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**Morphometric evaluation of hepatic hemosiderosis and necrosis in
Magellanic penguins (*Spheniscus magellanicus*) naturally infected
by *Plasmodium* spp.**

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RESUMO

EWBANK, A. C. **Avaliação morfométrica da hemossiderose e necrose hepática em pinguins-de-Magalhães (*Spheniscus magellanicus*) naturalmente infectados por *Plasmodium* spp.** [Morphometric evaluation of hepatic hemosiderosis and necrosis in Magellanic penguins (*Spheniscus magellanicus*) naturally infected by *Plasmodium* spp.]. 2016. 127 f. Dissertação (Mestrado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2016.

Durante sua migração invernal, pinguins-de-Magalhães permanecem na plataforma continental brasileira. Neste período, animais debilitados e/ou doentes são encaminhados a centros de reabilitação ao longo da costa do Brasil. Durante a estadia nesses centros, essas aves podem desenvolver malária aviária, doença causada por protozoários do gênero *Plasmodium* e transmitida por mosquitos vetores. Hemossiderose e necrose hepáticas já foram descritas em casos de malária aviária. Nesse estudo foram utilizadas técnicas morfométricas para avaliar a hemossiderose e necrose em lâminas de cortes histológicos de fígado de pinguins-de-Magalhães naturalmente infectados por *Plasmodium* spp. e nove pinguins-de-Magalhães comprovadamente negativos para *Plasmodium* spp. (grupo controle), utilizando as colorações de Perls e reticulina. Todos os animais utilizados nesse estudo foram mantidos sob as mesmas condições de manejo. Linhagens de *Plasmodium* spp. haviam sido previamente identificadas por esfregaço sanguíneo e/ou análise filogenética do gene *cyt-b* mitocondrial. O objetivo desse estudo foi avaliar a significância dos quadros de hemossiderose e necrose em pinguins-de-Magalhães infectados por *Plasmodium* spp. e entre as linhagens/espécies de *Plasmodium* spp. Fragmentos histológicos foram analisados sob microscópio equipado com um sistema digital de análise de imagens. Foi realizada captura fotográfica do centro de cada fragmento hepático, seguido por 8 capturas adicionais a 50µm do centro da lâmina, a intervalos de 45°, sob as mesmas condições de luminosidade. Áreas de hemossiderose e necrose foram semi-automaticamente delineadas, sob zoom máximo de 50%. A porcentagem das áreas ocupadas pela hemossiderina e fibras reticulares foram consideradas, respectivamente, como Índice de Hemossiderose Hepática (IHH) e Índice de Necrose Hepática (IHN). O IHN do grupo controle foi significativamente maior que o IHN do grupo positivo ($p > 0.001$).

Entretanto, não foi observada diferença entre o IHH dos dois grupos. Diferenças significativas também não foram observadas no IHH e IHN em relação a instituição, idade, sexo, contaminação por óleo, ou linhagem/espécie de *Plasmodium* ($p>0,05$). Não foram observadas correlações significativas entre o IHH e o IHN quanto ao período total de estadia em centro de reabilitação ou período de estadia em centro de reabilitação durante o verão (período de maior densidade do mosquito/vetor) ($p>0,05$). Hemossiderose hepática possivelmente foi causada por outros fatores, tais como alterações fisiológicas sazonais, ações antropogênicas levando a poluição e competição por alimento, alterações climáticas, doenças concomitantes e técnicas de manejo e tratamento durante a reabilitação. Necrose hepática foi significativa entre ambos os grupos, sugerindo relação entre esta patologia e *Plasmodium* spp. (por ex: hipóxia causada por obstrução mecânica da vascularização hepática, vasculite parasitária ou presença de nematódeos gastrointestinais). A malária aviária é uma das mais importantes afecções de cativeiro em pinguins, podendo comprometer seriamente a reabilitação de pinguins-de-Magalhães. Estudos futuros são necessários para esclarecer os mecanismos dessas hipóteses.

Palavras-chave: Malária aviária. Ferro. Fígado. Hemossiderina. Reabilitação.

