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PROTOZOOLOGY - ORIGINAL PAPER

Epidemiology, hematology, and unusual morphological characteristics of *Plasmodium* during an avian malaria outbreak in penguins in Brazil

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Abstract

Avian malaria is a mosquito-borne disease caused by *Plasmodium* spp. protozoa, and penguins are considered particularly susceptible to this disease, developing rapid outbreaks with potentially high mortality. We report on an outbreak of avian malaria in Magellanic penguins (*Spheniscus magellanicus*) at a rehabilitation center in Espírito Santo, southeast Brazil. In August and September 2015, a total of 89 Magellanic penguins (87 juveniles and 2 adults) received care at Institute of Research and Rehabilitation of Marine Animals. Over a period of 2 weeks, *Plasmodium* infections were identified in eight individuals (9.0%), four of which died (mortality = 4.5%, lethality = 50%). Blood smears and sequencing of the mitochondrial cytochrome *b* gene revealed the presence of *Plasmodium lutzi* SPMAG06, *Plasmodium elongatum* GRW06, *Plasmodium* sp. PHPAT01, *Plasmodium* sp. SPMAG10, and *Plasmodium cathemerium* (sequencing not successful). Two unusual morphological features were observed in individuals infected with lineage SPMAG06: (a) lack of clumping of pigment granules and (b) presence of circulating exoerythrocytic meronts. Hematological results (packed cell volume, plasma total solids, complete blood cell counts) of positive individuals showed differences from those of negative individuals depending on the lineages, but there was no overarching pattern consistently observed for all *Plasmodium* spp. The epidemiology of the outbreak and the phylogeography of the parasite lineages detected in this study support the notion that malarial infections in penguins undergoing rehabilitation in Brazil are the result of the spillover inoculation by plasmodia that circulate in the local avifauna, especially Passeriformes.

Keywords Hemosporida · Neotropics · Pathogen spillover · Seabird · Spheniscidae · Vector-borne disease

Introduction

Avian malaria is a disease caused by mosquito-borne protozoa of the genus *Plasmodium* (Valkiūnas 2005). These parasites were traditionally classified on the basis of their morphology in blood smears, with 55 recognized morphospecies (Valkiūnas and Iezhova 2018). The dawn of the molecular era in the twenty-first century, however, revealed that the

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genetic diversity of these parasites is far greater than could be recognized from their morphology (Bensch et al. 2009; Outlaw and Ricklefs 2014). Over 1200 mitochondrial cytochrome *b* gene (cyt-*b*) lineages of *Plasmodium* have been deposited in the MalAvi database to date, and this number continues to increase rapidly. However, only ~40 of these cyt-*b* lineages representing 23 morphospecies have been morphologically characterized (Valkiūnas and Iezhova 2018), and as a result our understanding of the relationship between cyt-*b* lineages and morphospecies is highly fragmented and their relation to the biological species concept remains unclear (Outlaw and Ricklefs 2014; Palinauskas et al. 2015).

Determining the cyt-*b* lineage of a malarial parasite provides valuable information on its host and geographic distribution, allowing for a deeper understanding on their ecology and host-parasite dynamics (Lacorte et al. 2013; Medeiros et al. 2013; Clark et al. 2015). However, because molecular methods can fail to detect mixed infections (Valkiūnas et al. 2006), favor the detection of some lineages over others (Bernotienė et al. 2016), or even fail to detect some lineages altogether (Zehtindjiev et al. 2012), these methods still need to be combined with morphological analyses to ensure the reliability of diagnostic results. Furthermore, since most of our knowledge about avian plasmodia was developed under the morphospecies paradigm in the twentieth century (Valkiūnas 2005), determining the relationships between genetic lineages and morphospecies unlocks a wealth of historical information about the biology, epidemiology, and pathology of these parasites.

The pathogenicity of plasmodial infections in birds can vary considerably depending on the parasite and host species, and some avian taxa are known to be particularly susceptible to these parasites, developing acute and severe infections with high morbidity and mortality (Valkiūnas 2005; Atkinson 2007). This seems to be a problem especially for avian groups that have historically inhabited environments with few or no mosquitoes, such as Hawaiian endemic birds (Atkinson and LaPointe 2009) and penguins (Grilo et al. 2016). Among penguins, avian malaria is a particularly relevant disease for zoos and rehabilitation centers in tropical and temperate regions, as avian malaria can cause rapid outbreaks resulting in high mortality (Grilo et al. 2016; Vanstreels et al. 2016a).

Magellanic penguins (*Spheniscus magellanicus*) are the most abundant penguin species in South America, with an estimated 1.3 million pairs breeding in Argentina, Chile, and the Falkland/Malvinas Islands (Boersma et al. 2013). The species migrates to Uruguayan and Brazilian waters during winter (Stokes et al. 2014), and a number of individuals stranding along the coast of these wintering grounds are admitted at rehabilitation centers (Dantas et al. 2013). However, these birds may be exposed to mosquito-borne pathogens while under care at these facilities, and the prevention of avian malaria outbreaks is a key challenge for these rehabilitation efforts (Vanstreels et al. 2014, 2015).

In previous studies, we investigated the epidemiology of avian malaria at several rehabilitation centers along the Atlantic coast of South America and found that the diversity of plasmodia that infect these birds is broader than previously thought (Silveira et al. 2013; Vanstreels et al. 2014, 2015, 2016b). Here, we report on an outbreak of avian malaria in Magellanic penguins at a rehabilitation center in Espírito Santo, southeast Brazil, combining hematological, molecular, and morphological methods to investigate the epidemiology and health impacts of these parasites.

Methods

The Institute of Research and Rehabilitation of Marine Animals (Instituto de Pesquisa e Reabilitação de Animais Marinhos – IPRAM) is a non-profit organization that rescues and rehabilitates marine animals. IPRAM is located at Cariacica, Espírito Santo, Brazil (20° 19′ 54″ S 40° 21′ 35″ W), and regularly receives Magellanic penguins rescued along the coast of Bahia, Espírito Santo, and Rio de Janeiro states (Supplemental File S1). From 24-Aug-2015 to 23-Sep-2015, a total of 89 penguins received care at the facility and were evaluated in this study, comprising 87 juveniles and 2 adults.

