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ORIGINAL ARTICLE

Hematologic parameters and the effect of hemoparasites of wild anurans in Northern Sinaloa, Mexico

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Abstract

Background: Few hematologic profiles for free-ranging amphibians are available. Hematologic evaluation is a useful tool for determining the health of amphibian populations and providing further knowledge for conservation actions.

Objectives: Hematologic variables and the presence and effect of hemoparasites in anuran species were evaluated in Northern Sinaloa, Mexico.

Methods: Blood samples were collected from wild anurans of eight species to perform blood cell counts, leukocyte differential counts, and serum protein concentrations using manual methods and refractometry. In addition, morphologic identification and quantification of the hemoparasites were performed on blood smears.

Results: Differences were observed by sex, age, and season for the hematologic values of *Incilius alvarius* (n = 23), *Incilius mazatlanensis* (n = 46), *Rhinella horribilis* (n = 64), *Leptodactylus melanonotus* (n = 46), *Lithobates forreri* (n = 135), *Lithobates catesbeianus* (n = 20), *Smilisca fodiens* (n = 42), and *Scaphiopus couchii* (n = 7). Intra- and extra-erythrocytic hemoparasites were found in 56.2% of amphibian hosts; the hemoparasite infection of *R. horribilis* and *L. melanonotus* was higher in the dry season, showing increases in erythroplastids and monocytes. For *L. forreri*, males were more infected than females, and increases in leukocytes were associated with infections of different types of hemoparasites species.

Conclusions: Hematologic values, hemoparasite prevalence, and the response to hemoparasite infection vary among amphibian species, sex, and age, as well as on season and hemoparasite type. This highlights the importance of hematologic evaluations in wild amphibian populations to determine the subclinical effects of hemoparasite infections.

KEYWORDS
amphibian, hemogregarines, microfilaria, *Trypanosoma*

1 | INTRODUCTION

Amphibians are one of the vertebrate taxa most threatened with extinction due to their biological characteristics and life cycle. In addition, they may serve as important sentinels of ecological health.[1] The evaluation of amphibian health could help in the detection of environmental toxins and contamination[2,3]; however, there are other factors that could affect the health status of wild amphibians, such as the presence of infectious agents. Despite the information on the clinical pathology of wildlife, amphibian hematology profiles are

not always available, particularly from free-ranging animals.[1] Most hematologic profiles are primarily obtained from captive animals.[1,2] However, these profiles are not sufficiently reliable because of the small sample size and differences in biological (ie, age or sex) or environmental (ie, captivity or climate) conditions.[1,2,4-7]

Hematologic analysis provides information on amphibian health, indicating physiologic processes, the presence of diseases, nutritional status, and the effects of toxins.[3] Some of these changes are primarily observed in hematologic analysis, indicating subclinical physiologic processes.[3,8] Blood parameters, including erythrocytes, hematocrits, and erythroplastids, indicate the oxygenation capacity or changes in the oxygenation efficiency,[8–11] while the leukogram may provide information on several conditions, including inflammation, infection, or stress responses.[5,12,13] Most hematologic parameters reported in amphibians come from the most abundant and morphologically large species,[7,14–19] while some threatened species (small or rare) are left behind.

The aim of this study was to provide information on the hematologic parameters and the effects of hemoparasites on these values for several wild amphibian species from Northern Sinaloa, Mexico.

2 | MATERIALS AND METHODS

2.1 | Study site

Free-raging anurans were captured monthly (from February to November 2021) from different areas in the municipalities of Guasave and Ahome in Northern Sinaloa, Mexico. Amphibians were collected at night between 22:00 and 01:00 h, near the water bodies of each site, using a hand net.[19] Handling was made with nitrile gloves, and each anuran was placed in an individual plastic container, which was marked with the site of collection. To prevent cross-contamination in the processing area, amphibians were ordered by site and species for sampling, and a Benzalkonium chloride solution was used after handling. All procedures were approved by the Institutional Animal Care and Use Committee, Universidad Nacional Autónoma de México (Protocol No.: 113) and under the Scientific Permit SGPA/DGVS/05264/21.

2.2 | Sample collection and clinical examination

Morphometric measurements were recorded for each individual, including body weight (W, in g), and snout-to-vent length (SVL, in cm). When possible, each species was differentiated by sex according to sexual dimorphism characters, and according to the SVL, they were categorized as adults or juveniles. In addition, a physical examination was performed by a qualified veterinarian for the identification of lesions or abnormalities. Blood samples were collected from a facial vein puncture[8] using 23G to 27G needles according to the size of the individual. Blood was collected in a capillary tube of 75 µL (Corning Mexicana), and blood volumes were between 15 to 500 µL according to weight.[13] A fresh blood smear was made and then air-dried. The blood was placed into EDTA microtainer tubes (Sarstedt, EDTA KE/1.3) until the amount of blood was sufficient to provide the correct EDTA ratio. When samples were small, blood was placed into heparinized cryovials.[13,20] Once all samples were taken, all amphibians were freed at the site of collection.

