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Effect of parasitism of *Lernaea cyprinacea* on tadpoles of the invasive species *Lithobates catesbeianus*

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ABSTRACT

The introduction of invasive species is one of the greatest threats currently faced by natural ecosystems, causing ecological imbalances between native populations and transmission of a variety of diseases. We reported the interaction between two exotic species given by the parasitic infestation of the copepod *Lernaea cyprinacea* in the early stages of the development of the American bullfrog *Lithobates catesbeianus* in the central area of Argentina. In this paper we analysed the leukocyte profile of parasitized and non-parasitized tadpoles of *L. catesbeianus* with *L. cyprinacea* and their body condition (BC) as biomarkers of the health status of organisms. A total of 27 tadpoles of *L. catesbeianus* were analysed (12 non-parasitized and 15 parasitized). The lower BC recorded in parasitized organisms show a lower health status in these tadpoles, which could be affecting the metamorphosis and therefore impact at the population level. Leukocyte response of *L. catesbeianus* tadpoles to the parasitism of *L. cyprinacea* was found. Mature and immature lymphocyte frequencies and hematocrit were higher in parasitized compared to non-parasitized tadpoles, which is a typical response to the presence of parasites. However, eosinophils and monocytes were recorded at high frequencies in not parasitized tadpoles, which could be due to the important role played by these leucocytes in the metamorphosis of frogs. The results of this study constitute a first antecedent on leukocyte profile in aquatic stages of anurans during an ectoparasitosis and its possible implications for environmental health. The parasitism of *L. cyprinacea* influences the biology of the American bullfrog at both the individual and population levels. Parasitized individuals are not killed directly by the parasite, but they can create conditions for secondary infections, growth retardation, behavioral changes and, ultimately, reduce populations.

1. Introduction

The introduction of invasive species is one of the greatest threats currently faced by natural ecosystems, causing ecological imbalances between native populations and transmission of a variety of diseases (Kraus, 2009). In a previous paper, we reported the interaction between two exotic species given by the parasitic infestation of the copepod *Lernaea cyprinacea* in the early stages of the development of the American bullfrog *Lithobates catesbeianus* in the central area of Argentina (Salinas et al., 2016). The bullfrog *L. catesbeianus* is naturally extended from eastern Canada through central and eastern United States to northeastern Mexico (Frost, 2014). However, new wild populations of bullfrogs were reported around the world due to their high value in the food trade (Mazzoni et al., 2003). Argentina and Brazil present the most extensive invasions of bullfrog in South America (Giovanelli et al., 2008; Akmentins and Cardozo, 2010). On the other hand, *L. cyprinacea* is an Eurasian species that has expanded worldwide. Although its name expresses it, it is not limited to cyprinids as hosts (Kupferberg et al., 2009). Like most parasitic copepods, *L. cyprinacea* has a direct life cycle that involves only one host, and the transmission occurs through free swimming nauplii and the first copepod larval stage. The first to the fifth copepod stage are located within the mouth of the tadpole and the gill chambers (Tidd & Shields 1963a,b). The copepods adhere with their maxillipeds and remain there sessile, eating underlying epithelial tissue and connective tissue (Shields and Tidd, 1974) until the fifth larval stage when copulation occurs. Post-copulation, the females undergo another molt, travel along the integument of the tadpole, and penetrate the tadpole tissue within their heads. Penetration sites can be located in any area, however,

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they most often adhere to the juncture between the tail and the body, and a number of tissues (eg., liver, lung, spinal cord) can be damaged (Tidd, 1962). These copepods can cause hemorrhages, ulcerations, muscle necrosis and intense inflammatory response on hosts (Khalifa and Post, 1976; Berry et al., 1991; Silva-Souza et al., 2000; Carnevia and Speranza, 2003; McAllister et al., 2011, Sayyadzadeh and Roudbar, 2014) whether directly or indirectly through interactions with other pathogens as bacteria (Schäperclaus, 1991; Koprivnikar et al., 2010; Koprivnikar et al., 2012).

