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WHITE BLOOD CELL DIFFERENTIALS OF NORTHERN CRICKET FROGS (*ACRIS C. CREPITANS*) WITH A COMPILATION OF PUBLISHED VALUES FROM OTHER AMPHIBIANS

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ABSTRACT: Herpetologists are becoming increasingly aware of the importance of hematological parameters for evaluating the welfare of their study animals. In particular, differential counts of white blood cells (from blood smears) are now commonly performed on amphibians, although interpretation of such counts relies on some knowledge of the normal range for the species in question, and for many species this range is not known. In this study we examined blood smears from 79 cricket frogs (*Acris c. crepitans*) from two ponds in northeastern Georgia to establish reference ranges of white blood cell differentials for this species and to explore possible differences in relative white blood cell numbers between sexes, ponds, and body sizes. For comparison, we also compiled values of many other amphibians from published and unpublished records. We found that lymphocytes made up the majority (68.3%) of white blood cells in cricket frogs, followed by neutrophils (22.4%). There was no difference in relative cell numbers of any type between sexes, nor was there an association with body size. Relative numbers of certain cell types (lymphocytes and neutrophils) varied between ponds. In general the values for cricket frogs resembled those of other amphibians, with high numbers of lymphocytes and neutrophils and low numbers of eosinophils, basophils and monocytes. However, in assembling the records for other species we discovered that all Ambystomatid salamanders examined thus far have markedly elevated values of eosinophils compared to other amphibian species. Moreover, their eosinophil counts may be the highest of all vertebrates, and clearly warrant further study to determine their significance.

Key words: *Acris c. crepitans*; Cricket frog; Eosinophils; Lymphocytes; Neutrophils; White blood cells

MODERN herpetologists have increasingly been incorporating hematological approaches in their investigations to aid in the evaluation of the welfare of their study subjects (Cabagna et al., 2005; Chiesa et al., 2006, Forson and Storfer, 2006; Solis et al., 2007; Davis and Maerz, 2008*a*; Gervasi and Foufopoulos, 2008). In particular, parameters such as differential white blood cell counts (i.e., the relative proportions of the five white blood cell types) are frequently being obtained because these counts can offer the investigator a number of pieces of information concerning the health status of the organism. For example, higher than normal proportions of neutrophils in circulation can point to infections (Jain, 1993; Thrall, 2004), while eosinophil numbers are thought to be associated with parasitism defense (Kiesecker, 2002). Monocytes are phagocytic cells that engulf foreign material and are commonly found in animals with bacterial infections (Davis et al., 2004; Turner, 1988). In addition, the ratio of two cell types, neutrophils to lymphocytes, has long been known to reflect levels of stress hormones in all vertebrates (reviewed in Davis et al., 2008*a*), and this approach has often been used by herpetologists to assess stress levels of amphibians in a variety of contexts including limb amputation (Bennett, 1986), captivity (Davis and Maerz, 2008*b*), reproduction (Davis and Maerz, 2008*a*), temperature (Bennett and Daigle, 1983) and after direct injection with stress hormones (Bennett and Harbottle, 1968; Bennett et al., 1972).

Interpreting white blood cell counts of any animal requires some knowledge about how many cells of each type are normally found in the species being studied, and for many species of herpetofauna, this is not yet known. One amphibian species for which there is little hematological data is the cricket frog (*Acris crepitans*). These small (2 cm), nonclimbing members of the tree frog family, are widely distributed throughout the eastern United States. The southern subspecies (*A. c. crepitans*) ranges from New Jersey to Texas (Conant and Collins, 1998) and is highly