2.3 | Hematologic analysis

Blood smears were fixed and stained with a Wright stain (SEALAB Instant Wright, IWRC-150, Lot: 3693086), mounted with Entellan (R; Hycel), and analyzed under a compound microscope (Steindorff S-1100, Mel Sobel Microscopes Ltd.). Differential leukocyte counts were performed by counting 100 leukocytes,[20,21] while erythrocyte morphology, the number of immature erythrocytes, and erythroplastids (anucleate erythrocytes) were obtained by counting in 100 fields at 40×. The Lobularity Index was calculated by dividing the number of neutrophil nuclear lobes by the number of neutrophils counted in 100 fields at 40×.[17] The Neutrophil/Lymphocyte Ratio (N/L) was calculated by dividing the number of neutrophils by the number of lymphocytes counted in the differential count.[5,21]

Microhematocrits were performed using a capillary tube (Minicaps 10 II; Hirschmann, Germany) and centrifuged at 6900*g* (LW Scientific ZIP Combo) for 5 min. Total proteins were measured using a refractometer (Kitlab rhc200). When the blood sample was more than 15 µL, red blood cells (RBC) and white blood cells (WBC) were counted manually using Natt-Herrick's solution and a modified Neubauer hemocytometer.[13]

2.4 | Hemoparasite detection

Hemoparasite structures were identified according to their morphologic characteristics following previous reports on hemoparasites in amphibians.[1,5,6,11,13] The identified parasite structures were counted from 100 fields at 40× magnification to obtain the intensity of infestation, and photographic records were obtained (Leica DM750).

2.5 | Statistical analysis

Reference limits of the hematologic values were calculated by the mean ± standard deviation (SD) of each variable overall and by groups (sex, age, and season) of each species. The distribution of each hematologic and morphologic variable was proved using Shapiro–Wilk. The nonparametric Kruskal Wallis tests were used for intra-species comparisons (between sex and age). For hemoparasites, prevalence was calculated as the number of infected hosts from the total host number, and the intensity of infection was calculated as the total number of hemoparasites in 100 fields at 40× magnification.[19] Contingency tables were performed for intra-species comparisons and prevalence of hemoparasites, considering all hemoparasites genera as a group

and each genus individually. Thereafter, the Kruskal–Wallis test was used to determine the effect of hemoparasites on the blood values. For all statistical analyses, R Commander (The R Foundation for Statistical Computing, R Commander 2.2–5, 2015) and Epidat software were used, and the significance was set at $P<0.05$.

3 | RESULTS

3.1 | Clinical examination

A total of 383 individuals were captured, corresponding to five families and eight species of anurans: Bufonidae (*Incilius alvarius*, n = 23; *Incilius mazatlanensis*, n = 46; and *Rhinella horribilis*, n = 64), Leptodactylidae (*Leptodactylus melanonotus*, n = 46), Ranidae (*Lithobates forreri*, n = 135, and *L. catesbeianus*, n = 20), Hylidae (*Smilisca fodiens*, n = 42), and Scaphiopodidae (*Scaphiopus couchii*, n = 7).

No sexual dimorphism was observed for *I. alvarius* and *R. horribilis*, and no juvenile individuals were collected. Morphometry by age and sex of each species were calculated, and statistical differences in SVL and W are listed in Table 1. Differences between sex (bigger females) were observed in only three species of which two are bufonids. Differences by age (bigger adults) were observed in six species and marginal differences were seen in *S. couchii* ($P=0.051$). Only four species were captured during rainy seasons (*I. alvarius*, *I. mazatlanensis*, *S. fodiens*, and *S. couchii*), while the two ranids, one toad and leptodactylid frogs were captured during both seasons. Higher densities of *L. melanontus*, *L. forreri*, and *L. catesbeianus* were observed during rainy seasons ($P=0.000$,

TABLE 1 Physiologic measurements (mean ± SD) by sex and age for eight species of anurans from North Sinaloa, Mexico.

Anuran	Adult	Juvenile	*P*	Female	Male	*P*
Incilius alvarius						
N	17	6 (<10.5)*				–
SVL	13.71 ± 3.42	9.05 ± 2.03	0.005			
W	301.56 ± 171.12	66 ± 41.28	0.002			
Incilius mazatlanensis						
N	35	11 (<6)*		12	21	
SVL	7.4 ± 0.82	5.1 ± 0.58	0.000	7.61 ± 0.98	7.15 ± 0.4	0.026
W	42.97 ± 13.99	13.09 ± 4.99	0.000	52.58 ± 16.17	36.48 ± 8.19	0.006
Rhinella horribilis						
N	64		–	6	57	
SVL	13.94 ± 1.7			16.13 ± 1.99	13.71 ± 1.51	0.011
W	93.62 ± 54.6			490 ± 146.31	204.42 ± 64.96	0.000
Leptodactylus melanonotus						
N	31	15 (<3.2)*		13	18	
SVL	4.01 ± 0.47	2.63 ± 0.42	0.000	4.27 ± 0.56	3.83 ± 0.3	0.017
W	5 ± 1.68	1.6 ± 0.63	0.000	6.17 ± 1.75	4.22 ± 1.11	0.003
Lithobates forrer						
N	109	26 (<6)*		55	54	N = 55
SVL	9.46 ± 1.33	4.99 ± 0.87	0.000	9.7 ± 1.46	9.22 ± 1.16	
W	82.72 ± 48.82	11.69 ± 6.31	0.000	90.95 ± 64.58	74.33 ± 21.58	
Lithobates catesbeianus						
N	8	12 (<7)*		3	5	
SVL	17.01 ± 2.36	4.43 ± 1.82	0.002	17.53 ± 1.56	16.7 ± 2.86	
W	452.88 ± 168.04	10.83 ± 14.08	0.002	557 ± 179.76	390.4 ± 142.31	
Smilisca fodiens						
N	23	19 (<3)*		2	3	
SVL	5.18 ± 1.66	2.38 ± 0.2	0.000	8.65 ± 3.46	5 ± 0.8	
W	12.09 ± 19.41	1.11 ± 0.32	0.000	58.5 ± 55.86	6.33 ± 2.31	
Scaphiopus couchii						
N	2	5 (<5.5)*		1	1	
SVL	6.5 ± 0.99	4.42 ± 0.68	0.051	5.8	7.2	
W	21.5 ± 4.95	7.8 ± 3.96	0.051	18	25	

Abbreviations: N, number; *P*, Significant differences with Kruskal Wallis; SVL, Snout-Vent Length (cm); W, weight (g).

