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Short Communication

Prevalence of haemosporidians in a Neotropical endemic bird area

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ABSTRACT. Haemosporidians are vector-transmitted intracellular parasites that occur in many bird species worldwide and may have important implications for wild bird populations. Surveys of haemosporidians have traditionally focused on Europe and North America, and only recently have they been carried out in the Neotropics, where the prevalence and impacts of the disease have been less studied and are not well understood. In this study we carried out a survey in the endemic bird area of the Sierra Nevada de Santa Marta (SNSM), an isolated coastal massif in northern Colombia that contains a large number of biomes and that is experiencing high rates of habitat loss. We sampled birds from 25 species at 2 different altitudes (1640 and 2100 m asl) and determined avian haemosporidian infection by polymerase chain reaction and sequencing a portion of the cytochrome b (cyt b) gene of the parasite. From the sampled birds, 32.1% were infected by at least 1 of 12 unique cyt b lineages of haemosporidian genera: *Plasmodium*, *Leucocytozoon*, *Haemoproteus*, and subgenus *Parahaemoproteus*. We found a higher prevalence of avian haemosporidians at low altitudes (1640 m asl). All endemic bird species we sampled had at least one individual infected with avian haemosporidians. We also found evidence of higher overall prevalence among endemic rather than nonendemic birds, suggesting higher susceptibility in endemic birds. Overall, our findings suggest a high haemosporidian species richness in the bird community of the SNSM. Considering the rate of habitat loss that this area is experiencing, it is important to understand how avian haemosporidians affect bird populations; furthermore, more exhaustive sampling is required to fully comprehend the extent of avian haemosporidian infection in the area.

Prévalence d'hémosporidies dans une région néotropicale d'endémisme aviaire

RÉSUMÉ. Les hémosporidies, parasites intracellulaires transmis par vecteur, se trouvent dans de nombreuses espèces d'oiseaux partout dans le monde et pourraient avoir d'importantes répercussions dans les populations d'oiseaux sauvages. Traditionnellement, la détection de la présence d'hémosporidies a surtout été réalisée en Europe et en Amérique du Nord, et c'est seulement récemment qu'elle a été faite en région néotropicale, où la prévalence et les impacts de la maladie ont été moins étudiés et sont mal compris. Dans la présente étude, nous avons procédé à la détection dans la région d'endémisme aviaire de la Sierra Nevada de Santa Marta (SNSM), un massif côtier isolé dans le nord de la Colombie qui comporte un grand nombre de biomes et subit un taux élevé de perte d'habitat. Nous avons échantillonné des oiseaux appartenant à 25 espèces à deux altitudes différentes (1 640 et 2 100 m asl) et avons déterminé l'infection par les hémosporidies à l'aide de la réaction en chaîne de la polymérase et du séquençage d'une portion du gène cytochrome b (cyt b) du parasite. Parmi les oiseaux échantillonnés, 32,1 % étaient infectés par au moins 1 des 12 souches du cyt b uniques aux genres d'hémosporidies *Plasmodium*, *Leucocytozoon*, *Haemoproteus*, et au sous-genre *Parahaemoproteus*. Nous avons détecté une plus grande prévalence d'hémosporidies aviaires à faible altitude (1 640 m asl). Toutes les espèces d'oiseaux endémiques que nous avons échantillonnées présentaient au moins un individu infecté par des hémosporidies aviaires. Nous avons aussi obtenu des indices d'une plus grande prévalence en général chez les espèces endémiques par comparaison aux espèces non endémiques, ce qui laisse entendre qu'une plus grande vulnérabilité existerait chez les oiseaux endémiques. Dans l'ensemble, nos résultats indiquent qu'une richesse élevée d'espèces d'hémosporidies s'observe dans la communauté aviaire de la SNSM. Considérant le taux de perte d'habitat que cette région subit, il est important de comprendre comment les hémosporidies aviaires affectent les populations d'oiseaux; en outre, un échantillonnage plus intensif est nécessaire si nous voulons comprendre pleinement l'étendue des infections par hémosporidies aviaires dans la région.

Key Words: *avian malaria*; *endemic bird area*; *haemosporidian*; *Sierra Nevada de Santa Marta*

INTRODUCTION

Understanding the threats of infectious disease in the wild is a central issue for wildlife conservation (Scott 1988, Thompson et al. 2010). Such understanding is becoming more important as climate change-induced habitat changes are quickly affecting

the distribution of parasites and hosts (Garamszegi 2011). In the last few decades an increase in the prevalence of some infectious diseases in wild populations has been documented (Daszak et al. 2000, Dobson and Foufopoulos 2001, Hatcher et al. 2012) and these can have a significant impact on wild populations (Scott 1988).

