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Original Contribution

Molecular Epidemiology of Avian Malaria in Wild Breeding Colonies of Humboldt and Magellanic Penguins in South America

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Abstract: Avian malaria is a disease caused by species of the genera *Haemoproteus*, *Leucocytozoon*, and *Plasmodium*. It affects hundreds of bird species, causing varied clinical signs depending on the susceptibility of the host species. Although high mortality has been reported in captive penguins, limited epidemiological studies have been conducted in wild colonies, and isolated records of avian malaria have been reported mostly from individuals referred to rehabilitation centers. For this epidemiological study, we obtained blood samples from 501 adult Humboldt and 360 adult Magellanic penguins from 13 colonies throughout South America. To identify malaria parasitaemia, we amplified the mtDNA cytochrome *b* for all three parasite genera. Avian malaria was absent in most of the analyzed colonies, with exception of the Punta San Juan Humboldt penguin colony, in Peru, where we detected at least two new *Haemoproteus* lineages in three positive samples, resulting in a prevalence of 0.6% for the species. The low prevalence of avian malaria detected in wild penguins could be due to two possible causes: A low incidence, with high morbidity and mortality in wild penguins or alternatively, penguins sampled in the chronic stage of the disease (during which parasitaemia in peripheral blood samples is unlikely) would be detected as false negatives.

Keywords: *Haemoproteus*, *Spheniscus*, South America, penguins

Introduction

Pathogen pollution by emerging infectious diseases (EIDs) is increasingly being recognized as a cause of biodiversity loss through outbreaks causing mass mortality and declines in naive species (Cunningham et al. 2003). The main processes that determine outbreaks of EIDs in wildlife can be categorized in the following way: natural or anthropogenic changes in ecosystems, movement of pathogens or vectors, evolutionary changes in pathogens and changes in

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recognition of these pathogens due to advances in epidemiological techniques (Williams et al. 2002). With the increase in globalization, humans have acted as vectors for a large number of diseases around the globe, broadening distributions of: chytridiomycosis worldwide, poxvirus in Scandinavia, *Ranavirus* in North America, distemper virus in Africa and avian malaria in Hawaii, causing large morbidities and mortalities in vulnerable and endangered wildlife (Deem et al. 2000; Daszak et al. 2003; Woodworth et al. 2005; Fisher and Garner 2007). Therefore, monitoring EIDs that might substantially affect vulnerable and susceptible wildlife is crucial.