Although we previously reported the prevalence, infestation and consequences (hemorrhages and diverse affections) of this copepod parasitism in *L. catesbeianus* of this same place (Salinas et al., 2016) the state of integral health of the individuals has not been evaluated. This becomes important since it is known that local people hunt this species in the wild and consume them, which could, considering hygiene and health, put human health at risk. In this sense, biomarkers are essential for assessing environmental health and the state of the population (NRC 1987; Zhelev et al., 2016; Salinas et al., 2017). In particular, studies with amphibians have begun to incorporate leukocyte count as an endpoint because of their value in assessing the health of these animals (Cabagna et al., 2005; Barni et al., 2007; Davis et al., 2010; Salinas et al., 2015). Leukocytes in most vertebrates represent one of the first lines of defense against infectious diseases (Roitt et al., 2001). The evaluation of leukocytes can be useful to measure physiological disorders in the parasitized organism and, therefore, provide information on the level of damage in the host and the prognosis of diseases (Tavares Dias and Moraes, 2007; Soberón et al., 2014). Circulating leukocytes can rapidly increase in number after bacterial, protozoan blood infection and/or larger macroparasites, and particular types of leukocytes can attack broad pathogenic groups (Roitt et al., 2001). In amphibians, the leukocyte component has been used for the evaluation of individuals in stressful situations (Davis et al., 2008; Davis and Maerz, 2008), as an indicator of populations of contaminated sites (Cabagna et al., 2005; Barni et al., 2007) and to a lesser extent the effect caused by parasites on the guests. Leukocytes, such as monocytes, heterophiles and some lymphocytes, may offer an important measure of non-specific immune function and health status of the host (Davis et al., 2004). Salinas et al. (2015) and Kiesecker et al. (2001) highlight the importance of using this type of biomarker to evaluate the response of individuals to a parasitic infection.

On the other hand, body condition is an endpoint used to know the health state of an adult and tadpoles (Bionda et al., 2012; Babini et al. 2018). The body condition of tadpoles can affect the survival and recruitment of organism and compromise the persistence of the population (Wilbur, 1980; Semlitsch et al., 1988; Gray and Smith, 2005). Furthermore, studies in insects, fish, amphibians and rodents show that the appearance of infections and intensity of parasites is likely and more serious in individuals with an underlying poor physical condition (Beldomenico and Begon, 2010). Infection itself results in further deterioration of the host creating a "vicious circle" (Beldomenico and Begon, 2010). Some parasites can cause lesions at their sites of infection by their location (for example, intracellular in *Eimeria* sp.), by their binding mode (for example, *Trichuris* sp.) or in different tissues during larval migration (Ezquiaga et al., 2014).

In this paper we analyzed the leukocyte profile of parasitized and non-parasitized tadpoles of *L. catesbeianus* with *L. cyprinacea* and their body condition as endpoints of the health status of organisms (Davis, 2009; Davis et al., 2010; Wood and Richardson, 2009). Since there are no studies on the effects of this host-parasite interaction, the present work will contribute with relevant information on the immunological response of the larval stages of *L. catesbeianus*, a key stage for the persistence of population. In addition, we will expand our knowledge about the resistance of this species to adverse conditions such as parasitism; taking into account that *L. catesbeianus* is an invasive exotic species with resistance to a wide range of environmental changes, without natural predators and that displaces native species according to the characteristics of its niche.

2. Materials and methods

2.1. Study area

"Río de los Sauces" is located southwest of Córdoba province (32°31′40″ N, 64°35′18″ W) corresponding to the department of Calamuchita. "Río de los Sauces" presents typical vegetation of the plain corresponding to the "Espinal" with a transition to vegetation of the mountains to the "Bosque Serrano". In this sense, arboreal and herbaceous native species of the "Espinal" as *Prosopis alba*, *P. nigra*, and *P. kuntzei*, *Zizyphus mistol*, *Geoffroea decorticans*, *Salix humboldtiana*, *Acacia caven*, *Baccharis articulata*, *Lippia turbinata*, *Setaria geniculata*, *Condalia microphylla*, *Colletia spinosissima*, *Poa sp.*, *Paspalum sp.*, *Festuca sp.*, are associated with typical vegetation of the "Bosque Serrano" as *Lithraea ternifolia*, *Fagara coco* and *Schinus molle* (Di Tada and Bucher, 1996).

