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ORIGINAL PAPER

Detection and prevalence of *Haemoproteus archilochus* (Haemosporida, Haemoproteidae) in two species of California hummingbirds

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Abstract Haemosporidian blood parasites are transmitted to a wide range of avian hosts via blood-sucking dipteran vectors. Microscopy has revealed an impressive diversity of avian haemosporidia with more than 250 species described. Moreover, PCR and subsequent sequence analyses have suggested a much greater diversity of haemosporidia than morphological analyses alone. Given the importance of these parasites, very few studies have focused on the charismatic hummingbirds. To date, three *Haemoproteus* species (*Haemoproteus archilochus*, *Haemoproteus trochili*, and *Haemoproteus witti*) and one *Leucocytozoon* species (*Leucocytozoon quynzae*) have been described in blood samples taken from hummingbirds (Trochilidae). Unconfirmed *Plasmodium* lineages have also been detected in hummingbirds. Here, we report the detection of *H. archilochus* in two hummingbird species (*Calypte anna* and *Archilochus alexandri*) sampled in Northern California and perform a phylogenetic analysis of mitochondrial cytochrome *b* (cyt *b*) gene lineages. A total of 261 hummingbirds (157 *C. anna*, 104 *A. alexandri*) were sampled and screened for blood parasites using PCR and microscopy techniques. Combining both methods, 4 (2.55%) haemosporidian infections were detected in *C. anna* and 18 (17.31%) haemosporidian infections were detected in *A. alexandri*. Molecular analyses revealed four distinct *H. archilocus* cyt *b* lineages, which clustered as a monophyletic clade. No species of *Plasmodium* or *Leucocytozoon* were detected in this study, raising the possibility of specific vector associations with hummingbirds. These results provide resources for future studies of haemosporidian prevalence, diversity, and pathogenicity in California hummingbird populations.

Keywords Avian blood parasites · Prevalence · Diversity · California · Hummingbirds · *Haemoproteus archilochus*

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Introduction

Hummingbirds (Trochilidae) are a New World avian family composed of 338 species that range from Alaska to South America, with 9 species of hummingbirds regularly occurring in California (Williamson 2001; McGuire et al. 2014). Across the Americas, hummingbirds provide vital ecosystem services, including insectivory and pollination, and were worshipped in belief systems of diverse societies ranging from the Aztecs to the Caribbean Taino (Buzato et al. 2000; Burton 2001; Yanega and Rubega 2004). Hummingbirds are also valuable sentinel species as landscape level "samplers" as changes in their health can be indicative of underlying environmental changes and overall ecosystem health (Godoy et al. 2014). Four of the seven hummingbird species that regularly breed in California are classified by the National Audubon Society as "species of conservation concern" (Audubon.org). Potential risks to hummingbird populations include infectious diseases, habitat degradation, climate change, and

invasive species (Godoy et al. 2014). Though chronic infections with haemoparasites were often thought to be relatively harmless, they have been experimentally demonstrated to be pathogenic in wild birds, and potential impacts on hosts vary among blood parasite species (Earlé et al. 1993; Merino et al. 2000; Marzal et al. 2005; Olias et al. 2011; Asghar et al. 2015). A baseline understanding of hummingbird population health and disease ecology will facilitate the detection and monitoring of future threats to sensitive species as well as determine ecological parameters underlying disease prevalence (Parashar et al. 2009).

We examined the diversity and prevalence of Haemosporidian parasites (Phylum: Apicomplexa) in two species of California hummingbirds: black-chinned hummingbird (*Archilochus alexandri*) and Anna's hummingbird (*Calypte anna*), hereafter referred to as BCHU and ANHU, respectively. Hummingbird blood samples were surveyed for the Haemosporidian genera *Plasmodium*, *Haemoproteus*, and *Leucocytozoon*. Hummingbirds are known to be infected by three *Haemoproteus* sp.: *Haemoproteus trochili*, *Haemoproteus archilochus*, and *Haemoproteus witti*, all of which appear to be specific to the family Trochilidae (White et al. 1979; Valkiūnas 2005; Moens et al. 2016). Recently, *Leucocytozoon quynzae* from Colombia was described as the first of the genus in hummingbirds (Matta et al. 2014). In South America, several *Plasmodium* and *Haemoproteus* lineages were recently reported in hummingbirds, suggesting that these birds are commonly infected, and that the diversity of parasites is probably higher than reported in the literature (Harrigan et al. 2014; Moens et al. 2016; Valkiūnas et al. 2004). To our knowledge, this is the first study using both microscopy and molecular techniques to investigate Haemosporidian infections of North American hummingbirds.