Blood samples (< 1% body mass) were collected from the metatarsal or jugular veins from all penguins. Thin blood smears were freshly prepared, and the remaining blood was stored in heparin tubes at 4–8 °C for hematological analyses or frozen at − 20 °C for molecular analysis. The presence of palpebral lesions consistent with mosquito bites (Fig. 1) and body mass (precision 10 g) were recorded upon physical examination.

All penguins under care at the study facility received a chloroquine treatment starting on 01-Sep-2015; a second group of 14 penguins admitted on 09-Sep-2015 also received this treatment starting on 10-Sep-2015. The 11-day chloroquine treatment protocol was adapted from Vanstreels et al. (2014): on the first day, oral administration of 10 mg/kg of chloroquine at 0 h, followed by additional oral doses of

Fig. 1 Juvenile Magellanic penguin (*Spheniscus magellanicus*) with palpebral lesions consistent with mosquito bites

5 mg/kg of chloroquine at 6, 12, and 18 h; starting on the second day (24 h after 0 h), oral administration of 5 mg/kg of chloroquine once a day for 10 days. Although primaquine is traditionally associated with chloroquine to treat malarial infections in penguins (Grilo et al. 2016), this drug is restricted only for human use in Brazil. Starting on 01-Sep-2015, measures were also implemented to prevent exposure to mosquitoes such as the placement of fans in all enclosures and the application of roll-on insect repellent (diethyltoluamide) on the head feathers of each penguin in the evening (Vanstreels and Parsons 2014).

Hematological analyses were conducted for samples obtained on 02-Sep-2015 ($n = 73$) and, for a small subset of birds that were admitted later, on 10-Sep-2015 ($n = 14$). A subset of these samples was frozen for molecular analyses. Packed cell volume was determined through centrifugation in heparin capillaries at 16,000*g* for 10 min; plasma total solids were determined with a clinical refractometer. Red blood cell and white blood cell counts were obtained through manual counts in hemocytometer using Natt-Herrick's solution with 1:200 dilution. Because lymphocytes and thrombocytes cannot be reliably differentiated in the hemocytometer in Magellanic penguins, only heterophils and eosinophils were counted and white blood cell count was derived from differential counts as obtained from blood smears (Dein et al. 1994). Mean corpuscular volume was derived from packed cell volume and red blood cell count. Blood smears were air-dried, fixed with absolute methanol, and stained with Giemsa or Wright-Rosenfeld, and a minimum 200 microscope fields were examined under × 1000 magnification (c. 40,000 erythrocytes). Differential counts (heterophils, eosinophils, basophils, lymphocytes, and monocytes) were produced from 200 leukocytes, and the heterophil-to-lymphocyte ratio was calculated. Blood parasites were quantified with the assistance of digital image analysis to count 1000 erythrocytes (Gering and Atkinson 2004) and parasites were morphologically identified using the keys and descriptions provided by Valkiūnas (2005) and Valkiūnas and Iezhova (2018).

Penguins that died during rehabilitation were refrigerated (4–8 °C) and examined within 4 h after death. Gross lesions were photographed and noted, and samples of organs and tissues were fixed in 10% buffered formalin or frozen at − 20 °C for molecular analysis. Formalin-fixed tissues were embedded in paraffin and sections of 3 or 5 μm were obtained, stained with hematoxylin-eosin and examined under light microscopy. Impression smears were obtained from the lungs, liver, spleen, and kidneys; slides were air-dried, fixed with absolute methanol, stained with Giemsa, and examined under × 1000 magnification for a minimum of 15 min.

Approximately 10 μL of blood was transferred to 1.5-mL microtubes and samples were dried at 37 °C for subsequent DNA extraction using the phenol–chloroform method with isopropanol precipitation (Sambrook and Russell 2001). The genomic DNA pellet was resuspended in 50 μL of ultrapure water and quantified using a NanoDrop 2000 (Thermo Scientific, Waltham, USA). All available samples were tested using a PCR protocol targeting a 154-bp segment of the 18S small ribosomal subunit gene of *Haemoproteus* and *Plasmodium* using the primers 343F and 496R described by Fallon et al. (2003) and the amplification conditions described by Roos et al. (2015). Samples from penguins that were positive to blood parasites in traditional diagnostic methods (blood smears, histopathology, or impression smears) or to the 18S PCR test were then tested with a nested PCR protocol targeting a 478-bp segment of the cyt-*b* gene of *Haemoproteus* and *Plasmodium* using the primers HaemNFI, HaemNR3, HaemF, and HaemR2 and the amplification protocols described by Hellgren et al. (2004). Gel electrophoresis (6% polyacrylamide with silver staining) was conducted to visualize PCR amplification products. Amplification products of the cyt-*b* PCR were purified with Polyethylene Glycol 8000 (Sambrook and Russell 2001). Bi-directional Sanger sequencing with dye-terminator fluorescent labeling (Applied Biosystems 4-337-455, Life Technologies, Carlsbad, USA) was performed using the HaemF and HaemR2 primers (Hellgren et al. 2004) and an automated sequencer (ABI Prism 3100, Applied Biosystems, Foster City, USA).

Obtained DNA sequences were aligned, checked for the presence of mixed infections (the presence of double peaks in the eletrochromatograms), and edited using ChromasPro 2.0.6 (Technelysium Pty Ltd., Helensvale, Australia). Cyt-*b* sequences were deposited in Genbank (accession codes MK867779-82) and were compared to hemosporidian sequences using the BLASTN algorithm (Zhang et al. 2000) as implemented in the MalAvi (Bensch et al. 2009) and Genbank databases. The host and geographic distribution of these lineages was compiled based on publicly available records in the MalAvi and Genbank databases (access: 20-Mar-2019). Phylogenetic relationships among *Plasmodium* lineages identified in this study and reference lineages from the MalAvi database were evaluated through a Bayesian phylogenetic tree produced using MrBayes 3.2.5 (Ronquist et al. 2012). The GTR+I+G model of nucleotide evolution was used, as recommended by jModelTest 2 (Darriba et al. 2012). Two Markov chains were run simultaneously for 5 million generations that were sampled every 1000 generations. The first 1250 trees (25%) were discarded as a burn-in step and the remaining trees were used to calculate the posterior probabilities.

