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ASSESSING POPULATION HEALTH OF THE TOLUCA AXOLOTL *AMBYSTOMA RIVULARE* (TAYLOR, 1940) FROM MÉXICO, USING LEUKOCYTE PROFILES

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***Abstract*.—World-wide declines of amphibians have heightened the need for information relating to their health status and immune function under natural conditions. Evaluation of differential white blood cell (leukocyte) counts from thin blood smears is one way to gain this information, and this approach is increasingly being used by herpetologists to gauge the integrity of amphibian populations. This approach is especially useful in natural settings because amphibian leucocyte profiles can vary depending on biological and physiological processes, including those caused by environmental factors. In this study we examined, for the first time, the leukocyte profile of the Toluca Axolotl (*Ambystoma rivulare*) in Central México, for the purposes of evaluating population health. This species is poorly studied with respect to its life history and biology. We collected 163 individuals over a 6 mo time span from a population in the municipality of Amanalco de Becerra, in the state of México. From microscopic evaluation of blood smears from each individual, we determined the average leukocyte profile of this population to be 77.1% lymphocytes, 7.3% neutrophils, 12.1% eosinophils 2.3% basophils, and 1.2% monocytes. These values are similar to that reported for other members of this genus, so these counts could be considered normal. Moreover, the average ratio of neutrophils to lymphocytes (N/L ratio), which reflects stress levels in vertebrates, appeared especially low in this population compared to other Ambystomatid salamanders. Relative numbers of all cell types varied by month and there were also gender differences in the density of certain cells.**

Key Words.—amphibians; conservation; N/L ratios; physiology

INTRODUCTION

In many zoological disciplines, including herpetology, researchers are often tasked with conducting assessments of animal populations that are at risk or declining. While assessment of population size or animal abundance can be useful for this task, a secondary approach is to conduct non-invasive hematological assessments of animals in the population, which can provide information on the health state of animals. In all vertebrates, blood is responsible for important bodily functions like immune response, nutrient transport, transport of gases and metabolic wastes (Solis et al. 2007; Álvarez-Mendoza et al. 2011). Thus, any alteration of these parameters can indicate potential health problems in the animal, and by extension, problems with the surrounding environment.

Counts of the number of circulating immune cells (white blood cells, or leukocytes) are especially useful for gauging the immune status of animals, and are increasingly being used by researchers in a variety of conservation and/or research settings (Rohr et al. 2008; Shutler and Marcogliese 2011; Peterson et al. 2013; Das and Mahapatra 2014). In these situations the numbers of each of the five white cell types (the leukocyte profile) are typically ascertained from microscopic examination of thin blood films. Each cell type performs a different function in the innate immune system. Neutrophils are the first phagocytic leukocytes that act and proliferate in the circulation in response to infections, inflammations, and stress (Jain 1993; Thrall et al. 2006; Davis et al. 2008). Lymphocytes are involved in several immunological functions such as the production of

FIGURE 1. Toluca Axolotl (*Ambystoma rivulare*) from the study site in Mexico. (Photographed by Oswaldo Hernández-Gallegos).

immunoglobulin and modulation of the immune defense. Eosinophils act in inflammatory processes and are associated with the defense against metazoan parasites. Monocytes are associated with defense against infections and bacteria. Finally, the exact role of basophils is not yet clarified, but they are known to be implicated in the inflammation process (Jain 1993; Thrall et al. 2006).

Counts of specific leukocyte types present in circulation can also provide information concerning the overall stress levels of vertebrate animals (reviewed in Davis et al. 2008). This stems from the unique physiological effect of elevated stress hormones on certain subsets of the leukocyte population. Specifically, increases in stress hormones cause neutrophils to increase in circulation while lymphocytes tend to decline, leading to a rise in the ratio of these cells (neutrophil-to-lymphocyte, or N/L ratio). While there are aspects of this relationship that remain unclear, this metric has been demonstrated to depict elevated stress levels in a variety of animals, including amphibians, under laboratory and field conditions (Davis and Maerz 2008b, 2009; Davis and Maerz 2010; Davis and Maerz 2011; Shutler and Marcogliese 2011), making it a useful tool for population health assessments.