* SLV in cm for age differentiation.

$P=0.000$, $P=0.025$), and for *L. catesbeianus*, more juveniles were seen in this season ($P=0.035$).

There were only 4.7% (18/383) individuals with lesions, including scars, absence of phalanges, limb absences, and deformities, such as digital protuberances (cutaneous grown shorter than the proximal digit) or duplications, small protuberances among the eyes (co-ossified cutaneous grown), and microphthalmia. The presence of lesions by species was 10% (2/20) for *L. catesbeianus*, 7.8% (5/64) for *R. horribilis*, 7.1% (3/42) for *S. fodiens*, 4.4% (1/23) for *I. alvarius*, 3.7% (5/135) for *L. forreri*, and 2.2% (1/46) for *I. mazatlanensis* and *L. melanonotus*.

3.2 | Hematology

The hematologic values for each species were established by age (Table 2) and sex (Table 3). Lymphocytes were the most abundant blood cell type, representing over 60% of leukocytes, followed by neutrophils, eosinophils, basophils, and monocytes. Some differences were detected among ages: juvenile *I. alvarius* had higher immature erythrocytes ($P=0.03$) than adults, and adult *I. mazatlanensis* showed higher counts of relative eosinophils ($P=0.038$) and monocytes ($P=0.048$) than juveniles. Adult *L. forreri* had higher total protein concentrations ($P=0.031$) than juveniles, and *L. catesbeianus* adults showed lower lobularity indices ($P=0.023$), and higher neutrophil/lymphocyte ratios ($P=0.028$) and relative neutrophil counts ($P=0.002$) than juveniles. Differences by sex were detected in male *R. horribilis* with higher relative and total lymphocyte counts ($P=0.024$, $P=0.021$) and lower relative neutrophils counts ($P=0.013$) and neutrophil/lymphocyte ratios ($P=0.014$) than females. Females of *Leptodactylus melanonotus* had higher total protein concentrations ($P=0.032$) and those of *L. forreri* had higher hematocrits ($P=0.030$) and lower relative and total monocyte counts ($P=0.000$ and $P=0.023$) than males.

For those species captured in different seasons (rainy and dry), differences were detected in *R. horribilis* for the rainy season compared with the dry season, with higher relative counts of basophils ($P=0.038$) and eosinophils ($P=0.045$), hematocrits ($P=0.033$), and total protein concentrations ($P=0.010$) and lower erythroplastids ($P=0.047$) and RBC counts ($P=0.006$). For *L. melanonotus*, in the rainy season, lower relative counts of monocytes ($P=0.037$) were observed compared with the dry season.

3.3 | Hemoparasites

Among the hemoparasites found, intraerythrocytic structures were seen, including *Hepatozoon*, *Hemolivia*, *Lankesterella*, and Haemogregarine-like organisms. Unidentified inclusions and extraerythrocytic structures, such as *Trypanosoma* and microfilaria, were also found (Figure 1). Overall, hemoparasite prevalence was

TABLE 2 Hematologic values (mean ± SD) for adults and juveniles for eight species of anurans from North Sinaloa, Mexico.

Species	*Incilius alvarius*		*Incilius mazatlanensis*		*Rhinella horribilis*	*Leptodactylus melanonotus*	
	A (n = 11)	J (n = 3)	A (n = 20)	J (n = 6)	A (n = 58)	A (n = 23)	J (n = 4)
Ht (L/L)	32.6 ± 3.67	0.67 ± 0.58	33.99 ± 10.97	26.8 ± 7.47	36.41 ± 14.9	27.46 ± 14.58	13.23 ± 1.61
TP (g/dL)	6.63 ± 1.08	5 ± 2.46	3.46 ± 1.52	2.34 ± 1.03	231.62 ± 112.6	2.25 ± 1.4	1.2 ± 0.85
N (%)	21.55 ± 18.3	19 ± 1.73	11.4 ± 7.22	9.67 ± 2.73	20.97 ± 8.03	53.74 ± 38.78	57.75 ± 66.09
L (%)	66.82 ± 16.8	75.33 ± 3.06	68.75 ± 16.11	80 ± 9.74	71.31 ± 9.83	81.57 ± 8.68	85 ± 5.72
B (%)	3.64 ± 3.67	0.67 ± 0.58	4.1 ± 6.14	3.67 ± 3.14	1.48 ± 2.76	0.17 ± 0.49	0.25 ± 0.5
E (%)	7.73 ± 5.08	5 ± 4.36	10.6 ± 5.31	6.33 ± 7.55	5.9 ± 4.54	1.7 ± 1.69	2 ± 2.16
M (%)	0.27 ± 0.65	0 ± 0	1.3 ± 1.08	0.33 ± 0.52	0.41 ± 0.82	0.39 ± 0.89	1.5 ± 2.38
N/L	0.52 ± 0.92	0.25 ± 0.01	0.2 ± 0.18	0.12 ± 0.05	0.31 ± 0.16	0.21 ± 0.14	0.13 ± 0.04
LI	0.44 ± 0.02	0.44 ± 0.02	0.57 ± 0.09	0.51 ± 0.05	0.45 ± 0.05	0.47 ± 0.04	0.49 ± 0.05
IE	21.82 ± 40.87	40 ± 16.64	160.4 ± 194.22	145.17 ± 166.67	27.16 ± 97.72	18.13 ± 22.64	68.75 ± 117.74
Ep	1.73 ± 1.42	3.33 ± 2.08	21.3 ± 25.79	9 ± 4.47	1.83 ± 3.94	5.09 ± 3.98	9 ± 12.19
	N = 5	N = 2	N = 1	–	N = 25	N = 7	–
RBC ($\times 10^{12}$/L)	0.67 ± 0.07	0.68 ± 0.18	0.67	–	0.4 ± 0.23	0.98 ± 0.14	–
WBC ($\times 10^{9}$/L)	10.12 ± 2.89	8.97 ± 2.57	1.76	–	8.68 ± 4.06	5.37 ± 2.09	–
N ($\times 10^{9}$/L)	2.18 ± 1.36	1.79 ± 0.51	0.16	–	1.78 ± 1.15	0.88 ± 0.45	–
L ($\times 10^{9}$/L)	6.67 ± 1.92	6.92 ± 2.1	1.43	–	5.88 ± 3.2	4.31 ± 1.89	–
B ($\times 10^{9}$/L)	0.24 ± 0.41	0.04 ± 0.05	0.04	–	0.16 ± 0.34	0.02 ± 0.05	–
E ($\times 10^{9}$/L)	0.94 ± 0.7	0.22 ± 0.0	0.14	–	0.56 ± 0.51	0.15 ± 0.16	–
M ($\times 10^{9}$/L)	0.03 ± 0.06	0 ± 0	0	–	0.03 ± 0.07	0.01 ± 0.02	–

