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Original Article

Comparative morphometric evaluation of hepatic hemosiderosis in wild Magellanic penguins (*Spheniscus magellanicus*) infected with different *Plasmodium* spp. subgenera

Avaliação morfométrica comparativa da hemossiderose hepática em pinguins-de-Magalhães (*Spheniscus magellanicus*) de vida livre infectados por diferentes subgêneros de *Plasmodium* spp.

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Abstract

Avian malaria is one of the most important diseases of captive penguins. We employed morphometric techniques to evaluate hepatic hemosiderosis in rehabilitating wild Magellanic penguins (*Spheniscus magellanicus*) that were negative (n = 9) or naturally infected by different subgenera of *Plasmodium* spp. (n = 24), according with: *Plasmodium* subgenera (*Haemamoeba, Huffia*, Other lineages, and Unidentified lineages), severity of *Plasmodium* histopathological lesions, and concurrent diseases, age class (juvenile or adult plumage), sex (male, female or not determined), body score (emaciated, thin, good, excellent, not available), molt, presence or absence of oil contamination upon admission, iron supplementation, and rehabilitation center. The percentage of the area occupied by hemosiderin was called 'Index of Hepatic Hemosiderosis (IHH)'. *Plasmodium*-positive females presented significantly higher IHH values (17.53 ± 12.95%) than males (7.20 ± 4.25%; p = 0.041). We observed higher levels of congestion (p = 0.0182) and pneumonia (p = 0.0250) severity between Unidentified lineages *vs*. *Huffia*. We believe that the hepatic hemosiderosis observed in this study was multifactorial, the result of pathological processes caused by malaria, molting, hemoglobin and myoglobin catabolism during migration, anemia, concomitant diseases, and iron supplementation, all possibly potentiated by decreased liver mass. Further studies are needed to clarify the mechanisms of these hypotheses.

Keywords: Avian malaria, iron, hemosiderin, liver, morphometry, penguin.

Resumo

Malária aviária é uma das mais relevantes doenças em pinguins cativos. Foram aplicadas técnicas morfométricas para avaliar a hemossiderose hepática em pinguins-de-Magalhães (*Spheniscus magellanicus*) de vida livre em reabilitação negativos (n = 9) e naturalmente infectados por diferentes subgêneros de *Plasmodium* spp. (n = 24), quanto a: subgênero de *Plasmodium* (*Haemamoeba*, *Huffia*, Outras Linhagens, e Linhagens não identificadas), severidade das lesões histopatológicas causadas por *Plasmodium* e doenças concomitantes, faixa etária (plumagem juvenil ou adulta), sexo (macho, fêmea, indeterminado), condição corporal (emaciado, magro, bom, excelente, indisponível), muda, presença/ausência de óleo a admissão,

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suplementação de ferro, e centro de reabilitação. A porcentagem da área ocupada por hemossiderina foi denominada "Índice de Hemossiderose Hepática (IHH)". Fêmeas *Plasmodium*-positivas apresentaram IHH significativamente mais elevado que machos, respectivamente, 17,53 ± 12,95% e 7,20 ± 4,25% (p = 0,041). Níveis mais elevados de congestão (p = 0,0182) e pneumonia (p = 0,0250) foram observados entre Linhagens não identificadas *vs*. *Huffia*. Possivelmente, a hemossiderose hepática observada nesse estudo seja multifatorial, resultado de processos patológicos causados por malária, muda, catabolismo de hemoglobina e mioglobina durante a migração, anemia, doenças concomitantes e suplementação de ferro, potencialmente intensificados por massa hepática reduzida. Estudos complementares são necessários para esclarecer os mecanismos de tais hipóteses.

Palavras-chave: Malária aviária, ferro, hemosiderina, fígado, morfometria, pinguim.

Introduction

The Magellanic penguin (*Spheniscus magellanicus*) breeds in colonies located on the coasts of Chile, Argentina, and the Falkland (Malvinas) Islands. During their winter migration, individuals from the Atlantic Ocean colonies migrate along the continental shelf of northern Argentina, Uruguay, and southern Brazil (STOKES et al., 2014), where they face many challenges (PETRY & FONSECA, 2002; GARCÍA-BORBOROGLU et al., 2006; BRANDÃO et al., 2011; BALDASSIN et al., 2012; REZENDE et al., 2013; KEHRIG et al., 2015). Birds stranded alive are rescued and referred to local rehabilitation centers along the Brazilian coast (GARCÍA-BORBOROGLU et al., 2010; RODRIGUES et al., 2010; RUOPPOLO et al., 2012)

While under rehabilitation, these birds may be exposed to avian malaria, one of the most significant threats to the conservation of penguins, able to seriously compromise their survival in captivity (JONES & SHELLAM, 1999; VANSTREELS et al., 2016). Avian malaria is transmitted by Culicidae mosquitoes infected with *Plasmodium* spp. protozoans (VALKIŪNAS, 2005), and is relatively asymptomatic in most avian species, but potentially pathogenic for those species that have not co-evolved with the parasite, such as penguins (Spheniscidae) (VALKIŪNAS, 2005; VANSTREELS et al., 2016). Penguins are highly susceptible to avian malaria. Rapid and severe outbreaks with high mortality (VANSTREELS et al., 2016) have been reported worldwide in populations of several penguin species in the wild (FANTHAM & PORTER, 1944; LAIRD, 1950; LEVIN et al., 2009), in captivity (GRINER & SHERIDAN, 1967; BAK et al., 1984; FIX et al., 1988), and undergoing rehabilitation (PARSONS & UNDERHILL, 2005). In Brazil, reports of avian malaria in Magellanic penguins include birds in zoos (BUENO et al., 2010) and rehabilitation centers (SILVEIRA et al., 2013; VANSTREELS et al., 2014, 2015).