Avian malaria (*Plasmodium* sp.) and other haemosporidians (*Leucocytozoon*, *Haemoproteus*, and subgenus *Parahaemoproteus* spp.) are vector-transmitted intracellular blood parasites (hereafter haemosporidians) that occur on every continent apart from Antarctica (Valkiūnas 2004). These parasites occur in many bird species, but are mainly found infecting passerines (Valkiūnas 2004). Traditionally, the detection and classification of avian haemosporidians have been done using light microscopy. However, the advent of molecular polymerase chain reaction (PCR)-based detection methods has revealed many insights about parasite genetic diversity (Hellgren et al. 2004, Waldenström et al. 2004) and has allowed the detection of over 1300 unique avian haemosporidian lineages (MalAvi database, Bensch et al. 2009). These haemosporidian lineages vary in the degree of avian host specificity (Ricklefs et al. 2004, Fallon et al. 2005), with some lineages restricted to individual host species, whereas others have a wide range of hosts (Hellgren et al. 2009). Recent evidence suggests that *Plasmodium* lineages are more generalist, whereas specialist associations are more common in *Haemoproteus* (Bensch et al. 2000, Beadell et al. 2009). No studies have been done on the degree of host specialization of *Leucocytozoon* lineages.

The different genera of haemosporidians differ in some features of their life cycle, but clinical aspects of infection in vertebrate hosts are very similar (Van Riper et al. 1994). Once infected, birds undergo an acute phase where parasitaemia (number of individual parasites) increases to a peak approximately 6 — 12 days after infection (Van Riper et al. 1994). After the acute phase, surviving birds may completely clear an infection or alternatively infection may become chronic with very low levels of parasitaemia (Van Riper et al. 1994). Infection induces an antibody and cell-mediated immune response in hosts (Atkinson et al. 2001), and when birds are re-exposed to the same haemosporidian they may quickly mount an effective immune response to control the infection (Cellier-Holzem et al. 2010).

Apart from the role of host immune response in the risk of haemosporidian infection, the environment also modulates infection risk. Avian haemosporidian infection has been associated with environmental variables such as temperature, altitude, and precipitation (reviewed in Sehgal 2015). This is due to the effects of these variables on parasite and vector development (LaPointe et al. 2010, 2012). An elevation limit of avian haemosporidians was documented in Hawaii at approximately 1500 m during the 1970s (van Riper et al. 1986); as a consequence, susceptible hosts have suffered range restrictions, and can only be found above this altitude. However, there have been recent reports of avian haemosporidians at much higher altitudes in this island (Freed et al. 2005), and in continental areas (Rodríguez et al. 2009), a phenomenon probably related with global warming (Garamszegi 2011) that could have grave implications for birds with restricted distributions.

The recent methodological advances in the detection of avian haemosporidian infection have led to an increase in the number of published surveys across the world (reviewed in Clark et al. 2014). Some biogeographical patterns of haemosporidian distribution have been inferred. For example, continental avian diversity hotspots tend to have higher avian haemosporidian species richness estimates compared to non-hotspots. Another

interesting pattern is that continental South America harbours the largest number of lineages of both *Plasmodium* and *Haemoproteus* (Clark et al. 2014). However, much of the research that has been done is restricted to European and North American passeriform communities, and only a few sites have been sampled in continental Neotropics (Lacorte et al. 2013, Clark et al. 2014, González et al. 2015, Marzal et al. 2015). Thus, species richness for continental Neotropics is likely to be underestimated.

Introduced haemosporidians in isolated islands may pose a threat to naïve endemic birds that have not evolved ways to counteract the infection. Such is the case of some Hawaiian birds that suffered large population declines after the haemosporidian vectors were accidentally introduced to the islands (Warner 1968, van Riper et al. 1986). Negative impacts caused by haemosporidian infection include death (Atkinson et al. 2000, Sol et al. 2003), reduced body condition (Merino et al. 2000, Calero-Riestra and García 2016), increased risk of predation (Møller and Nielsen 2007), lower reproductive success (Merino et al. 2000, Marzal et al. 2005), and reduced lifespan (Asghar et al. 2015). The Neotropical region has a large number of endemic bird areas (42) and avian haemosporidians may pose a greater threat to continental endemic birds because they have restricted distributions and populations cannot avoid infection. The study of avian haemosporidians in areas of high bird endemism has been restricted to islands and little attention has been paid to continental areas of high bird endemism.

The aim of this study was to conduct a preliminary survey of the prevalence of avian haemosporidian parasites in the endemic bird area of the Sierra Nevada de Santa Marta in northern Colombia, home to a variety of biomes and one of the world's most important continental avian centers of endemism (Cracraft 1985, Carbono and Lozano-Contreras 1997). Only 15% of the original forest cover in the Sierra Nevada remains (Strewe and Navarro 2004), and despite having a large proportion within national and private protected areas, it is still experiencing high rates of habitat loss. In addition to habitat-related threats, the local avifauna could also be exposed to infectious diseases, which could exacerbate the effects of habitat reduction on the local populations.