Abbreviations: A, adult; B, basophil; E, eosinophil; Ep, erythroplastid; IE, immature erythrocyte; J, juvenile; Ht, hematocrit; L, lymphocyte; LI, lobularity index; M, monocyte; N/L, neutrophil/lymphocyte ratio; N, neutrophil; RBC, red blood cells; TP, total protein; WBC, white blood cells.

56.2% (131/233), and *Hepatozoon* was the most prevalent organism at 24.9% (58/233), followed by haemogregarine-like structures at 21.5% (50/233). The prevalence of extracellular hemoparasites, microfilaria, and *Trypanosoma* was 16.7% (39/233) and 12% (28/233), respectively. The less prevalent hemoparasites were inclusions, *Hemolivia*, and *Lankesterella*, with a prevalence of 6.4% (15/233), 5.6% (13/233), and 0.9% (2/233), respectively. Microfilaria were only found in *L. forreri*, and no hemoparasites were detected in *L. catesbeianus and Scaphiopus couchii*. In addition, *L. forreri* had a greater number of parasite species found, with a higher prevalence than the other anurans (Table 4). Co-infections were detected in only two amphibians; one *L. melanonotus* (1/7) individual had a co-infection with *Trypanosoma*, haemogregarines, and inclusions, and for *L. forreri* (48/80), 29 individuals had two parasites, 13 had three parasites, and 6 had four parasites. The most frequent co-infections were *Hepatozoon*-microfilaria (n = 13), Haemogregarine-microfilaria (n = 7), *Trypanosoma*-Haemogregarine (n = 7), and *Trypanosoma*-Haemogregarine (n = 4); the remaining co-infections (n = 17) were present in three or fewer anurans.

Differences in hemoparasites infections among ages were also seen. Adult *I. mazatlanensis* had a greater number of infections with hemoparasites ($P = 0.042$), and adult *L. forreri* had a higher prevalence of *Hepatozoon* ($P = 0.009$), and microfilariae ($P = 0.019$), and a higher prevalence and total hemoparasite counts ($P = 0.002$, $P = 0.007$) then their juvenile counterparts. But for *L. melanonotus*, higher prevalences and total counts of Haemogregarines were seen in juveniles ($P = 0.015$, $P = 0.027$) than in adults. Differences regarding infections among sexes were only seen in the *L. forreri* haemogregarine infections, with males having greater infection rates than females ($P = 0.043$). Seasonal effects on the infections were observed in *R. horribilis* and *L. melanonotus*, with high prevalences ($P = 0.008$, $P = 0.011$) and total hemoparasite counts ($P = 0.030$, $P = 0.033$) in the dry season compared with the wet season.

3.4 | Effect of hemoparasites on blood parameters

Only *I. alvarius* showed no hematologic variations among infected and noninfected individuals for the six species infected with hemoparasites. Hemoparasite infections increase the relative eosinophil counts in *I. mazatlanensis* ($P = 0.031$). For *R. horribilis*, higher numbers of erythroplastids ($P = 0.008$) and lower total protein concentrations ($P = 0.006$) were detected compared with the other anurans. Differences in specific parasite effects were detected in *L. melanonotus* and *L. forreri* individuals infected with Haemogregarine. There were higher relative counts of monocytes ($P = 0.037$) for the *L. melanonotus* and higher relative and total counts of basophils ($P = 0.018$, $P = 0.008$), total lymphocytes ($P = 0.044$), relative monocytes ($P = 0.015$), and WBC counts ($P = 0.015$) for *L. forreri*. For individuals with inclusions, infected *L. melanonotus* and *L. forreri* showed