Health assessment of amphibian populations are becoming increasingly important because of global-scale declines in their populations due largely to diseases and habitat degradation (Stuart et al. 2004). In fact some species are declining even before they can be fully studied. Many species of salamanders within the Ambystomatidae family are examples of this. This family has two genera and 31 species, and these are distributed from southern Canada and Alaska into the Trans-Mexicana volcanic belt. Nineteen species of the genus *Ambystoma* are only found in Mexico, and most of these are poorly studied with respect to their life history, biology, and physiology (Bille 2009; Parra-Olea et al. 2012).

The Toluca Axolotl, *Ambystoma rivulare* (Fig. 1), is distributed in the states of México, Michoacán, and Guerrero (in the country of México), and it is a species defined by the IUCN Red List as Data-Deficient (Shaffer et al. 2008). It is considered microendemic because it is only found above 2,800 m elevation, mainly in the lotic ecosystems within Pine, Pine-Oyamel, and Pine-Oak forests (Huacuz 2001; Casas-Andreu et al. 2004). In a recent report by the Secretaria del Medio Ambiente y Recusos Naturales of México (Secretary of Environment and Natural Resources), its status was listed as threatened (SEMARNAT 2010). In this study we conducted a hematologic characterization (i.e., leukocyte assessment) of a population of *Ambystoma rivulare*, occurring in the Los Hoyos stream, within the Corral de Piedra watershed, which is located in the municipality of Amanalco de Becerra in the state of México. This study represents the first description of leukocyte profiles of this species, and these data should provide useful information for evaluating the health status of this population.

MATERIALS AND METHODS

Study site.—The study was conducted in the Los Hoyos stream, which is located in the basin of the

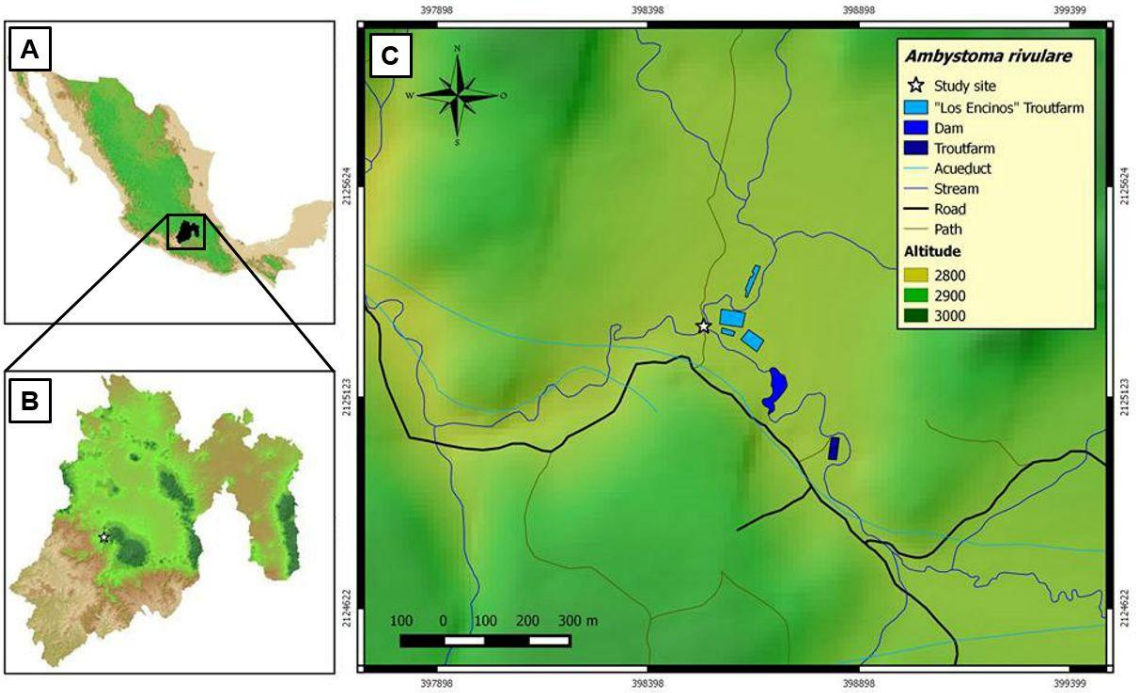

FIGURE 2. Map of the study site for the Toluca Axolotl (*Ambystoma rivulare*) where we collected salamanders. The study site was within the "Los Hoyos" stream, which is located in the Corral de Piedra Ranch property, Amanalco Becerra, State of Mexico.