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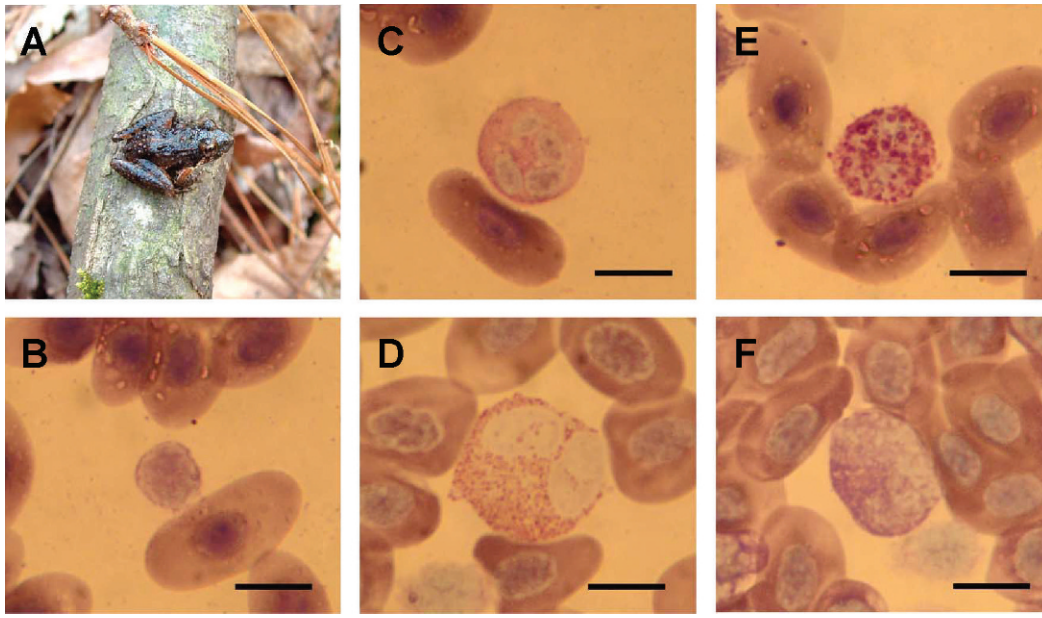

FIG. 1.—(A) Northern cricket frog (*Acris c. crepitans*); (B) typical lymphocyte, with large nucleus and faint ring of light blue cytoplasm; (C) neutrophil with multilobed nucleus and pink-colored cytoplasm; (D) large eosinophil, showing bilobed nucleus and orange granules in cytoplasm; (E) basophil with nucleus nearly obscured by purple-colored granules; and (F) monocyte, with large reticulated nucleus and faint grey-colored cytoplasm. Black bars in cell images are 10 μm.

abundant in aquatic areas where they can be seen basking on sunny days. Cricket frogs have recently been used as subjects in experiments addressing questions of immunity (McCallum and Trauth, 2007), though their innate immune system (i.e., white blood cells) has not yet been examined.

The white blood cells of a selected number of other amphibian species have been examined since the early 1900s, although these records are often scattered throughout the veterinary, herpetological and ecological literature. Compiling these records would provide future hematological investigations a baseline set of values for each cell type. The objectives of this study therefore were twofold. First, we sought to establish a normal range of white blood cell differentials for northern cricket frogs and to statistically explore possible differences in cell numbers between sexes and locations and across body sizes. The secondary objective was to compile the existing published records of white blood cell differentials from other amphibian species, as well as several other unpublished data sets from our lab, for comparison with the cricket frog values and to serve as a reference for future hematological investigations of amphibians.

MATERIALS AND METHODS

Collecting and Processing Frogs

In April of 2008 we hand-captured 79 northern cricket frogs (Fig. 1A) from two permanent ponds in Clarke County, GA, USA. Both ponds were within 300 m of each other. Frogs were placed in plastic containers and transported to the lab for processing. Later the same day, each frog was anesthetized via immersion in MS-222, and its snout–vent length (SVL) was measured to the nearest mm. It was then killed by decapitation, and the blood that welled from the exposed heart was dripped onto a clean microscope slide and a blood smear made with a second slide. The body cavity was then dissected and the sex was determined from the presence of testes or developing eggs. For 30 frogs the sex was not recorded. Blood smears were air-dried then stained with giemsa.

TABLE 1.—Summary of white blood cell differentials (% of total cells) for 79 northern cricket frogs examined in this study.

	Mean	Minimum	Maximum	SD
Lymphocytes	68.3	28.3	93.9	18.1
Neutrophils	22.4	1.0	67.3	16.8
Eosinophils	1.6	0.0	8.6	1.8
Basophils	5.0	0.0	15.4	3.4
Monocytes	2.7	0.0	17.6	3.7

White Blood Cell Examination

Slides were examined under 1000× oil immersion following established procedures used for counting white blood cells of amphibians and birds in our lab (Davis and Maerz, 2008*a*,*b;* Davis et al., 2008*b*). Cells were identified as neutrophils, lymphocytes, eosinophils, basophils and monocytes following Thrall et al. (2004), Hadji-Azimi et al. (1987) and Turner (1988). All cells were counted until at least 100 cells were counted, or when 150 fields of view had been examined. Only fields of view with even distributions of red blood cells were used. The relative number (i.e., proportion) of each cell type was calculated based on the number of cells of that type counted divided by the total number of leukocytes counted.