Lithobates forreri		*Lithobates catesbeianus*		*Smilisca fodiens*	*Scaphiopus couchii*
A (n = 89)	J (n = 15)	A (n = 8)	J (n = 6)	A (n = 14)	A (n = 2)
35.86 ± 15.2	27.48 ± 11.43	25.84 ± 11.73	25.79 ± 12.03	28.61 ± 19.3	18.6 ± 4.28
4.85 ± 2.74	1.9 ± 2.24	3.34 ± 1.73	2	3.06 ± 3.57	3.4 ± 0.85
13.29 ± 7.16	14 ± 9.97	28.25 ± 9.22	12.67 ± 2.73	16.79 ± 15.31	22 ± 8.49
69.64 ± 10.75	72.14 ± 9.26	60 ± 9.97	68.83 ± 12.06	77 ± 17.14	77.5 ± 9.19
5.11 ± 5.66	5.14 ± 6.18	8.25 ± 7.03	8.17 ± 9.87	0.57 ± 2.14	0 ± 0
10.71 ± 6.34	7.86 ± 5.49	2.88 ± 2.42	9.67 ± 8.82	5.43 ± 7.89	0.5 ± 0.71
1.16 ± 2.16	0.57 ± 0.53	0.63 ± 0.92	0.67 ± 0.82	0.21 ± 0.43	0 ± 0
0.21 ± 0.15	0.21 ± 0.17	0.51 ± 0.27	0.24 ± 0.13	0.29 ± 0.39	0.29 ± 0.14
0.41 ± 0.06	0.4 ± 0.05	0.4 ± 0.03	0.44 ± 0.01	0.42 ± 0.06	0.65 ± 0.11
118.89 ± 171.09	207.71 ± 372.22	1.88 ± 4.52	20.8 ± 21.8	67.93 ± 79.76	208 ± 285.67
2.03 ± 2.08	3.86 ± 3.08	1.38 ± 2	2.4 ± 3.36	4.71 ± 6.51	5.5 ± 7.78
N = 42	–	N = 5	–	N = 5	N = 2
0.41 ± 0.09	–	0.34 ± 0.1	–	0.49 ± 0.36	0.55 ± 0.24
6.98 ± 3.41	–	3.32 ± 1.68	–	5.68 ± 1.92	1.32 ± 0.62
0.94 ± 0.69	–	1.04 ± 0.9	–	1.13 ± 1.31	0.26 ± 0.02
4.94 ± 0.69	–	1.91 ± 0.67	–	4.13 ± 1.88	1.05 ± 0.6
0.3 ± 0.45	–	0.27 ± 0.3	–	0 ± 0	0 ± 0
0.76 ± 0.57	–	0.08 ± 0.1	–	0.41 ± 0.72	0 ± 0
0.05 ± 0.09	–	0.02 ± 0.03	–	0.01 ± 0.03	0 ± 0

TABLE 3 Hematologic values (mean ± SD) for females and males of seven species of anurans from North Sinaloa, Mexico.

	Incilius mazatlanensis		Rhinella horribilis		Leptodactylus melanonotus	
Species	F (n = 8)	M (n = 10)	F (n = 5)	M (n = 53)	F (n = 23)	M (n = 4)
Ht (L/L)	33.67 ± 3.42	35.74 ± 13.3	49.83 ± 18.26	34.29 ± 13.38	27.46 ± 14.58	13.23 ± 1.61
TP (g/dL)	4.43 ± 0.25	3.35 ± 1.54	6.47 ± 2.2	4.01 ± 2.27	2.25 ± 1.4	1.2 ± 0.85
N (%)	9.5 ± 6.19	12.6 ± 8	31.2 ± 9.15	20 ± 7.29	15.74 ± 9.02	9.2 ± 5.07
L (%)	66.25 ± 24.78	71.8 ± 6.91	60.4 ± 10.19	72.34 ± 9.25	81.57 ± 8.68	85 ± 5.72
B (%)	3.63 ± 6.35	3.3 ± 5.36	0.2 ± 0.45	1.6 ± 2.86	0.17 ± 0.49	0.25 ± 0.5
E (%)	9.63 ± 4.07	11 ± 6.58	8 ± 5.57	5.7 ± 4.44	1.7 ± 1.69	2 ± 2.16
M (%)	1.75 ± 1.04	1 ± 1.05	0.2 ± 0.45	0.43 ± 0.84	0.39 ± 0.89	1.5 ± 2.38
N/L	0.22 ± 0.24	0.18 ± 0.15	0.55 ± 0.25	0.29 ± 0.13	0.21 ± 0.14	0.13 ± 0.04
LI	0.6 ± 0.11	0.55 ± 0.07	0.43 ± 0.03	0.45 ± 0.05	0.47 ± 0.04	0.49 ± 0.05
IE	127.88 ± 132.3	198.4 ± 245.92	16 ± 27.06	28.21 ± 101.97	18.13 ± 22.64	68.75 ± 117.74
Ep	17.75 ± 12.88	26.8 ± 34.47	1.6 ± 1.34	1.85 ± 4.11	5.09 ± 3.98	9 ± 12.19
	–	N = 1	N = 3	N = 22	N = 6	N = 1
RBC ($\times 10^{12}$/L)	–	0.67	0.22 ± 0.17	0.42 ± 0.23	1 ± 0.14	0.85
WBC ($\times 10^{9}$/L)	–	1.76	6.05 ± 1.77	9.04 ± 4.17	5.61 ± 2.19	3.96
N ($\times 10^{9}$/L)	–	0.16	1.89 ± 1.05	1.76 ± 1.18	0.87 ± 0.49	0.95
L ($\times 10^{9}$/L)	–	1.43	3.4 ± 0.61	6.22 ± 3.27	4.54 ± 1.97	2.97
B ($\times 10^{9}$/L)	–	0.04	0.01 ± 0.02	0.18 ± 0.36	0.03 ± 0.05	0
E ($\times 10^{9}$/L)	–	0.14	0.73 ± 0.42	0.53 ± 0.52	0.17 ± 0.17	0.04
M ($\times 10^{9}$/L)	–	0	0.01 ± 0.02	0.04 ± 0.07	0.01 ± 0.02	0

Abbreviations: B, basophil; E, eosinophil; Ep, erythroplastid; F, female; Ht, hematocrit; IE, immature erythrocyte; L, lymphocyte; LI, lobularity index; M, male; M, monocyte; N/L, neutrophil/lymphocyte ratio; N, neutrophil; RBC, red blood cells; TP, total protein; WBC, white blood cells.

higher immature erythrocytes ($P = 0.011$) and total neutrophil counts ($P = 0.014$), respectively.

