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Leukocyte Reference Intervals for Free-Ranging Hummingbirds in Northern California, USA

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ABSTRACT: Hummingbirds are specialized nectarivores and important ecological pollinators that are the focus of conservation efforts as well as scientific investigations of metabolism and flight dynamics. Despite their importance, basic information is lacking about hummingbird blood cells. We aimed to establish reference intervals for total and differential leukocyte counts from healthy free-ranging hummingbirds in northern California. Hummingbirds were captured in four counties in spring and summer of 2012. A drop of blood was used to prepare smears for total white blood cell estimate and 200-cell differential leukocyte counts. Reference Value Advisor was used for descriptive statistics and calculation of reference intervals. Blood smears from 42 Anna's Hummingbirds (*Calypte anna*) and 33 Black-chinned Hummingbirds (*Archilochus alexandri*) were included. The only significant differences in leukocyte counts were due to age, and juvenile hummingbirds had significantly higher lymphocyte counts than adult hummingbirds ($P<0.0001$). These reference intervals provide robust baseline data to evaluate health status and disease in free-ranging hummingbirds.

Key words: Archilochus alexandri, avian, *Calypte anna*, hematology, white blood cells.

The hummingbird family (*Trochilidae*) are nectarivores with major ecological roles in pollination (Martín González 2015). Hummingbirds have been the focus of diverse scientific investigations, including analyses of flight kinetics (Goller and Altshuler 2014), muscle physiology (Song et al. 2015), energy regulation (Suarez et al. 2011), and migration and population dynamics (Hobson et al. 2003). Despite the scientific attention that hummingbirds have received, we lack basic information about hummingbird leukocytes, the quantity and morphology of which can provide important information about bird health and disease (Owen 2011).

Our aim was to determine total and differential leukocyte counts for clinically healthy free-ranging hummingbirds sampled in northern California in 2012 and to evaluate differences based on species, sex, age, weight, and season. Mostly Anna's Hummingbirds (*Calypte anna*) and Black-chinned Hummingbirds (*Archilochus alexandri*) were sampled. Hummingbirds were captured using standard drop nets around hummingbird feeders at four banding site locations (locations are approximate to protect the privacy of land owners): Winters (Solano County; 38°31′49″N, 121°51′2″W), Big Creek Reserve near Big Sur (Monterey County; 36°4′20″N, 121°35′46″W), Placerville (El Dorado County; 38°44′36″N, 120°56′6″W), and Oregon House (Yuba County; 39°19′54″N, 121°16′25″W), California. The majority of samples were collected in the spring (March–May) or summer (June–July) of 2012 within 6 h of sunrise. All procedures were approved by the University of California (Davis, California) Institutional Animal Care and Use Committee (no. 18605).

Body weights (range 2.3–6.0 g) were measured using a mini-electronic digital weight scale (Harbor Freight, Calbasas, California, USA). A limited physical examination was done to confirm gross clinical health. Species, age, classified as after-hatch year (AHY) or hatch year (HY), and sex were determined as previously described (Russell 2001). Blood samples were collected after a conservative toenail clip using commercial cuticle scissors (Revlon Co., New York, New York, USA); a silver nitrate stick was used for hemostasis (Owen 2011). Blood was first

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spotted onto filter paper for PCR analysis of hemoparasites (Bradshaw et al. 2017), followed by the placement of a drop onto a coverslip slide for smear preparation. Smears were air-dried and stored in a slide box for up to 2 wk prior to Wright's staining with an automatic slide stainer (Wescor Inc., Logan, Utah, USA).

We scanned smears for the presence and distribution of leukocytes; we excluded smears if large numbers of lysed cells or clots were observed. We estimated total white blood cells (WBCs) by averaging the number of cells in 15 fields, using a 100× oil objective. Different areas within each smear were evaluated to minimize potential bias caused by uneven distribution of leukocytes. The average number of WBCs was multiplied by 10,000 and rounded to the nearest integer to obtain the total WBC estimate (cells/μL). A 200-cell differential count was done by a single trained individual (R.B.), as previously described (Lilliehöök et al. 2004; Papasouliotis et al. 2006). Leukocytes were categorized as heterophils, lymphocytes, monocytes, eosinophils, and basophils. Percentage values were multiplied by the total WBC estimate to obtain absolute counts (cells/μL). Hemoparasites were reported if seen during smear examination.

Data were recorded in an Excel spreadsheet (version 14.7.3, Microsoft Corporation, Redmond, Washington, USA) and analyzed for outliers, distribution, descriptive statistics, and reference intervals by using Reference Value Advisor (Geffré et al. 2011). Outliers were flagged based on a Tukey's test and confirmed using the Dixon-Reed test to evaluate the distance from the outlier to the nearest value, divided by the whole range of values. Gaussian distribution was assessed by the Anderson-Darling test.

