Journal of Parasitology 2021 107(5) 794–798

Published 13 October 2021

DOI: 10.1645/20-168

Contents and archives available through www.bioone.org or www.jstor.org

Journal of Parasitology

journal homepage: www.journalofparasitology.org

LOW PREVALENCE OF HAEMOSPORIDIANS IN BLOOD AND TISSUE SAMPLES FROM HUMMINGBIRDS

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KEY WORDS

Bird
Blood parasite
Haemosporidian
Hummingbird
Blood
Tissue
Prevalence
Detection

ABSTRACT

Hummingbirds are vital members of terrestrial ecosystems, and because of their high metabolic requirements, they serve as indicators of ecosystem health. Monitoring the parasitic infections of hummingbirds is thus especially important. Haemosporidians, a widespread group of avian blood parasites, are known to infect hummingbirds, but little is known about the prevalence and diversity of these parasites in hummingbirds. The prevalence of haemosporidians in several hummingbird species was examined and we compared 4 different tissue types in detecting parasites by polymerase chain reaction (PCR). Blood samples from 339 individuals of 3 different hummingbird species were tested, and 4 individuals were found positive for haemosporidian infection, a prevalence of 1.2%. Hummingbird carcasses ($n = 70$) from 5 different hummingbird species were also sampled to assess differences in detection success of haemosporidians in heart, kidney, liver, and pectoral muscle tissue samples. Detection success was similar among tissue types, with haemosporidian prevalence of 9.96% in heart tissue, 9.52% in kidney tissue, 10.76% in liver tissue, and 11.76% in pectoral muscle tissue. All tissue samples positive for haemosporidian infection were from the Black-chinned Hummingbird (*Archilochus alexandri*). Possible reasons for low prevalence of these blood parasites could include low susceptibility to insect vectors or parasite incompatibility in these hummingbirds.

Hummingbirds (Family: Trochilidae) make up 1 of the most diverse families of birds in the world, with over 330 species occurring in habitats throughout the Americas (Bleiweiss, 1998; Williamson, 2001). Hummingbirds have an important role in the ecosystems they inhabit, serving as both pollinators of plants and as consumers of various insect species (Buzato et al., 2000; Yanega and Rubega, 2004). Because of their high metabolic requirements, hummingbirds are also indicators of ecosystem health and are susceptible to changing environmental conditions (Sarwar, 2020). Currently, over 14% of all hummingbird species are considered vulnerable, threatened, or endangered (Wethington and Finley, 2009), and 4 of the 7 species that breed in California are classified as "species of conservation concern" by the Partners in Flight Landbird Conservation Plan (Rosenberg et al., 2016). Habitat loss and degradation appear to contribute broadly to declines in hummingbird populations, although the impacts of other stressors have not been well studied (Wethington and Finley, 2009). Infectious agents and diseases are known to play a large role in avian population declines, although little is known about how they affect hummingbirds specifically (Godoy et al., 2014).

Hummingbirds are prone to exposure to a wide range of pathogens and diseases (Magagna et al., 2019), including avian pox virus (Godoy et al., 2013; Baek et al., 2020), West Nile virus (Backus et al., 2019), *Chlamydia* spp., *Salmonella* spp., *Aspergillus* spp., numerous helminth infections, various mites (Yamasaki et al., 2018), lice, ticks, and several species of protozoan blood parasites, including haemosporidians (Godoy et al., 2014; Bradshaw et al., 2017; Magagna et al., 2019). Haemosporidians are of significance because of their high diversity across the avian tree of life, with over 250 species of haemosporidian blood parasites infecting a wide variety of avian hosts (Valkiūnas et al., 2004). The pathogenicity of these parasites varies depending on avian host and parasite species, ranging from commensal to parasitic, where host reproduction and survival is adversely impacted (Valkiūnas et al., 2004; Merino et al., 2008; Asghar et al., 2015).