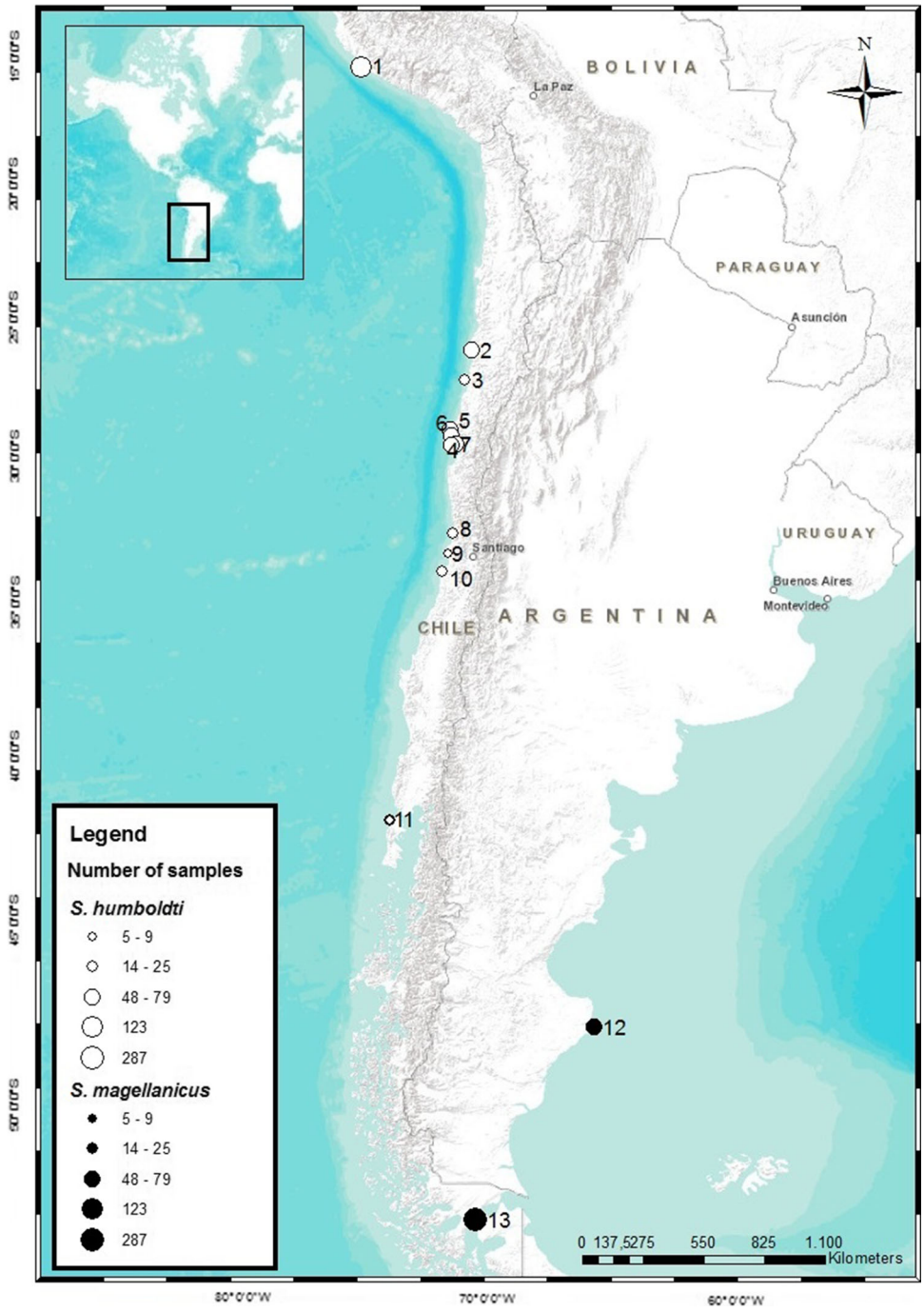

Figure 1. Map of South America indicating penguin colony localities and sampling intensity. Circle size represents sample frequency per colony and adjacent numbers represent the penguin colonies sampled according to Table 1 (White circles = *S. humboldti*; Black circles = *S. magellanicus*; colony 11 is a mixed colony).

Avian malaria and other blood parasitic infections of birds constitute increasingly popular model systems in ecological and evolutionary host–parasite studies (Knowles et al. 2011). However, the presence of the disease has not been thoroughly evaluated in wild birds, and thus it is cataloged as an emerging infectious disease in many countries and species. In this study, we define avian malaria as a disease caused by the multiplication of haemosporidian protozoa of mainly three genera of the order Haemosporida: *Plasmodium*, *Haemoproteus*, and *Leucocytozoon* (Braga et al. 2011). These protozoa have a complex life cycle: most of them fulfill their sexual stage in an invertebrate vector (their definitive host), which are exclusively hematophagous dipteran insects belonging to 17 different genera (Valkiūnas 2005; Braga et al. 2011). The genus *Haemoproteus* presents two subgenera, *Haemoproteus* and *Parahaemoproteus* (Bennett et al. 1965; De Guisti et al. 1973; Orkun and Güven 2013). The main difference between the subgenera is the vectors they parasitize; *Parahaemoproteus* is only transmitted by the family Ceratopogonidae, while *Haemoproteus* is transmitted by Hippoboscidae dipterans (Outlaw and Ricklefs 2010; Valkiūnas 2005). However, some authors treat *Parahaemoproteus* as a different genera altogether (Bennett et al. 1965; Outlaw and Ricklefs 2010).

A classic example of EIDs is the introduction of the avian malaria vector *Culex quinquefasciatus* to the island of Hawaii, resulting in mortality rates of 65–90% in naive endemic hosts (Atkinson et al. 1995; Woodworth et al. 2005). As research on this disease increases, we now know that this pathogen has a worldwide distribution except for Antarctica (Braga et al. 2011). Furthermore, the disease has an acute and chronic presentation depending on the susceptibility of the host and the pathogenesis of the parasite, which depends on the genus as well as the species of the haemosporidian that is infecting the host. When a malaria parasite infects a host, the infection usually begins with a primary acute (high parasitaemia) phase, followed by a chronic phase (low parasitaemia) which is more benign (Asghar et al. 2012). The acute phase of the disease usually is accompanied with clinical signs where the most common ones are lethargy, fever, anorexia, reduced weight gain, anemia, green feces, and often death (Williams 2005). On the other hand, the chronic stage of the disease tends to present low or null levels of parasitaemia with no clinical signs in the infected individual (Braga et al. 2011).