The aims of this study were to (1) identify and determine the prevalence of Haemosporidian blood parasite infections in blood samples from hummingbirds captured in Northern California, and (2) determine the phylogenetic relationship of detected *Haemoproteus* cyt *b* lineages to previously classified *Haemoproteus* species.

Materials and methods

Sample collection and processing

To determine the prevalence of Haemosporidian infections among ANHU and BCHU in California hummingbirds, a total of 261 individuals (ANHU n = 157; BCHU n = 104) were captured using Hall drop nets at feeders from January 2012 to August 2013 at locations throughout the state. Most birds were sampled at three primary sites in Central California: Putah Creek, Winters (38°32′03″N, 121°50′37″W); Big Creek UC Nature Reserve, Big Sur (36°1′30″N, 121°31′26″W); and Oregon House (39°20′3″N, 121°16′33″W). Each bird sampled was physically examined to determine its health status and was classified by species, sex, and age (relative to hatch year) based on anatomic features and plumage. Hummingbirds were banded with unique identifying bands following UC Davis IACUC approved protocol #18605 under Federal Bird Banding Laboratory Permit #22157, Federal Scientific Collecting Permit MB55944B-1, and California State Scientific Collecting Permit #13066.

Blood was obtained by clipping a distal toenail, a safe method associated with a very low risk of harm to hummingbirds (Owen 2011). Blood was collected on Nobuto filter strips (Advantec, Dublin, CA) and stored at room temperature (Dusek et al. 2011). When possible, additional blood was used to make a blood smear and stained with Wrights-Giemsa (Campbell and Dein 1984). To compare haemoparasite lineages from this study across a broader landscape, 29 additional samples of ANHU and BCHU dating back to 2008 from California, Texas, and Arizona were analyzed concurrently.

Molecular screening and sequencing

DNA was extracted from blood dried on Nobuto strips using a DNAeasy Blood and Tissue kit (Qiagen, Valencia, CA) and tested for parasites using nested polymerase chain reaction (PCR) protocols (Valkiūnas et al. 2008). We used primers to amplify a section of the mtDNA cytochrome *b* gene (Hellgren et al. 2004). Primers HaemNF and HaemNR2 were used to amplify a 478-bp fragment (excluding the primers) from *Haemoproteus* and *Plasmodium*. Primers HaemF and HaemR were then used to perform a second nested PCR (Hellgren et al. 2004; Waldeonstöm et al. 2004). To amplify *Leucocytozoon* spp., we conducted another nested PCR in which primers DW2 and DW4 were used for the first round and primers Leuco Cyt F and Leuco Cyt R for the nested round (Perkins and Schall 2002; Sato et al. 2007). Reaction conditions and reagents were as described in Carlson et al. (2016).

To detect *Haemoproteus* and *Plasmodium* spp., the cycling profile for both PCR reactions consisted of an initial denaturation for 3 min at 94 °C, followed by 20 cycles of denaturing at 94 °C for 30 s, annealing at 50 °C for 30 s, and extension at 72 °C for 45 s, followed by a final extension at 72 °C for 10 min. The cycling profile for both PCR reactions testing for *Leucocytozoon* spp. consisted of an initial denaturation for 3 min at 94 °C, followed by 35 cycles of denaturing at 94 °C for 30 s, annealing at 54.5 °C for 30 s, and extension at 72 °C for 60 s, followed by a final extension at 72 °C for 10 min. PCR products were viewed on 1.8% agarose gels and visualized using GelStar (Lonza, Basel, Switzerland) or SYBR Safe (Life Technologies, Carlsbad, CA). Positive PCR products were sent to QuintaraBio (Richmond, CA) for purification and bi-directional sequencing.

Bi-directional sequences were aligned and manually edited with Geneious v. 7.1.9 (http://www.geneious.com; Kearse et al. 2012). Sequence analysis showed no evidence of double peaks in the chromatograms, indicating no mixed infections. Genera were distinguished by comparing sequences to their closest matches in GenBank, using NCBI nucleotide BLAST (Basic Local Alignment Search Tool) (Altschul et al. 1990).