Several haemosporidian species in the genera *Haemoproteus* and *Plasmodium* are described in hummingbirds, though little is

known about the prevalence or effects of these parasites on hummingbird populations (White et al., 1979; Griner, 1983; Matta et al., 2014). The majority of these descriptions are from tropical areas in Central and South America, though infected hummingbirds have also been reported in more temperate climates in Canada and the United States (Williams, 1978; White et al., 1979). Recently, *Haemoproteus archilochus* was described in 2 species of hummingbirds found in California (Bradshaw et al., 2017). Of the 261 hummingbirds sampled by Bradshaw et al. (2017), only 2.55% of the Anna's Hummingbird (*Calypte anna*) and 17.31% of the Black-chinned Hummingbird (*Archilochus alexandri*) were infected. The low prevalence in these 2 species differs from some findings of higher haemosporidian prevalence in hummingbirds from other regions, and in birds from other avian families. Harrigan et al. (2014) screened multiple hummingbird species in the Andes and found several distinct haemosporidian lineages, and a haemosporidian prevalence of 31% from 1 hummingbird species, the White-whiskered hermit (*Phaethornis yaruqui*, Harrigan et al., 2014). Another study, also in the Andes, found that some hummingbird species harbored prevalences of up to 80% (Moens et al., 2016). Several more recent studies in South America have again found much higher prevalences of haemosporidian infections, up to 66% in certain species (Barrow et al., 2019; Fecchio et al., 2019). And 1 study, by Abad et al. (2021), found an unusually high prevalence of *Plasmodium* in 12 different hummingbird species from Ecuador, with 96% and 100% of hummingbirds in the 2 study areas infected. Although several hummingbird species from Central and South America were shown to have much higher prevalences of haemosporidians than what is known from North American hummingbirds, it is important to note that there were also many hummingbird species that had very low prevalence or lacked infections altogether (Moens et al., 2016; Barrow et al., 2019; Fecchio et al., 2019). It appears that haemosporidian prevalence within hummingbirds is highly dependent on species, and it remains unclear why certain species harbor higher parasite loads. Multiple factors could possibly be influencing this pattern, including host competency, vector specificity, and/or possible inconsistencies in sampling or detection methods (Godoy et al., 2014).

All of these studies, and the majority of studies on avian haemosporidia in general, use blood samples (analyzed by both microscopy and polymerase chain reaction [PCR]) as the primary sampling method for detection. In addition, several tissue types, including heart, liver, pectoral muscle, and kidney samples, have infrequently been incorporated with nonhummingbirds (Edwards et al., 2005; Svensson-Coelho et al., 2016). Recently, several studies have looked at the differences in detection of avian haemosporidia across different tissue and blood samples, although none have focused on detection in hummingbirds specifically. Svensson-Coelho et al. (2016) found detection rates of *Plasmodium* to be similar across blood, heart, liver, and pectoral muscle samples in 46 White-shouldered Fire-eyes (*Pyriglena leucoptera*), although a combination of all 3 tissue types did yield a higher detection rate compared to that of blood alone (Svensson-Coelho et al., 2016). Similarly, Fecchio et al. (2019) found that blood, muscle, and liver samples yielded similar detection rates for *Haemoproteus* and *Leucocytozoon* infections, but that blood samples had a higher prevalence for *Plasmodium* infections (Fecchio et al., 2019). Harvey and Voelker (2017) compared detection rates from paired blood and pectoral muscle samples from 220 birds from 7 different orders, comprising 78 different species, and found higher prevalence and genetic diversity from blood samples in regard to *Haemoproteus* infections but higher prevalence in pectoral muscle in regard to *Plasmodium* infections. These inconsistencies indicate that further research into the best method of detecting haemosporidia in avian hosts is needed and could be dependent on both avian host and parasite lineage. Host-specific studies on best source materials for detection are lacking and could provide valuable information when assessing the prevalence of haemosporidia in specific groups of birds.