Because the PCR protocols employed in this study do not differentiate between *Plasmodium* and *Haemoproteus* unless the amplification products are sequenced and because detection of *Plasmodium* DNA at low levels can occur in abortive

inoculation without clinical significance (see Valkiūnas et al. 2014), individuals for which no parasites were detected in blood smears and PCR tests were positive but no cyt-*b* sequences could be obtained were classified as "inconclusive." The individual outcome (died, survived) was determined relative to 23-Sep-2015 (30 days after the first positive diagnosis). Pearson's chi-square test was used to determine whether the categories of *Plasmodium* diagnosis (positive, negative, inconclusive) were homogeneously distributed relative to the outcome and to the presence of palpebral lesions consistent with mosquito bites. Hematological variables were tested for normality using the Kolmogorov-Smirnov test; the Johnson transformation was applied to some variables. Normally distributed variables were compared between the categories of *Plasmodium* diagnosis using ANOVA, whereas Kruskal-Wallis tests were used for variables that could not be normalized. Significance level was 0.05 for all tests. Individual hematological results were considered "abnormal" when they were outside the central 90% interval (i.e., 5th to 95th percentile) of the results of negative individuals.

Results

The chronology of the outbreak and of sample collection and diagnostic results are summarized in Supplemental File S1. Briefly, most penguins at the facility had been admitted from June to August 2015, and the first cases of avian malaria were detected on 24-Aug-2015. Starting on 01-Sep-2015, chloroquine treatment was administered for all birds at the facility and measures were implemented to prevent exposure to mosquitoes. A systematic health screening was conducted on 02-Sep-2015, including physical examination, complete cell counts, and blood smear examination. An additional 14 penguins were admitted from another facility on 09-Sep-2015 and were subjected to the same health screening and treatment as the remaining birds that were already in the facility. Supplemental File S2 provides a detailed break-down of the individual history and diagnostic results for each penguin.

When blood smear and molecular analyses are considered altogether, eight of 89 individuals (9.0%) in this study were ultimately classified as positive, and another 15 individuals (16.9%) were classified as inconclusive (i.e., PCR-positive but no parasites were seen in blood smears and cyt-*b* sequences could not be obtained) (Table 1). Only two individuals in this study were adults, both of which were positive. Palpebral lesions consistent with mosquito bites were recorded with similar frequency in positive (62.5%), inconclusive (66.7%), and negative individuals (60.6%) ($\chi^2 = 0.19$, df = 2, $P = 0.908$). No clinical signs that could be attributed to avian malaria were seen, and the three *Plasmodium*-positive individuals that died appeared to be healthy up until they were found dead. Four of the eight positive individuals died (lethality = 50%), compared to three out of 66 negative individuals (4.5%) and one out of 15 individuals with an inconclusive diagnosis (6.7%) ($\chi^2 = 18.139$, df = 2, $P < 0.001$).

Table 2 summarizes the result of morphological and molecular identification of parasites for the eight positive individuals. Gene sequencing identified four cyt-*b* lineages of *Plasmodium*: GRW06 (three individuals), SPMAG06 (two individuals), PHPAT01 (one individual), and SPMAG10 (one individual). There was no evidence of double peaks in the chromatograms. Publicly available information on the host and geographic distribution of these lineages was compiled in Supplemental File S3. Phylogenetic analysis (Fig. 2) showed the following: (a) PHPAT01 and SPMAG06 cluster with reference lineages of *Plasmodium lutzi* and *Plasmodium matutinum*; (b) GRW06 is closely related to ERIRUB01, a *Plasmodium elongatum* reference lineage; and (c) SPMAG10 was not clustered with any reference lineages.

In the two cases where the lineage SPMAG06 was detected, the blood smears revealed the presence of an organism that formed large round gametocytes, with small round hemozoin granules distributed randomly or along the margins of the parasite's cytoplasm (Fig. 3a–d, h). These parasites markedly displaced the host cell nucleus (Fig. 3a, c–f, h) or occasionally enucleated the host cell (Fig. 3b, g), and often showed cytoplasmic vacuolation (Fig. 3d–g) or fragmentation (Fig. 3d, h) suggestive of degeneration due to chloroquine treatment. Trophozoites were occasionally seen in polychromatic erythrocytes (Fig. 3e). In one SPMAG06-infected individual (case 2), circulating exoerythrocytic meronts were also seen in erythroblasts (Fig. 3i, j) and mononuclear leukocytes (Fig. 3k–n). One individual infected with GRW06 showed parasites that were consistent with *P. elongatum*, and in three instances there were erythrocytic meronts with elongated merozoites (Fig. 3p). Unfortunately, SPMAG10 could not be morphologically characterized due to the low parasitemia, but some morphological characteristics were preliminarily documented (Supplemental File S4).

Hematology, histopathology, and tissue impression smears

No significant differences were identified among positive, inconclusive, and negative individuals for the body mass ($P = 0.138$) nor for any hematological parameters (all $P > 0.06$). However, several *Plasmodium*-positive individuals had "abnormal" hematological results depending on the morphospecies or lineage (Table 3).

Table 1 Summary of the rescue location and diagnostic results for *Plasmodium* sp. in Magellanic penguins (*Spheniscus magellanicus*)

Blood smear	PCR	Cyt-*b* sequencing	Rescue location					Total
			Northern Bahia	Southern Bahia	Espírito Santo	Northern Rio de Janeiro	Southern Rio de Janeiro	
Positive	Positive	Successful	0	3	2	0	1	6
		Not successful	0	0	0	0	1	1
Negative	Positive	Successful	0	1	0	0	0	1
		Not successful	3	2	4	3	3	15
	Negative	Not applicable	11	5	22	2	26	66
Total			14	11	28	5	31	89

Avian malaria was confirmed as the cause of death of cases 2 and 4 based on the high density of tissue meronts and the generalized vasculitis and endothelial damage, severe lung congestion and edema, mild to moderate granulocytic pneumonia, periportal mononuclear hepatitis, and multifocal granulocytic necrotic splenitis. Histopathology revealed the presence of tissue meronts in the lungs, cerebrum, heart, and kidneys of cases 2 and 4, and also in the trachea, liver, spleen, cerebellum, and coprodeum of case 2 (Fig. 4). Tissue impression smears of six individuals decreased during the study period identified only one individual as *Plasmodium*-positive (case 2). Tissue meronts were only detected in the lung impression smear (Fig. 3o) and were not seen in spleen impression smears despite the presence of large clusters of exoerythrocytic meronts (Fig. 4c). *Plasmodium* infection was not detected in the tissue impression smears of two individuals that were otherwise known to be *Plasmodium*-positive (cases 3 and 4). Unfortunately, some individuals that died with a positive *Plasmodium* diagnosis could not be analyzed by histopathology (cases 3 and 5) or tissue impression smears (case 5).