Iron homeostasis in birds, as in other vertebrates, is regulated mainly through intestinal absorption and excess iron is stored as intracellular aggregations of ferritin or hemosiderin, particularly in hepatic and splenic cells (NORAMBUENA & BOZINOVIC, 2009). Hemosiderosis is the accumulation of iron in hepatic and splenic sinusoidal macrophages and hepatocytes without significant architectural, cellular, or functional alterations (LOWENSTEIN & MUNSON, 1999). Iron absorption is influenced by age, iron and health status, gastrointestinal conditions, the amount and chemical form of the iron ingested, and the amount and proportions of various other organic and inorganic components of the diet (CRISSEY et al., 2000; SHEPPARD & DIERENFELD, 2002; PEREIRA et al., 2010). Although there is no conclusive evidence that hepatic hemosiderosis has any clinical significance in birds, it has been associated with concurrent infections, neoplasia, parasitism, anemia, hepatopathies, intoxications, starvation, oil exposure, trauma, lead poisoning, and the physiological cycle (e.g., egg production and laying, migration, and molting) (CORK et al., 1995; GOTTDENKER et al., 2008; PEREIRA et al., 2010; KLASING et al., 2012).

Hemosiderosis, especially hepatic, has been previously reported in *Plasmodium*-infected penguins (FIX et al., 1988; GRILO et al., 2016), believed to be caused by the build up of hemosiderin in visceral macrophages following intravascular hemolysis and phagocytosis of parasitized cells (VANSTREELS & PARSONS, 2014). However, these studies did not evaluate if such condition varied among *Plasmodium* spp. subgenera or considered other possible causes of hemosiderosis aside from *Plasmodium* spp. infection. Considering the importance of avian malaria to penguin conservation and the limited amount of information regarding iron metabolism and the pathogenesis of avian malaria in birds, our objective was to quantify the hepatic hemosiderosis in *Plasmodium* spp.- negative and - naturally infected Magellanic penguins that died while under care in rehabilitation centers in Brazil, in regards to the presence of *Plasmodium* and *Plasmodium* species/lineages, and within the context of these birds' biology and individual history.

Material and Methods

The Magellanic penguins evaluated in this study represent all *Plasmodium*-positive (n = 24) and some of the *Plasmodium*-negative (n = 9) birds, that died while under rehabilitation along 2500 km of the Brazilian coastline between January 2009 and February 2013 during studies on the prevalence of *Plasmodium* in Magellanic penguins (SILVEIRA et al., 2013; VANSTREELS et al., 2014, 2015). Physical and postmortem examinations, including necropsy and hematology findings, are described elsewhere (VANSTREELS et al., 2015). The positive group comprised 24 *Plasmodium*-positive animals: 21 individuals naturally infected by *Plasmodium* spp., previously diagnosed by morphologic (blood smears, histopathology) and molecular techniques (nested PCR, genetic sequencing) (VANSTREELS et al., 2015); and 3 additional natural infection cases identified using the same methods (cases 3, 14, and 15). Positive birds were divided into 4 study groups: (1) *Haemamoeba* (*P. cathemerium* and *P. tejerai*), (2) *Huffia* (*P. elongatum*), (3) Other lineages (*P. nucleophilum*, *P. unalis,* and *Plasmodium* sp. lineages E, G, and H), and (4) Unidentified lineages (*Plasmodium* sp. that

could not be morphologically or molecularly characterized). The positive birds evaluated in this study either (a) died suddenly, (b) presented antemortem malaria-associated clinical signs, dying after unsuccessful treatment, or (c) were euthanized for reasons unrelated to malaria (e.g., aspergillosis). Nine *Plasmodium*-negative Magellanic penguins were selected based on individual history and a negative diagnosis for *Plasmodium* spp. on both nested PCR and blood smears. Selected *Plasmodium*-negative birds died without presenting any clinical signs of concurrent infections, and remained in captivity for similar periods as the positive birds. All birds from both groups (positive and negative) were kept under similar infrastructure and husbandry conditions, and diet and iron supplementation varied among rehabilitation facilities (Table 1).

All birds were examined *postmortem*, and liver samples were collected and fixed in 10% formalin for a minimum of 72 hours before processing. Paraffin embedded liver fragments were sectioned at 5 µm and stained with haematoxylin-eosin and Perls stains, as described by Luna (1992) (Figure 1). The Index of Hepatic