This area is an undisturbed piedmont environment where natural characteristics are preserved. Considering that this is a touristic place, there are different conservation policies of the environment in order to preserve the native conditions of the place. Another aspect to keep in mind is the fact that the sewage effluents are not discharged in the river, but to blind wells. In addition, anthropic activities such as agriculture and livestock are far from this site (2000 meters away).

The study area is characterized by humid and temperate climate that transitions to semi-wet or dry to the West (Di Tada and Bucher, 1996). The maximum temperature is 34 °C with average minimum 9 °C. Maximum rainfall occurs from October–March, with an annual average of 901 mm (Salinas et al., 2016).

2.2. Data collection

The sampling period started in December 2013 to March 2014. The individuals of *Lithobates catesbeianus* were captured in the "Río de los Sauces" river (32°31′53″ N, 64°35′27″ W) and "El Toledo" stream (32°28′8″ N, 64°35′27″ W). Environmental variables registered in each site are presented in Salinas et al. (2016). Visual Encounter Survey (VES) methodology was used for sampling of individuals (Heyer et al., 1994). Tadpoles were captured with a net on the banks of the river and streams where the water is calmer and therefore are not washed away by the current. The individuals were associated with a lot of floating vegetation such as *Azolla*, *Hydrocotyle sp.*, *Lemna sp.*, and submerged as *Ceratophyllum demersum*.

Individuals captured were anesthetized with a solution at 0.5% of MS 222 or Methanesulfonate Salt (3-Aminobenzoic Acid Ethyl Ester Sigma-Aldrich™) and several measures were recorded for each one: development stage, following Gosner (1960); weight using an Ohaus digital balance (0.01 g); total length (TL; length from the snout to the end of the tail); body length (BL; snout-vent length); body maximum width (BMW: maximum width of the body, dorsal view); tail length (TAL); maximum tail height (MTH: maximum height of the tail, side view); body maximum height (BMH: maximum height of the body, side view); tail muscle height (TMH; tail muscle height bundle the vent tube, lateral view); and eye diameter (ED). In advanced larval stages, femur length (F), tibia length (T) and Shine bone length (W) leg length (L); (TMH) Muscle width of the tail; Eye diameter (ED) were measured. Morphometric measurements (MM) were performed with a digital caliper Mahr 16 (0.01 mm). Tadpoles were inspected to detect parasites of *L. cyprinacea* using a binocular loupe Zeiss West Germany and the count of the amount of parasites was done in the adult females of the species. According to the presence of parasites the tadpoles were divided into two groups: not parasitized (NP) and parasitized (P) (Fig. 1).

Blood samples were obtained from each tadpole by cardiac puncture (Babini et al., 2015) and blood smears were prepared on clean slides, fixed, and stained by means of the May-Grünwald/Giemsa method (Dacie and Lewis, 1995; Barni et al., 2007). Blood smears were observed with Zeiss Primo Star iLED and five types of leukocytes were counted (Fig. 2):

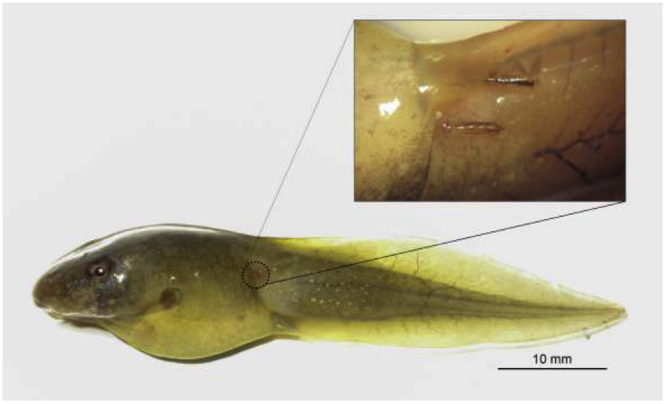

Fig. 1. Tadpol of *Litobathes catesbeianus* infected by the copepod *Lernaea cyprinacea*.

mature and immature lymphocytes, monocytes, neutrophils, basophils and eosinophils, main leukocytes that respond to a parasitism. Leucocytes were distinguished following Hadji-Azimi et al. (1987) and Coppo (2003). For each individual a differential count of 100 leucocytes (Davis et al., 2004), Hematocrit (H) and index "N/L" were completed.