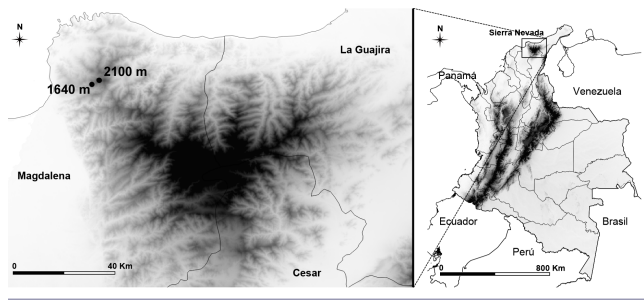
METHODS

Study area

The Sierra Nevada de Santa Marta (SNSM) is an isolated mountain range located in northern Colombia 42 km off the Atlantic coast (Fig. 1). It reaches an altitude of 5775 m above sea level (asl) and encompasses 17,000 km² in the departments of Magdalena, Cesar, and La Guajira. The climate in the SNSM is characterized by temperatures that vary between 0°C and 28°C and mean annual rainfall of approximately 1500 mm at elevations of 500 m. Rainfall regime is bimodal with two peaks in September—December and May—June and two dry periods in January—April and July—August (Instituto Geográfico Agustín Codazzi [IGAC] 1993). The SNSM is characterized by a large variety of habitats resulting from the altitudinal range and its location; the area presents a mosaic of different biomes including mangroves, semideserts, tropical dry forests, tropical wet forests, montane forests, and páramos (Strewe and Navarro 2004). Because of its isolation and variety of habitats the SNSM is constituted as an important Endemic Bird Area (Stattersfield et al. 1998) with 18

endemic species and 55 endemic subspecies. Our study site is located in the San Lorenzo ridge in the northwest slope of the SNSM in the department of Magdalena where two collection points were chosen, one at 1640 and another at 2100 meters asl (Fig. 1).

Fig. 1. Location of the sampling sites in the San Lorenzo ridge within the Sierra Nevada de Santa Marta in northern Colombia.



Sampling and molecular procedures

Birds were captured in February 2014 and June 2015 using mist nets (12-m-long \times 3-m-high). Each captured bird was marked for subsequent identification when recaptured by clipping the tail feathers. A small ($\sim 20 \mu\text{l}$) blood sample was obtained by brachial venipuncture using a sterile hypodermic needle. Blood was collected in Queen's lysis buffer (Seutin et al. 1991) in 1.5 ml microcentrifuge tubes at room temperature.

Genomic DNA was extracted from blood using a salt extraction method (Richardson et al. 2001). Quality of the DNA was assessed by PCR of a fragment of approximately 950 bp of the avian mitochondrial gene ND4 using primers ND4 and LEU (Arévalo et al. 1994) and PCR conditions previously described (McGuire et al. 2007). Only DNA samples that successfully amplified the avian mitochondrial marker were used in the haemosporidian screening. Haemosporidian infection was screened using a nested PCR method that amplifies a section of the cytochrome b gene of the haemosporidian genome. PCR was performed in 10 μl volumes containing ~ 30 ng of genomic DNA (universal PCR) or amplicon (nested PCRs), 2 mM each dNTP, 0.5 μM each primer, 2 mM MgCl_2 , 1 X PCR buffer, and 0.03 units of Taq polymerase (Thermo Scientific). Universal PCR was done using primers HAEMNF1 and HAEMNR3 (Hellgren et al. 2004); the thermal profile started with 3 min at 96°C followed by 20 cycles of 96°C for 20 sec, 50°C for 30 sec, and 72°C for 45 sec, and ended with an extension step at 72°C for 10 min. Nested PCR was done with primers HAEMF and HAEMR2 for *Plasmodium* and *Haemoproteus* (Bensch et al. 2000) and HAEMFL and HAEMR2L for *Leucocytozoon* (Hellgren et al. 2004). Thermal profiles started with 3 min at 96°C, followed by 40 cycles of 96°C for 30 sec, 55°C (for *Plasmodium*/*Haemoproteus*) or 57°C (for *Leucocytozoon*) for 45 sec, and 72°C for 45 sec, and ended with a final extension at 72°C for 10 min. PCR products were visualized in 2% agarose gels stained with GelRed (Biotium). We routinely used negative controls. All samples were screened at least twice and only samples that amplified twice were considered to be infections.

PCR products were sequenced in Macrogen Inc. using the BigDye reaction (Thermo Scientific) and running products on an automated sequencer. Sequences were visually checked and edited in Bioedit 7.0.9 (Hall 1999), and aligned to sequences from the National Center for Biotechnology Information GenBank database and the MalAvi database for avian malaria (Bensch et al. 2009).