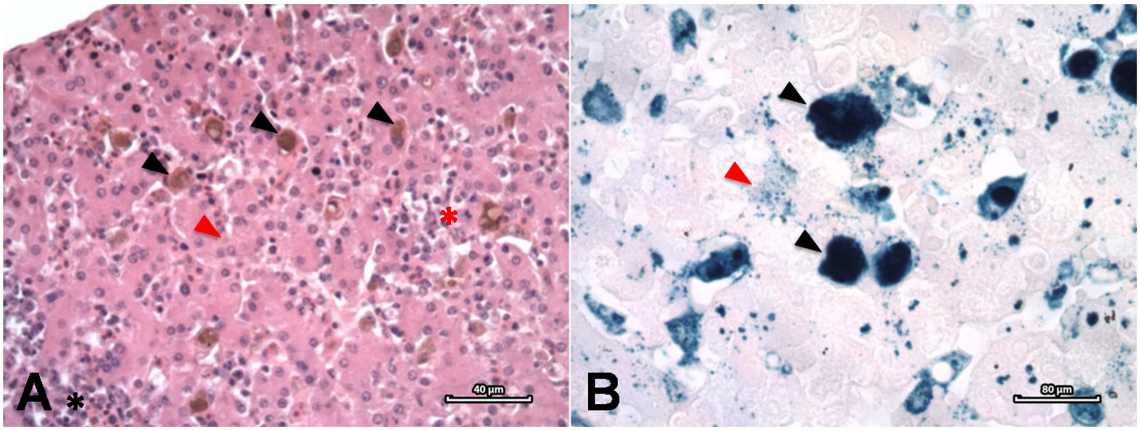

Figure 1. Hepatic hemosiderosis in a Magellanic penguin (*Spheniscus magellanicus*). A. Areas of intracellular hemosiderin deposition in Kupffer cells (black arrowheads) and hepatocytes (red arrowhead), diffuse cordonal derangement (red asterisk) and inflammatory infiltrate (black asterisk). H&E stain. 40 µm. B. Intracellular hemosiderin deposits within Kupffer cells (black arrowheads) and hepatocytes (red arrow). Perls stain. 80 µm.

Table 1. *Plasmodium*-positive and control Magellanic penguins (*Spheniscus magellanicus*) according with rehabilitation center, iron supplementation, administration criteria and diet.

Rehabilitation Center	Iron supplementation (Dose/Route/Frequency)	Administration Criteria	Diet (whole fish-based)
CRAM[a]	Ferrodex[d] → 0.2mg/kg/IM – single dose (until 2013)	Hematocrit < 35%	banded croaker (*Paralonchurus brasiliensis*)
	Ferrodex → 0.2mg/kg/IM – single dose (from 2013 to the present)	Hematocrit < 30% until the parameter reaches the acceptable interval for the species[f]	stripped weakfish (*Cynoscion guatucupa*) acoupa weakfish (*Cynoscion acoupa*)
	Ferrodex → 0.2mg/kg/IM – single dose in association with Hemolitan pet[e] → 0.1mg/kg PO – q24h	Hematocrit < 35%until the parameter reaches the acceptable interval for the species[f]	Argentine croaker (*Umbrina canosai*)
	(from 2013 to the present)		whitemouth croaker (*Micropogonias furnieri*)
R3 Animal[b]	Ferrodex → 1ml/IM– single dose	Upon admission	Brazilian sardine (*Sardinella brasiliensis*)
IPRAM[c]	Hemolitan pet → 1ml/PO – q24h	Penguins treated as huddles. Medication administered upon admission and maintained for days to weeks, until the birds exhibited normal colored oral mucosae	Brazilian sardine (*Sardinella brasiliensis*)

[a]CRAM = Centro de Recuperação de Animais Marinhos (Rio Grande do Sul state, Brazil). [b]R3 = Associação R3 Animal (Santa Catarina state, Brazil). [c]IPRAM = Instituto de Pesquisa e Reabilitação de Animais Marinhos (Espírito Santo state, Brazil). [d]Ferrodex® (Tortuga, Companhia Zootécnica Agrária, São Amaro, São Paulo, Brazil). [e]Hemolitan pet (Indústria e Comércio de Produtos Veterinários Ltda., Louveira, São Paulo, Brazil). [f]Reference interval for the species (SILVA-FILHO & RUOPPOLO, 2014).

Hemosiderosis (IHH) was evaluated according with the presence of infection, *Plasmodium* species/lineage, malaria severity (based on the classification of the histopathological findings observed in heart, lung, liver, and spleen samples: mild, mild to moderate, moderate, moderate to severe, and severe - Table 2), concurrent diseases and/or significant lesions, individual history (age class

Table 2. *Plasmodium*-positive and control Magellanic penguins according with the Index of Hepatic Hemosiderosis (IHH), study group, *Plasmodium* lineage, *Plasmodium* species and malaria severity.

Case	Study group	*Plasmodium* lineage	IHH (%)	Malaria severity
1	*Haemamoeba*	*P. cathemerium*	0.4	Moderate pulmonary congestion
				Moderate granulocytic pneumonia
				Moderate-severe monocytic hepatitis
2	*Haemamoeba*	*P. cathemerium*	14.8	Moderate-severe pulmonary congestion
				Moderate to severe granulocytic pneumonia
				Mixed moderate-severe splenitis
				Mild-moderate monocytic hepatitis
3	*Haemamoeba*	*P. cathemerium*	1.1	Mild-moderate acute diffuse granulocytic pneumonia
				Parasitic pneumonia compatible with *Schistosoma* sp.
				Diffuse necrotic splenitis
				Splenic limphocytolysis, histiocytolysis and lymphoid hypoplasia
				Parasitic granulomatous hepatitis
				Diffuse renal degeneration
				Mild cerebellar congestion
				Mild necrotic focal myocarditis
				Mild monocytic diffuse myocarditis
4	*Haemamoeba*	*P. cathemerium*	18.3	Moderate pulmonary congestion
				Moderate granulocytic pneumonia
				Moderate-severe granulocytic splenitis
				Moderate mixed hepatitis
5	*Haemamoeba*	*P. cathemerium*	4.2	Moderate-severe pulmonary congestion
				Mild granulocytic pneumonia
				Moderate-severe mixed splenitis
				Moderate-severe monocytic hepatitis
6	*Haemamoeba*	*P. cathemerium*	5.8	Moderate pulmonary congestion
				Moderate granulocytic pneumonia
				Mild-moderate granulocytic splenitis
				Mild-moderate mixed hepatitis
7	*Haemamoeba*	*P. tejerai*	10.8	Moderate-severe pulmonary congestion
				Moderate granulocytic pneumonia
				Mild-moderate granulocytic splenitis
				Moderate monocytic hepatitis
				Hepatic ductal hyperplasia
8	*Haemamoeba*	*P. tejerai*	1.5	Moderate-severe pulmonary congestion
				Moderate mixed hepatitis
				Hepatic ductal hyperplasia
9	*Haemamoeba*	*P. tejerai*	38.2	Moderate pulmonary congestion
				Moderate granulocytic pneumonia
				Mild-moderate granulocytic splenitis
				Mild-moderate granulocytic hepatitis
				Hepatic ductal hyperplasia
10	*Haemamoeba*	*P. tejerai*	31.9	Moderate-severe pulmonary congestion
				Severe granulocytic pneumonia
				Mild-moderate mixed splenitis
				Moderate monocytic hepatitis
				Hepatic ductal hyperplasia
				Mild monocytic myocarditis