After the euthanasia, the animals were discarded and deposited in the Herpetological Collection of Ecology, Department of Natural Sciences, Faculty of Exact, Physical-Chemical and Natural Sciences, National University of Río Cuarto, Córdoba Province, Argentina. The care, treatment and sampling of animals used in this study followed the Animal Care Regulations of University National of Río Cuarto and state law "Protection and Conservation of Wild Fauna" (Argentina National Law N° 22.421). In addition, the bioethics committee approved, through protocol number 38/11, the manipulation of captured individuals.

2.3. Statistic analysis

Star graph and Principal Component Analysis (PCA) based on MM was performed. We standardized the data set before plotting the PCA because variables have different units. The biplot was performed with Principal Component 1 and 2 (PC1 and PC2). In addition, a multivariate variance analysis was made with the MM to differentiate between P and NP tadpoles.

Measures of weight and TL were used to calculate the BC of tadpoles. Values of weight were regressed on TL and the residuals were taken as an index of BC (Wood and Richardson, 2009).

Linear Mixed Models (LMMs) were used for analyse BC. We included presence of parasite as fixed factor and Gosner Stage (GS) as a random factor. The null model with the intercept only was also evaluated. The best model was selected using the Akaike information criterion (AIC) and Bayesian information criterion (BIC) methods (AIC and BIC minors implies a better fit of the data to the model).

Data of frequency of mature and inmature lymphocytes, monocytes, neutrophils, eosinophils and basophils were adjusted to a generalized linear model (GLM). These response variables were adjusted to a binomial distribution and log it link function (Nelder and Wendderburn, 1972; Myers et al., 2002). Then, the post-hoc DGC test (Test of Di Rienzo, Guzmán & Casanoves) was used to test differences between means (Di Rienzo et al., 2002). This test uses the multivariate cluster analysis technique, mean chain or UPGMA (unweighted pair-group method using an arithmetic average) in a distance matrix obtained from the sampling means (Balzarini et al., 2008). InfoStat (Di Rienzo et al., 2017) and R 3.3.2 (R Core Team, 2016) were used for all analyses.

3. Results

We captured a total of 27 tadpoles: 12 individuals without parasite and 15 individuals with the parasite. *Lernaea* parasites were usually found in the cloaca, with a maximum of two parasites per individual. We observed clinical symptoms of inflammation, haemorrhage, and ulcers in the skin with mucus formations in the attachment of the parasites. The tadpoles captured in the "Rio de los Sauces" river were in the larval stage of Gosner 31, 36, 37, 40, 41; while those captured in "El Toledo" stream: 27, 28, 29, 30, 34.

Biplot of morphometric parameters obtained by PCA indicate a positive association of all morphometric variables with the NP tadpoles and the star graph shows that P tadpoles had lower mean morphometric parameters with respect to NP tadpoles (Fig. 3). Multivariate variance