Phylogenetic analyses

A phylogenetic tree was constructed using 25 cyt *b* lineages (478 bp) of haemosporidian parasites consisting of 4 sequences of *Haemoproteus* spp. from this study and 20 *Haemoproteus* spp. sequences from GenBank. For a comparison with the only other *Haemoproteus* spp. sequenced from hummingbirds, a single sequence of *Haemoproteus witti* was included in the phylogeny. A single sequence of *Leucocytozoon quynzae* (also from hummingbirds) was used as an outgroup. The phylogeny was constructed using Bayesian analysis with MRBAYES v. 3.2 (Ronquist and Huelsenbeck 2003) using the general time-reversible model (GTR + I + T). Two Markov Chain Monte Carlo simulations were run simultaneously for 10 million generations, with sampling every 200 generations, creating 100,000 trees. Twenty-five percent of trees generated were discarded as burn-in. The remaining 75,000 trees were used to construct a majority rule consensus tree and to estimate posterior probabilities of individual nodes. Genetic distances between cyt *b* lineages were also calculated using Geneious v. 7.1.9 (http://www.geneious.com; Kearse et al. 2012).

Microscopy

Blood smears were examined at low magnification (×500) light microscopy followed by the evaluation of parasite-infected cells under oil emersion at high magnification (×1000). Microscopy allowed haemoparasites to be identified by an experienced clinical laboratory specialist from the hematology laboratory at the UC Davis Veterinary Medicine Teaching Hospital. All resulting images and data were confirmed by the authors. Representative slides are deposited in the Queensland Museum, Queensland, Australia (G466197-G466200).

Statistical analysis

Prevalence calculations among species, sex, location, and age were compared using Fisher's Exact test. A p value of 0.05 or less was considered significant. Birds recaptured ≥5 months apart were included, but counted only once, unless their infective stage changed.

Results

Diversity and prevalence

A total of 261 hummingbirds (ANHU n = 157; BCHU n = 104) were sampled over the course of our study. Thirteen birds (ANHU n = 2; BCHU n = 11) were positive for the presence of parasite cyt *b* DNA by PCR (Table 1). Sequence analysis revealed four distinct *Haemoproteus* cyt *b* lineages, but no *Leucocytozoon* spp. or *Plasmodium* spp. amplicons were detected (Table 1). Within-species prevalence of *Haemoproteus* spp. detected by PCR was 1.27% in ANHU and 10.58% in BCHU (Table 1). Five birds were recaptured, 5 months later, but none exhibited a change in infection status.

Seventy-nine blood smear slides (ANHU n = 42; BCHU n = 37) were examined for the presence of haemosporidia. Sixteen blood smear slides were positive for *H. archilochus* (Table 1) as identified by the characteristic circumnuclear gametocytes (Fig. 1) (White et al. 1979; Valkiūnas 2005). Within-species prevalence detected by microscopy was determined to be 4.76% in ANHU and 37.84% in BCHU (Table 1).

As expected, some discordance between blood smear slide and PCR results was seen in this study. Of the four ANHU positive samples, two were detected only by PCR while the other two were detected only by microscopy. Of the 18 BCHU positive samples, 3 were detected by PCR only, 7 were detected by microscopy only, and 7 were detected by both techniques.

BCHU were significantly more likely to be infected than ANHU (p = 0.00004) and after hatch years were significantly more likely to be infected than hatch years (p = 0.015). No other significant differences in prevalence were found between sexes or geographical locations. Evaluating PCR positive or slide positive samples separately or in combination did not significantly affect the statistical results.

Phylogenetic relationships

Four distinct *H. archilochus* cyt *b* lineages were identified with sequence divergence percentages among them of ≤0.84% (Table 2). The closest matching lineage was a *Haemoproteus* sp. (11PMALO, GenBank accession number KJ661248) from a green-fronted lancebill (*Doryfera ludoviciae*, family Trochilidae) captured in Peru (Harrigan et al. 2014) that was 0.44% divergent (Table 2) and grouped into a single clade with *H. archilochus* lineages from this study (Fig. 2). All four *H. archilochus* lineages from this study were at least 6.64% divergent from all other described species lineages catalogued in GenBank (as viewed on October 11, 2016). A study by Moens et al. (2016) recently provided a molecular characterization of *Haemoproteus witti*, one of three *Haemoproteus* species to infect hummingbirds. Our analysis show that *H. witti* is 6.90% divergent (Table 2) and

