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RELATIONSHIPS BETWEEN LEUKOCYTES AND *HEPATOZOON* SPP. IN GREEN FROGS, *RANA CLAMITANS*

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ABSTRACT: There are few published data on amphibian leukocyte profiles, and relationships between amphibian leukocytes and parasites are even less well known. Using counts from 35 pairs of blood smears taken 2 days apart, we tested for correlations between leukocyte proportions and infection intensities of *Hepatozoon* spp. (either *Hepatozoon catesbianae* or *Hepatozoon clamatae*) in green frogs (*Rana clamitans*). On average (SE), we counted 65.4 (1.7) lymphocytes, 14.0 (1.3) neutrophils, 19.3 (1.6) eosinophils, 0.9 (0.1) monocytes, and 0.4 (0.1) basophils per 100 leukocytes. All frogs harbored *Hepatozoon* spp. (median seven parasites per 100 leukocytes; range 1–250). Significant relationships were not observed between numbers of leukocytes and infection intensities of *Hepatozoon* spp. Among the possible explanations for these null results are that *Hepatozoon* spp. is benign, that *Hepatozoon* spp. is able to evade detection by the immune system, that *Hepatozoon* spp. is able to manipulate leukocyte investment, or that other unmeasured or undetected parasites were more important in affecting immune response.

Key words: Green frog, *Hepatozoon* spp., immunity, neutrophil:lymphocyte, parasitism, *Rana clamitans*.

INTRODUCTION

The immune system of captive amphibians is poorly studied (Gentz, 2007), and that of wild amphibians is even less well characterized. Although laboratory studies (Du Pasquier et al., 1989; Brodkin et al., 1992; Haynes et al., 1992; Miodonski et al., 1995) have provided insight into amphibian immune function, extrapolation to wild animals often fails (Shutler et al., 1996). We studied relationships between immune function and blood parasitism in wild green frogs, *Rana clamitans*.

Leukocyte ratios provide one measure of immune function (Norris and Evans, 2000). Neutrophils are generally associated with investment in the innate immune response, whereas lymphocytes represent investment in the acquired immune response. Eosinophils are cytotoxic cells that stimulate other white blood cells to release histamines and protect hosts from parasitic invaders (Edwards, 1994). In mammals, higher neutrophil (heterophils in birds)-to-lymphocyte ratios are indicative of stress (Gross and Siegel, 1983; Vleck et al., 2000; Shutler et al. 2004), including disease (Work et al., 2001). Possibly, the same is true of neutrophil:lymphocyte ratios in amphibians (Forbes et al., 2006).

Hepatozoon spp. (phylum Apicomplexa) are intraerythrocytic parasites of tetrapods, and over 40 species have been recorded from anurans worldwide (Smith, 1996). Two species, *Hepatozoon catesbianae* and *Hepatozoon clamatae*, commonly infect ranid frogs in Nova Scotia (Boulianne et al., 2007). Parasites develop in the Malpighian tubules of the mosquito definitive host, *Culex territans*, after these insects take a blood meal from adult frogs that carry infective gamonts in their circulation. Transmission occurs when mosquitoes, infected with mature oocysts of the parasite, are ingested by adult frogs. After one round of asexual development in a frog's liver, parasites emerge and infect erythrocytes, where they form gamonts (Desser et al., 1995). In this study, we tested for relationships between leukocytes of green frogs, *Rana clamitans*, and naturally occurring infections of *Hepatozoon* spp.

METHODS

All procedures were approved by the Acadia Animal Care Committee. Thirty-

five adult frogs were collected opportunistically from Gaspereau Lake ($n=5$; 44°58′N, 64°31′W, a large reservoir system in cottage country surrounded by natural vegetation), Starr's Point ($n=1$; 45°07′N, 64°23′W, an artificial wetland excavated for improving waterfowl habitat in an agricultural matrix), Wolfville ($n=11$; 45°05′N, 64°20′W and 45°05′N, 64°22′W, two ponds in hayfields bordered by residential areas), Coldbrook ($n=6$; 45°02′N, 64°35′W, temporary wetlands in a quarry surrounded by mixed forested), and South Alton ($n=12$; 45°00′N, 64°31′W, wetlands adjoining a paved road and surrounded by mixed forest), Kings County, Nova Scotia, Canada, between 31 May 2006 and 4 August 2006. Frogs' exposure histories were unknown. Green frogs in our study area spend one winter as tadpoles, but do not completely lose their tails to become full-fledged adults until late July or early August (Gilhen, 1984; Smith, pers. obs.). Thus, most if not all frogs we sampled were likely in or beyond their third summer. Frogs were transported to the lab in 40×30×20-cm polypropylene containers covered with vinyl screening.