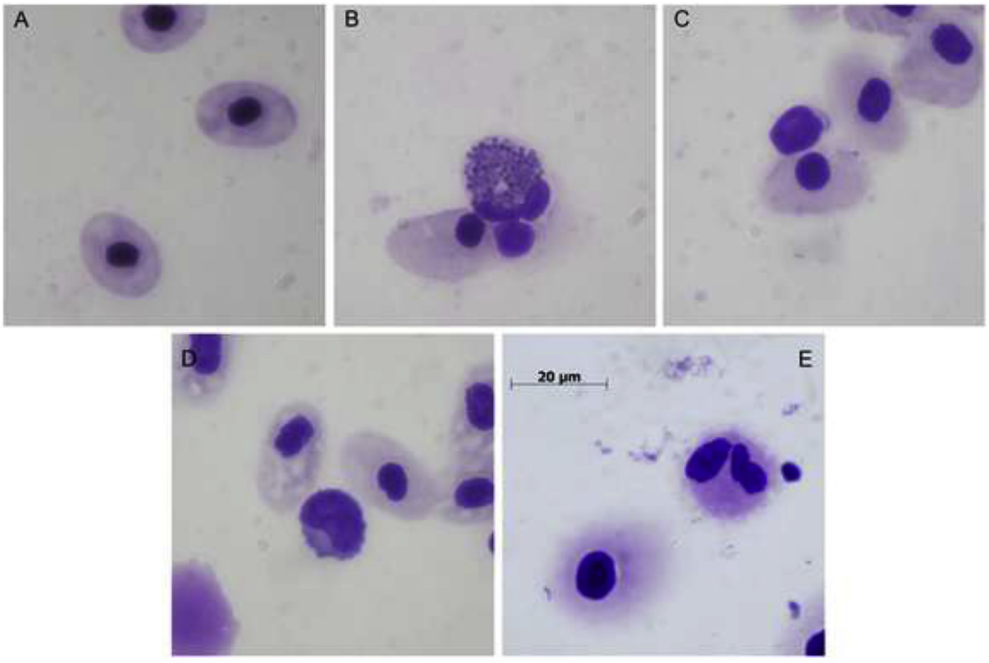

Fig. 2. Different types of leucocytes from *Lithobates catesbeianus* tadpoles. A) Erythrocytes. B) Eosinophil with granulates. C) Lymphocyte. D) Monocyte. E) Neutrophil.

analysis did not indicate significant differences between P and NP tadpoles.

The model that best fit the data of BC was: P (presence of Parasite; fixed factor) + GS (Gosner Stage; random factor, heterocedastic variance); AIC: 101, BIC: 107 (Model P: AIC = 104, BIC = 108. Model P + GS; homocedastic variance: AIC = 106, BIC = 111). BC of the parasitized tadpoles was significantly lower than the BC of not parasitized tadpoles (LMMp-value <0.0001).

The higher frequencies of immature and mature lymphocytes were recorded in P tadpoles (Table 1) and were statistically different between P and NP tadpoles (GLM IL: $F_{1, 25}$: 6.6; GLM ML: $F_{1, 25}$: 17.98). The higher frequencies of eosinophils and monocytes were recorded in NP tadpoles (GLM E: $F_{1, 25}$: 13.81; GLM M: $F_{1, 25}$: 13.86). Mean frequencies of neutrophils and basophils and the mean of the N/L ratio were higher in NP tadpoles; however, the statistical analysis did not detect significant differences.

Table 1
Mean values and standard error of the leukocyte formula of tadpoles parasitized and not parasitized with *Lernea cyprinacea*; p-value of analysis and post-hoc DGC test.

	Not Parasitized	Parasitized	P-valor	DGC test
Immature Lymphocytes	20.7 ± 5.28	24.89 ± 3.76	0.0102[a]	NP < P
Mature Lymphocytes	33.22 ± 5.69	41.19 ± 4.79	<0.0001[c]	NP < P
Neutrophils	25.68 ± 3.1	23.24 ± 3.82	0.1418	
Eosinophils	9.09 ± 1.4	5.31 ± 0.87	<0.0002[b]	NP > P
Basophils	2.3 ± 0.38	2.08 ± 0.54	0.7026	
Monocytes	6.39 ± 2.31	3.29 ± 0.9	0.0002[b]	NP > P
N/L	0.53 ± 0.08	0.44 ± 0.12	0.6129	
Hematocrit	25.89 ± 2.11	35.25 ± 5.67	0.073	

[a] p < 0,05.
[b] p < 0,01.
[c] p < 0,0.

4. Discussion

The results of this study present a clear picture of how the number of leukocytes changes when larval anurans amphibians are parasitized. In addition, they constitute a first antecedent on leukocyte profile in aquatic stages of anuran during an ectoparasitosis and its possible implications for environmental health.

Body size is a parameter of great importance due to its impact on adult populations. Low values of BC indices corresponding to early stages are extended to adult ages (Harris et al., 2000), and population groups composed of smaller individuals are more likely to decline than those composed of large individuals (Gray and Smith, 2005). Although P and NP tadpoles did not show statistical differences in the morphometric parameters recorded, there was a difference in the BC index. The relationship between weight and TL of an individual as the BC index are originally non-lethal rates applied to fish species. It is assumed that within a species, larger animals are in better condition. This results in an indicator of the health status of the animal, in terms of energy reserve and the individual's ability to tolerate the effects of environmental stressors (Mayer et al., 1992), as it is in this case. Therefore, the lower BC recorded in parasitized organisms show a lower health status in these tadpoles caused by *L. cyprinacea* (Wood and Richardson, 2009; Babini et al., 2016).