Table 2. Continued...

Case	Study group	*Plasmodium* lineage	IHH (%)	Malaria severity
11	*Haemamoeba*	*P. tejerai*	9.4	Mild-moderate pulmonary congestion Mild-moderate granulocytic pneumonia Mild-moderate mixed splenitis Mild granulocytic myocarditis
12	*Huffia*	*P. elongatum*	22.8	Moderate pulmonary congestion Mild granulocytic pneumonia Moderate monocytic hepatitis
13	*Huffia*	*P. elongatum*	1.4	Mild-moderate congestion Acute granulocytic diffuse myocarditis Moderate cerebellar congestion Moderate diffuse acute granulocytic pneumonia Moderate coalescent multifocal histiocytosis, moderate lymphoid hypoplasia, lymphocytolisis, extramedullary erythropoiesis, mild-moderate mixed diffuse necrotic splenitis Mixed multifocal interstitial nephritis Moderate-severe multifocal necrotic hepatitis Extramedullary hepatic erythropoiesis Hepatic ductal hyperplasia
14	*Huffia*	*P. elongatum*	6.9	Mild cardiac congestion Mild acute diffuse myocarditis Mild to moderate cerebellar congestion Moderate mixed diffuse pneumonia Moderate pulmonary congestion Spleen tissue was not available for examination Diffuse renal degeneration Moderate-severe mixed coalescent multifocal necrotic periportal hepatitis and mild extramedullary erythropoiesis
15	Other lineages	*P. nucleophilum*	27.6	Mild pulmonary congestion Mild-moderate monocytic pneumonia Mild-moderate monocytic splenitis Moderate-severe mixed hepatitis
16	Other lineages	*P. unalis*	15	Moderate-severe pulmonary congestion Moderate-severe monocytic pneumonia Mild monocytic hepatitis
17	Other lineages	*Plasmodium* sp. *E*	6.2	Moderate pulmonary congestion Moderate granulocytic pneumonia Moderate granulocytic splenitis Mild monocytic hepatitis Hepatic ductal hyperplasia
18	Other lineages	*Plasmodium* sp. *G*	4.8	Moderate-severe pulmonary congestion Moderate granulocytic pneumonia Moderate granulocytic splenitis Moderate-severe monocytic hepatitis
19	Other lineages	*Plasmodium* sp. *G*	45.4	Moderate-severe pulmonary congestion Severe granulocytic pneumonia Mild granulocytic splenitis Mild-moderate granulocytic hepatitis Severe myocardial necrosis
20	Other lineages	*Plasmodium* sp. *H*	14.6	Moderate pulmonary congestion Moderate mixed pneumonia Moderate monocytic hepatitis

Table 2. Continued...

Case	Study group	*Plasmodium* lineage	IHH (%)	Malaria severity
21	Unidentified lineages	*Plasmodium* sp.	10.7	Moderate-severe pulmonary congestion
				Moderate-severe monocytic pneumonia
				Moderate granulocytic splenitis
				Moderate-severe monocytic hepatitis
				Mild-moderate monocytic myocarditis
22	Unidentified lineages	*Plasmodium* sp.	12.8	Moderate-severe pulmonary congestion
				Moderate-severe granulocytic pneumonia
				Moderate-severe granulocytic splenitis
				Moderate granulocytic hepatitis
				Mild granulocytic hepatitis
23	Unidentified lineages	*Plasmodium* sp.	8.3	Severe pulmonary congestion
				Severe monocytic pneumonia
				Mild-moderate mixed splenitis
				Moderate monocytic hepatitis
				Mild granulocytic myocarditis
24	Unidentified lineages	*Plasmodium* sp.	13.8	Moderate-severe pulmonary congestion
				Moderate granulocytic pneumonia
				Mild-moderate monocytic hepatitis
				Hepatic ductal hyperplasia
25	*Plasmodium* - negative	Negative	0.9	-
26	*Plasmodium* - negative	Negative	18.1	-
27	*Plasmodium* - negative	Negative	11.6	-
28	*Plasmodium* - negative	Negative	2.9	-
29	*Plasmodium* - negative	Negative	0.9	-
30	*Plasmodium* - negative	Negative	5.6	-
31	*Plasmodium* - negative	Negative	13.4	-
32	*Plasmodium* - negative	Negative	6.5	-
33	*Plasmodium* - negative	Negative	12.5	-