Plasmodium infection is the major cause of mass mortality of penguins in captivity worldwide (Silveira et al. 2013), reaching approximately 50% mortality (Cranfield

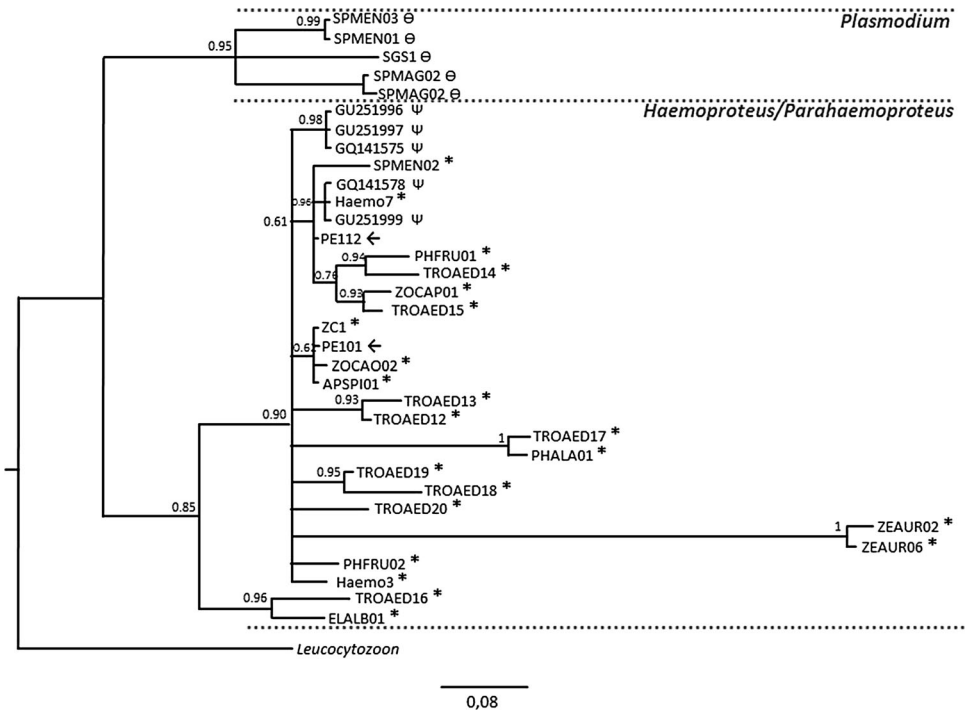

Figure 2. Bayesian phylogenetic reconstruction of 318 bp haemosporidian cytochrome *b* sequences from positive penguin samples compared to other hemoparasite lineages of South America (PE112 and PE101 are samples from this study). Posterior support values are shown for each node greater than 0.5. *Haemoproteus *θ* Plasmodium Ψ Parahaemoproteus ← New lineages.

Table 1. Sampled Breeding Colonies of *Spheniscus* penguins, with the Number of Samples Obtained for Each Colony and the Parasitaemia Present in the Samples.

Site number	Locality	Latitude	Longitude	Samples	Results	Sampled species
1	Punta San Juan	15°22′	75°12′	115	3 Positive	*S. humboldti*
2	Isla Pan de Azucar	26°09′	70°41′	68	Negative	*S. humboldti*
3	Isla Grande de Atacama	27°14′	70°58′	15	Negative	*S. humboldti*
4	Isla Chañaral	29°02′	71°34′	55	Negative	*S. humboldti*
5	Isla Choros	29°16′	71°32′	79	Negative	*S. humboldti*
6	Isla Tilgo	29°32′	71°20′	50	Negative	*S. humboldti*
7	Isla Pajaros	29°35′	71°32′	76	Negative	*S. humboldti*
8	Isla Cachagua	32°35′	71°27′	15	Negative	*S. humboldti*
9	Algarrobo	33°21′	71°41′	9	Negative	*S. humboldti*
10	Pupuya	33°58′	71°53′	14	Negative	*S. humboldti*
11	Puñihuil	41°55′	74°02′	5 & 25	Negative	*S. humboldti and S. magellanicus*
12	Puerto Deseado	47°54′	65°42′	48	Negative	*S. magellanicus*
13	Isla Magdalena	52°55′	70°34′	287	Negative	*S. magellanicus*

et al. 1991). Malaria parasites have been reported as causing high mortality rates in Magellanic penguins (*Spheniscus magellanicus*; Fix et al. 1988), Humboldt penguins (*S. humboldti*; Huff and Shiroishi 1962), and African penguins (*S. demersus*; Grim et al. 2003). All of these cases were captive animals or wild-caught individuals admitted to rehabilitation centers. Two *Plasmodium* species, *P. relictum* and *P. elongatum*, have been implicated in the majority of fatal malaria cases reported in these studies. Two separate reports of *Plasmodium* infection in Magellanic penguins (*S. magellanicus*) in rehabilitation centers have been recently described: both involving two individuals, one in southern Brazil (Silveira et al. 2013) and the other in the coastal region of Valdivia in Chile (Raffo and Munoz 2009). All four individuals subsequently died as a result of their infection.