Discussion

Despite a high lethality (50%), the overall infection rate and mortality in this study were low (9.0% and 4.5%, respectively) compared to other outbreaks reported in captive penguins, where mortality as high as 50 to 80% have been reported (Fix et al. 1988; Bueno et al. 2010; Vanstreels et al. 2014). This is likely the result of the early implementation of chloroquine treatment to all penguins as soon as the first cases were diagnosed. Considering that our results also demonstrate that clinical signs and palpebral mosquito bites are not reliable diagnostic indicators for malarial infections in penguins, and that hematological results may vary considerably among *Plasmodium* lineages, we believe that as soon a positive case

Table 2 Summary of the history and diagnostic results of confirmed cases of *Plasmodium* spp. infection in Magellanic penguins (*Spheniscus magellanicus*) sampled at a rehabilitation center in Espírito Santo, Brazil

Case	Origin	Age group	Outcome	Blood smear	Cyt-*b* lineage
1	Rio de Janeiro [a]	Juvenile	Survived	*P.* (*Haemamoeba*) sp.	*P. lutzi* SPMAG06
2	Bahia [b]	Juvenile	Died	*P.* (*Haemamoeba*) sp. [c]	*P. lutzi* SPMAG06
3	Bahia [b]	Adult	Died	No parasites seen [d]	*Plasmodium* sp. PHPAT01
4	Bahia [b]	Juvenile	Died	*Plasmodium* sp.	*Plasmodium* sp. SPMAG10
5	Bahia [b]	Juvenile	Died	*P. elongatum*	*P. elongatum* GRW06
6	Espírito Santo	Juvenile	Survived	*P. elongatum* + *P.* (*Haemamoeba*) sp.	*P. elongatum* GRW06
7	Espírito Santo [e]	Adult	Survived	*P. cathemerium* + *Plasmodium* sp.	*P. elongatum* GRW06
8	Rio de Janeiro [1]	Juvenile	Survived	*P. cathemerium*	Sequencing unsuccessful

[a] Individual spent 5–7 days at a stabilization facility in Araruama, Rio de Janeiro, prior to being transferred for rehabilitation at the study facility

[b] Individual spent 2–8 weeks at a rehabilitation facility in Porto Seguro, Bahia, prior to being transferred for rehabilitation at the study facility

[c] Presence of exoerythrocytic meronts in blood smears

[d] No parasites were seen in a blood smear from 24-Aug-2015, and the individual died on 31-Aug-2015 before another blood smear was produced

[e] Individual had a flipper band that indicated it had previously been rehabilitated at a facility in Rio Grande (32° 01′ 34″ S 52° 06′ 21″ W), southern Brazil