ABSTRACT

EWBANK, A. C. **Morphometric evaluation of hepatic hemosiderosis and necrosis in Magellanic penguins (*Spheniscus magellanicus*) naturally infected by *Plasmodium* spp.** [Avaliação morfológica da hemossiderose e necrose hepática em pinguins-de-Magalhães (*Spheniscus magellanicus*) naturalmente infectados por *Plasmodium* spp.]. 2016. 127 f. Dissertação (Mestrado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2016.

While foraging on the Brazilian continental shelf during winter migration, sick and/or debilitated Magellanic Penguins (*Spheniscus magellanicus*) found ashore are directed to rehabilitation centers along the coast. While under care, these birds may develop avian malaria, a mosquito-transmitted disease caused by protozoans of the genus *Plasmodium*. Hepatic hemosiderosis and necrosis have been previously described in avian malaria. We used morphometric techniques to evaluate hemosiderosis and necrosis in Perls- and reticulin-stained liver samples from 24 Magellanic penguins naturally infected by *Plasmodium* spp. and nine *Plasmodium*-negative Magellanic penguins (control group). All birds were kept under similar housing and husbandry regimens. *Plasmodium* lineages had been identified through blood smear morphology and/or phylogenetic analysis of the mitochondrial *cyt-b* gene. Our goal was to evaluate the significance of hepatic hemosiderosis and necrosis in Magellanic penguins infected with *Plasmodium* sp. and between *Plasmodium* lineages/species. Histological sections were analyzed under a microscope equipped with a digital system for image analysis. A high power-field of the center of each sample was captured, and eight additional images were captured 50 μm from this point, at 45° intervals, under the same lighting conditions. Areas of hemosiderin and reticulin fibers were semi-automatically outlined, under a maximum zoom of 50%. The percentage of the area occupied by hemosiderin and reticular fibers were respectively considered the index of hepatic hemosiderosis (IHH) and index of hepatic necrosis (IHN). IHN was significantly higher in the control group in comparison with the positive group ($p < 0.001$), however, no difference was detected between the IHH of both groups. Significant differences were not detected between IHH and IHN regarding institution, age, sex, oil contamination, and *Plasmodium* lineages/species ($p > 0.05$). There were also no correlation between IHH and IHN

regarding the total period of stay in the rehabilitation center or period of stay in the rehabilitation center during summer (period of highest mosquito/vector density) ($p > 0.05$). Hepatic hemosiderosis was possibly related to other causes, such as seasonal physiological changes, anthropogenic disturbance leading to pollution and food competition, climatic changes, concurrent diseases, and management and treatment while under care. Hepatic necrosis was significant between both groups, suggesting a relationship between this pathology and *Plasmodium* spp. (e.g., hypoxia due to mechanical obstruction of the hepatic vasculature, parasitic vasculitis leading to hepatic necrosis, or presence of gastrointestinal nematodes). Avian malaria is one of the most important diseases of captive penguins, and may seriously compromise the rehabilitation of Magellanic penguins. Further studies are still needed to clarify the mechanisms of these hypotheses.

Keywords: Avian malaria. Iron. Liver. Hemosiderin. Rehabilitation.

1 INTRODUÇÃO

The behavior and population dynamics of seabirds reflect natural and anthropogenic changes to the marine environment and provide insights into patterns of regional ocean productivity, long-term climate variation, and pollution (BOERSMA 2008; WAGNER; BOERSMA, 2011). Penguins correspond to 50-80% of the Antarctic Ocean's avian biomass, playing a vital role on the energetic transfer between the marine and terrestrial ecosystems of the Southern Hemisphere (VANSTREELS et al., 2014). Therefore, penguins are considered sentinels of the marine environment, indicators of biological productivity, and oceanic and coastal ecosystem health (BOERSMA 2008; GARCÍA-BORBOROGLU et al., 2011; MARINAO et al., 2014; SKEWGAR; BOERSMA; SIMEONE, 2014). Many penguin species are also economically important because their breeding colonies are tourist attractions, currently generating important revenues at local and regional scales. In addition, penguins are flagship charismatic species, able to create public and political support to protect habitats and other species under the requirements of their large marine habitat (BOERSMA 2008; GARCÍA-BORBOROGLU et al., 2011; MARINAO et al., 2014).