Malarial parasites have also been widely reported in wild penguin populations; however, the implications of these findings are unclear. Recently, the prevalence of malaria has been evaluated in wild Galapagos penguins (*S. mendiculus*) in 7 islands of the Galapagos Archipelago. Although a 1996 study failed to demonstrate *Plasmodium* in Galapagos penguins ($n = 94$) using PCR (Miller et al. 2001), a prevalence of 3–7% was later detected in asymptomatic penguins in 2003–2005 (Levin et al. 2009). Similarly, high seroprevalence of malarial antibodies was documented in wild New Zealand penguins (Graczyk et al. 1995; McDonald 2003) and can be associated with population declines in wild Yellow-eyed penguins (*Megadyptes antipodes*; Gill and Darby 1993). Furthermore, this protozoan parasite has also been detected in a variety of wild temperate and sub-Antarctic species, including African, Yellow-eyed, Rockhopper (*Eudyptes chrysocome*), and Chinstrap (*Pygoscelis antarcticus*) penguins (Clarke and Kerry 1993).

Although these reports show the susceptibility of penguins to avian malaria disease leading to mortality, there is a lack of knowledge about these parasites in the wild for most penguin species. It is widely known that endemic species may be particularly vulnerable to exotic disease agents (Clarke and Kerry 1993). Furthermore, it is not clear how and where penguins that arrive in rehabilitation centers become infected with the disease. Hypotheses include that haemosporidian infections may occur in captivity, developing an acute stage of the disease due to penguins' high susceptibility (Vanstreels et al. 2014). On the other hand, penguins infected in the wild with the chronic stage of the disease become immunosuppressed in captivity, developing an acute stage with parasitaemia (Palmer et al. 2013).

The introduction of exotic diseases such as malaria can lead to the extinction or population loss of native avian species. *Plasmodium, Haemoproteus*, and *Leucocytozoon* have been described in varied native avian orders in Chile (Passeriformes, Apodiformes and Piciformes), and evidence of in situ trans-species infection has been evaluated; however, no studies related to the dipteran vector have been done in the country (Forrester et al. 1977, 2001; Merino et al. 2008; Vianna et al. Unpub. Data). All cited studies for Chile have focused mainly on parasite records of

terrestrial bird species (but not on prevalence of the disease in a species), and have included only parts of their distribution (Forrester et al. 1977, 2001; Merino et al. 2008). Comparatively, less (if any) information is available on avian malaria parasite genera, species, and lineages present in seabird populations and the risk for species survival.

Penguins have been found to be extremely susceptible to avian malaria, and due to their extensive South American distribution, Magellanic and Humboldt penguins inhabit areas where other avian species have been found to be positive to these haemoparasites. The breeding range of the Humboldt penguin extends from Isla Foca (5°S) in northern Peru to Metalqui (42°S) on the southern Pacific coast of Chile (Paredes et al. 2003; Hiriart-Bertrand et al. 2010; De la Puente et al. 2013). The breeding range of Magellanic penguins extends from 41°S on the eastern coast of South America, down around Cape Horn and north to 40°S on the Pacific coast, and includes the Malvinas–Falkland Islands (Boersma et al. 2013). Adults show strong colony fidelity and philopatry (Araya et al. 2000, Simeone and Wallace 2014) and extreme nest site fidelity (Teare et al. 1998). However, this behavior does not completely agree with recent population genetic results, which showed little or no population structure (Schlosser et al. 2009). Similarly, high levels of heterozygosity and low population structure were found for Magellanic penguins using four microsatellite loci and mitochondrial DNA COI sequences from six colonies on the South Atlantic coast (Bouzat et al. 2009). Due to their proximity to human activities, both of these species show a decreasing population trend, and the Magellanic penguin is cataloged as near threatened while the Humboldt penguin is cataloged as vulnerable (IUCN 2014).

We evaluated the prevalence of avian malaria in the main breeding colonies of Humboldt and Magellanic penguins along most of the species' distribution in the Pacific and Atlantic Oceans using molecular procedures. We also studied the evolutionary relationship between the detected genera and lineages of avian malaria with other lineages described for penguins and avian species in South America.

Methods

Sampling

Samples were collected during penguin breeding seasons from the years 2010–2013. A total of 501 adult Humboldt penguins and 360 adult Magellanic penguins (861 individuals in total) from 13 breeding colonies in Peru, Chile, and Argentina (Table 1, Fig. 1) were sampled. Penguins were quietly approached, and a noose pole 1.5 meters in length was used to lead the penguins out of their nests, and then captured manually (Penguin Taxon Advisory Group 2005). Penguins were handled following the standard methods described by the CCAMLR Ecosystem Monitoring Program (2004). Blood (1 cc) samples were obtained from the internal metatarsal vein or the brachial vein using a 23G needle and 3 ml syringe and stored in 96% sterile ethanol for genetic analysis. To avoid re-sampling, penguins were marked temporarily with water-resistant color markers. Each penguin was classified as adult or juvenile according to Martínez and González (2005), and only adult penguins (more than 2 years of age) were sampled. To estimate health and body condition, each penguin was weighed, measured and clinically examined by a wildlife veterinarian. The bioethics permit was provided by the Pontificia Universidad Católica de Chile following CONICYT Bioethics Guidelines.