Table I. Number of hummingbird blood samples that tested positive for *Haemoproteus*/*Plasmodium* infection over total number of samples taken for each age and sex category based on species. The samples were tested using a nested polymerase chain reaction protocol. Abbreviations: ALHU, Allen's Hummingbird; RUHU, Rufous Hummingbird; ANHU, Anna's Hummingbird.

Species	ALHU	RUHU	ANHU	Hybrid*
Hatch year male	0/25	0/13	0/0	0/0
Hatch year female	0/12	0/2	0/0	0/0
After hatch year male	0/105	2/23	0/2	0/42
After hatch year female	2/73	0/19	0/1	0/22
Total	2/215	2/57	0/3	0/64

* Hybrid refers to hummingbirds that were identified as ALHU × RUHU hybrids via hybrid index/linear discriminant function analysis.

Our study aimed to expand on the knowledge regarding haemosporidian prevalence in North American hummingbirds and investigated the likelihood of detection of haemosporidia from different tissue types. We screened blood samples from 339 live individuals from 3 different hummingbird species to detect haemosporidian prevalence. We then screened 70 carcasses from 5 hummingbird species from various wildlife rehabilitation centers in California, Oregon, and Tennessee to study differences in detection success of haemosporidians in heart, kidney, liver, and pectoral muscle tissue samples.

To detect haemosporidian prevalence in hummingbirds we screened 215 Allen's Hummingbird (*Selasphorus sasin*), 57 Rufous Hummingbird (*Selasphorus rufus*), 64 Allen's × Rufous Hummingbird hybrids (*Selasphorus sasin* × *rufus*), and 3 Anna's Hummingbird (*Calypte anna*) (Table I). Individuals were captured using drop nets at feeders from March 2016 to September 2017 at multiple localities throughout California and Oregon. Allen's × Rufous Hummingbird hybrids were diagnosed via hybrid index/linear discriminant function analysis following methods described in Myers et al. (2019). Upon capture, each individual was inspected physically to assess health status, species, age (either hatch year or after hatch year), and sex (Pyle and Ruck, 1997; Russell and Russell, 2019). Blood was collected by clipping the distal toenail of the third digit and stored on Nobuto filter strips (Advantec, Dublin, California) at room temperature, following methods from Bradshaw et al. (2017). DNA was extracted from the blood using a DNAeasy Blood and Tissue Kit (Qiagen, Valencia, California), and extractions were tested in triplicate for haemosporidian infection using standard nested PCR protocols to amplify a known region of the mtDNA cytochrome *b* gene (Waldenström et al., 2004; Walther et al. 2015). PCR products

Table II. Number of tissue samples that positive for *Haemoproteus*/*Plasmodium* infection over total tissue samples for 6 hummingbird species. (In some cases, not all tissues could be dissected from each individual bird.) Abbreviations: ALHU, Allen's Hummingbird; RUHU, Rufous Hummingbird; ANHU, Anna's Hummingbird.

Species	Heart	Pectoral muscle	Kidney	Liver	Total birds positive
RTHU	0/10	0/10	0/10	0/10	0/10
ANHU	0/33	0/32	0/28	0/29	0/34
RUHU	0/1	0/1	0/1	0/1	0/1
Hybrid*	0/1	0/1	0/1	0/1	0/1
CAHU	0/1	0/1	0/1	0/1	0/1
BCHU	6/21	8/23	6/22	7/23	8/23
Total	6/67	8/68	6/63	7/65	8/70

* Hybrid refers to hummingbirds that were identified as ALHU × RUHU hybrids via hybrid index/linear discriminant function analysis.

were visualized on agarose gels, with 1 positive triplicate confirming a haemosporidian infection.

