ± 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 ± 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 ± 96	1557 ± 92	*1610 ± 66*
Kidney	280 ± 40	338 ± 42	*309 ± 29*
Cholinesterases			
Liver	13.5 ± 0.8	13.8 ± 1.2	*13.7 ± 0.7*
Muscle	6.8 ± 0.7	6.3 ± 0.8	*6.5 ± 0.5*
Plasma	7.5 ± 0.8	10.6 ± 1.2*	
Glutathione-S-transferase			
Liver	8.8 ± 0.6	8.3 ± 0.6	*8.5 ± 0.4*
Kidney	2.0 ± 0.1	2.2 ± 0.2	*2.1 ± 0.1*
Muscle	0.029 ± 0.003	0.0028 ± 0.003	*0.029 ± 0.002*
Plasma	0.015 ± 0.004	0.021 ± 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 μmol/min/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 ± 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 ± 0.03	0.90 ± 0.06*	
PCV (%)	21.0 ± 1.2	20.1 ± 1.3	*20.6 ± 0.9*
Hb (g/dL)	6.5 ± 0.2	6.9 ± 0.3	*6.7 ± 0.2*
MCV (fL)	314.6 ± 34.3	246.0 ± 21.9	*281.5 ± 21.0*
MCH (pg)	93.7 ± 5.3	86.5 ± 6.8	*90.1 ± 4.3*
MCHC (g/dL)	34.5 ± 2.5	39.5 ± 3.1	*36.9 ± 2.0*
Total WBCs[a]	39.3 ± 2.5	38.3 ± 3.3	*38.8 ± 2.0*
Total Thrombocytes[b]	18.7 ± 1.6	16.1 ± 1.2	*17.4 ± 1.0*
Neutrophils (%)	5.1 ± 0.8	6.9 ± 1.1	*6.1 ± 0.7*
Lymphocytes (%)	87.8 ± 1.5	86.1 ± 1.5	*86.9 ± 1.0*
Monocytes (%)	2.2 ± 0.3	2.9 ± 0.4	*2.6 ± 0.3*
Eosinophils (%)	1.4 ± 0.5	1.1 ± 0.3	*1.2 ± 0.3*
Basophils (%)	3.2 ± 0.4	2.8 ± 0.4	*3.0 ± 0.3*
N/L Ratio	0.06 ± 0.01	0.09 ± 0.02	*0.08 ± 0.01*

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

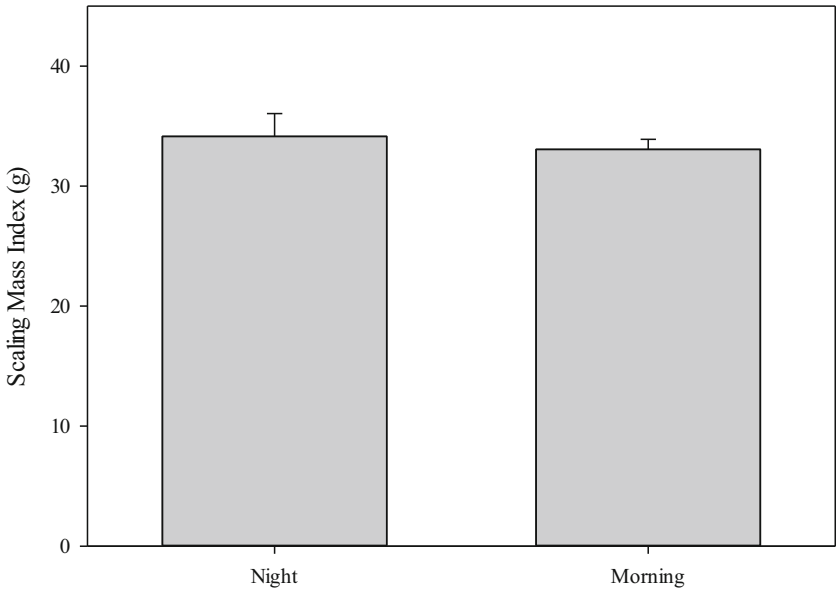

Fig. 1 Body condition (mean ± S.E.) of *Leptodactylus latrans* frogs when sampled the night of their capture or the next morning

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 suggests 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 undergoing 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 plasmatic 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.

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