The Magellanic Penguin (*Spheniscus magellanicus*) Forster, 1781, belongs to the order *Sphenisciforme*, family *Spheniscidae*, genus *Spheniscus* (BALDASSIN et al., 2010; MADER; SANDER; CASA JR, 2010; RUOPPOLO et al., 2012; REZENDE, 2013). Their breeding colonies are located in the temperate coasts of Chile, Argentina, and the Falkland (Malvinas) Islands. During their winter migration (March to September), individuals from colonies on the Atlantic coast of South America, on islands in the Beagle Channel, and on the Falkland/ Malvinas Islands migrate to the continental shelf off the coast of northern Argentina, Uruguay, and southern Brazil (PÜTZ et al., 2000, 2007; BALDASSIN et al., 2010; MADER; SANDER; CASA JR, 2010; RUOPPOLO et al., 2012; STOKES; BOERSMA; DAVIS, 1998, 2014; SKEWGAR; BOERSMA; SIMEONE, 2014). There is no accurate data on the size of the Magellanic penguin population that migrates to Brazil, but a great mortality incidence is observed in Brazil, especially on the southern coast (Rio Grande do Sul and Santa Catarina), recently estimated at 30-31 dead penguins/km every year (approximately 19.500 corpses) (MADER; SANDER; CASA JR, 2010; BRANDÃO;

BRAGA; LUQUE, 2011). The vast majority of these birds are first-year juveniles, emaciated and cachectic, with no recent food in their stomachs, dehydrated, hypothermic, sustaining trauma, or covered in oil. Live birds are rescued and referred to rehabilitation centers along the southwest Atlantic coast (GARCÍA-BORBOROGLU et al., 2006; PINTO; SICILIANO; DIBENEDITTO, 2007; XAVIER et al., 2007; GARCÍA-BORBOROGLU et al., 2010; MADER; SANDER; CASA JR, 2010; CARDOSO et al., 2011; GARCÍA-BORBOROGLU et al., 2011; BALDASSIN et al., 2012).

While under rehabilitation, these birds are exposed to avian malaria, one of the most significant threats to penguin conservation (CLARK; KERRY, 1993; JONES; SHELLAM, 1999; LEVIN; PARKER, 2011; VANSTREELS et al., 2014). Avian Malaria is caused by a hemoprotozoan, *Plasmodium* sp., transmitted through the bite of infected *Culicidae* mosquitoes (VALKIUNAS 2005; VANSTREELS et al., 2014). Malaria outbreaks in several penguin species have been reported worldwide in captive (GRINER; SHERIDAN, 1967; FLEISCHMAN et al., 1968; BAK; PARK; LIM, 1984; BROSSY, 1992; GRACZYK; CRANFIELD; MCCUTCHAN, 1994; PENRITH, 1994; LOMBARD; BROSSY; BLACKBEARD, 1999), wild populations (FANTHAM; PORTER, 1944; LAIRD, 1950; HUFF; SHIROISHI, 1962; FIX; WATERHOUSE; GREINER, 1988; BROSSY, 1992; GRACZYK et al., 1995; ALLEY, 2001; CARVAJAL; ALVARADO, 2009; LEVIN et al., 2009), and in birds undergoing rehabilitation (GRIM et al., 2003; CARVAJAL; ALVARADO, 2009; CAPELLINO et al., 2013). In Brazil, reports in Magellanic penguins include birds in captivity (BUENO et al., 2010) and in rehabilitation (RUOPPOLO et al., 2004; OSÓRIO et al., 2011; SILVEIRA et al., 2013; VANSTREELS et al., 2014). Avian malaria 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*) (ATKINSON; VAN RIPER, 1991; VALKIUNAS 2005; ATKINSON, 2008; VANSTREELS et al., 2014). These birds are highly susceptible to avian malaria, an infirmity able to produce rapid and severe outbreaks in penguins, with as much as 50–80% mortality within few weeks (VANSTREELS et al., 2014).