Table 1 *Haemoproteus archilochus* prevalence and lineages (HUMHA1–HUMHA4) identified in this study among hummingbird host species (ANHU and BCHU)

Hummingbird (Trochilidae) host Species	Total			PCR				Microscopy		
	Number of individuals sampled[a]	Number of unique individuals infected	Prevalence	Number of positives	Prevalence	GenBank accession number	Lineage	Number of lineages	Number of individuals sampled	Number of positives	Prevalence
Calypte anna (ANHU)	157	4	2.55%	2	1.27%	KY560444	HUMHA1	1	42	2	4.76%
						KY560447	HUMHA4	1			
Archilochus alexandri (BCHU)	104	18	17.31%	11	10.58%	KY560445	HUMHA2	1	37	14	37.84%
						KY560446	HUMHA3	1			
						KY560447	HUMHA4	9			

[a] All individuals sampled were tested using PCR

does not group in the clade with *H. archilochus* and 11PMALO (Fig. 2). *H. archilochus* lineages from this study have been deposited in GenBank and assigned accession numbers KY560444–KY560447.

Discussion

By identifying molecular markers of the mitochondrial cytochrome *b* gene, we were able to match sequences and blood film samples from this study to a haemosporidian morphospecies. We found that ANHU and BCHU were hosts to four distinct lineages of *H. archilochus*, a parasite previously described only by morphology. DNA sequences from this study will aid in diagnostics of other less studied Haemosporidian parasite infections. In addition, the identification and molecular characterization of *H. archilochus* in hummingbirds of the western United States will clarify pathogenic risks to hummingbird species of the region.

H. archilochus, along with *H. trochili* and *H. witti*, are the only three *Haemoproteus* sp. known to infect the host family Trochilidae (White et al. 1979; Valkiūnas 2005; González et al. 2015; Moens et al. 2016). *H. archilochus* was originally described from a single ruby-throated hummingbird (*Archilochus colubris*) in Nebraska (Coatney and West 1938). More recently, *H. archilochus* was redescribed from a blood film from a black-chinned hummingbird (BCHU) (a focal species in this study) (Greiner et al. 1975; White et al. 1979). This parasite was also recorded by Williams (1978) in British Columbia from two rufous hummingbirds (*Selasphorus rufus*).

We found that *H. archilochus* is most similar to *Haemoproteus* spp. infecting passerines, as well as small numbers of hummingbird species, catalogued in South America. Sequence similarity to species found on continents

Fig. 1 Light microscopy image of *Haemoproteus archilocus*-infected erythrocytes from an adult black-chinned hummingbird (*Archilochus alexandri*). Note the circumnuclear gametocyte characteristic of the species (*arrow*). Magnification = ×1000

Table 2 The sequence divergence (in percentage) among 4 unique *Haemoproteus archilochus* mitochondrial cytochrome *b* (478 bp) lineages detected in this study and 8 *Haemoproteus* spp.

Haemoproteus sp. lineage	1	2	3	4	5	6	7	8	9	10	11	12
H. archilochus (KY560444)[a]	–											
H. archilochus (KY560445)[a]	0.84	–										
H. archilochus (KY560446)[a]	0.63	0.63	–									
H. archilochus (KY560447)[a]	0.42	0.42	0.21	–								
H. sp. (KJ661248)	0.88	0.44	0.44	0.44	–							
H. majoris (AF254977)	7.53	7.32	7.32	7.11	7.68	–						
H. balmorali (DQ060767)	8.72	8.94	9.36	9.15	9.21	5.32	–					
H. minutus (DQ060772)	6.64	7.06	7.06	6.85	7.02	4.13	4.89	–				
H. witti (KU364540)[b]	7.11	7.11	7.11	6.90	7.24	3.77	5.32	3.09	–			
H. parabelopolskyi (AY831750)	8.79	9.00	8.79	8.58	9.21	7.53	7.87	6.85	5.65	–		
H. belopolskyi (DQ000321)	7.74	7.32	7.53	7.32	7.24	6.07	7.02	5.81	4.60	6.07	–	
H. multipigmentatus (GU296214)	13.81	13.60	13.60	13.39	12.72	12.76	13.83	13.13	12.76	13.39	10.67	–

Sequence divergence percentages were calculated using the GTR + I + G substitution model

[a] Lineages of *Haemoproteus archilochus* from this study

[b] *H. witti* (KU364540) from hummingbird (Trochilidae) host *Adelomyia melanogenys*

other than North America is of considerable interest, suggesting possible co-speciation of parasites and their hosts across great distances.