Of the 339 individual hummingbirds tested for haemosporidians via blood sample, 4 (2 Allen's and 2 Rufous Hummingbirds) tested positive for *Haemoproteus*/*Plasmodium* infection by PCR. Prevalence of haemosporidians by species was 0.93% in Allen's Hummingbird, 3.5% in Rufous Hummingbird, and 0% in both Anna's Hummingbird and Allen's × Rufous Hummingbird. All 4 infected individuals were aged as after-hatch-year birds. The 2 Allen's Hummingbird infected were both females captured from the same locality, Inverness (38°6′3.5994″N, 122°51′25.1994″W), in northern California. Both infected Rufous Hummingbird were males, 1 captured from Sunset Bay State Park, Oregon (43°20′2.4″N, 124°22′15.6″W) and 1 from William M. Tugman State Park, Oregon (43°36′3.5994″N, 124°10′33.6″W).

To assess differences in detection across sample types, tissue samples from 34 Anna's Hummingbirds, 23 Black-chinned Hummingbirds (*Archilochus alexandri*), 10 Ruby-throated Hummingbirds (*Archilochus colubris*), 1 Rufous Hummingbird, 1 Calliope Hummingbird (*Selasphorus calliope*), and 1 Allen's × Rufous Hummingbird hybrid carcasses were used (Table II). For the majority of individual hummingbirds, all 4 tissue types (heart, liver, kidney, and pectoral muscle) were analyzed for haemosporidian infections. In total, 67 heart tissues, 63 kidney tissues, 65 liver tissues, and 68 muscle tissues were used for this analysis (Table II). We were unable to dissect each of the tissues from every individual. DNA was extracted from tissues and tested for presence of *Haemoproteus* or *Plasmodium* DNA using the same methods outlined previously.

Of the 70 individual hummingbirds screened, 8 tested positive for *Haemoproteus*/*Plasmodium* infection by PCR. All positive tissue samples came from Black-chinned Hummingbirds. Positive carcasses came from several different wildlife rehabilitation centers in the following locations: 1 from Solano, California; 1 from Yolo, California; 1 from Los Angeles, California; 2 from Tucson, Arizona; 1 from Paradise, Arizona; 1 from Benson, Arizona; and 1 from Green Valley, Arizona. This finding corroborates earlier results of a slightly higher prevalence of *Haemoproteus* found in Black-chinned Hummingbird compared to other species (Bradshaw et al., 2017). Six individuals tested positive across all 4 tissue types, 1 individual testing positive for haemosporidian infection only in pectoral muscle tissue, and 1 testing positive for infection in liver and pectoral muscle tissue. From all 70 individuals screened, haemosporidian prevalences were 9.96% from heart tissue, 9.52% from kidney tissue, 10.76% from liver tissue, and 11.76% from pectoral muscle tissue.

Several Haemosporidians are known to infect members of the Trochilidae family. In South America, unidentified *Plasmodium* species also infect hummingbirds (Griner, 1983; Bennett et al., 1993; Abad et al., 2021). However, *Plasmodium* have not yet been found in North American hummingbirds, although North American hummingbirds have been identified with infection from *Haemoproteus* (Godoy et al., 2013). Worldwide, *Haemoproteus archilochus*, *Haemoproteus trochili*, and *Haemoproteus witti* are the 3 known species that infect members of the Trochilidae family (White et al., 1979; González et al., 2015; Moens et al., 2016). *Haemoproteus archilochus* is the only species previously identified in North American hummingbirds, and is also known to infect South American hummingbirds, as well as several passerine species (Coatney and West, 1938; Bradshaw et al., 2017). Although we were unable to sequence the PCR results of these samples, we expect the parasites are of this same species, *H. archilochus,* as found in other North American hummingbirds.