While studying *Plasmodium* sp. in naturally infected Magellanic penguins, VANSTREELS 2014 observed significant hepatic hemosiderosis and necrosis. Hemosiderosis associated with malaria has been reported in birds (GOTTDENKER et al., 2008; VANSTREELS 2014; VANSTREELS et al., 2015; GRILO et al., 2016)

and humans (BARSOU 2000; DAS, 2008). Iron-containing BROWN pigment occurs frequently in the livers of birds of several orders and families, and has been observed in both wild and domestic birds, reported in a wide range of species from zoo and private collections, in regions all over the world (CORK; ALLEY; STOCKDALE, 1995; SHEPPARD; DIERENFELD, 2002). The pathophysiology of iron storage disease has not been well described in the avian patient (MATHESON, 2007). Several studies have shown that both diet and physiology must be considered as contributing factors to the development of hemosiderosis (SHEPPARD; DIERENFELD, 2002). However, variable susceptibility of individuals within species and distribution of stainable iron in tissues are an indication that multiple etiological factors may apply (LOWENSTINE; PETRAK, 1980; WARD et al., 1991; SHEPPARD; DIERENFELD, 2002). Hepatic necrosis has been reported in *Macaca mulatta* experimentally infected with *Plasmodium* spp., in *P. falciparum* infection in humans, (COOK, 1995), and in *P. vinckei* infected mice (CLARK et al., 1987). In birds, hepatic necrosis has been described in Magellanic penguins (VANSTREELS et al., 2015), white leghorn chickens (FREVERT et al., 2008), a saddleback and a stitch bird (CORK; ALLEY; STOCKDALE, 1995), and in a female eider duck (*Somateria mollissima*) (WADSWORTH; JONES; PUGSLEY, 2008).

Considering the importance of avian malaria to penguin conservation, the limited amount of information regarding the pathogenesis of *Plasmodium* sp., and the occurrence of hepatic hemosiderosis and necrosis in these animals, the aim of this study was to use histologic (Perls and Reticulin staining) and morphometric techniques to quantify the hepatic hemosiderosis and necrosis in naturally infected Magellanic penguins within the context of these birds' biology, individual history, and parasite lineage, in an attempt to characterize the differences between *Plasmodium* species on their hosts in a rehabilitation setting.

7 FINAL COMMENTS

Magellanic penguins are very charismatic birds, sources of touristic attractions in the wild and popular flag species for public education and commitment when under care in rehabilitation centers or in zoological parks. This species' natural behavior and biology has challenged studies regarding their health and population status while on breeding colonies, but mainly, their migratory behavior, including patterns, foraging and diet. Further studies are needed in order to understand these aspects of their natural life cycle, but also the physical conditions in which they arrive to Brazilian shores, and how affected they are by the challenges – either natural or anthropogenic – faced during migration.

Our results show that *Plasmodium* sp in naturally infected Magellanic penguins may be associated with hepatic necrosis, as previously reported by other authors in birds, but for the first time in penguins. Although we did not find a direct correlation between avian malaria and hemosiderosis, several possible causes for this clinical findings have been raised and require further studies to be elucidated. In spite of that, relevant topics on penguin rehabilitation have been raised, in the hope of expanding our current knowledge and understanding of Magellanic penguin strandings in Brazil and more importantly, the role and significance of iron in avian malaria infections for penguin rehabilitation and conservation.

An important topic has also been discussed: the current use of iron as a first option for anemia treatment, in birds highly susceptible to avian malaria while under rehabilitation. The use of iron should be carefully considered in these situations, and a risk-benefit analysis needs to be undertaken to ascertain whether the current guidelines of penguin iron treatment and supplementation are appropriate. Finally, we would like to address the need of further research into the topics of hepatic necrosis and hemosiderosis, and the conscious use of iron supplementation both in birds infected by *Plasmodium* sp. and in those species susceptible to avian malaria.

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