For microfilaria infections, infected individuals of *L. forreri* showed higher relative eosinophil counts ($P = 0.001$) and lower immature erythrocyte ($P = 0.003$) and relative lymphocyte ($P = 0.018$) counts compared with uninfected individuals. Finally, in trypanosome infections of *S. fodiens*, there were higher relative eosinophil ($P = 0.042$) and total monocyte counts ($P = 0.046$) compared with those not infected with trypanosomes.

4 | DISCUSSION

The hematologic evaluation of seven wild anurans species in Northern Sinaloa, Mexico, allowed the assessment of subclinical changes associated with hemoparasite infections. In some cases, a relationship between season, sex, and infection was seen. Season is the main factor associated with the reproductive cycles of amphibians, influencing the presence of juveniles or reproductive adults in water bodies.[22] This seasonal effect was observed in seven of the eight species, except for *R. horribilis*. These reproductive interactions, together with other factors like predation or the effect of environmental contaminants due to agricultural activity, could cause abnormalities or lesions, which may be prevalent in wild amphibians.[22]

As previously reported, lymphocytes are the most abundant leukocytes in amphibians,[6,17,23] followed by neutrophils, monocytes, eosinophils, and basophils.[3] During this study, monocytes were the least abundant leukocyte.[22] Amphibian hematology is extremely variable due to external (season, temperature, and humidity) and internal (sex, age, and reproductive state) factors.[4,5,24] Differences were identified for immature erythrocytes between adult and juvenile *I. alvarius*, which could be explained by the physiologic response of metamorphosis, where tadpole erythrocytes are a substitute for adult erythrocytes.[25] For *I. mazatlanensis*, *L. forreri*, and *L. catesbeianus*, high leukocyte and total protein values were present in adults, which could be explained due to definitive populations of leukocytes appearing in circulation during late development,[25] and adults are more exposed to stress factors like disease, mating, and pollution.[6]

Hematologic differences associated with sex were observed in three amphibian species. For *R. horribilis*, differences in lymphocytes, neutrophils, and N/L ratios were detected. In contrast, Forbes et al[16] and Brown and Shine[23] did not find differences in leukocyte numbers and sex in toads of the genus *Rhinella*; this could be explained by the sampling method used (cardiac puncture) that may not show the peripheral distribution of leukocytes. For the other two species, *L. melanonotus* and *L. forreri*, differences were observed for total protein concentrations and hematocrits, respectively, which could be associated with the reproductive stage of females. In reptiles, increased total protein concentrations could be observed in yolk-producing females.[26]

Some seasonal changes found in *R. horribilis* and *L. melanonotus* may be related to the effects on hemoparasite infections, as seasonal

Lithobates forreri		Lithobates catesbeianus		Smilisca fodiens		Scaphiopus couchii	
F (n = 46)	M (n = 41)	F (n = 3)	M (n = 5)	F (n = 2)	M (n = 2)	F (n = 1)	M (n = 1)
39.27 ± 16.41	32.21 ± 12.99	16.83 ± 11.97	32.6 ± 6.13	53.49 ± 24.74	27.57 ± 4.29	21.62	15.57
5.33 ± 3.08	4.13 ± 1.98	2.53 ± 1.51	3.95 ± 1.83	11	1.9	4	2.8
14.63 ± 8.65	11.78 ± 4.66	26 ± 11.53	29.6 ± 8.73	26.5 ± 24.75	11 ± 1.41	16	28
70.54 ± 12.41	68.63 ± 8.55	61 ± 13.11	59.4 ± 9.32	55.5 ± 12.02	75.5 ± 6.36	84	71
4.57 ± 6.08	5.73 ± 5.16	9.67 ± 8.74	7.4 ± 6.77	4 ± 5.66	0 ± 0	0	0
9.74 ± 5.9	11.8 ± 6.69	3.33 ± 1.53	2.6 ± 2.97	13.5 ± 17.68	13 ± 8.49	0	1
0.5 ± 0.94	1.9 ± 2.82	0 ± 0	1 ± 1	0.5 ± 0.71	0.5 ± 0.71	0	0
0.23 ± 0.19	0.18 ± 0.09	0.47 ± 0.31	0.53 ± 0.27	0.54 ± 0.56	0.15 ± 0.01	0.19	0.39
0.4 ± 0.05	0.41 ± 0.07	0.39 ± 0.01	0.41 ± 0.03	0.33 ± 0.07	0.47 ± 0.02	0.57	0.73
97.35 ± 105.39	143.1 ± 222.04	0.33 ± 0.58	2.8 ± 5.72	141 ± 188.09	28 ± 9.9	410	6
1.98 ± 2.07	2.1 ± 1.12	3 ± 2.65	0.4 ± 0.55	6.5 ± 2.12	16 ± 14.14	11	0
N = 28	N = 14	N = 2	N = 3	N = 1	–	N = 1	N = 1
0.42 ± 0.08	0.39 ± 0.11	0.25 ± 0.03	0.4 ± 0.09	0.5	–	0.72	0.39
7.49 ± 3.43	5.96 ± 3.25	2.31 ± 1.24	4 ± 1.78	6.49	–	1.76	0.88
1.02 ± 0.78	0.77 ± 0.46	0.47 ± 0.32	1.41 ± 1.08	0.58	–	0.28	0.25
5.38 ± 2.7	4.05 ± 2.47	1.61 ± 1.01	2.11 ± 0.49	4.15	–	1.48	0.62
0.33 ± 0.53	0.23 ± 0.26	0.12 ± 0.17	0.36 ± 0.36	0	–	0	0
0.73 ± 0.54	0.82 ± 0.63	0.1 ± 0.08	0.07 ± 0.12	1.69	–	0	0.1
0.03 ± 0.08	0.09 ± 0.09	0 ± 0	0 ± 0	0.06	–	0	0