Since none of the data were normally distributed, we reported median, minimum, and maximum values. Mann-Whitney *U*-tests were used to compare leukocyte results based on species (Anna's Hummingbird vs. Black-chinned Hummingbird), sex (female vs. male), age (HY vs. AHY), and season (spring vs. summer) and to compare birds with and without hemoparasites observed in smears. A Kruskal-Wallis test was used to compare the results based on county (El Dorado, Yuba, Monterey, and Solano). GraphPad Prism 5.04 software (GraphPad Software, La Jolla, California, USA). Linear regression analysis was used to assess the relationship between total WBC counts and body weight. After adjustment for multiple comparisons by Bonferroni correction, *P*-values of <0.05 were considered to be statistically significant (JMP®, Version 11, SAS Institute Inc., Cary, North Carolina, USA). We excluded 22 samples because of clots, and 10 smears because of lysed cells. Single samples were also excluded: one from an Allen's Hummingbird (*Selasphorus sasin*), one from a Rufous Hummingbird (*Selasphorus rufus*), and one from a bird whose species was not identified. Blood smears from 42 Anna's Hummingbirds (*Calypte anna*) and 33 Black-chinned Hummingbirds (*Archilochus alexandri*) were included. The hummingbirds were from El Dorado (n=14), Yuba (n=13), Monterey (n=10), and Solano (n=38) counties and were sampled in summer (n=32) or spring (n=43). Hummingbirds included HY (n=13) and AHY (n=59) birds; the age of three hummingbirds could not be determined.

Reference intervals were calculated for all hummingbirds combined and for cell counts that differed significantly by age group (Table 1). Since data in all groups were non-Gaussian and asymmetrical, the nonparametric method was used to calculate reference intervals. When samples size was insufficient for the nonparametric method (<40), minimum and maximum values were reported as the reference interval (Friedrichs et al. 2012).

Morphologic features of hummingbird leukocytes were documented (Fig. 1). Fourteen of 75 (19%) hummingbirds had *Haemoproteus archilochus* observed within rare or a few erythrocytes. Leukocyte results for birds with hemoparasites were not statistically different from those of birds without parasites (P=0.360). No significant correlation was identified between the body weights and total WBC counts (Pearson correlation coefficient, P=0.412).

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TABLE 1. Descriptive statistics and reference intervals for total white blood cell (WBC) and differential leukocyte counts in visually healthy free-ranging Anna's Hummingbirds (*Calypte anna*) and Black-chinned Hummingbirds (*Archilochus alexandri*) collected in spring and summer of 2012 in northern California, USA. Values that differed significantly between after hatch year (AHY) and hatch year (HY) are presented separately.

			Cells/μL						
Group	Analyte	*n*	Mean	Median	SE	Minimum–maximum	Reference interval[a,f]	95% CI[b] (lower limit)[f]	95% CI[b] (upper limit)[f]
AHY	Total WBC estimate	59[c]	3,303	3,000	252.6	600–10,666	890–9,458	600–1,000	7,000–10,666
	Lymphocytes	59	1,504	1,546	121.3	315–6,186	340–5,812	315–415	4,159–6,186
AHY and HY	Heterophils	74	1,009	848	72.4	87–3,010	103–2,888	87–191	2,284–3,010
	Monocytes	74	87	58	19.4	0–520	0–430	0	245–520
	Eosinophils	74	67	46	16.5	0–300	0–283	0	208–300
	Basophils	74	183	159	19.7	0–805	0–626	0–7	446–805
HY	Total WBC estimate	13[d]	6,423	7,000[e]	658.4	2,500–12,000	ND	ND	ND
	Lymphocytes	13	4,280	3,845[e]	257.3	1,675–8,100	ND	ND	ND

[a] Calculated using the nonparametric method. One outlier each was removed in calculating reference intervals for heterophils (4913/μL), monocytes (960/μL), eosinophils (810/μL), and basophils (1,000/μL).

[b] 95% CI = 95% confidence interval around the lower and upper limits of the reference interval.

[c] *Calypte anna* (*n*=29), *Archilochus alexandri* (*n*=30).

[d] *Calypte anna* (*n*=10), *Archilochus alexandri* (*n*=3).

[e] Significantly higher than in after-hatch year birds (Mann-Whitney *U*-tests with Bonferroni correction, $P<0.0001$).

[f] ND = not determined because of small sample size. Minimum–maximum values should be used as the reference interval.