Though broad surveys and the hundreds of distinct haemoparasite lineages already published indicate that host-switching events do occur, and that Haemosporidians can be specialists or generalists, there is evidence that closely related haemoparasite species infect closely related hosts, leading to a strong host-lineage effect on parasite distributions (Ricklefs and Fallon 2002; Waldeonstöm et al. 2004; Fallon et al. 2003). This latter model is potentially supported by our observation that Trochilidae in California were only found infected with closely related lineages of *H. archilochus*. Similarly, Moens et al. (2016) found that while hummingbirds are predominantly infected by generalist parasites such as *H. witti*, these parasites can be highly host dependent and be thought of as hummingbird specialists. The difficulty discerning host specialization in *Haemoproteus* sp. infecting hummingbirds could be the result of their unique physiology as a host.

We saw significant differences in the prevalence of *H. archilochus* between species and age classes. Of particular interest is the significantly higher prevalence of *H. archilochus* infection among BCHU both when analyzed using PCR and microscopy (Table 1). Based on their close phylogenetic relationship with other *Haemoproteus* (*Parahaemoproteus*) species, the *H. archilochus* reported here are likely transmitted by biting midges (Ceratopogonidae) (Valkiūnas and Iezhova 2004), which often inhabit riparian zones. Since both BCHU and ANHU were commonly found in riparian habitats during sampling for this study, causes of the discrepancy in *H. archilochus* prevalence between the BCHU and ANHU remain unclear. A potential explanation might be differences in exposure to vectors at their wintering habitats. While ANHU typically stay along the Pacific coast of North America year round with minimal migratory movement (Williamson 2001), BCHU are medium- to long-distance migrants. During migration and at wintering habitats in western Mexico (Baltosser and Russell 2000), BCHU vector exposure could be higher than in habitats used by both species at more northern latitudes.

As expected, adult birds are significantly more likely to be infected than juveniles because adults have had more time to come into contact with parasite vectors. Adults are also likely to have encountered more potential vectors during seasonal migrations, which juveniles had yet to undertake. Additionally, by virtue of having already survived to adulthood, adult hummingbirds may have more robust immune systems than juveniles and thus may be less likely to succumb to parasitemia-induced mortality. Though we did not observe geographical differences in prevalence, this could be the result of sampling over a small geographic range or at a scale not capable of discerning geographical patterns.

Actual prevalence of Haemosporidia is likely higher than reported in this study due to multiple factors. The amount of blood collected was limited by the small size of our focal species, thus it was not possible to make a blood smear for every individual sampled. Blood smears were often of low volume and not fixed immediately due to field locations, which might decrease the success of haemoparasite detection via microscopy (Valkiūnas et al. 2008). Similarly, the amount of blood available to test via PCR was often limited. Finally, extended PCR protocols require high-quality DNA and though Nobuto paper is an efficacious storage medium for blood (Michaud et al. 2007), DNA degradation rates during long-term storage at room temperature are not