DNA Extraction and PCR Techniques

DNA was extracted using a simple salt method with ammonium acetate 9 M modified from Sambrook et al. (1989). DNA quality and quantity (ng/µl) was estimated using a microplate reader (Epoch Microplate Spectrophotometer, BioTek, USA).

We amplified the cytochrome *b* segment of the haemosporidian parasites using non-specific primers 3760F and 4292Rw to detect *Haemoproteus*/*Plasmodium* (Beadell et al. 2004). To detect mixed infections, we also used specific primers for *Plasmodium* (PF and 4292Rw, Merino et al. 2008), *Haemoproteus* (HML and HMR, Merino et al. 2008), and *Leucocytozoon* (HaemFL and HaemR2L, Hellgren et al. 2004).

For all primer pairs, the PCR was performed with a total volume of 30 µl, of which 2 µl corresponded to the template DNA, 1X reaction buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.5 µM of each primer, and 1.25 units of Taq Platinum (Invitrogen®). PCR cycles were followed according to the authors of each primer pair. DNA from other terrestrial bird species containing each of the three avian parasite genera was used as a positive control.

To evaluate positive and negative individuals, all PCR products were visualized on 0.8% agarose gels with SB 1X buffer (Brody and Kern 2004). Five different PCR reactions

Table 2. Avian Malaria Haplotypes Used in Phylogenetic Reconstruction, with Genbank Accession Number, Host and Locality.

Haplotype in tree	Malavi lineage	Genbank Accession Number	Parasite Genus	Country	Host	Paper
PE101	–	KJ561806	*Haemoproteus*	Peru	*Spheniscus humboldti*	This one
PE112	–	KJ561807	*Parahaemoproteus*	Peru	*Spheniscus humboldti*	This one
ZC1	–	KC486265	*Haemoproteus*	Peru	*Zonotrichia capensis*	Jones et al. (2013)
SGS1	SGS1	AB474378	*Plasmodium*	Japan	*Spheniscus humboldti*	Ejiri et al. (2009)
SPMEN01	SPMEN01	GQ395640	*Plasmodium*	Ecuador	*Spheniscus mendiculus*	Levin et al. (2009)
PSMEN02	PSMEN02	GQ395686	*Haemoproteus*	Ecuador	*Spheniscus mendiculus*	Levin et al. (2009)
SPMEN03	SPMEN03	JF833046	*Plasmodium*	Ecuador	*Spheniscus mendiculus*	Levin et al. (2009)
SPMAG01	SPMAG01	JX272844	*Plasmodium*	Brazil	*Spheniscus magellanicus*	Silveira et al. (2013)
SPMAG02	SPMAG02	HQ591361	*Plasmodium*	Brazil	*Spheniscus magellanicus*	Silveira et al. (2013)
APSPI01	APSPI01	EF153652	*Haemoproteus*	Chile	*Elaenia albiceps*	Merino et al. (2008)
ELALB01	ELALB01	EF153647	*Haemoproteus*	Chile	*Elaenia albiceps*	Merino et al. (2008)
PHALA01	PHALA01	EF153650	*Haemoproteus*	Chile	*Phrygilus alaudinus*	Merino et al. 2008
PHFRU01	PHFRU01	EF153654	*Haemoproteus*	Chile	*Phrygilus fruticeti*	Merino et al. 2008
PHFRU02	PHFRU02	EF153653	*Haemoproteus*	Chile	*Phrygilus fruticeti*	Merino et al. (2008)
ZOCAP01	ZOCAP01	EF153649	*Haemoproteus*	Chile	*Zonotrichia capensis*	Merino et al. (2008)
ZOCAP02	ZOCAP02	EF153648	*Haemoproteus*	Chile	*Zonotrichia capensis*	Merino et al. (2008)
TROAED12	TROAED12	KF767421	*Haemoproteus*	Peru	*Troglodytes aedon*	Gahen & Witt unpub. Data.
TROAED13	TROAED13	KF767419	*Haemoproteus*	Peru	*Troglodytes aedon*	Gahen & Witt unpub. Data.
TROAED14	TROAED14	KF767418	*Haemoproteus*	Peru	*Troglodytes aedon*	Gahen & Witt unpub. Data.
TROAED15	TROAED15	KF767417	*Haemoproteus*	Peru	*Troglodytes aedon*	Gahen & Witt unpub. Data.
TROAED16	TROAED16	KF767424	*Haemoproteus*	Peru	*Troglodytes aedon*	Gahen & Witt unpub. Data.
TROAED17	TROAED17	KF767416	*Haemoproteus*	Peru	*Troglodytes aedon*	Gahen & Witt unpub. Data.
TROAED18	TROAED18	KF767423	*Haemoproteus*	Peru	*Troglodytes aedon*	Gahen & Witt unpub. Data.
TROAED19	TROAED19	KF767425	*Haemoproteus*	Peru	*Troglodytes aedon*	Gahen & Witt unpub. Data.
TROAED20	TROAED20	KF767420	*Haemoproteus*	Peru	*Troglodytes aedon*	Gahen & Witt unpub. Data.
ZEAUR02	ZEAUR02	FJ462665	*Haemoproteus*	Peru	*Zenaida auriculata*	Santiago-Alarcon et al. 2010
ZEAUR06	ZEAUR06	GU296212	*Haemoproteus*	Peru	*Zenaida auriculata*	Valkiūnas et al. 2010
GU251997	–	GU251997	*Parahaemoproteus*	Mexico	*Dendroica magnolia*	Outlaw & Ricklefs, 2010
GU251996	–	GU251996	*Parahaemoproteus*	Mexico	*Dendroica magnolia*	Outlaw & Ricklefs, 2010
GQ141575	–	GQ141575	*Parahaemoproteus*	–	–	Outlaw & Ricklefs, 2010
GU251999	–	GU251999	*Parahaemoproteus*	–	–	Outlaw & Ricklefs, 2010
Haemo7	–	GQ395651	*Haemoproteus*	Ecuador	–	Levin et al. (2009)
GQ141578	–	GQ141578	*Parahaemoproteus*	–	–	Outlaw & Ricklefs, (2010)
Haemo3	–	GQ395671	*Haemoproteus*	Ecuador	–	Levin et al. (2009)
Leucocytozoon	–	AB302215	*Leucocytozoon*	Japan	*Gallus gallus*	Omori et al. (2008)