On the day of capture, the maxillary vein of each frog was punctured with a sterile 27-gauge needle (Smith et al., 2000), and approximately 20–40 μL of blood was collected in a heparinized capillary tube before being smeared on a microscope slide (Bennett, 1970). Punctures were treated with Bactine® (Bayer Inc., Robinson, Pennsylvania, USA) to prevent infection. Once air-dried, slides were fixed and stained with Protocol® Hema 3 (Biochemical Sciences Inc., Swedesboro, New Jersey, USA), and affixed with cover slips. Frogs were kept in the Acadia Animal Care facility for 2 additional days, at which point a second smear was made. No gross evidence of inflammation was noted at initial puncture sites. The 2-day interval between smears was somewhat arbitrary because we are unaware of any published data on how long it takes green frogs to mount an immune response to *Hepatozoon* spp. However, observations from other vertebrates suggest that immunity may respond to parasites within hours of infection (Gross and Siegel, 1983; Dufva and Allander, 1995; Ots and Hõrak, 1996; Vleck et al., 2000; Ruiz et al., 2002) and elevated immune responses may persist for weeks thereafter (Ali and Behnke, 1985; Garside et al., 1989). Moreover, other apicomplexans can persist in vertebrate hosts for weeks to years (Atkinson and van Riper, 1991; Desser and Bennett, 1993). Once a second smear had been made, frogs were returned to their point of capture.

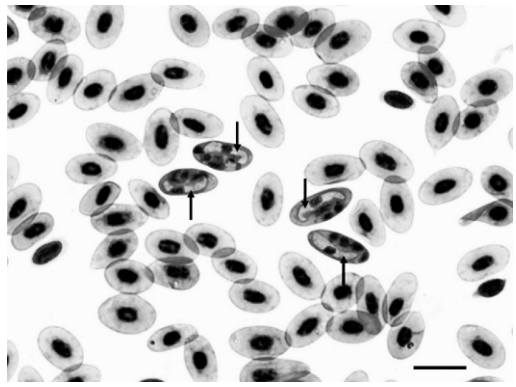

FIGURE 1. Typical blood film used to detect *Hepatozoon* species and for counts of blood cells. Arrows show the location of intracellular gamonts of *Hepatozoon* spp. Scale bar=20 μm.

We examined each smear under a microscope at 400× and distinguished the first 200 white blood cells (neutrophils [including neutrophilic, or agranular, granulocytes and the small eosinophilic granulocytes], eosinophils, lymphocytes, basophils, and monocytes) encountered, and enumerated the number of *Hepatozoon* spp. (pooling *H. catesbianae* and *H. clamatae* because they are not always reliably distinguished), *Trypanosoma* spp., and *Lankesterella* spp. in the fields that were examined (Fig. 1). Recounts of 10 randomly chosen slides were made to test repeatability. We also computed neutrophil:lymphocyte ratios as a measure of stress.

Statistical analyses were carried out in

SAS Version 9.1 (SAS Institute, Cary, North Carolina, USA). If leukocyte investment eventually reduces blood parasite infection intensities (hereafter, intensities), we predict a negative association between day-1 leukocyte counts and day-3 intensities. If, on the other hand, infections eventually provoke leukocyte investment, we predict a positive association between day-3 leukocyte counts and day-1 intensities. If these relationships are more immediate (within hours), we predict relationships between day-1 leukocytes and day-1 intensities, and between day-3 leukocytes and day-3 intensities. Intensity data for parasite intensities were not normally distributed, so we report medians rather than means, and used nonparametric Spearman rank correlations (r_S). Repeated measures analyses may be appropriate for some of our tests. However, based on the broken-stick model (Jackson, 1993), the first principle component of leukocyte counts explained less variation than expected by chance. Thus, each leukocyte count should be considered as independent, which would mean running a separate repeated measures general linear model for each of the five leukocytes; this would increase the chance of committing a Type I statistical error, which is the purpose of a repeated measures analysis. However, a repeated measures analysis was carried out when we treated *Hepatozoon* spp. intensity as the response variable.

RESULTS

There was significant repeatability for lymphocytes (r_S=0.51, P=0.002), neutrophils (r_S=0.42, P=0.01), eosinophils (r_S=0.72, P<0.0001), and *Hepatozoon* spp. (r_S=0.90, P<0.0001), but not for monocytes (r_S=0.23, P=0.18) or basophils (r_S=−0.31, P=0.07). Thus monocytes and basophils were not analyzed further. Our leukocyte counts had more lymphocytes and fewer neutrophils than has been reported in some studies (Table 1), possibly because of differences in sampling techniques. Prevalence was 100% for *Hepatozoon* spp., 26% for *Lankesterella* spp., and 4% for *Trypanosoma* spp. A median of 14.0 *Hepatozoon* spp. parasites was counted per 100 leukocytes, whereas medians were 0 for the other two parasites. Because of their lower prevalences and intensities, *Lankesterella* spp. and *Trypanosoma* spp. are not discussed further; their inclusion in subsequent analyses did not influence results reported below.