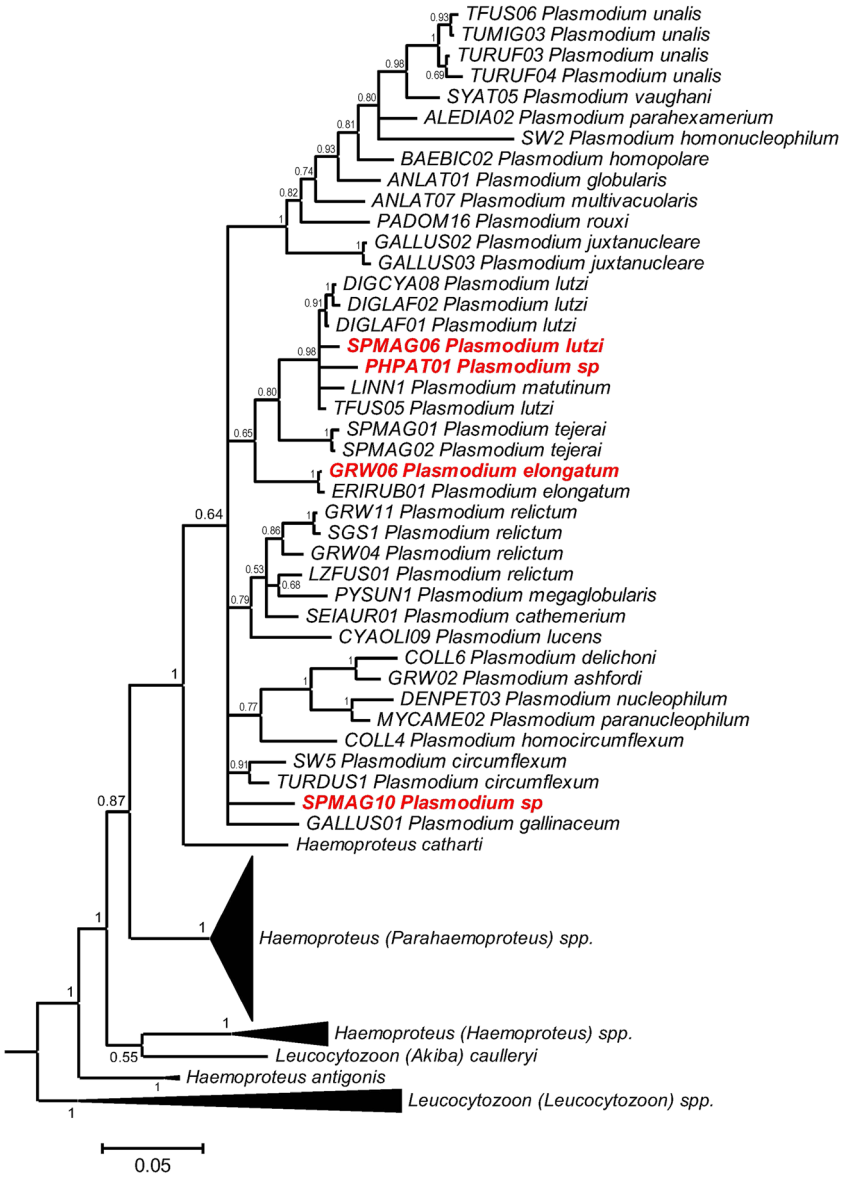

Fig. 2 Bayesian phylogenetic tree of the cyt-*b* gene of the *Plasmodium* lineages detected in this study (red) in relation to reference lineages from the MalAvi database. Branch lengths are drawn proportionally to the extent of changes (scale bar is shown). Values adjacent to nodes represent posterior probabilities

is detected, all individuals at the facility should be treated regardless of their individual diagnostic results or clinical signs. It is also interesting to note that the outbreak in this study occurred in late austral winter, whereas in a previous study we had identified spring-summer (October to April) as the peak malaria season for penguins at rehabilitation centers in Brazil (Vanstreels et al. 2015), suggesting that facilities at lower latitudes might face a more extended malaria season.

Previous studies have established that SPMAG06 is morphologically consistent with *Plasmodium* (*Haemamoeba*) *lutzi* (de Oliveira et al. 2019). However, we observed two morphological features that are inconsistent with previous descriptions of *P. lutzi* and lineage SPMAG06, namely the lack of clumping of pigment granules and the presence of circulating exoerythrocytic meronts. The clumping of pigment granules into a single spot (or coalescing into a few spots) is one of the key diagnostic characteristics of *P. lutzi* (Mantilla et al. 2013; González et al. 2015; Valkiūnas and Iezhova 2018; de Oliveira et al. 2019) but was not observed in gametocytes in this study. The presence of exoerythrocytic meronts in the circulating blood is highly unusual among avian plasmodia and has never been documented in *P. lutzi*; this characteristic was previously reported only in *Plasmodium* (*Novyella*) *paranucleophilum* (Manwell and Sessler 1971; Valkiūnas 2005). We propose three hypotheses to explain these unusual morphological characteristics, possibly in combination: (a) they were induced by the chloroquine treatment; (b) they are related to undetected coinfections with one or more *Plasmodium* species; (c) they are atypical features related to the fact that the Magellanic penguin is an accidental host. Although we saw a number of degenerative changes (vacuolation and fragmentation) in the erythrocytic parasites (Fig.