were conducted; isolated DNA (that ranged between 230 ng/μl-1222 ng/μl) was used for three reactions using each primer pair, and other two reactions with DNA concentration at 200 ng/μl and 100 ng/μl. Samples were considered positive when the parasite DNA was amplified in at least two reactions. All positive samples were purified and sequenced by Macrogen (Korea). The Cyt-*b* sequences were deposited in Genbank, accession numbers: KJ561806 and KJ561807.

Phylogeny Reconstruction

Forward and reverse sequences were aligned and edited using Sequencher v.5.1 (GeneCodes Corporations). Polymorphic sites were evaluated by eye using Clustal X2 (Larkin et al. 2007). The parasite sequences obtained were compared with other South American lineages found in the MALAVI database (http://mbioserv4.mbioekol.lu.se/avianmalaria/index.html; Bensch et al. 2009) and deposited in

Genbank, for parasites found in avian species and other penguin species. We used Bayesian phylogenetic reconstruction to estimate the evolutionary relationship between the lineages detected for penguins and the other avian malaria lineages described for South America (Table 2). A total of 418 bp were sequenced; however, 318 bp were used for phylogenetic reconstruction due to sequence lengths from other *Haemoproteus* lineages previously described in South America. As an outgroup, we used a cytochrome *b* sequence obtained from *Leucocytozoon caulleryi* (Omori et al. 2008) (Table 2). The best substitution model suitable for Bayesian phylogenetic reconstruction, selected by the Akaike Information Criterion (AIC) and Bayesian Information Criterion (BIC) using J-ModelTest v.2.1.4 program (Posada 2008), was GTR + I + G. The Bayesian analysis was run using MrBayes v.3.3 (Huelsenbeck and Ronquist 2001). Four independent Markov chains, each beginning with a random tree, were run for 500,000 generations until reaching a split value of 0.009. To visualize the consensus tree, we used FigTree v.1.4.0 (Rambaut 2009).

Prevalence and Confidence Intervals

Prevalence and their confidence intervals were calculated for each species and population selecting a binomial proportion using the function "binconf" (Harrell et al. 2013) and the Wilson's score interval in R (R Core Team 2013).

Results

Three out of 861 blood samples from free-ranging penguins were found to be positive by PCR techniques for haemoparasites of the genus *Haemoproteus/Parahaemoproteus* (Table 1). All positive samples came from one locality in Peru (Punta San Juan) and belonged to Humboldt penguins. Avian malaria prevalence for Humboldt penguins with parasitaemia was thus 0.6% (CI 0.2–1.8%), while within the Punta San Juan colony this species showed a prevalence of 2.6% (CI 0.9–7.4%). Chile had no positive Humboldt penguin samples. Furthermore, no positive Magellanic penguins were found, and no penguin was found to be positive for parasitaemia of *Plasmodium* or *Leucocytozoon*.