There is a leukocyte response of the tadpoles of *L. catesbeianus* to the

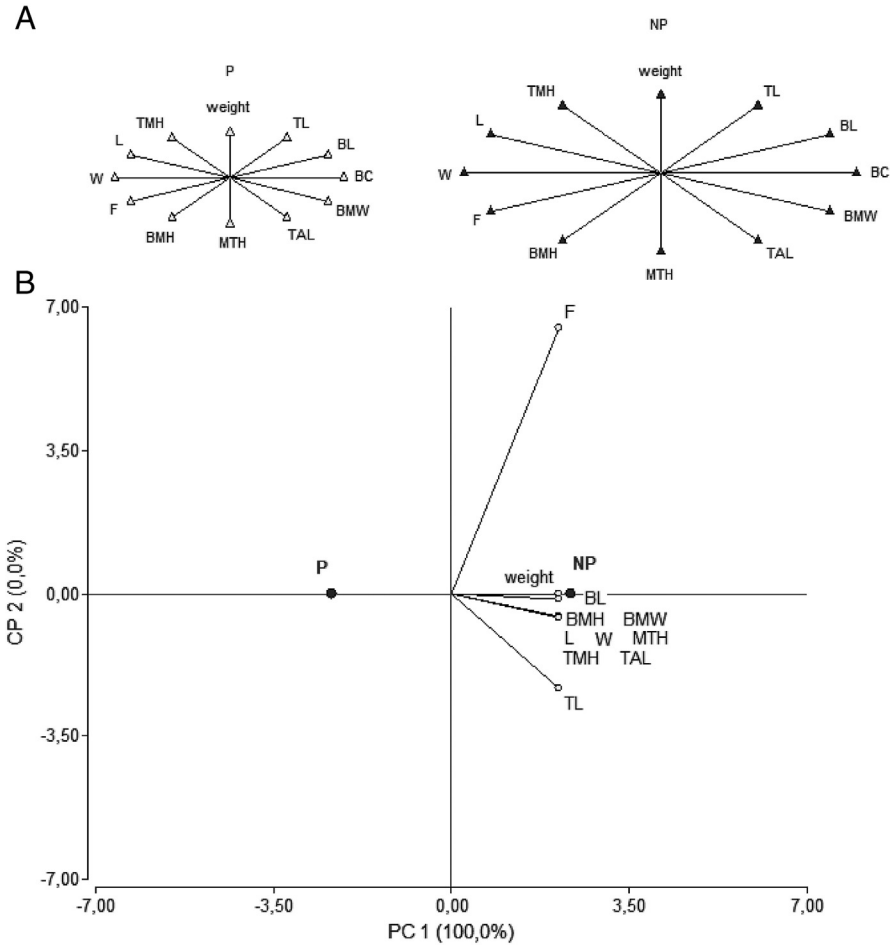

Fig. 3. A. Star graph with the morphometric parameters of tadpoles parasitized with *Lernea cyprinacea* (P) and not parasitized (NP). B. Biplot of morphometric parameters obtained by PCA of tadpoles parasitized (P) and not parasitized (NP). Morphometric parameters: Weigth; Total length (TL); Body length (BL); Body condition (BC); Body maximum width (BMW); Tail length (TAL); Maximum tail height (MTH); Body maximum height (BMH); Femur length (F); Shine bone length (W); Legs length (L); Tail muscle height (TMH); Eye diameter (ED).

parasitism of *L. cyprinacea*. The higher frequencies of immature and mature lymphocytes were recorded in P tadpoles. With respect to lymphocytes, the most frequent were the mature lymphocytes coinciding with Cabagna Zenklusen et al. (2011). According to Copete-Sierra (2013) in juvenile animals of some species, lymphocytes may be the predominant subpopulation within the differential leukocyte count. However, according to Panjvini et al. (2016) stress mechanisms such as the presence of parasites promote an increase in the percentage of lymphocytes.