Within frogs, day-1 and day-3 counts were similar for neutrophils (r_S=0.42, P=0.01), lymphocytes, (r_S=0.51, P=0.002), eosinophils (r_S=0.72, P<0.0001), and *Hepatozoon* spp. (r_S=0.90, P<0.0001). However, neither leukocyte counts nor neutrophil:lymphocyte ratios were significantly associated with *Hepatozoon* spp. intensities (Table 2), although the relationship between day-1 eosinophils and day-1 *Hepatozoon* spp. intensities was close (P=0.053), and each of the four possible correlations was negative. *Hepatozoon* spp. infections did not differ significantly among sites on either day 1 ($F_{5,29}$=0.7, all P>0.64) or day 3 ($F_{5,29}$=0.9, all P=0.50; results were also not significant if we excluded Starr's Point where we had only captured a single frog). We also failed to detect significant associations between intensity and leukocytes with repeated measures general linear models (all $F_{1,28}$<0.9, all P>0.37).

DISCUSSION

Our leukocyte proportions differed from other published data (Table 1). The location from which blood is extracted may be associated with leukocyte ratios; in particular, based on Table 1, peripheral blood appears to have more lymphocytes. However, because there are so few published data for amphibian leukocyte profiles, and because profiles appear to change in response to a variety of environmental cues (Foxon, 1964; Duellman and Trueb, 1986; Maniero and Cary, 1997), it is too early to make generalizations.

TABLE 1. Published leukocyte percentages for amphibians.

Species	*n*	Lymphocytes	Neutrophils	Eosinophils	Basophils	Monocytes	Blood source	Source
Bufo americanus[a]	27	20±1.7	68±2.0	3±0.6	7±1.0	2±0.4	Cardiac	Forbes et al. (2006)
Rana pipiens[b]	19	53 (29–75)	27 (11–24)	7 (4–11)	4 (0–9)	11 (5–24)	Aorta	Rouf (1969)
Rana pipiens[c]	30	11–23	48–58	3–10	22–25	3–5	Cardiac	Maniero and Careyn (1997)
Rana lessonae and *Rana ridibunda*[d]	137	51–66	10–20	8–19	10–16	0.5–0.7	Peripheral	Romanova and Romanova (2003)
Rana clamitans[a]	35	66±1.7	16±1.3	17±1.6	1±0.1	1±0.1	Maxillary vein	This study

[a] Mean±SE.
[b] Mean (range).
[c] Range from 2 separate temperature treatments.
[d] Data pooled in the paper for these species, ranges from five different study sites; method of blood sampling not specified.

TABLE 2. Spearman rank correlations among *Hepatozoon* spp. counts in 35 green frogs (*Rana clamitans*) 1 and 3 days postcapture, and white blood cell counts and neutrophil to lymphocyte ratios 1 and 3 days postcapture.

	Day-1 *Hepatozoon* intensity		Day-3 *Hepatozoon* intensity	
	r_S	*P*	r_S	*P*
Day-1 neutrophils	0.17	0.34	0.19	0.26
Day-1 eosinophils	−0.33	0.05	−0.28	0.10
Day-1 lymphocytes	0.26	0.14	0.20	0.24
Day-1 neutrophils: lymphocytes	0.06	0.74	0.09	0.60
Day-3 neutrophils	0.20	0.26	0.16	0.36
Day-3 eosinophils	−0.22	0.18	−0.13	0.45
Day-3 lymphocytes	0.23	0.18	0.17	0.34
Day-3 neutrophils: lymphocytes	0.10	0.56	0.09	0.63

Significant relationships were not found between immune response, indexed by leukocytes and leukocyte ratios, and infection with *Hepatozoon* spp. Few other studies have assessed relationships between hematozoa and leukocytes; these studies often detect elevated numbers of lymphocytes (Rose et al., 1979; Dufva and Allander, 1995; Ots and Hõrak, 1998; Figuerola et al., 1999). We have not found such studies for amphibians. There are a number of possible reasons for these null results, and we discuss here only a few. First, *Hepatozoon* spp. may be benign, and not worth host investment in altered ratios of circulating leukocytes. Indeed, experimental infections inducing high parasitemias of *Hepatozoon* spp. in ranid frogs caused no apparent morbidity (Kim et al., 1998; Smith et al., 2000). Second, *Hepatozoon* spp. may be able to evade immune detection, perhaps by staying primarily within erythrocytes. Third, *Hepatozoon* spp. may be able to modulate immune response, as has been reported for other parasites (Garside et al., 1989; Eckert, 1991; Maizels et al., 1993). Fourth, other unmeasured or undetected parasites including helminths or intestinal protozoa may be more important to green frogs, so that the more important relationships were not tested. Further experimental

manipulation of parasitism or immune function would help to tease apart these possibilities.

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