Analyses

To determine the relationship between the avian haemosporidian parasite lineages identified we constructed maximum likelihood trees with the unique sequences using 1000 bootstrap replications and the general time reversible substitution model using MEGA 6 (Tamura et al. 2013). The tree was rooted using a published sequence of *Babesia gibsoni* (GenBank accession number AB685190.1)

Given that the two sites had different sample sizes both for hosts and parasites (see results), and in order to assess haemosporidian species richness and to compare values of this parameter at the two sampling sites, we performed a rarefaction analysis (Gotelli and Colwell 2001). For rarefaction we used the package 'vegan' (Oksanen et al. 2013) in R (R Development Core Team 2011). Rarefaction calculates the expected number of species in a random subsample of individuals from a single, large collection (Gotelli and Colwell 2001). Rarefaction curves were then built using subsample size on the x axis and expected number of species in the y axis using custom R scripts (obtained from <http://www.jennajacobs.org/R/rarefaction.html>). To test for possible specialist parasite-host associations we used the hypergeometric distribution function in R (R Development Core Team 2011) to calculate the probability of drawing the number of individuals of a parasite species (the observed number of such species) from the pool of individuals (all individuals of all bird species) and finding the same parasite species.

RESULTS

A total of 53 birds from 25 species were sampled with 1-7 individuals per species (mean \pm SD = 2.12 \pm 1.51); 14 of these birds (11 species) were caught at 1640 m asl and 39 (18 species) at 2100 m asl, (Table 1). Seventeen birds (32.1%) belonging to 11 species were identified as infected by at least 1 genus of avian haemosporidian. Among these, the most common genus was *Leucocytozoon* (detected in 58.8% of individuals belonging to 9 bird species), followed by *Haemoproteus* in 41.2% (belonging to 3 species), *Plasmodium* in 23.5% (belonging to 4 bird species) and *Parahaemoproteus* in 5.9% (only 1 individual infected). Five individuals (9.4%) presented infections by 2 genera. At an altitude of 1640 m asl 57.1% of birds captured were infected by at least 1 genus of haemosporidian, compared to a 23.7% of infected birds at 2100 m asl. The most common genus of haemosporidian found at 1640 m asl was *Leucocytozoon* (45.4%), followed by *Plasmodium* (27.3%), *Haemoproteus* (18.2%), and subgenus *Parahaemoproteus* (9.1%). Double infections were found in 27.3% of birds caught at 1640 m asl. At an altitude of 2100 m asl *Haemoproteus* and *Leucocytozoon* accounted for 45.4% of infections each and *Plasmodium* was found in only 1 individual (9.1%). Double infections were found in 18.2% of individuals at 2100 m asl.

Table 1. Bird species captured at two elevation points in the Sierra Nevada de Santa Marta and occurrence of haemosporidians of the genera *Plasmodium*, *Leucocytozoon*, and *Haemoproteus*, and subgenus *Parahaemoproteus*. Number of birds infected by each haemosporidian genera (or subgenus) for each species is shown.

Family	Species name	N	Altitude (N)	N <i>Plasmodium</i>	N <i>Leucocytozoon</i>	N <i>Haemoproteus</i>	N <i>Parahaemoproteus</i>
Trochilidae	<i>Amazilia saucerrotti</i>	1	2100				
	<i>Colibri thalassinus</i>	1	2100				
	<i>Metallura tyrianthina</i>	1	2100			1	
Ramphastidae	<i>Aulacorhynchus prasinus</i>	1	2100				
Furnariidae	<i>Anabacerthia striaticollis</i>	2	2100		1		
	<i>Automolus rubiginosus</i>	1	1640				
	<i>Lepidocolaptes lacrymiger</i>	3	1640 (1) 2100 (2)				
Tyrannidae	<i>Elaenia flavogaster</i>	3	2100				
	<i>Elaenia frantzi</i>	2	2100				
	<i>Mionectes olivaceus</i>	3	1640 (1) 2100 (2)		1		
	<i>Myiodynastes maculatus</i>	1	1640				
	<i>Ochtoeca diadema</i>	1	2100				
	<i>Pyrrhomyias cinnamomeus</i>	1	1640				
Vireonidae	<i>Vireo leucophrys</i>	1	1640				1
Trogloditidae	<i>Henicorhina leucophrys</i>	4	1640 (2) 2100 (2)				
Turdidae	<i>Turdus flavipes</i>	2	1640	1	1		
	<i>Turdus olivater</i>	1	1640	1			
Parulidae	<i>Basileuterus culicivorus</i>	1	2100				
	<i>Myioborus flavivertex</i> [†]	5	2100		1		
	<i>Myiothlypis conspicillata</i>	1	2100				
	<i>Vermivora peregrina</i> [‡]	2	2100			1	
Emberizidae	<i>Atlapetes melanocephalus</i> [‡]	7	1640 (2) 2100 (5)		1 1	2 3	
	<i>Arremon basilicus</i> [†]	2	1640	1	2		
Thraupidae	<i>Diglossa albilatera</i>	3	2100				
	<i>Diglossa humeralis</i>	3	2100	1	1		
Total	25	53		4	9	7	1