The low prevalence of haemosporidians we found in Allen's, Rufous, Anna's and Ruby-throated Hummingbird corroborates what was previously observed in Anna's and Black-chinned Hummingbird in California (Bradshaw et al., 2017). Although similarly low prevalences of haemosporidia have been found in other species of hummingbirds closer to the equator, the same regions also contained hummingbird species with much higher prevalences. The hummingbird community as a whole in North America appears to have a much lower prevalence of haemosporidians than that of hummingbird communities in other regions (Barrow et al., 2019; Fecchio et al., 2019). One possible explanation for the low prevalence of haemosporidians in North American hummingbirds could be because of the interaction between the vectors, biting midges, and hummingbird hosts. Although sampling for vectors in concurrence with hummingbird sampling was not conducted, *Haemoproteus* readily infects many other avian species in North America and other regions with similar temperate climates. Thus, it seems likely that *Haemoproteus* vectors are present in the locations we sampled from (Clark et al., 2014). A hummingbird-specific life history trait that promotes lower exposure of nestlings compared with other birds could explain this, but further studies into the possible mechanisms behind this need to be done. Vector avoidance by hummingbirds is not likely, as hummingbirds enter torpor at night, which makes them vulnerable to biting insects. A lack of biting midges specific to hummingbirds in North America could be a possible explanation and needs to be further investigated.

Another possible explanation for the low prevalence of *Haemoproteus* in North American hummingbirds compared to South American hummingbirds could be related to the competency of North American hummingbird species as hosts for haemosporidians. North American hummingbirds could potentially be nonadapted hosts, causing abortive development of haemosporidians, which would still be picked up as a positive infection via PCR (Valkiūnas et al., 2014). We were unable to obtain blood smears for these samples, but they would be imperative to examine in the future, in addition to analyzing blood via PCR, to confirm that these parasites are actively completing their life cycle in these hummingbirds. Moens et al.

(2016) found that of the lineages of *Haemoproteus* in several South American hummingbird species sampled, all were generalists known to infect a wide array of hosts across many avian orders. This suggests that infections of *Haemoproteus* in hummingbirds are the result of multiple recent spillover events. Perhaps these same events have yet to occur in North American hummingbirds, where diversity and richness of avian species is much lower. One *Haemoproteus* species examined, *H. witti*, was found to have a high prevalence in hummingbirds and appeared to rely on hummingbirds as a reservoir host (Moens et al., 2016). The colonization of hummingbirds by *H. witti* as new hosts and subsequent exploitation of hummingbirds as a reservoir host in South America suggests that the same process may occur within North American hummingbirds.

Differences between hummingbird species life history traits could also explain the variability in haemosporidian prevalence between hummingbird species. Certain factors, such as body size and migration routes, could affect parasite prevalence. All positive tissue samples in our study came from Black-chinned hummingbird, a species that appears to harbor a higher prevalence of haemosporidians than other sympatric hummingbird species (Bradshaw et al., 2017). One potential reason for this difference could be due to habitat usage. Black-chinned Hummingbirds utilize riparian habitats, as do biting midges, the usual vector or *Haemoproteus* sp. (Valkiūnas et al., 2004; Bradshaw et al., 2017). Although other hummingbird species included in the study, such as Anna's Hummingbird, are also found in riparian habitats, Black-chinned Hummingbird are migratory, traveling to wintering grounds in Mexico, where they could potentially be exposed to more vectors and parasites (Baltosser and Russell, 2000).

The drivers of lower prevalence of haemosporidian infections in hummingbirds in North America remain unclear. However, identifying these drivers is important in order to address the future distribution of haemosporidians in hummingbirds, the movement of haemosporidian parasites in the environment, and the ability of closely related species to harbor different competencies and risks of infection for haemosporidian pathogens. A larger amount of blood smears and blood samples from live hummingbirds of multiple different species across the Americas is a crucial next step to inform the current state of this pathogen. Future experimental infection studies looking at changes in hummingbird physiology and behavior are also important to determine the effects of these parasites on hummingbird health.

The authors thank the Lindsay Wildlife Experience, Cynthia Routledge (Southeastern Avian Research), and Dr. Susan Wethington (Hummingbird Monitoring Network) for providing hummingbird carcasses (birds that did not survive rehabilitation) for this study.

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