The analyzed sequences (418 bp in length) showed that all three positive penguins for the parasite presented parasitaemia by different *Haemoproteus* lineages. One sample displayed polymorphism at two sites, suggesting a mixed infection with at least two parasite lineages; therefore, this sample was excluded from further analyses. We found in a total 8 polymorphic sites between the two identified sequences (418 bp). All three analyzed parasite lineages were *Haemoproteus/Parahaemoproteus,* compared with the sequences database. Comparing the 418 bp sequences of the other two lineages (PE101 and PE112) with other sequences available in Genbank, PE101 was identified as a new haplotype that was closely related (99% similarity) to a *Haemoproteus* infection found in a Rufous-collared sparrow (*Zonotrichia capensis*) in Peru. On the other hand, PE112 also had a new haplotype most closely related to a designated *Parahaemoproteus* and two *Haemoproteus* lineages found in birds from the Galapagos Islands (99% similarity). In the phylogenetic analysis, the *Haemoproteus* and *Parahaemoproteus* lineages found in penguins belonged to two different clades of South American haemosporidians (Fig. 2).

Discussion

The *Haemoproteus/Parahaemoproteus* lineages detected for Humboldt penguins in Punta San Juan (Peru) were unique and had not been described before for any other avian species. The phylogenetic reconstruction showed no clear phylogeographical pattern associated with avian host species or geographical location. The two lineages belong to two distinct clades and are both similar to other lineages detected for different species in South America (with posterior support values of 0.6–0.96). One lineage (PE101) belongs to a clade composed of *Haemoproteus* from Chile and Peru, including a closely related lineage from Peru (Jones et al. 2013) found in a widely distributed passerine bird, as is the Rufous-collared sparrow (*Zonotrichia capensis*). The other lineage (PE112) belongs to a clade not only composed of several lineages from different localities, but also includes *Haemoproteus* and *Parahaemoproteus* sequences analyzed and cataloged by Outlaw and Ricklefs (2010). Although it was not the goal of this study, and we used only one molecular marker, we did not observed distinct monophyletic clades between the possible *Haemoproteus* and *Parahaemoproteus* genera or subgenera.

We detected *Haemoproteus* prevalence for Humboldt penguins of approximately 0.6% (CI 0.2–1.8%), and 2.6% (CI 0.9–7.4%) for the Punta San Juan colony. These results are similar to the low prevalence found for Galapagos penguins also using PCR (Levin et al. 2009). The authors

described a reduced prevalence ranging from 3% to 7% for *Plasmodium* (n = 362), and 0.3% for *Haemoproteus*. Similarly, Brossy (1992) found a single possible case of *Plasmodium relictum* in a sample of 140 free-ranging African penguins in southern Africa. The fact that none of the penguins from Chile and Argentina presented parasitaemia in their blood samples strongly suggests a low prevalence for both penguin species. However, this low prevalence could also be explained by two main factors: (1) the known high levels of morbidity and mortality of this disease in captive penguins (Huff and Shiroishi 1962; Fix et al. 1988; Grim et al. 2003) could also affect wild penguins; or (2) the penguins are infected with the chronic stage of the disease, where the parasite remains latent in the liver and infection is thus undetectable by PCR of peripheral blood samples. Traditional ornithological capture techniques, where birds are captured and sampled during the species' breeding season, may also contribute to the first hypothesis. That is, sampled individuals are healthy reproductive penguins, while other individuals may have died from primary acute haemosporidian infection (Valkiūnas 2005) prior to breeding and remain unsampled. The second hypothesis is that the sampled penguins in the breeding colonies are infected and in the chronic stage of the disease with a higher prevalence. This implies that captured infected birds are usually at the chronic (relatively benign) stage, with low or null levels of parasitaemia in the circulatory system (Braga et al. 2011; Silveira et al. 2013). The use of molecular techniques, such as PCR, can increase the sensitivity for identifying these parasites in blood samples compared to blood smears (Perkins and Schall 2002). However, neither method is able to detect infection if the animals do not present parasitaemia. Liver biopsy and histopathology could be an adequate method to diagnose chronically infected penguins; however, Cannell et al. (2013) in necropsies of Little penguins (*E. minor*) found that *Haemoproteus* parasites were not found in histologically normal hepatic tissue and only in areas affected by significant pathological changes. Therefore, this technique could be effective for recent mortalities, since the complete liver can be examined macroscopically and later histopathologically (Cannell et al. 2013). Furthermore, the clinical signs and symptoms of the disease are only expressed in the acute stage of infection, when a high degree of parasitaemia is present (Braga et al. 2011). In our study, sampled penguins were in apparently good health and did not present any clinical signs corresponding to an acute stage of avian malaria disease.