Data Analysis

Relative numbers of each white blood cell type were arcsine square-root transformed prior to analyses to approximate normal distributions. We then used a general linear modeling approach (with multiple dependent variables) to compare the relative numbers of each cell type between sexes and ponds and across body sizes. Since there were 30 frogs that were not sexed, we conducted a preliminary analysis using only the data from sexed individuals. This analysis examined how sex, pond (both as dichotomous variables), as well as snout–vent length (a continuous variable), and the interaction of sex by body size influenced the relative numbers of each cell type (i.e., all cell types were included as dependent variables). We then conducted a full analysis using all individuals and without examining the effect of sex. In this case, the relative numbers of all five cell types were again the dependent variables, and pond and SVL were the independent variables. When significant effects were found for dichotomous variables, Tukey's post-hoc tests were performed to elucidate the effect. All analyses were conducted using Statistica 6.1 software (Statistica, 2003).

RESULTS

General

Of the 79 frogs examined, 48 were from pond 1 and 31 were from pond 2. Sizes of all frogs varied from 15.4 mm to 24.2 mm, with an average of 19.6 mm, and there was an approximate normal distribution of sizes. There was no significant difference in body size between ponds (two-sample t-test, $t = 0.422$, df = 77, $P = 0.674$). There was also no difference in body sizes of males and females, of the individuals assigned to sex ($t = -0.395$, df = 47, $P = 0.694$). Of the frogs determined to be female, developing eggs were seen in two.

White Blood Cells

All cell types in this species appeared to show typical morphology for amphibians (Hadji-Azimi et al., 1987; Thrall 2004). Lymphocytes (Fig. 1B) were the most commonly-encountered white blood cell type (averaging 68.3% across all individuals, Table 1), followed by neutrophils (Fig. 1C, 22.4%). Eosinophils, basophils and monocytes (Figs. 1D, 1E, 1F, respectively) each made up less than or equal to 5% of white blood cells (Table 1). There was no significant difference in relative cell numbers (of any type) between sexes; in our preliminary analysis, there was no main effect of sex ($F_{5,40} = 0.509$, $P = 0.767$), nor a significant interaction of sex by length ($F_{5,40} = 0.491$, $P = 0.781$). In the full analysis with all 79 individuals (without sex as a variable), there was no support for the inclusion of the pond*SVL interaction effect ($F_{5,71} = 1.404$, $P = 0.233$). Results of a model

with the main effects only revealed no significant variation due to body size ($F_{5,72} = 1.007$, $P = 0.420$), but there was significant variation between ponds ($F_{5,72} = 2.756$, $P = 0.025$). Further exploration of this pond effect revealed that the relative number of lymphocytes was higher in pond 2 than pond 1 (Tukey's post-hoc test, $P = 0.03$), and the relative number of neutrophils was greater in frogs from pond 1 than pond 2 ($P = 0.009$). No other cell type differed significantly.

Differentials of Other Species

Leukocyte proportions of other amphibian species reported in the published literature as well as from certain unpublished data sets from our lab are shown in Table 2. In most cases, lymphocytes and neutrophils are the most commonly seen cell types within amphibians, which is comparable to that found in cricket frogs. However, which of these two cells is most common depends on the species and even the investigator, as evidenced by strikingly different reference values obtained for leopard frogs, *Rana pipiens* and bullfrogs, *Rana catesbeiana*. Also of note are the records for salamanders in the genus *Ambystoma* (mole salamanders), which are grouped together. In each of these species examined, the relative number of eosinophils is dramatically higher than what appears to be the norm for other amphibian species (32.1% on average for *Ambystoma*, 4.7% for other salamanders, 7.0% for anurans). Comparing the eosinophil data among these three groups shows this difference is significant statistically (one-way ANOVA, $F_{2,33} = 39.46$, $P < 0.001$).