[†]Endemic

[‡]Migratory

Prevalence of avian haemosporidians in endemic species

Within the bird species we captured, 3 were endemic to the SNSM: Santa Marta Brush Finch, *Atlapetes melanocephalus* (7 individuals); Yellow-crowned Redstart, *Myioborus flavivertex* (4 individuals) and Colombian Brush Finch, *Arremon basilicus* (2 individuals). Of the individuals from endemic species 61.5% were infected with avian haemosporidians compared to a 22.5% of nonendemics (Table 1). Both individuals of the species *Arremon basilicus* were infected by the genus *Leucocytozoon*. *Atlapetes melanocephalus* had a haemosporidian prevalence of 71.4%; *Haemoproteus* sp. accounted for 100% of these infections, while *Leucocytozoon* was found in 40% of infected birds (2 individuals presented infections by both genera). One individual of *Myioborus flavivertex* was infected with *Leucocytozoon* sp. (25% prevalence).

Species of haemosporidians

Sequencing of the 22 PCR products that were positive for haemosporidian infection revealed a total of 12 unique sequences that grouped into 9 lineages (Table 2, Figure 2). All sequences identified had a sequence identity of at least 99% to sequences

reported in GenBank (Table 2), 7 of these had not been previously reported and have been deposited in GenBank (accession numbers KX130084-KX130090). Four lineages had 100% match to sequences previously reported in the Neotropics including: *Haemoproteus witti* PA182, *Leucocytozoon lutzii* PA286, *Plasmodium* sp. HMA2012, and *Leucocytozoon* sp. LT-011 (Table 2). One lineage had 100% match to *Plasmodium* sp. POMFER01, a lineage that has previously been reported in China (Table 2). The phylogenetic analysis of the identified sequences revealed 2 lineages of *Haemoproteus*, 1 of *Parahaemoproteus*, 3 of *Plasmodium*, and 3 of *Leucocytozoon* (Fig. 2). The tree had well-supported nodes, except for the node separating *Leucocytozoon* lineages ChL11 and L-T011 (Fig. 2). The most abundant lineage identified was 99% identical (1 or 2 bp different) to the previously described *Haemoproteus* lineage DICER01 (González et al. 2015), accounting for 22.7% of infections, all of which were found in the same host species, the SNSM endemic *A. melanocephalus*. The probability of finding the DICER01-similar lineage always infecting *A. melanocephalus* just by chance was 0.0082; thus, it is likely that there is a specialist association between the DICER01-similar lineage and the bird species *A. melanocephalus*. The other lineages that were detected in more than 1 individual were present in several host species (Table 2, Fig. 2).

Table 2. Avian haemosporidian lineages detected in this study and their sequence identity to previously detected lineages published in GenBank. Host species where lineages have been detected (in this study and previous ones) are listed.

Genus	closest GenBank match (sequence identity)	Reference (closest GenBank match)	GenBank # (Closest match)	Host species (this study)	Host species (previous studies)	Locality (previous studies)
<i>Haemoproteus</i>	<i>H. witti</i> PA182 (100%)	González et al. 2015	KC121053.1	<i>Metallura tyrianthina</i> , <i>Vermivora peregrina</i>	<i>Eriocnemis vestitus</i> , <i>Eriocnemis cupreiventris</i> , <i>Eriocnemis derbyi</i> , <i>Eriocnemis vestita</i>	Colombia Andes, Perú
<i>Haemoproteus</i>	sp. DICER-01 (99%)	González et al. 2015	KM2113-49.2	<i>Atlapetes melanocephalus</i>	<i>Buhtraupis montana</i> , <i>Diglossa caerulescens</i>	Colombia, Galápagos
<i>Leucocytozoon</i>	sp. ChL11 (99%)	Martínez et al., unpublished manuscript [†]	EF153665.1	<i>Mionectes olivaceus</i> , <i>Anabacerthia striaticollis</i>	<i>Zonotrichia capensis</i>	Chile
<i>Leucocytozoon</i>	sp. L-T011 (100%)	C. C. Witt and S. M. McNew, unpublished manuscript	JQ988120.1	<i>Diglossa humeralis</i> , <i>Atlapetes melanocephalus</i> , <i>Arremon basilicus</i>	<i>Diglossa humeralis</i>	Perú
<i>Leucocytozoon</i>	sp. SPOW6 (99%)	Ishak et al. 2008	EU627802.1	<i>Vireo leucophrys</i> , <i>Turdus flavipes</i> , <i>Myioborus flavivertex</i>	<i>Strix occidentalis occidentalis</i>	USA, California
<i>Parahaemoproteus</i>	<i>P. vireonis</i> (99%)	González et al. 2015	KF537331.1	<i>Vireo leucophrys</i>	<i>Vireo olivaceus</i>	Colombia, Perú
<i>Plasmodium</i>	<i>P. lutzi</i> PA286 (100%)	González et al. 2015	KJ780795.1	<i>Diglossa humeralis</i>	<i>Diglossa cyanea</i>	Colombia Andes
<i>Plasmodium</i>	sp. HMA-2012 (99-100%)	Archer et al., unpublished manuscript [‡]	JN819328.1	<i>Turdus flavipes</i> , <i>Turdus olivater</i>	<i>Tangara icterocephala</i> , <i>Turdus assimilis</i>	Costa Rica
<i>Plasmodium</i>	sp. POMFER01 (100%)	Zhang et al. 2014	KJ145051.1	<i>Arremon basilicus</i>	<i>Ficedula monileger</i>	China

[†]Martínez, J., S. Merino, J. Moreno, R. Vasquez, I. Sanchez-Monsalvez, C. F. Estades, S. Ippi, P. Sabat, R. Rozzi, S. McGehee, M. A. Rodríguez-Girones, J. Martínez-de la Puente, S. García-Fraile, and E. Lobato.