Eosinophils are the primary cell responsible for protection against metazoan parasites (Belden and Kiesecker, 2005). Further, in anurans the eosinophils appeared directly associated with metamorphosis, generally increasing in abundance to a peak at metamorphic climax. This is due to their role in the process of tissue lysis during metamorphosis, which resembles their role in modulating inflammation responses (Jordan and Speidel, 1922, 1923; Ussing and Rosenkilde, 1995; Davis, 2009; Hota et al., 2013). However, in this study we recorded a lower frequency of eosinophils in tadpoles parasitized with *L. cyprinacea*. NP tadpoles would have an adequate frequency of eosinophils to reach their metamorphic climax. On the contrary, P tadpoles may be investing less resource in the production of these cells, affecting the metamorphosis process.

Furthermore, Davis (2009) found evidence that monocytes are involved in the final stages of metamorphosis and these act to rid of cellular debris. This would explain the higher frequency of monocytes that we recorded in NP tadpoles.

For other leukocytes, copepod infestations have induced alterations in the number of neutrophils in the blood of the hosts (Corrêa et al., 2016). However in this study, high frequencies of this leukocyte were not recorded in P tadpoles. Respect to high values of basophiles, the role in the immune system of amphibians is not clear (Salinas et al., 2015) and they do not appear to be involved in metamorphosis (Davis, 2009). These cells do vary greatly in abundance among amphibian species from as much as 57% of leukocytes in *Cynops pyrrhogaster* (Pfeiffer et al., 1990) to 1% in *Bufo alvarius* (Cannon and Cannon, 1979).

Finally, hematocrit was higher in P tadpoles, coinciding with values reported by Soberón et al. (2014) and Peña-Rehbein et al. (2013) in *Ligaria cuneifolia* and *Jodina rhombifolia*. In addition, the registered value was higher to the results found by Coppo (2003) in *L. catesbeianus* and lower to hematocrit reported by Corrêa et al. (2016) in fishes also parasitized by *L. cyprynacea*.

Parasitism by *L. cyprinacea* could be influencing the biology of American bullfrog both to individual and population levels. As seen in fish the infected individuals are not eliminated directly by the parasite, but it may open routes for secondary infection, growth retardation, behavioural changes, and finally, reduce the populations (Sayyadzadeh and Roudbar, 2014). That is to say, parasites negatively influence host fitness, and in response, hosts develop anti-parasitic defences, for example, a functional immune system, to reduce the fitness cost induced by parasitism (Peña-Rehbein et al., 2013). In fact, in amphibian these parasites tend to adhere to areas such as the mouth and cloaca, affecting their feeding and develop of hind legs. Furthermore, the epithelial of tadpoles compared to adults is more sensitive and wet in these areas, facilitating adhesion of parasites (Salinas et al., 2016). According to some studies, as parasite infestation increases, of host mortality also increases (Martins and Souza, 1996). A long-term monitoring of the parasites and amphibian in this basin is highly recommended (Sayyadzadeh and Roudbar, 2014). Consequently, a more exhaustive spatial and temporal study of the environmental conditions in which this parasite-host interaction is found, as well as the health status of the anuran individuals, would be necessary. These studies could provide relevant information to control the invasive species *L. catesbeianus*.

5. Conclusion

In conclusion, there is a leukocyte response of the tadpoles of *L. catesbeianus* to the parasitism of *L. cyprinacea*, evidenced by high frequencies of mature and immature lymphocytes in parasitized tadpoles. Eosinophils and monocytes were recorded at high frequencies in not parasitized tadpoles, which could be due to the important role played by these leucocytes in the metamorphosis of frogs. The results of the study not only represent an important contribution to knowledge of the immune response of anuran amphibians during parasitism, but also for invasive species such as *L. catesbeianus*. Because it is still consumed in the wild, it is essential to avoid consequences on human health. We recommend that the latest research in this direction do what is necessary to help maintain awareness of the health status of these edible species.

Declarations

Author contribution statement

Z.A. Salinas: Conceived and designed the experiments; Performed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

M.S. Babini: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

P.R. Grenat, F.G. Biolé: Conceived and designed the experiments; Wrote the paper.

A.L. Martino, N.E. Salas: Contributed reagents, materials, analysis tools or data.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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