Discussion

As this study represents the first hematological investigation of northern cricket frogs, the white blood cell differentials we obtained can serve as general reference values for future investigations involving this species. In comparison with other amphibians, white blood cell proportions of cricket frogs appeared generally typical, with the most abundant cell type being lymphocytes (68% of white blood cells), followed by neutrophils (22%), then low numbers of eosinophils, basophils and monocytes. Researchers should keep in mind, however, that such values are inherently variable for many reasons, including general procedural differences among labs. This point is highlighted in Table 2, where vastly different values for the same species (i.e., adult bullfrogs and leopard frogs) were reported by different labs. These may highlight either the variability among labs in assessing white blood cell numbers microscopically or, alternatively, in processing animals. In the latter case, largely different cell numbers could be obtained if animals are unduly stressed before blood samples are obtained. The effect of stress hormones on white blood cells is well-known in amphibians, where increases in stress cause an influx of neutrophils into circulation and an egress of lymphocytes out of circulation (Bennett, 1986; Bennett and Alspaugh, 1964; Bennett and Daigle, 1983; Bennett and Harbottle, 1968; Bennett and Johnson, 1973; Bennett and Newell, 1965; Bennett and Reap, 1978; Bennett et al., 1972; Davis et al., 2008*a*). Further, recent work shows that wild-caught specimens held in captivity for 10 days leads to high neutrophil and low lymphocyte counts in salamanders (Davis and Maerz, 2008*b*). This phenomenon may well be the reason for the differences among labs in reference values of bullfrogs and leopard frogs, especially given that the main differences among labs appear to be in the neutrophils and lymphocytes (Table 2). This rationale may also explain the differences we found in relative lymphocyte and neutrophil numbers between the two ponds; pond 1 frogs had more neutrophils and fewer lymphocytes than pond 2 frogs, indicating the pond 1 frogs had higher stress levels (Davis et al., 2008*a*).

Our compilation of published and unpublished records from ambystomatid salamanders uncovered a surprising trend, and one that certainly warrants future study. In all of the ambystomatid species examined, there appears to be a considerably high number of eosinophils (on average 32.1% of all white blood cells). Not only is this number larger than in other amphibians, to our knowledge it is larger than that found in most vertebrates, including mammals (Jain, 1986), birds (Rupley, 1997), and reptiles (Raskin, 2000), though among the reptiles, turtles can have as much as 20% eosinophils (Campbell, 1996; Raskin,

TABLE 2.—Compilation of published and unpublished (from various unrelated investigations by the author, AKD) white blood cell differentials in amphibians. Values shown represent percentages of all leukocytes. Values from control animals only are given from studies involving experimental manipulations. If multiple populations were sampled, the average of all is presented. Ambystomatid salamanders are shown separately to highlight their high eosinophil counts. Online, updated version of this table available at http://www.wildlifehematology.uga.edu.

Species	Age	n	Lymphocytes	Neutrophils	Eosinophils	Basophils	Monocytes	Source
Acris c. crepitans	**Adult**	**79**	**68.3**	**22.4**	**1.6**	**5.0**	**2.7**	**This study**
Rana catesbeiana	Adult	14	62.9	22.0	8.9	2.5	0.6	Cathers et al., 1997
Rana catesbeiana	Adult	302	26.8	60.9	5.8	3.5	2.9	Coppo et al., 2005
Rana catesbeiana	Larvae‡	40	73.0	23.8	3.6	2.3	0.3	Davis, 2009
Rana pipiens	Adult	50	53.4	26.5	7.3	4.4	11.0	Rouf, 1969
Rana pipiens	Adult	18	55.4	11.3	10.1	19.2	4.1	Maniero and Carey, 1997
Rana pipiens	Adult	14	25.4	61.8	7.0	1.8	5.2	Bennett and Alspaugh, 1964
Rana esculenta	Adult	*	75.2	17.1	5.7	1.9	0.0	Friedsohn, 1910 (cited in Jordan, 1938)
Rana esculenta	Adult	136	57.6	15.2	14.4	12.4	0.5	Romanova and Romanova, 2003
Rana clamitans	Adult	35	66.0	16.0	17.0	1.0	1.0	Shutler et al., 2009
Bufo arenarum	Adult	12	60.9	27.3	3.7	3.8	1.7	Chiesa et al., 2006
Bufo arenarum	Adult	24	64.0	20.9	13.7	0.0	1.3	Cabagna et al., 2005
Bufo americanus	Adult	27	20.0	68.0	3.3	7.4	1.5	Forbes et al., 2006
Bufo alvarius	Adult	*	37.0	48.0	9.0	1.0	5.0	Cannon and Cannon, 1979
Bufo fowleri	Adult	6	72.3	8.5	8.5	9.8	0.8	A. K. Davis, unpublished data
Bufo vulgaris	Adult	*	73.0	18.3	1.3	6.7	0.0	Friedsohn, 1910 (cited in Jordan, 1938)
Bombina bombina	Adult	31	51.6	25.0	3.9	7.6	12.6	Wojtaszek and Adamowicz, 2003
Xenopus laevis	Adult	10	30.1	26.5	1.2	40.5	1.6	Hadji-Azimi et al., 1987
Glyphogloossus molossus	Adult	18	41.6	26.3	1.1	8.3	22.7	Ponsen et al., 2008
Average for all anurans			**52.6**	**29.1**	**7.0**	**7.5**	**4.0**	
Standard Deviation			**18.0**	**17.6**	**4.7**	**9.3**	**5.7**	
Cryptobranchus alleganiensis	Adult	27	63.7	23.3	4.5	0.0	8.9	Jerrett and Mays, 1973
Cryptobranchus alleganiensis	Adult	78	42.6	38.9	11.8	6	0.6	Solis et al., 2007
Notophthalmus viridescens	Adult	120	63.5	24.3	6.2	3.2	2.8	Bennett and Daigle, 1983
Notophthalmus viridescens	Adult	13	55.6	37.6	1.8	0.8	4.2	Bennett and Johnson, 1973
Taricha granulose	Adult	13	88.0	7.0	2.0	1.0	1.0	Friedmann, 1970
Triton cristatus	Adult	*	42.9	52.2	3.7	1.2	0.0	Friedsohn, 1910 (cited in Jordan, 1938)
Cynops pyrrhogaster	Adult	23	3.0	28.0	4.0	57.0	6.0	Pfeiffer et al., 1990
Plethodon cinereus	Adult	63	65.0	21.7	3.6	8.8	0.8	A. K. Davis, unpublished data
Average for all urodeles			**53.0**	**29.1**	**4.7**	**9.8**	**3.0**	
Standard Deviation			**24.8**	**13.6**	**3.2**	**19.3**	**3.1**	
Ambystoma maculatum	Adult	1	51.1	19.6	19.6	9.8	0.0	A. K. Davis, unpublished data
Ambystoma maculatum	Adult†	10	31.7	18.1	25.5	24.2	0.6	Davis and Maerz, 2009
Ambystoma maculatum	Larvae	4	51.7	14.1	26.9	6.8	0.6	A. K. Davis, unpublished data
Ambystoma tigrinum	Adult	1	46.5	14.0	23.3	16.3	0.0	A. K. Davis, unpublished data
Ambystoma mexicanum	Adult	7	20.1	21.7	52.0	4.9	1.0	Ussing and Rosenkilde, 1995
Ambystoma mexicanum	Adult	15	59.0	13.5	22.5	4.0	1.0	Deparis and Beetschen, 1967