[‡]Archer, H. M., C. D. Mendenhall, C. H. Sekercioglu, and R. N. M. Sehgal.

In most cases where several hosts presented infections with the same haemosporidian lineage, these were found at both altitudes (Fig. 2). The exception was one lineage (*Haemoproteus witti* PA182; González et al. 2015), which was detected in only 2 individuals of different species at 2100 m asl.

Rarefaction curves clearly show that haemosporidian species richness is higher at low altitudes after correcting for difference in sample size between the two sampling areas (Appendix 1). At a subsample size of 9 (the minimum number of single haemosporidians observed at a specific site) rarefaction gave a species richness of 6.45, 5.58, and 8.20 for all samples, only samples from 2100 m asl and only samples from 1640 m asl, respectively. The slopes of the rarefaction curves also suggest that the actual number of haemosporidian lineages is likely to be much higher than the observed value and sampling effort needs to be more intense to identify all species/lineages of haemosporidians present in the area.

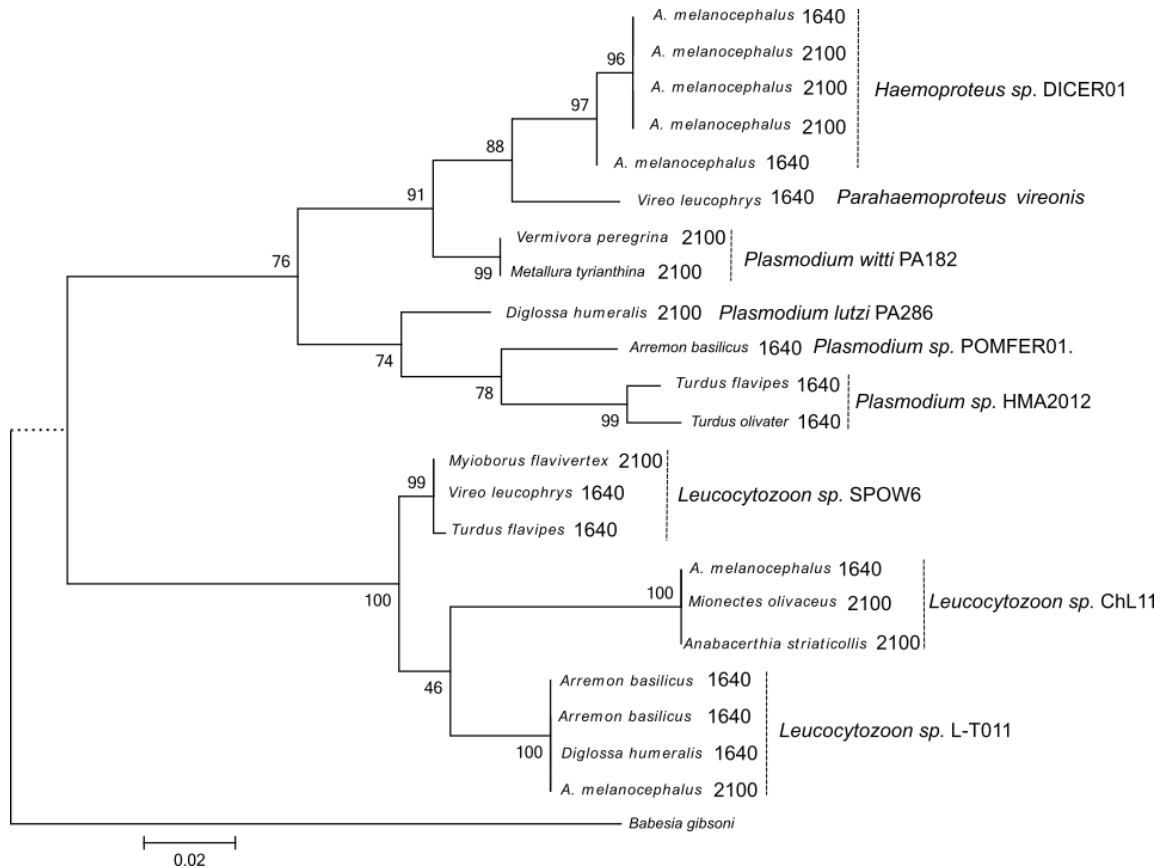
DISCUSSION

In this study we assessed infection by haemosporidians in birds from the Sierra Nevada de Santa Marta (SNSM), an important endemic bird area, and report a possible new parasite-host association. We also found that the three endemic species we sampled were infected by at least one genus of haemosporidian with a high prevalence. Our results clearly show that the actual

number of haemosporidian lineages in this area is likely to be much larger than the numbers we found, and considering the conservation concern of the SNSM and its endemic species, the ecology of these parasite - host associations needs to be further explored.