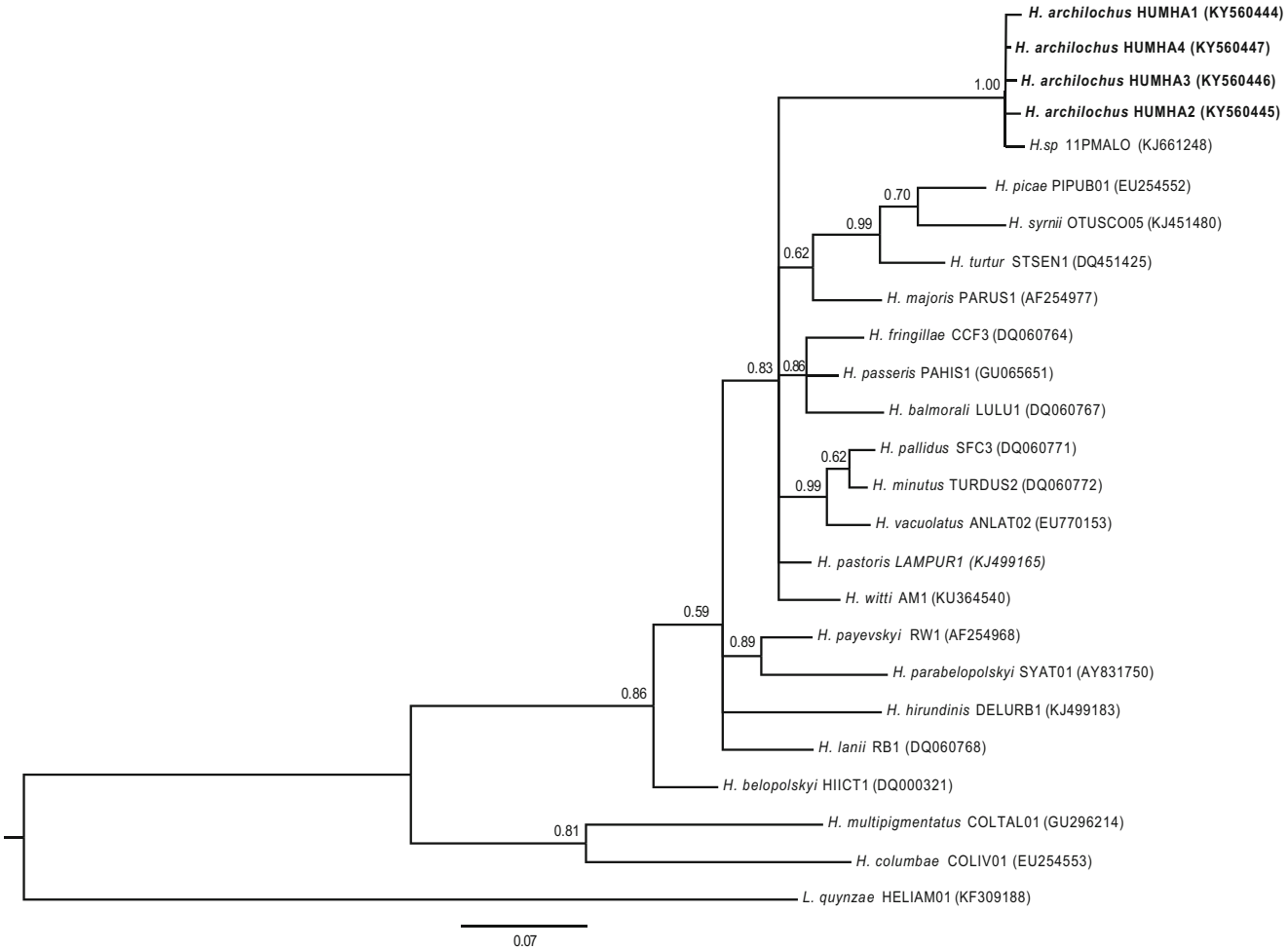

Fig. 2 Bayesian phylogeny of 24 *Haemoproteus* spp. cytochrome *b* (cyt *b*) gene sequences created in MrBayes v3.1.2 using the GTR + I + G substitution model. Posterior probabilities are depicted above each node. *Haemoproteus archilochus* lineages identified in this study are given in *bold*. MalAvi lineage codes and GenBank accession numbers (in *parentheses*) follow parasite species. A single *Leucocytozoon quynzae* lineage was used as an outgroup. Branch lengths are proportional to the amount of change. *Scale bar* shows substitutions per site

known (Valkiūnas et al. 2008). We did not examine temporal effects on the prevalence of infection because the seasonal status of each species has been only qualitatively described in our study region, thus making the designation of temporal bins too subjective at present.

The pathogenicity of the detected *H. archilochus* infections on hummingbird hosts is not known. Though sampled hummingbirds were considered to be healthy based on field-based physical examinations, the possibility that chronic Haemosporidian infection can be recurrent or cause subtle health effects with a deleterious impact on fitness cannot be ruled out (Merino et al. 2000; Martínez-de la Puente et al. 2010; Godoy et al. 2014; Asghar et al. 2015). With a rapidly changing global climate and anthropogenic alterations of these species' natural habitats, infectious disease risks are likely to be above historic levels and can be expected to potentially increase even further (Aguirre and Tabor 2008). Detecting pathogens that reduce individual fitness and threaten population health, identifying those that may emerge in the future, and determining the reservoirs and vectors of such infections, will aid biologists and conservationists alike in their efforts to better understand and protect these species.

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