Lymphocytes were the predominant leukocytes in hummingbirds, similar to avian species such as budgerigars, canaries, cockatiels, finches, rose-ringed parakeets, and many species of Amazon parrots (Latimer and Bienzle 2011). Rare reactive lymphocytes were noted in six of the samples. Basophil counts exceeded eosinophil counts, similar to findings in other healthy birds (Vinkler et al. 2010; Latimer and Bienzle 2011; Campbell 2015). Significant differences in leukocyte counts were not observed between Anna's and Black-chinned hummingbirds ($P=0.261$).

The only significantly different values were greater lymphocyte counts in HY hummingbirds compared to AHY hummingbirds ($P<0.0001$; Table 1). This finding is consistent with physiologic age-related antigenic stimulation followed by lymphocyte proliferation in the bone marrow, thymus, and bursa of Fabricius (Latimer and Bienzle 2011). Lymphocytes from these organs are found in the circulation when they redistribute to populate peripheral lymphoid tissues. Increased numbers of lymphocytes in healthy juveniles are observed in other avian species as well as mammalian (Latimer and Bienzle 2011).

We did not find sex-based differences in the two species of hummingbirds in our study, which was similar to what has been found for Mallard (*Anas platyrhynchos*; Fairbrother and O'Loughlin 1990) but differs from what has been documented in Golden Quail (*Coturnix coturnix*) and in Orange-winged Amazon parrots (*Amazona amazonica*; Muhammad 2013; Vergneau-Grosset et al. 2016).

We report age-specific leukocyte reference intervals for free-ranging Anna's and Black-chinned hummingbirds in northern California. In the advent of population decline, this information will be useful for monitoring health or progression of disease and can be used for comparative analysis with other bird species. Our findings expand the cumulative data on avian blood leukocyte populations and distributions.

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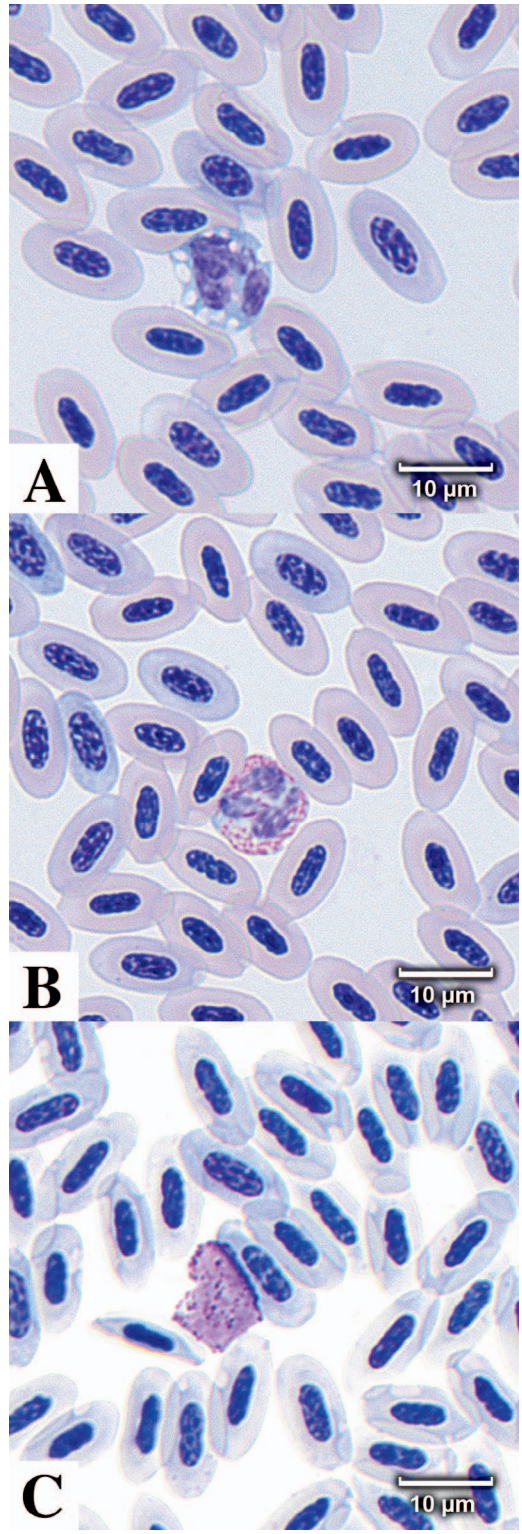

FIGURE 1. Images of leukocytes from Wright-stained blood smears from blood samples taken from visually healthy free-ranging Anna's Hummingbirds (*Calypte anna*) and Black-chinned Hummingbirds (*Archilochus alexandri*) collected in spring and summer of 2012 in northern California, USA. (A) Eosinophil; (B) heterophil; and (C) basophil.

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