All three genera (and one subgenus) of haemosporidians were represented in our sample of 53 birds. We found an overall prevalence of haemosporidians of 32.1%. This result concurs with prevalence reported for the Neotropics (Latta and Ricklefs 2010, Ricklefs et al. 2011, Lacorte et al. 2013, Marzal et al. 2015). In contrast, a previous large-scale study (1487 birds screened) across Neotropical highlands in Colombia (González et al. 2015) found an overall prevalence of 10%, although this study did not screen for *Leucocytozoon* infections and this genus was the most common in our study (see below). However, the prevalence in our study was similar to that reported in another small-scale study in the Chingaza Park, Colombia at elevations above 3000 m asl, which included *Leucocytozoon* in the screening, and reported an overall haemosporidian prevalence of 27.9% (Rodríguez et al. 2009). Furthermore, the most common haemosporidian genus found in the cited study was *Leucocytozoon* with a prevalence of 21.3% (Rodríguez et al. 2009). This result is similar to ours where the most common haemosporidian genus was also *Leucocytozoon* with an overall prevalence of 17%, and contrasts with previous reports in the Neotropics, where prevalence for this genus has

Fig. 2. Phylogenetic tree of cytochrome b sequences of haemosporidians detected in birds sampled in the Sierra Nevada de Santa Marta at two different altitudes (1640 and 2100 m asl). Each line is composed of the host species name of each sample (in smaller case), followed by the altitude where the individual was caught and the name of the closest haemosporidian lineage, as shown in Table 2, in larger font. Vertical dashed lines span individuals infected with each haemosporidian lineage. Bootstrap values are displayed at each node. The tree was rooted with a cytochrome b sequence from *Babesia gibsoni*.



been as low as 0.2% (White et al. 1978), 0.3% (Basto et al. 2006), 2% (Valkiūnas et al. 2003) or completely absent (Rodríguez and Matta 2001). This is probably a reflection of the ecological constraints of *Leucocytozoon*, which is transmitted by blackflies (Fallis et al. 1951). The larvae of these vectors require streams with constant current rates (Zahar 1951), which are common in mountainous areas such as the SNSM.

Our haemosporidian cytochrome b phylogeny revealed a total of nine unique lineages with well-supported nodes, except for the node separating *Leucocytozoon* lineages ChL11 and L-T011 (Fig. 2). To separate these two lineages we would probably need to include additional molecular markers or to increase the size of the cytochrome b fragment sequenced. Given the small sample size of the birds screened in the present study, the number of lineages found is a considerable value and is comparable with other studies in the Neotropics that have found a large number of lineages (Fallon et al. 2005, Lacorte et al. 2013, Clark et al. 2014). The high species richness we report agrees with the hypothesis that haemosporidian diversity may be a function of avian and/or vector host diversity, both of which are high in

tropical continental regions (reviewed in Clark et al. 2014). Given the high bird species diversity of the SNSM, the number of avian haemosporidian species in this Neotropical region could be considerably high. In fact, the rarefaction curve built with our haemosporidian species presence data shows that the actual species richness is likely to be much higher than the one we observed. Host switching has been shown to be a key factor in haemosporidian speciation (Ricklefs et al. 2014) and the high bird species richness in the SNSM could accelerate this process because more hosts are available and host switching would be more likely to occur. A more exhaustive screening of haemosporidian parasites in the avifauna of the SNSM is required to confirm this.

Apart from diversification by host switching, another possibility for the high haemosporidian diversity found in the SNSM is that parasites could have been introduced by non-native birds that migrate through the area. Invasion of haemosporidians has previously been reported for highly generalist species, such as *Plasmodium relictum* (Marzal et al. 2015). The lineages detected in this study are found in several host species, and they have been reported elsewhere suggesting that they could have been

introduced easily from migrating bird species. In this study we only captured two individuals of the migrating species *Vermivora peregrina*, resident of North America, one of which was infected with the previously reported *Haemoproteus witti* lineage PA182 (González et al. 2015). The same PA182 sequence was detected in *Metallura tyrianthina* in our study, a species of hummingbird that is widespread across northern South America (del Hoyo et al. 1999). Distribution of both species during the wintering period for *V. peregrina* overlaps over a large area, therefore we cannot make any assumptions about the transmission of this parasite lineage between the two species or about the direction of transmission. A more comprehensive screening including more individuals and more species would be needed to confirm this.