effects were also observed on the prevalence of infection. However, some authors reported seasonal leukogram changes increased circulating lymphocytes due to stress in *L. caerulea* and *Cynops pyrrhogaster*. In contrast, *Rana perezi* showed a decrease in lymphocytes and an increase in neutrophil counts.[3] For *R. horribilis*, an increase in hematocrit was observed in the rainy season, which could be associated with increased hematopoiesis at ambient temperatures. Even when the increased values of basophils, eosinophils, and proteins are linked to parasite infection (not seen in our study), such a result could be associated with immune responses to pollution.[3]

Genuses such as *Trypanosoma*, Haemogregarine, *Hepatozoon*, *Lankesterella*, and microfilariae are common hemoparasites in amphibian populations of different countries.[27–32] Hemoparasites are indirect lifecycle parasites that need a hematophagous vector to complete the transmission to the anuran host.[30] Amphibians spend a large portion of their life cycle in aquatic environments and are, therefore, more susceptible to interactions with vectors and more prone to hemoparasite infections.[28] This was observed in the aquatic hosts (*Lithobates* and *Leptodactylus*) in contrast to the terrestrial amphibians (*Incilius* and *Rhinella*) in this study. We found more adult infections by some hemoparasites, which may be explained by the capacity of adults to move from aquatic to terrestrial environments. Reproductive behavior, like vocalizations in males, may also attract mosquitoes, facilitating the transmission of hemoparasites.[33,34]

Hemoparasite indirect lifecycles require a vector, which differs according to the genus of the parasite, but many include mosquitos, mites, or leeches.[3,6,31] During sampling nights, mosquitoes and midges were observed at the sites and were more abundant in the wet season, but we did not identify blood-sucking insects in this study. Seasonal influences on vector populations could reflect seasonal hemoparasite infections,[3] as hemoparasite lifecycles require a developmental stage inside the anuran host, with tissue development[30] before a parasitemia. This could explain why there were more hemoparasite infections in *R. horribilis* and *L. melanonotus* during the dry season. As inclusions have different sizes and morphologic characteristics, they could have different etiologic origins, which include viruses and, possibly, alterations due to agrochemicals.

As no clinical signs were observed, subclinical hematologic changes could have been associated with the presence of parasites.[26,31,35,36] The effects of hemoparasite infections were observed in five amphibian species; however, no other factors (sex, age, or season) affected hematologic values except in *S. fodiens*, where the infection with *Trypanosoma* demonstrated increased eosinophils and monocytes. Eosinophils are associated with parasite infections, while monocytes may indicate chronic inflammation.[36] In *I. mazatlanensis*, increased eosinophils might be associated with parasitic infections,[36] as this was found in adults with haemogregarine infections.

During dry seasons, an increase of erythroplastids (erythrocytes with no nucleus) in infected *R. horribilis* may help in the efficiency of oxygenation.[10] In addition, a decrease in total protein concentrations was observed in infected toads and could