[juvenile or adult plumage] (SILVA-FILHO & RUOPPOLO, 2014), sex [male, female or unknown], body mass on admission (emaciated, thin, good, excellent, not available [N/A]), body score, hematocrit, presence or absence of oil upon admission [oiling], molt and iron supplementation [Fe]) (Table 3). To establish the IHH, slides were evaluated by light microscopy and captured images where analyzed by a computerized image analyses software (Image Pro® Plus, version 5.1.2.59, Media Cybernetics) to determine the percentage of liver tissue occupied by hemosiderin pigment. The intersection of the two longest axis of each bird's hepatic section was determined as the center of the sample, and used as the reference field to capture images at 20X magnification. Another 8 images were captured as TIFF files, at 50 µm of this point, and 45° intervals, under the same lighting conditions and magnification. The malarial pigment, called hemozoin, stains brown with Perls stain, while hemosiderin stains bright turquoise blue, promoting an easy visual distinction between the two pigments. Hemosiderin deposits were semi-automatically delineated on the Perls stained slides, starting at the lowest blue intensity to the most intense, to avoid the inclusion of artifacts and the background. When overlapping areas of formolic pigment and hemosiderin deposition where present, the areas of formolic pigment deposition where individually marked and deducted from such areas. In order to standardize the technique, a maximum digital zoom of 50% was stipulated. The average of the 9 microscopic fields was considered the IHH.

One-way ANOVA/Kruskal-Wallis test followed by Dunn's multiple comparisons test and Mann-Whitney tests were used to verify differences between the evaluated parameters and groups. The Spearman test was used to evaluate the correlation between quantitative variables. The significance level was defined as 5%. Data were analyzed with GraphPad Prism software (Version 4.02 for Windows, GraphPad Software, Inc.).

All samples used in this study were collected in full compliance with specific federal permits issued by the Brazilian Ministry of Environment and approved by the Biodiversity Information and Authorization System (SISBIO 20825-8). All procedures were performed according to the Ethical Committee in Animal Research of the School of Veterinary Medicine and Animal Sciences, University of São Paulo (Process numbers 1757/2009 and 9411100414).

Table 3. *Plasmodium*-positive and control Magellanic penguins according with the Index of Hepatic Hemosiderosis (IHH), rehabilitation center, age class, sex, body mass on admission (g), body score, hematocrit (Ht), presence or absence of oil upon admission (oil), moult, iron supplementation (Fe) and concurrent disease and/or significant lesion.

Case	IHH (%)	Rehab center[a]	Age class[b]	Sex[c]	Body mass (g)	Body score	Ht[d]	Oil	Moult	Fe	Concurrent disease and/or significant lesion
1	0.4	IPRAM	J	F	1915	Thin	N/A	No	No	No	Aspergillosis
2	14.8	IPRAM	J	F	2290	Thin	N/A	No	No	No	Spleen hemorrhage and rupture
3	1.1	CRAM	J	M	N/A	N/A	N/A	No	No	N/A	-
4	18.3	R3	J	F	2560	Good	44%	No	Yes	No	Helminthiasis
5	4.2	R3	J	M	N/A	Good	40%	No	Yes	No	N/A
6	5.8	R3	J	M	3240	Good	N/A	No	Yes	No	Avipoxvirus
7	10.8	R3	J	M	2400	N/A	32%	No	No	Yes	N/A
8	1.5	R3	J	ND	2600	N/A	N/A	No	No	Yes	Disseminated intravascular clotting
9	38.2	R3	J	ND	3450	N/A	N/A	No	Yes	Yes	N/A
10	31.9	R3	A	F	3300	Good	N/A	No	No	No	Helminthiasis (small intestine)
11	9.4	R3	J	M	2100	Good	N/A	No	No	N/A	N/A
12	22.8	IPRAM	J	F	2260	Good	N/A	No	No	No	Biliary stasis, Unidentified cysts (myocardium)
13	1.4	CRAM	A	F	N/A	Good	N/A	No	No	N/A	-
14	6.9	CRAM	A	M	N/A	Good	N/A	No	No	N/A	-
15	27.6	CRAM	J	F	2802	Good	50%	Yes	No	N/A	Amyloidosis, helminthiasis (gastrointestinal, lungs, liver)
16	15	CRAM	J	F	2810	Good	40%	Yes	No	N/A	Aspergillosis, Amyloidosis
17	6.2	R3	A	F	3220	Good	N/A	No	No	No	-
18	4.8	R3	J	M	3060	Good	27%	No	Yes	No	Amyloidosis, helminths (air sacs)
19	45.4	R3	J	F	2700	Bad	19%	No	Yes	No	Aspergillosis, pancarditis
20	14.6	R3	A	M	3260	Good	N/A	No	Yes	No	Helminthiasis
21	10.7	CRAM	J	ND	3890	Good	36%	No	No	Yes	-
22	12.8	CRAM	J	F	2910	N/A	33%	No	No	N/A	Spleen hemorrhage and rupture
23	8.3	CRAM	J	ND	2645	Thin	53%	Yes	No	Yes	-
24	13.8	CRAM	A	F	2750	Thin	46%	Yes	No	Yes	-
25	0.9	IPRAM	J	F	1750	Cachectic	N/A	Yes	No	Yes	-
26	18.1	IPRAM	J	F	1865	Thin	N/A	Yes	No	Yes	-
27	11.6	IPRAM	J	F	2015	Thin	N/A	Yes	No	Yes	-
28	2.9	IPRAM	J	F	2250	Thin	N/A	Yes	No	Yes	-
29	0.9	R3	J	ND	N/A	Good	N/A	Yes	No	Yes	-
30	5.6	R3	A	ND	N/A	Good	N/A	Yes	No	Yes	-
31	13.4	CRAM	J	M	2248	N/A	N/A	Yes	No	N/A	-
32	6.5	CRAM	J	M	2268	N/A	N/A	Yes	No	N/A	-
33	12.5	CRAM	J	M	3110	N/A	N/A	Yes	No	N/A	-