Balsas, within the Corral de Piedra watershed (Fig. 2). This watershed is west of the state of México in the municipality of Amanalco de Becerra between coordinates 19°10′29′′N and 99°53′27′′W. This area represents the highest point of the natural Los Hoyos stream (Salinas 1999). At this site the average annual temperature is 13.4° C and the mean annual precipitation is 1,200 mm. The site where we collected most of the salamanders was in the Los Encinos trout-farm drainage system (Fig. 2).

Capturing salamanders.—We sampled between the months of October 2011 and May 2012, capturing between 20 and 30 individuals per month (except April). This time frame included the dry and rainy seasons of this region. We captured salamanders using aquatic nets and we temporarily placed all specimens in 20 L plastic containers filled with stream water. Shortly thereafter, we collected blood samples from each individual (below) and we recorded weight, length snout-vent (SVL), tail length, sex, and time of capture.

We collected a small blood sample (less than 1 ml) from each individual through a caudal vein puncture using a 3 ml heparinized syringe (23G needle) following Homan et al. (2003). We prepared thin blood films from the sample and we let them air-dry. Blood films were later stained with Wright stain for microscopic evaluation. We recorded the time from capture to blood sampling for all individuals. The average time from capture to blood sampling of the salamanders for the project was 55 min. We released all salamanders at the site of capture.

Blood smear analysis.—In the lab, we examined all blood films with a light microscope to obtain a differential leukocyte count for each salamander. We identified leukocytes based on morphological description given in other studies (e.g. Friedmann 1970; Davis and Durso 2009; Davis and Maerz 2010; Das and Mahapatra 2014). Counting was performed by a single person to avoid observer variation, using 400 × magnification, and the slide was moved in a zigzag pattern under the microscope without looking at the sample. We considered only fields with even distribution of cells (erythrocytes and leukocytes). We counted at least 100 leukocytes for each individual. From these data we calculated the percentage of each leukocyte type, as well as the ratio of neutrophils to lymphocytes (N/L ratio), which is an indicator of stress in amphibians and other vertebrates (Davis et al. 2008).

Data analysis.—While the primary goal of this study was to obtain baseline data on leukocyte profiles of this population (i.e. to generate average values of all cell types), we also evaluated the degree of seasonal (i.e., monthly) and sexual variation in the cell types, as well as

TABLE 1. Results of statistical analysis examining factors influencing the proportions of all types of leukocytes (except monocytes, due to their low abundance) and N/L ratios for *Ambystoma rivulare*. N/L is the ratio of neutrophils to lymphocytes, an indicator of stress (Davis et al. 2008). All cell data were transformed prior to analyses (arcsin-squareroot).

DEPENDENT	INDEPENDENT	DF	MS	*F*	*P*
LYMPHOCYTES	Month	6	0.40	56.78	< 0.001
	Sex	1	0.13	18.88	< 0.001
	SVL	1	0.01	0.84	0.361
	Month*Sex	6	0.02	2.95	0.010
	Error	149	0.01		
NEUTROPHILS	Month	6	0.02	5.09	0.001
	Sex	1	0.00	0.99	0.322
	SVL	1	0.00	0.00	0.966
	Month*Sex	6	0.01	2.12	0.055
	Error	149	0.00		
BASOPHILS	Month	6	0.03	10.49	< 0.001
	Sex	1	0.00	0.32	0.573
	SVL	1	0.00	0.11	0.740
	Month*Sex	6	0.00	0.17	0.985
	Error	149	0.00		
EOSINOPHILS	Month	6	0.43	76.12	< 0.001
	Sex	1	0.16	29.16	< 0.001
	SVL	1	0.01	1.97	0.163
	Month*Sex	6	0.01	2.40	0.031
	Error	149	0.01		
N/L RATIO	Month	6	0.09	12.93	< 0.001
	Sex	1	0.04	6.03	0.015
	SVL	1	0.00	0.35	0.553
	Month*Sex	6	0.03	4.48	< 0.001
	Error	149	0.01		