Jarvi et al. (2002) described the use of serological techniques to study antibodies for *Plasmodium relictum* in experimentally infected Passerines, and found that serology was the most sensitive test to identify chronic infections. Furthermore, a recent study in wild Galapagos penguins clarified the possibility that this species is able to survive the acute phase of the malaria infection (Palmer et al. 2013). The authors described high seroprevalence (97.2%) of malarial antibodies in Galapagos penguins, which contrasted with the 9.2% prevalence of *Plasmodium* detected by PCR (Palmer et al. 2013). Animals were apparently healthy and were resampled over several years. Hence, it is possible that Humboldt and Magellanic penguins could have a higher prevalence but would be presenting the disease at a chronic stage with high levels of seropositivity and low levels of parasitaemia. Although Palmer et al. (2013) studied only seropositivity for *Plasmodium*, it is possible that *Plasmodium* and *Haemoproteus* are present in Humboldt and Magellanic penguins along their distribution).

High prevalence of *Plasmodium* and *Haemoproteus* has been found in native terrestrial avifauna across different latitudes in Chile and Peru (Perkins and Schall 2002; Merino et al. 2008; Santiago-Alarcon et al. 2010; Galen and Witt unpubl. data. Vianna et al. unpubl. data). It is thus feasible that these bird species could be acting as reservoirs for the disease and in this way affect penguin colonies when both bird ensembles and the vectors of the parasites come into contact (e.g., at colonies on islands). Penguins that might be infected with avian malaria but present the chronic stage of the disease would not display clinical signs, but when faced with stressful situations (human-induced or not) in rehabilitation centers or zoological facilities, could evidence a relapse into the acute stage of the disease, which was the case for African penguins infected with *Plasmodium juxtanucleare* (Grim et al. 2003).

Moreover, as with many EIDs, changes in the natural habitat of South American *Spheniscus* penguins might increase the occurrence of acute stages of avian malaria due to immunosuppression of the hosts, since it has been shown that these dormant parasites can resume their haemoparasitic stage when hosts are stressed (Scheuerlein and Ricklefs 2004). Due to their distribution, Humboldt penguins are extremely affected by El Niño events, which normally implies dramatic reductions of food supplies for birds, mainly anchovies (*Engraulis ringens*) and sardines (*Sardinops sagax*) (Barber and Chávez 1983, Alamo and Bouchon 1987). This increases mortality and stress in

penguins by diminishing food sources and increasing foraging with large-scale movements (Tovar and Guillén 1987, Valle et al. 1987). Climate change can affect the intensity and frequency of El Niño events, causing augmented stress for the penguin colonies. Recently, climate change has increased rainfall in the Magellanic penguin colonies such as Punta Tombo in Argentina, with a rise in chick mortality (50%) (Boersma and Rebstock 2014). These changes are stressful for penguin colonies, and therefore these individuals have a greater probability of becoming immunosuppressed, which could consequently boost the prevalence of diseases such as avian malaria in wild populations. Likewise, immunosuppression can revert the disease from the chronic to the acute phase revealing clinical signs, causing their arrival to rehabilitation centers (Cranfield et al. 1994; Palinauskas et al. 2011).

This highlights the necessity of epidemiological monitoring of wild populations as the introduction of another disease could also increase possibilities of a chronic avian malaria infection changing into an acute one (Braga et al. 2011). The study of EIDs usually begins when severe outbreaks and mortality have already occurred. The keys to understanding, controlling, and balancing ecosystem health, human health, and animal health are important through preventive and monitoring programs, which analyze and study possible outbreaks before they occur. The underlying aim of projects involving EIDs such as avian malaria in susceptible species is to provide information that can be used to predict and control the emergence or spread of the disease as well as to predict future emergence of related pathogens (Williams et al. 2002). This study evaluated a large number of penguins in their complete distribution to monitor a disease that could possibly affect the conservation status of both species. However, avian malaria prevalence in Humboldt and Magellanic penguin breeding colonies could be overlooked using blood samples and PCR procedures. The chronic infection hypothesis could be explored in future studies using liver biopsies for parasite detection by PCR and histopathology, with antibody serological studies. The aim of conservation medicine is ultimately to develop a solution-oriented, practice-based approach in addressing health problems derived from environmental change (Daszak et al. 2004). Therefore, ongoing monitoring of vulnerable *Spheniscus* penguins needs to include more detailed sample collection and analyses if we are aiming for a 'solution based approach.'

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