[a]IPRAM = Instituto de Pesquisa e Reabilitação de Animais Marinhos (Espírito Santo state, Brazil); R3 = Associação R3 Animal (Santa Catarina state, Brazil); CRAM = Centro de Recuperação de Animais Marinhos (Rio Grande do Sul state, Brazil); [b]J = Juvenile; A = adult; [c]F = female; M = male; ND = not determined; [d]N/A = Information not available.

Results and Discussion

The pulmonary congestion, intense inflammatory response (especially in liver and spleen), and hemosiderosis found in our birds are in agreement with previous microscopic descriptions of *Plasmodium* lesions in penguins (FIX et al., 1988; VANSTREELS & PARSONS, 2014; GRILO et al., 2016). However, there were no significant differences neither in the presence of moderate to severe hepatic hemosiderosis or in IHH values between *Plasmodium*-negative *vs*. *Plasmodium*-positive individuals or in regards to the *Plasmodium* species/lineage. The epidemiology and pathogenicity of the *Plasmodium* lineages previously diagnosed in our birds (VANSTREELS et al., 2015) raised the question on whether the *Plasmodium* subgenus *Haemamoeba* was more pathogenic to these penguins than other *Plasmodium* subgenera. Unfortunately, we were not able to find a clear answer to that question; these lineages are not completely understood and have been recorded only recently. However, we found higher levels of

congestion (p = 0.0182) and pneumonia severity (p = 0.0250) between Unidentified lineages *vs*. *Huffia* (Figure 2).

Plasmodium-positive females presented significantly higher IHH values (17.53 ± 12.95%) than males (7.20 ± 4.25%; p = 0.041) (Figure 3), in contrast with findings in toucans and aracaris (Ramphastidae family) (CUBAS, 2008) and in red-spectacled amazons (*Amazona pretrei*) (Psittacidae family) (PEREIRA et al., 2010), who failed to observe correlation between the rate of hepatic hemosiderosis and sex. Hemosiderosis has been previously associated with egg production (GOTTDENKER et al., 2008), which could not have played a role in our findings based on the age and underdeveloped gonads observed in necropsy. It is not clear why females presented higher IHH values than males; however, one may not eliminate the possibility of sampling influence, either due to the limited number of evaluated birds or higher number of females than males, which reflects previous studies documenting a female-biased sex ratio in Magellanic penguins stranded along the Brazilian coast (VANSTREELS et al., 2013). Comparison between the Pneumonia score (A) and Congestion Score (B) among *Plasmodium* species/lineages, and IHH in female *vs*. male *Plasmodium*-positive Magellanic penguins (*Spheniscus magellanicus*) (C) are shown in Figure 3.

Concurrent diseases were not statistically relevant. According to the institutions' protocols, many of the birds analyzed in this study were considered anemic, and received iron supplementation, which we believe may have also played a role in the hepatic hemosiderosis observed in this study. The physiological values of circulating iron concentration in Magellanic penguins have been previously evaluated (GHEBREMESKEL et al., 1989); however, to the authors' knowledge, there are no available data regarding iron hepatic levels for this species. Furthermore, the posology of iron supplementation is usually *ad-hoc*, and might not address the physiological requirements of these birds. In addition, iron represents a critical nutrient for pathogens and tight regulation of iron ("iron-withholding") is a paramount defense strategy of the host's innate immune system (KOSKI & SCOTT, 2003; JOHNSON, 2008; BEERNAERT et al., 2010; CASSAT & SKAAR, 2013). Thus, individuals suffering from iron overload have an enhanced risk of infection (BEERNAERT et al., 2010). Some of our specimens presented gastrointestinal nematodes, air sac parasites, and aspergillosis. It has been suggested that chronic infection or repeated acute infections could lead to greater accumulation of iron in the liver of birds (KLASING et al., 2012). Unfortunately, the low number of concurrent diseases observed in this study prevented statistical evaluation. Aspergillosis and parasitosis could have not only contributed to hemosiderosis, but also thrived upon iron supplementation, further debilitating the affected birds and contributing to their death. Due to all the factors discussed above, we recommend caution in the employment of iron supplementation to Magellanic penguins undergoing rehabilitation.