the possible effect of body size (Davis and Maerz 2011). All cell count data, including N/L ratios, were arcsin-squareroot transformed to approximate normal distributions. Then we used analysis-of-covariance to simultaneously examine the effects of these three parameters on the (transformed) percentage of each cell type (response variables). In each test, sex (male or female) and month were independent variables and SVL was a covariate. We also included the sex*month interaction term. This test was performed on all cell types except monocytes, due to their low abundance, and including the N/L ratio. All analyses were done using the Statistica 12.0 software program, and tests were considered significant if $P < 0.05$.

RESULTS

Salamander collection.—We captured 164 individuals (111 females and 53 males) for this project with 21–27 individuals captured each month. The average SVL for the population was 90.4 ± 6.34 mm (SD) with maximum SVL of 112 mm and a minimum of 74 mm. The average weight was 43.56 ± 7.95 g with a maximum of 63 g and a minimum of 21 g.

Blood cell results.—We completed leukocyte counts on 163 of the 166 captured individuals. Of these, the average for each cell type was as follows: 77.4% (13.2 SD) lymphocytes, 7.9% (3.6 SD) neutrophils, 11.2% (10.6 SD) eosinophils, 2.3% (2.1 SD) basophils, and 1.2% (0.9 SD) monocytes. The average N/L ratio for this collection was 0.11 (0.1 SD). The relative abundance of each leukocyte varied significantly by month ($P < 0.001$ for all; Table 1). We note that although there was statistically significant monthly variation in all cell types, it is clear that the largest variation occurred primarily in the counts of lymphocytes and eosinophils (Fig. 3). Lymphocyte counts were notably lower and eosinophils higher during January and February. Counts of other cell types varied between 2 to 10% throughout most months. Moreover, we also found significant monthly variation in N/L ratios ($F_{6,149}$=12.93, $P < 0.001$; Table 1).

In terms of sexual variation, we detected significant differences in counts of lymphocytes and eosinophils between males and females (Table 1), although there were also significant sex*month interaction terms in