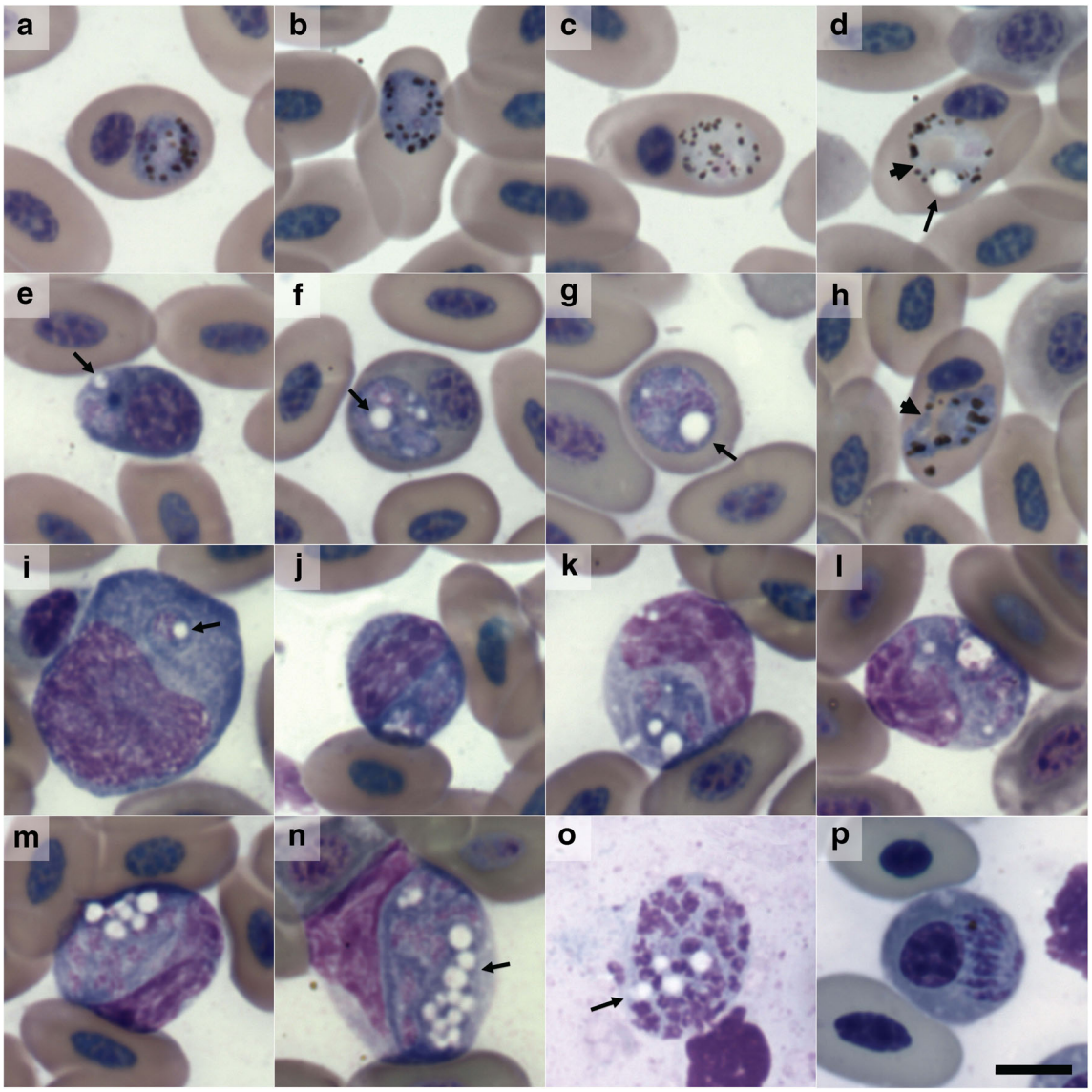

Fig. 3 Photomicrographs of *Plasmodium* spp. in the blood smears of Magellanic penguins (*Spheniscus magellanicus*). Case 1 (lineage SPMAG06, blood smear, Wright-Rosenfeld stain): **a**, **b** macrogametocytes, **c**, **d** microgametocytes. Case 2 (lineage SPMAG06, blood smear, Wright-Rosenfeld stain): **e** trophozoite in polychromatic erythrocyte, **f**, **g** erythrocytic meronts, **h** macrogametocyte, **i**, **j** exoerythrocytic meronts in erythroblasts, **k**–**n** exoerythrocytic meronts in mononuclear leukocytes. Case 2 (lineage SPMAG06, lung impression smear, Wright-Rosenfeld stain): **o** exoerythrocytic meront in unidentified cell. Case 6 (lineage GRW06, lung impression smear, Giemsa stain): **p** erythrocytic meront with elongated merozoites. Cytoplasmic vacuolation (thin arrows) and the loss cytoplasmic continuity (thick arrows) are indicated. Scale bar = 5 μm

3d–h) that are likely due to chloroquine treatment (Vanstreels et al. 2014), the lack of pigment clumping was not necessarily accompanied by these changes (Fig. 3a–c). With regard to the vacuolation of circulating exoerythrocytic meronts, this characteristic was also seen in *P. paranucleophilum* in the absence of chloroquine treatment (Manwell and Sessler 1971) and might therefore be a normal characteristic of these parasite forms. It is worth noting, nonetheless, that the significant polymorphism of *P. lutzi* documented in this and other studies (de Oliveira et al. 2019) suggests that the morphological characteristics traditionally attributed to this taxon might not always be reliable and the species boundaries of this taxon might require reconsideration in the future.

Lineage SPMAG06 has only been reported from three bird species in South America (Supplemental File S3). The rufous-bellied thrush (*Turdus rufiventris*) appears to be this parasite's most frequent host, having been recorded at three sites in Brazil and Peru (de Oliveira et al. 2019; Fecchio et al. 2019). The other two recorded hosts appear to be accidental infections: a violet turaco (*Musophaga violacea*) at a zoo in southeast Brazil (Chagas et al. 2017), and Magellanic penguins at the same zoo (Bueno et al. 2010) and at a rehabilitation center in

Table 3 Hematology of Magellanic penguins (*Spheniscus magellanicus*) in relation to the diagnostic results for *Plasmodium* spp. Asterisks indicate hematological results identified as abnormal (relative to negative individuals). Note that no hematological analyses were conducted for case 3

Values	Case 1 (SPMAG06)	Case 2 (SPMAG06)	Case 4 (SPMAG10)	Case 5 (GRW06)	Case 6 (mixed)	Case 7 (mixed)	Case 8 (*P. cathemerium*)	Inconclusive ($n = 15$)	Negative ($n = 65$)
Parasitemia (10^{-2} cell)	< 0.1	0.4	< 0.1	7.4	0.8	0.1	0.5	–	–
Packed cell volume (%)	50.0 *	48.0	35.0 *	23.0 *	43.0	40.0	38.0	43.5 ± 5.3	44 ± 3.5
Plasma total solids (g L^{-1})	60.0	72.0	80.0 *	52.0 *	80.0 *	75.0	67.0	67.1 ± 6.6	66.1 ± 8.2
Red blood cells (10^{-12} L)	2.4	2.0	1.7	1.0 *	1.8	1.9	1.6	2.0 ± 0.4	1.9 ± 0.3
Mean corpuscular volume (fL)	212.8	241.2	204.7	230.0	235.0	207.3	243.6	224.9 ± 30.5	234 ± 47.8
White blood cells (10^{-9} L)	14.9	16.2	13.9	12.0	8.0	2.5 *	20.5	17.7 ± 9.5	17.8 ± 11
Heterophils (10^{-9} L)	5.1	5.0	4.0	5.1	3.3	1.5 *	11.3	8.5 ± 5	9.3 ± 5.8
Lymphocytes (10^{-9} L)	9.2	10.4	8.9	6.5	4.5	0.9 *	9.0	8.8 ± 6.2	8.1 ± 6.5
Eosinophils (10^{-9} L)	0.0	0.3	0.8 *	0.4	0.1	0.0	0.0	0.4 ± 0.5	0.2 ± 0.4
Basophils (10^{-9} L)	0.4	0.2	0.1	0.0	0.1	0.0	0.2	0.1 ± 0.1	0.1 ± 0.2
Monocytes (10^{-9} L)	0.1	0.3	0.0	0.0	0.0	0.0	0.0	0.0 ± 0.1	0.1 ± 0.2
Heterophils (%)	34	31 *	29 *	43	42	60	55	49.6 ± 12	53.2 ± 12.4
Lymphocytes (%)	62	64 *	64 *	54	56	38	44	48.2 ± 12.1	44.4 ± 11.7
Eosinophils (%)	0	2	6 *	3	1	1	0	1.5 ± 2.4	1.4 ± 2
Basophils (%)	3 *	1	1	0	1	0	1	0.4 ± 0.7	0.6 ± 0.9
Monocytes (%)	1	2	0	0	0	1	0	0.3 ± 0.6	0.3 ± 0.7
Heterophil-lymphocyte ratio	0.55	0.48 *	0.45 *	0.80	0.75	1.58	1.25	1.18 ± 0.68	1.39 ± 0.83

Salvador, northeast Brazil (Vanstreels et al. 2015). It is also worth noting that the lineage TUAMA01, a lineage whose cyt-*b* sequence differs from that of SPMAG06 by a single base-pair, has also been recorded exclusively in *Turdus* spp. in Brazil and Peru (Lacorte et al. 2013; Ferreira Junior et al. 2017; Fecchio et al. 2019), with the exception of a record in Magellanic penguins at a zoo in southern Brazil (Taunde et al. 2019). It therefore seems that SPMAG06 and TUAMA01 are highly specialized parasites of *Turdus* spp. in tropical South America that can occasionally spill over to Magellanic penguins brought to this region. The fact that one of the penguins infected by SPMAG06 in this study died due to avian malaria (case 2) corroborates previous indications that this pathogen can be lethal to penguins (Bueno et al. 2010). A decreased heterophil-to-lymphocyte ratio was also noted in infected penguins, consistently with previous studies on other *Plasmodium* species (Graczyk et al. 1994). Surprisingly, however, this lineage did not seem to cause anemia but actually led to an elevated hematocrit, presumably due to dehydration.