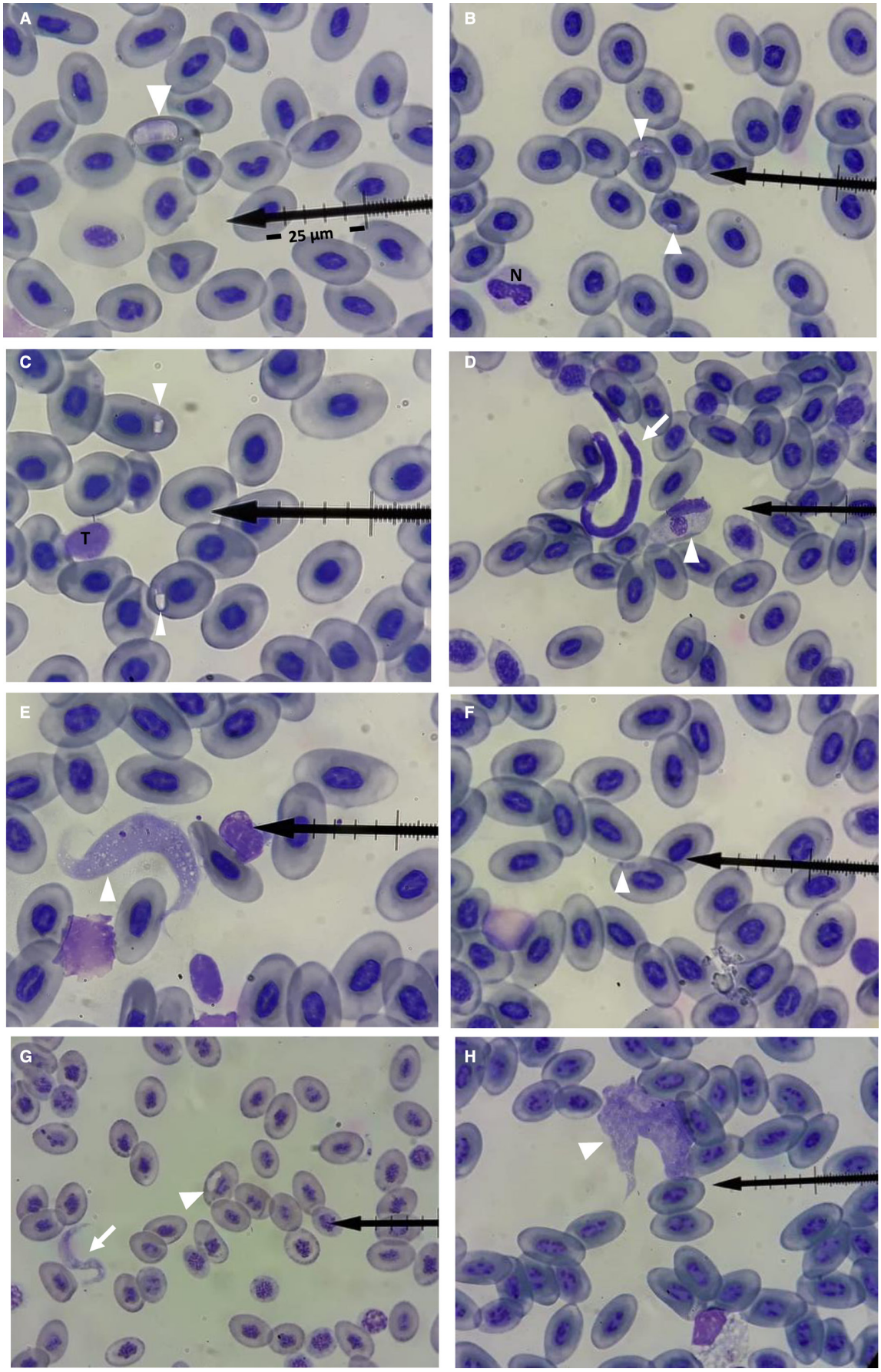

FIGURE 1 Blood smears from Mexican anurans. Wright's stain, 100×; black arrow with scale = 25 µm. A, *Incilius mazatlanensis*; intraerythrocytic haemogragarine (Arrow head). B, *Rhinella horribilis*, *Lankesterella* sp. (arrow head), and a neutrophil (N). C, *R. marina*, intracytoplasmic structure (arrow head) and a thrombocyte (T). D, *Lithobates forreri*, *Hepatozoon* sp. (arrow head) and microfilaria (arrow). E, *L. forreri*, *Trypanosoma* sp. (arrow head). F, *L. forreri*, *Lankesterella* sp. (arrow head). G, *Leptodactylus melanonotus*, *Trypanosoma* sp. (arrow) and *Hemolivia* sp. (arrow head). H, *Smilisca fodiens*; *Trypanosoma* sp. (arrow head).

TABLE 4 Hemoparasite prevalence for six species of anurans of North Sinaloa, Mexico

Anuran	N	*Trypanosoma*	Microfilaria	*Hepatozoon*	Haemogregarine	*Hemolivia*	*Lankesterella*	Other structures	Total
Incilius alvarius	15	–	–	–	20%	–	–	6.7%	26.7%
Incilius mazatlanensis	26	–	–	7.7%	23.1%	–	–	3.8%	34.6%
Rhinella horribilis	58	–	–	1.7%	24.1%	20.7%	1.7%	–	50%
Leptodactylus melanonotus	27	11.1%	–	–	3.7%	3.7%	–	14.8%	25.9%
Lithobates forreri	94	24.5%	41.5%	58.5%	27.7%	–	1.1%	9.6%	85.1%
Smilisca fodiens	13	15.4%	–	–	–	–	–	–	15.4%

be associated with malnutrition, protein-losing enteropathies, chronic hepatic or renal diseases,[27] and hemoparasite infiltration of the liver and spleen.[30]

An increase in infected individuals and monocyte counts was observed during the dry season for *L. melanonotus*; monocytes were influenced by season, age, and hemoparasite infections. However, an increase in immature erythrocytes was identified with other parasite structures and was defined as polychromasia (variable staining of erythrocytes) associated with erythrocyte damage and regeneration.[36]

Lithobates forreri males demonstrated a higher prevalence of some hemoparasite infections, which could be related to reproductive vocalization behaviors.[33,34] Due to these infections, increased WBC counts (higher basophil, lymphocyte, and monocyte counts) were observed. In reptiles, high counts of lymphocytes are related to hematozoan parasite infections,[27] but for metazoan parasites (ie, microfilariae), leukocyte responses are different.[36] in this study, an increased number of eosinophils and fewer lymphocytes were identified.

Co-infections were observed in the present study, as reported for hemoparasites in amphibians from other countries.[19,26,28,30,31,37,38] Nevertheless, host–parasite interactions are affected by factors such as the types of parasites, the timing of arrival to the host, and the intensity of infection. Further research is needed for a better understanding of the infection dynamics in amphibians, as other pathogens (like viruses or fungi) can be present, affecting the hematologic values. Clinical signs of disease could not be identified in any sampled amphibian. Nevertheless, pathogens were present in subclinical infections. Laboratory tests need to be performed to address the health status of wildlife amphibians, considering factors such as sex, age, and the environment of the hosts. As no generalizations on hematologic changes could be made, the hematologic information provided by this study may contribute to further evaluations of wild amphibian health, for taxonomic purposes or species in similar environmental conditions.

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CONFLICT OF INTEREST

The authors have no conflict of interest.

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