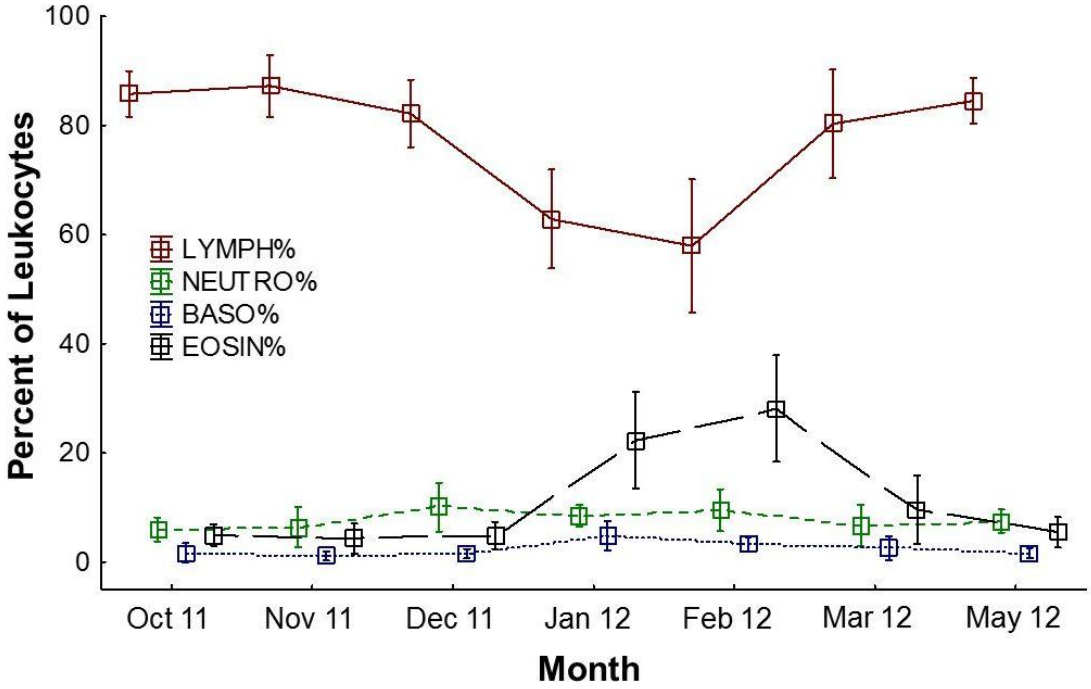

FIGURE 3. Seasonal distribution of leukocyte profiles of the Toluca Axolotl (*Ambystoma rivulare*) from Mexico. Each line depicts the average percentage of a cell type (monocyte numbers not shown). Note that no salamanders were examined in April 2012. Whiskers represent one standard deviation.

these models suggesting that the magnitude of sexual differences depended on the month of capture. Males tended to have more eosinophils than females ($F_{1,149}$=29.16, $P < 0.001$) and females tended to have more lymphocytes than males ($F_{1,149}$=18.88, $P < 0.001$). There was also a significant sexual difference in N/L ratios ($F_{1,149}$=6.03, $P = 0.015$), with males tending to have slightly higher average values (mean = 0.12, SD = 0.09) than females (mean = 0.10, SD = 0.06).

DISCUSSION

The collective results from this hematological investigation indicate the population of Toluca axolotls we examined appeared to be in optimal health. This is based on our interpretation of their leukocyte profiles, and from comparison to other published reports of salamanders in this genus (Table 2). For example, the relative number of neutrophils was low in this population, which by itself is very informative; this cell responds to infections and stress (Thrall 2004; Allender and Fry 2008; Davis et al. 2008; Davis et al. 2010) so lower numbers in circulation reflect a lack of these issues. Furthermore, low counts of monocytes (also a phagocytic cell) also reflect the absence of infections (Wright 2001). Finally, the ratio of neutrophils to lymphocytes in this population (0.11) was the lowest of all studied ambystomatid salamanders thus far, which suggests individuals in this population are not exposed to stress-inducing situations such as chemical contaminants or other anthropogenic pressures. From prior studies of related ambystomatid salamanders and other amphibians, it has become clear that most unstressed populations or groups of individuals tend to have average N/L ratios close to 0.30 (Davis and Durso 2009). Also, when amphibians in such populations become stressed, their average N/L ratios increase by a factor of three (Davis and Maerz 2011), so that stressed individuals or populations would have N/L ratios that are closer to 1.0 or greater. The maximum N/L ratio we observed in this population was only 0.45, which further suggests the absence of stressors to these individuals.

In general, the many ambystomatid salamanders that are endemic to Mexico are not well-studied as a group, and this is especially true with respect to their hematology. Given that this was the first hematological investigation of *A. rivulare*, our basic observations of its cell morphology would be useful for comparative purposes. The appearance of leukocytes in this species generally was consistent with that reported from other amphibians (Friedmann 1970; Pfeiffer et al. 1990; Allender and Fry 2008): lymphocytes were spherical with a large nucleus. Neutrophils were larger than lymphocytes with multilobulated nucleus and pink cytoplasm. Eosinophils were large, rounded cells with bilobed nuclei and reddish cytoplasmic granules.

TABLE 2. Summary of leukocyte profile data obtained for the Toluca Axolotl (*Ambystoma rivulare*) population in this study, along with published and unpublished data from other related species for comparison. Shown are the average cell counts (% of all leukocytes). When possible, only values from wild-collected or otherwise unstressed individuals are shown from comparison datasets. The number of individuals sampled is n, and N/L is the ratio of neutrophils to lymphocytes, an indicator of stress (Davis et al. 2008). For the study by Ussing and Rosenkilde (1995), all individuals in this group were sampled while being induced to metamorphose in a laboratory.