TABLE 2.—Continued.

Species	Age	n	Lymphocytes	Neutrophils	Eosinophils	Basophils	Monocytes	Source
Ambystoma talpoideum	Adult††	34	41.5	12.7	45.7	0.0	0.2	Davis and Maerz, 2008*a*
Ambystoma talpoideum	Adult††	16	39.0	5.7	51.2	3.9	0.1	Davis and Maerz, 2008*b*
Ambystoma talpoideum	Larvae	45	52.1	16.7	22.0	8.5	0.7	A. K. Davis, unpublished data
Average for all Ambystoma			**43.6**	**15.1**	**32.1**	**8.7**	**0.5**	
Standard Deviation			**12.0**	**4.7**	**13.4**	**7.4**	**04**	

* Sample size not reported
† Recently metamorphosed adults (1–2 days)
‡ Gosner stage 26–39
†† Paedomorhic adults

2000). We cannot readily explain this phenomenon. Eosinophils are generally thought to be involved in the innate immune response to parasites (Kiesecker, 2002; Thrall, 2004; Turner, 1988), and ambystomatid salamanders should be examined for evidence of high parasite loads compared to other species (if these values represent a heightened response to current parasitic infections), or alternatively, of relatively low loads (if the numerous eosinophils act to prevent infections). Indeed, it is within the amphibians where there is the best evidence for the anti-parasite role of eosinophils. Kiesecker (2002) showed suppression of eosinophil numbers by a pesticide causes increased susceptibility to trematode infections in frogs. Less known is the role of eosinophils in amphibian metamorphosis. In a study of axolotls (*Ambystoma mexicanum*), Ussing and Rosenkilde (1995) discovered that eosinophils increased from 52% in neotenes to 75% of cells during metamorphic climax, then declined to 6% one month after metamorphosis. Similar results have been found with anurans (Rosenkilde et al., 1995; Davis, 2009). However, the records of *Ambystoma* salamanders in Table 2 reflect those of fully-transformed adults as well as larvae and paedomorphic individuals, so the metamorphosis-related increase in eosinophils is probably not the reason for the high numbers in this group.

Finally, it should also be pointed out that this discovery of high eosinophil numbers in ambystomatid salamanders is itself an example of the importance of reporting reference values for hematological parameters. When we compared values across the many published records of amphibian blood cells (along with others from our lab), this large difference was readily seen, and its discovery will no doubt lead to an exciting new avenue of exploration.

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