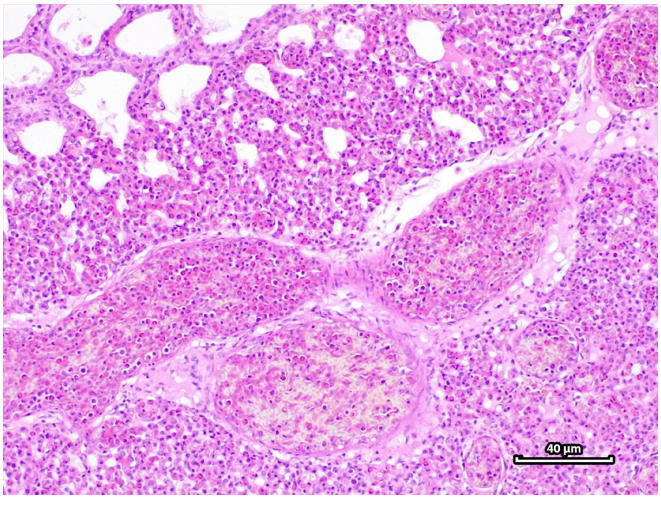

Figure 2. Moderate to severe pulmonary congestion and moderate to severe diffuse granulocytic pneumonia in a Magellanic penguin (*Spheniscus magellanicus*). H&E stain. 40 µm.

The IHH values in relation to presence of infection, *Plasmodium* species/lineage and pathology are summarized in Table 3.

Individual history parameters were not statistically relevant to the IHH values either. Magellanic penguins molt soon after breeding season, immediately prior to their winter migration (SILVA-FILHO & RUOPPOLO, 2014). Anseriformes and Passeriformes of the Sturnidae family showed an association between the hepatic level of hemosiderosis in this same period, with increased amounts of positive iron staining primarily located within lysosomic organelles of parenchymal hepatic cells (WARD et al., 1988; CORK et al., 1995). Such findings are possibly related to an increased hematopoietic activity and consequent erythrocyte production required to enhance oxygen uptake during periods of increased thermogenesis or changes in the levels of thyroid hormone involved in the molting process (WARD et al., 1988; CORK et al., 1995; OSBORN, 1979; CORK, 2000). Although not statistically relevant, some birds (4, 5, 6, 9, 18, 19, and 20) were molting during the study, so it is not possible to completely discard the hypothesis that residual iron from molting might have contributed to the hepatic hemosiderosis observed in our birds.

Fat can be severely depleted during migration, forcing birds to use body protein from the heart, pectoral, and leg muscles and the digestive organs (intestine, gizzard, and liver) as a second fuel option (LINDSTRÖM & PIERSMA, 1993; BATTLEY et al., 2000; BAUCHINGER & BIEBACH, 2001; SCHWILCH et al., 2002; MCWILLIAMS et al., 2004). Protein metabolism stems to a large extent from the skeletal muscles, which in penguins are especially rich in myoglobin, releasing the iron present in heme group of myoglobin, and possibly contributing to the development of hemosiderosis (SCHWARTZ et al., 1973; BAUCHINGER & BIEBACH, 2001; PONGANIS et al., 2010). The majority of migrating Magellanic penguins stranded in Brazil are in poor body condition, with markedly atrophied skeletal muscles and no subcutaneous fat deposits (CARDOSO et al., 2011). We assessed the true nutritional condition of these birds: body mass (ranging from 3.5-5 kg in wild specimens) and body condition score (SILVA-FILHO & RUOPPOLO, 2014). The only 2 emaciated birds (1 and 19), however, presented IHH values within the range found in this study. Nevertheless, increased iron deposition has been reported in malnourished black-necked swans (*Cygnus melanocoryphus*) (NORAMBUENA & BOZINOVIC, 2009), in the liver of rats