Previous studies have established that GRW06 is morphologically consistent with *Plasmodium* (*Huffia*) *elongatum* (Valkiūnas et al. 2008; Vanstreels et al. 2014; Palinauskas et al. 2016). Lineage GRW06 is one of the most frequently reported *Plasmodium* lineages worldwide and has been detected in all continents except Antarctica, including in wild terrestrial birds (*Malacoptila striata* and *Turdus leucomelas*) approximately 60 km from the rehabilitation facility in this study (Lacorte et al. 2013). To date, this lineage has been detected in 78 avian species in 10 orders, especially Passeriformes (61 spp.) (Supplemental File S3). Among penguins, GRW06 had already been documented in Magellanic penguins at a rehabilitation center in southern Brazil (Vanstreels et al. 2014, 2015), in wild Galapagos penguins (*Spheniscus mendiculus*) at the Galapagos islands (Levin et al. 2009), in African penguins (*Spheniscus demersus*) at a zoo in the USA (Pacheco et al. 2017), and in a blue penguin (*Eudyptula minor*) at a zoo in New Zealand (Sijbranda et al. 2017). This lineage is therefore a generalist parasite that is highly capable of infecting penguins; considering that this parasite is also known to occur in South Africa (Okanga et al. 2014), it seems plausible that African penguins could be exposed to this lineage in the wild. One GRW06-infected individual in this study died with necropsy findings suggestive of avian malaria. Additionally, the high parasitemia, marked anemia, and hypoproteinemia in case 5 also corroborate that this lineage can have significant pathogenicity to penguins. It is worth noting that we documented instances where GRW06 formed elongated merozoites; this had not been seen in our previous studies on avian malaria in penguins in Brazil (Vanstreels et al. 2014, 2015) and corroborates the interpretation that this otherwise common morphological feature of *P. elongatum* is rare when infecting unusual hosts such as penguins (Valkiūnas 2005).

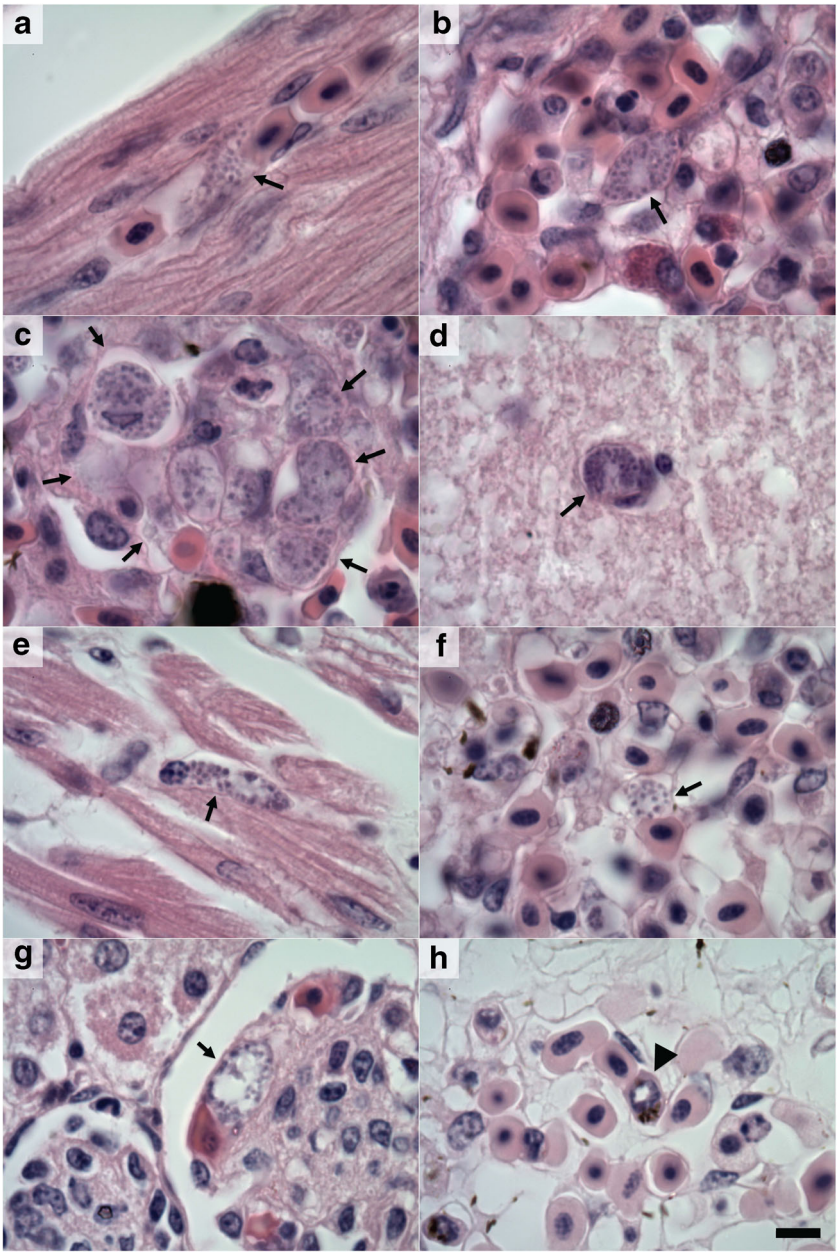

Fig. 4 Photomicrographs of *Plasmodium* spp. in the tissues of Magellanic penguins (*Spheniscus magellanicus*). Case 2 (lineage SPMAG06): **a** heart, **b** lung, **c** spleen, **d** brain. Case 4 (lineage SPMAG10): **e** heart, **f** lung, **g** kidney, **h** lung. Exoerythrocytic meronts (arrows) and erythrocytic parasites (arrowhead) are indicated. Hematoxylin-eosin. Scale bar = 10 μm