Avian haemosporidians could potentially affect the distribution of host species if the pathogens decrease the fitness of hosts (Warner 1968, Lachish et al. 2011a). This effect could be more drastic in endemic species that have restricted distributions and cannot, therefore, disperse to disease free areas. Although our sample size is not large enough to make statistical comparisons between the levels of infection between endemic and nonendemic birds, our result of higher prevalence in endemic birds concur with those of a previous community-level study that found higher prevalence of avian haemosporidians in endemic species than in nonendemics in the Dominican Republic (Latta and Ricklefs 2010). This finding has important implications for the conservation of the endemic species in the SNSM and poses the question whether this phenomenon is widespread among the SNSM endemic bird species and other areas and altitudes of the SNSM. Greater sampling effort across a larger altitudinal range in this and other areas of the SNSM is needed.

Host specificity is key in the biology of parasites. Host specialization might increase the risk of extinction for the parasite, but allows increased contact among individuals of a parasite species that is restricted to a few species, since parasite transmission between host congeners is more likely (Beadell et al. 2009). Long-term localized associations of a vertebrate host and a parasite may result in coevolution and a subsequent specialist association as has been previously suggested in an avian haemosporidian system where specialist haemosporidians were restricted to resident and endemic avian hosts (Latta and Ricklefs 2010). In line with this, the most common lineage detected in our study (*Haemoproteus* DICER01-closely related lineage) was only detected in one species, the SNSM endemic *Atlapetes melanocephalus*, thus, this could represent a host specialization strategy of this lineage. This observation is consistent with the hypothesis that lineages of *Haemoproteus* are more constrained at the level of host species than other haemosporidian genera (Fallon et al. 2005, Beadell et al. 2009), and might suggest a long-term localized coevolution of a parasite-host relationship, which is more likely to occur in endemic host species. However, to confirm unequivocally the existence of a specialist association it is necessary to screen blood smears to determine whether the infections are viable and to screen a larger number of host species to confirm that no other host species is infected with this lineage. The lineage DICER01 has been reported in *Buthraupis montana* and *Diglossa caerulescens* (González et al. 2015). Nonetheless, it is difficult to know whether our DICER01-closely related lineage corresponds to a new species or it in fact represents intraspecific genetic diversity of DICER01 that had not previously been

reported. Sequencing longer cytochrome b fragments of the mitochondrial genomes of both the original DICER01 lineage and the one detected in this study would be needed to clarify this.

Avian haemosporidian transmission is affected by the environmental variation at different scales, both in space (Wood et al. 2007, Lachish et al. 2011b) and time (Fallon et al. 2004, Lalubin et al. 2013). We detected a lower prevalence at high altitudes despite having screened a larger bird sample at 2100 m asl. This is in agreement with evidence of the effects of temperature and altitude on the abundance and activity patterns of vectors (Ahumada et al. 2004, Gilioli and Mariani 2011, Zamora-Vilchis et al. 2012) and on the development of haemosporidian parasites (Valkiūnas 2004, LaPointe et al. 2010). Further sampling is needed across a larger elevation gradient to further support our findings. We did not measure other environmental variables that could potentially affect the prevalence of haemosporidians in this area. The SNSM possesses a large variety of biomes and habitats, hence providing a great opportunity for assessment of environmental variables that could affect the distribution of the different haemosporidian lineages. Future work should focus on sampling hosts in areas with different environmental conditions in the SNSM.

Our data revealed high prevalence of avian haemosporidians in all captured endemic bird species in the SNSM during our study. Given that the Sierra Nevada in Northern Colombia is considered a center of endemism (Cracraft 1985, Carbono and Lozano-Contreras 1997), high prevalence and diversity of haemosporidians suggests a threat for wildlife. Global changes such as alterations in landscape and habitat, and climate change-induced shifts in wildlife populations have important implications in disease ecology (Deem et al. 2001). In the SNSM human settlement, the advancing agricultural and cattle ranching frontier, and the cultivation of illegal crops have led to loss of more than 70% of the original forest cover (Álvarez 2002). The landscape change combined with disease threat could have adverse consequences for the persistence of wild bird populations (Sehgal 2010). Further work is urgently needed to assess whether haemosporidians represent a threat for the conservation of the local avifauna. More exhaustive sampling across a larger altitudinal range, including other types of habitats and different time periods is of utter importance to estimate the extent of the presence of the disease in this important endemic bird area and to further understand the evolutionary and ecological dynamics of the disease in areas of high host and parasite species richness. Once a comprehensive screening of haemosporidians in the wild avifauna is performed, further studies should focus on determining the factors that modulate infection and transmission in the area and assessing the effects of haemosporidian infection on various aspects of host fitness.

Responses to this article can be read online at:
<http://www.ace-eco.org/issues/responses.php/834>

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Appendix 1 Rarefaction curves of malaria species richness at 2100 m asl (high), 1640 m asl (low), or pooling samples at both locations (all). Each subsample was iterated as many times as possible given the number of samples in each subsample. Bars denote standard error of the iterations at each subsample.