Species	n	Lymphocytes	Neutrophils	Eosinophils	Basophils	Monocytes	N/L	Source
A. rivulare	163	77.4	7.9	11.2	2.3	1.2	0.11	This study
A. maculatum	10	31.7	18.1	25.5	24.2	0.6	0.57	Davis and Maerz 2009
A. maculatum	15	40.2	14.6	29.7	15.1	0.2	0.44	Davis 2012
A. mexicanum	7	20.1	21.7	52	4.9	1.0	1.07	Ussing and Rosenkilde 1995
A. mexicanum	1	63.0	22.0	15.0	0.0	0.0	0.35	Wright 2001
A. talpoideum	34	41.5	12.7	45.7	0.0	0.2	0.31	Davis and Maerz 2008b
A. opacum	45	61.0	13.6	8.5	15.8	1.0	0.26	Davis and Maerz 2011
A. tigrinum	1	46.5	14	23.3	16.3	0.0	0.30	Andrew Davis, unpubl. data

Basophils had a darkened nucleus with purple granules in the cytoplasm, and monocytes were large pleomorphic cells with no cytoplasmic granules. In addition to the cell morphology, the leukocyte profile data gathered in this study should also prove useful to other researchers from a comparative standpoint (i.e., as comparative baseline data from a wild population). Such comparative data is becoming increasingly valuable for helping to interpret hematological data obtained in other research projects. In fact, it is only from prior compilations of published leukocyte data of amphibians that the normal values for all of their white blood cells have been realized (Davis and Durso 2009).

This study also adds to the body of knowledge on the nature of circulating leukocytes in amphibians by demonstrating their inherent temporal variation. In this population, the counts of all cell types varied significantly by month. Most studies on amphibian hematology have generally not accounted for the seasonal nature of immune cell distributions. Some exceptions include studies of seasonal trends in erythrocyte and leukocyte abundance in certain frogs (Harris 1972) and toads (Liu et al. 2013). The seasonal nature of the reproductive period is one factor that could affect hematology; in a related species (*A. talpoideum*), reproductively active adults experience an increase in the neutrophil-lymphocyte ratio (Davis and Maerz 2008a). This is likely caused by elevated levels of stress hormones during the reproductive season (Moore and Jessop 2003). Seasonal variations in the environment is another influence. For example, the Corral de Piedra watershed area has a marked seasonality (drought between the months of November to May and rain from June to September), which affects water flow during these times, although the effects of varying water flow on the amphibian community in this region are not known.

Other results from this project also improve current knowledge of amphibian immune systems, such as the sex-based differences in specific cell counts of *A. rivulare*. Males had relatively more eosinophils and fewer lymphocytes than did females. Hematologic variations due to gender have not been well-studied in amphibians; or when these have been examined, few differences have been found (Forbes et al. 2006; Cabagna Zenklusen et al. 2011). One exception is that breeding female *A. talpoideum* tend to have higher N/L ratios than males (Davis and Maerz 2008a), and in certain tree frogs there have been sexual differences in cell abundance (Das and Mahapatra 2014). We do point out, however, that the magnitude of the sexual differences we found in our collection were small (such as the minor difference in N/L ratios), so these differences may or may not be biologically meaningful.

Finally, it should be reiterated that at a global level, there are many amphibian populations that are currently experiencing rapid declines as a result of habitat degradation, disease, or environmental pollution at a

global level (Stuart et al. 2004). The immune system of amphibians is sensitive to most of these scenarios (Christin et al. 2004; Cabagna et al. 2005; Forson and Storfer 2006; Vatnick et al. 2006; Rohr et al. 2008). For this reason, monitoring populations for abnormalities in immune parameters can provide an early warning of potential threats to the population. Moreover, the establishment of baseline data on an apparently healthy population, such as those obtained in this study, can be especially valuable for evaluating effects of any adverse conditions the population faces in the future.

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