The morphospecies of PHPAT01 has yet to be determined, but the phylogenetic analysis suggests it is most closely related to *P. lutzi* and *P. matutinum*, suggesting that it probably belongs to the *Haemamoeba* subgenus and may share morphological characteristics with those morphospecies. Lineage PHPAT01 has been recorded in 13 avian species in the Americas, predominantly Passeriformes (10 spp.), ranging from the southern Chile to northern USA (Supplemental File S3). Although PHPAT01 has not been recorded yet in Espírito Santo state, it was abundantly documented in wild Passeriformes in the adjacent state of Minas Gerais (Lacorte et al. 2013; Ferreira Junior et al. 2017). It should be noted that the penguin infected with PHPAT01 in this study had been transferred 13 days earlier from a facility in Porto Seguro, Bahia, and it is possible that the infection had occurred before the penguin was transferred to the study facility in Espírito Santo. This lineage has also been documented infecting Magellanic penguins at a rehabilitation center in Santa Catarina state, southern Brazil (Vanstreels et al. 2015). Although histopathology could not be conducted to determine whether or not avian malaria caused the death of the PHPAT01-infected penguin in this study, the post-mortem findings in that case were suggestive of avian malaria (splenomegaly, hepatomegaly, severe lung congestion) and this lineage was already determined to have caused the death of a Magellanic penguin in a previous study (Vanstreels et al. 2015).

Little is known about lineage SPMAG10, and its morphology has yet to be characterized. Unfortunately, the lack of a close phylogenetic relationship to any known-morphospecies

lineages precludes speculation in this regard. SPMAG10 had only been recorded once until now, infecting a Magellanic penguin that had been at a rehabilitation center in Salvador, Bahia (Vanstreels et al. 2015). As for PHPAT01, because the penguin infected with SPMAG10 in this study had been transferred 13 days earlier from a facility in Porto Seguro, it is possible that the infection occurred before the penguin was transferred. The host and geographic distribution of SPMAG10 therefore remains unclear, and further studies on the blood parasites of wild birds in coastal Bahia are warranted. The penguin infected with this lineage showed microcytic anemia, hyperproteinemia, decreased heterophil-to-lymphocyte ratio, and ultimately died from avian malaria, demonstrating that this is a significant pathogen to penguins.

We detected *P. cathemerium* in blood smears but could not produce a cyt-*b* sequence to determine its lineage. However, in a previous study, we detected the lineage PADOM09 in two Magellanic penguins undergoing rehabilitation at the same facility (Vanstreels et al. 2015), so it is likely that the same lineage was involved in this case. It is worth noting that similarly to PHPAT01, the lineage PADOM09 appears to be restricted to the Americas and infects predominantly Passeriformes (Marzal et al. 2011; Lacorte et al. 2013; Ferreira Junior et al. 2017; Fecchio et al. 2018) but has also been recorded in Magellanic penguins at other rehabilitation centers in Brazil (Vanstreels et al. 2015).

Although avian malaria has been extensively reported in penguins at zoos and rehabilitation centers worldwide (Grilo et al. 2016; Vanstreels et al. 2016a), Magellanic penguins on the Patagonian coast of Argentina do not appear to have malarial infections (Vanstreels et al. 2017; Gallo et al. 2019). Epidemiological data from rehabilitation centers in Brazil corroborates the interpretation that *Plasmodium* infections are acquired only after admission to these facilities (Vanstreels et al. 2015). In this sense, the phylogeography of the parasite lineages detected in this study supports the notion that these infections are the result of the spillover inoculation by plasmodia that circulate in the local avifauna, especially Passeriformes. Although some of the *Plasmodium* lineages recorded in Magellanic penguins in rehabilitation centers are well-known generalists (e.g., GRW06), it would also seem that even otherwise specialist lineages (e.g., SPMAG06) are also able to infect penguins opportunistically. Additionally, the high frequency of mixed infections detected in this and previous studies (Vanstreels et al. 2014, 2015, 2016b) further supports the hypothesis that the immune system of penguins is particularly ill-equipped to combat malarial parasites. Furthermore, the fact that some lineages such as SPMAG10 are only known from penguins undergoing rehabilitation in areas of Atlantic forest and were never detected on wild birds from this biome suggests that penguins might be more prone to develop detectable infections by *Plasmodium* lineages that might otherwise be difficult to detect in their natural hosts.

Our results therefore corroborate that penguins are highly susceptible to malarial parasites and may develop acute and severe infections. At rehabilitation centers, there are a number of preventative measures that can be implemented to reduce the occurrence of avian malaria outbreaks such as mosquito netting, repellants, fans, and primaquine prophylaxis (Vanstreels and Parsons 2014; Grilo et al. 2016; Botes et al. 2017). In wild penguins, however, this heightened vulnerability to *Plasmodium* spp. can have dramatic consequences for the conservation of these species, as demonstrated by the recent outbreaks of avian malaria in yellow-eyed penguin (*Megadyptes antipodes*) in New Zealand (Webster et al. 2018). In this sense, avian malaria may become one more mechanism through which human activities push penguin populations towards extinction, via the introduction of mosquitoes to islands (e.g., Galapagos Islands and New Zealand; Tompkins and Gleeson 2006; Levin et al. 2009) or by inducing shifts in mosquito distribution through climate change and urbanization (Garamszegi 2011; Vanstreels et al. 2017). Further studies on the epidemiology and health effects of malarial parasites at penguin breeding colonies will therefore be valuable for the efforts concerning the conservation of these species, especially in tropical and subtropical regions.

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Compliance with ethical standards All procedures in this study were approved by the Ethics Committee on Animal Use of the School of Veterinary Medicine and Animal Science of the University of São Paulo (CEUA 601415) and were authorized by the Brazilian authorities (SISBIO 20825-6).

Conflict of interest The authors declare that there is no conflict of interest.

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