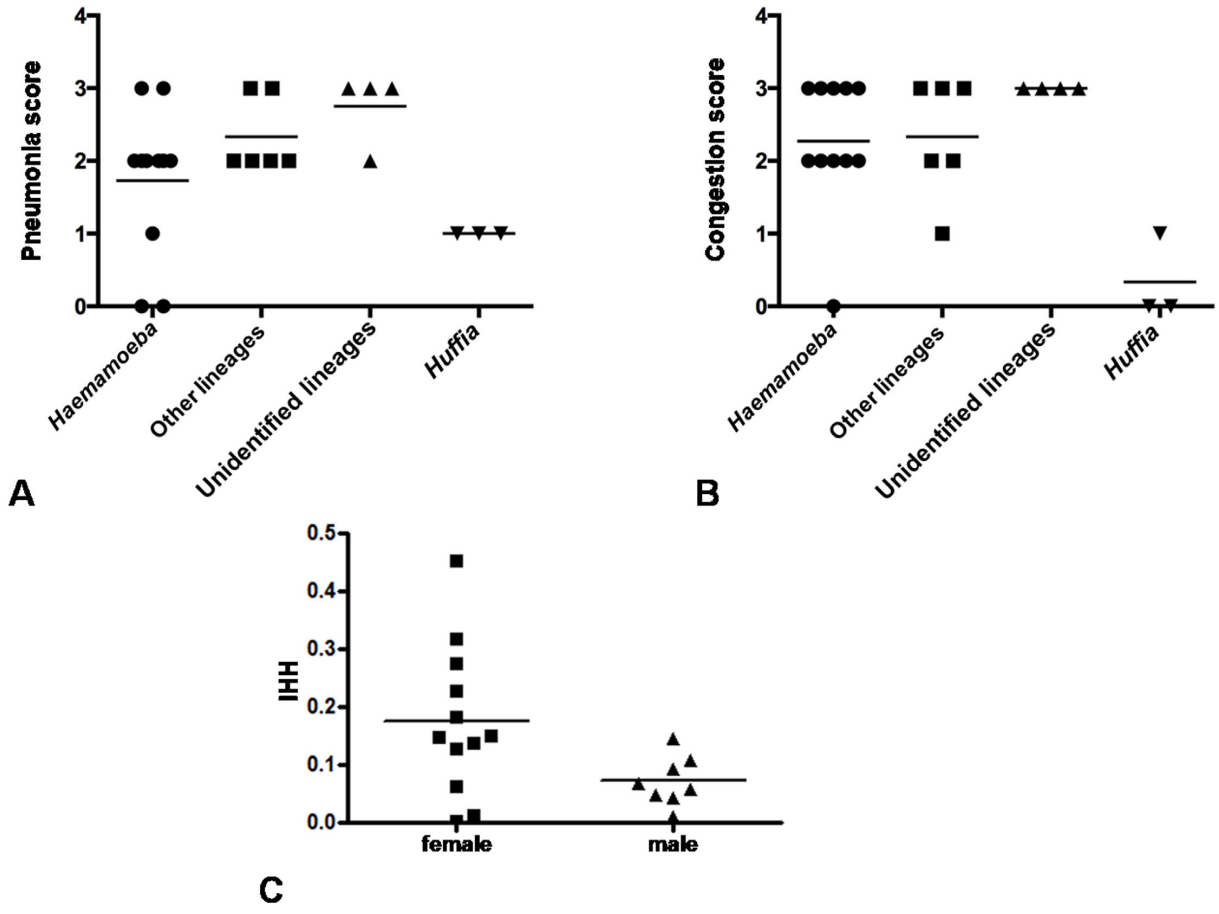

Figure 3. Comparison between the Pneumonia score (A) and Congestion Score (B) among *Plasmodium* species/lineages, and the index of hepatic hemosiderosis (IHH) in female *vs*. male *Plasmodium*-positive Magellanic penguins (*Spheniscus magellanicus*) (C).

subjected to food restriction (ROZENTAL et al., 1967), and in some severe cases of prolonged malnutrition in obese humans subjected to a variety of fasting periods (MITCHELL & JOHNS, 2008). It is important to consider that malnutrition may have a role in hemosiderosis in migratory bird species.

Intoxication (e.g., petroleum products) and hemoparasitosis are known causes of hemolytic anemia (FRY & LOWENSTINE, 1985). Systemic absorption of petroleum and its derivatives following acute or chronic oil exposure causes hemolytic anemia in birds (LEIGHTON, 1986; KHAN & RYAN, 1991; DINHOPL et al., 2011). Oil pollution is an important conservation threat to Magellanic penguins on wintering grounds (GARCÍA-BORBOROGLU et al., 2006). In this study, however, IHH was not significantly different between individuals with and without history of acute oil contamination upon arrival at the rehabilitation centers, suggesting that (a) oil exposure may have been too superficial or rapidly mitigated and therefore systemic absorption was minimal, and/or (b) other factors may have played a greater role in producing/modulating hemosiderosis in a manner that surpassed or contradicted any detectable effects from oiling. Nevertheless, these birds' history during migration is unknown, so we were not able to completely exclude the potential influence of chronic oil exposure in the hepatic hemosiderosis seen in this study.

Although previous studies (CRANFIELD et al., 1994; ONG et al., 2006) suggested that the low parasitaemia levels seen in *Plasmodium*-infected penguins are not sufficient to cause clinical anemia, *Plasmodium*-infected erythrocytes may suffer hemolysis or induce erythrocyte sequestration and extravascular hemolysis (VALKIŪNAS, 2005), releasing iron, which is subsequently stored in macrophages, as ferritin and hemosiderin (RATLEDGE, 2007). Eventually, cases 2 and 22 presented spleen hemorrhage and rupture, whereas case 8 presented disseminated intravascular coagulation - acute vascular complications likely associated with avian malaria that if not the primary cause, contributed to the development of secondary hemosiderosis. Another possible cause of anemia was the infestation by gastrointestinal nematodes (cases 4, 10, 15, 18 and 20).

Liver mass changes caused by the spontaneous long-term fasting characteristic of the annual cycle of penguins during reproduction and molt (GROSCOLAS & ROBIN, 2001) and/or due to concurrent diseases could have interfered with the morphometric evaluation of hepatic hemosiderin. It is unclear if changes in digestive organs of birds during migration are related to cell size (hypertrophy/atrophy) and/or cell number (hyperplasia/hypoplasia) (WAGENMAKERS et al., 1984). Further morphometric studies are needed in order to test these possibilities.

Both the amount of dietary iron and length of exposure to dietary iron are factors in hepatic iron accumulation in birds (CRISSEY et al., 2000; HELMICK et al., 2011). Unfortunately, data regarding their diet while in the Brazilian continental shelf during the winter migration is limited and there is no such information regarding the species offered to the studied birds while under care. To our knowledge, the only suggested nutritional iron concentration for adult penguin diets in a dry matter basis (60 - 80mg/kg) is based on requirements of domestic poultry, cats, and inferences from wild foods composition (WALLACE & WALSH, 2005; AZA Penguin Taxon Advisory Group, 2014). Further studies are needed to determine the nutritional iron requirements of Magellanic penguins.

Finally, our findings show that other factors aside from avian malaria likely played a role in the presence of hepatic hemosiderosis in *Plasmodium*-infected and *Plasmodium*-negative Magellanic penguins under rehabilitation in Brazil, and the importance of considering the species life cycle and clinical history when evaluating hepatic hemosiderosis. We propose that the increased hepatic hemosiderosis noted in this study was multifactorial, the result of pathological processes caused by malaria, molting, hemoglobin and myoglobin catabolism promoted by the intense energetic demands of Magellanic penguins' winter migration, anemia, concomitant diseases, and iron supplementation while under care, all possibly potentiated by an altered liver mass.

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