ANA CAROLINA EWBANK

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

Dissertação apresentada ao Programa de Pós-Graduação em Patologia Experimental e Comparada da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo para a obtenção do título de Mestre em Ciências

Departamento:

Patologia

Área de concentração:

Patologia Experimental e Comparada

Orientador:

Prof. Dr. José Luiz Catão-Dias

De acordo:_____
Orientador(a)

São Paulo

2016

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Programa de Pós-Graduação: Patologia Experimental e Comparada.

Área de concentração: Patologia Experimental e Comparada.

Orientador: Prof. Dr. José Luiz Catão-Dias.

1. Malária aviária. 2. Ferro. 3. Fígado. 4. Hemossiderina. 5. Reabilitação. I. Título.

São Paulo, 13 de maio de 2016
CEUA N 9411100414

Ilmo(a). Sr(a).
Responsável: José Luiz Catão Dias
Área: 0
Prof. Dr. José Luiz Catão Dias (orientador)

Título do projeto: "AVALIAÇÃO MORFOMÉTRICA DA HEMOSSIDEROSE E NECROSE HEPÁTICA EM PINGUINS-DE-MAGALHÃES (Spheniscus magellanicus) NATURALMENTE INFECTADOS POR Plasmodium sp.".

Parecer Consubstanciado da Comissão de Ética no Uso de Animais FMVZ/USP

A Comissão de Ética no Uso de Animais da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo, no cumprimento das suas atribuições, analisou e **APROVOU** a Notificação (versão de 28/abril/2016) do protocolo de estudo acima referenciado.

Resumo apresentado pelo pesquisador: "Bom dia. Gostaria de solicitar a alteração do título da minha tese de mestrado para AVALIAÇÃO MORFOMÉTRICA DA HEMOSSIDEROSE E NECROSE HEPÁTICA EM PINGUINS-DE-MAGALHÃES (Spheniscus magellanicus) NATURALMENTE INFECTADOS POR Plasmodium sp./ Morphometric Evaluation of Hepatic Hemosiderosis and Necrosis in Magellanic Penguins (Spheniscus magellanicus) naturally infected by Plasmodium sp. , por considerar que a inclusão do estudo da necrose nos pinguins infectados por malária, trouxe informações relevantes e contributivas para o meu estudo. Pretendo defender minha tese no final de Maio e gostaria que o título fosse totalmente claro quanto ao enfoque da tese. Muito obrigada. Att., Ana Carolina Ewbank ".

Comentário da CEUA: "Solicitação aprovada.".

Profa. Dra. Denise Tabacchi Fantoni
Presidente da Comissão de Ética no Uso de Animais
Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo

Roseli da Costa Gomes
Secretaria Executiva da Comissão de Ética no Uso de Animais
Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo

Ministério do Meio Ambiente - MMA
Instituto Chico Mendes de Conservação da Biodiversidade - ICMBio
Sistema de Autorização e Informação em Biodiversidade - SISBIO

Autorização para atividades com finalidade científica

Número: 20825-8	Data da Emissão: 03/05/2016 13:31	Data para Revalidação*: 02/06/2017

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Dados do titular

Nome: José Luiz Catão Dias	CPF: 029.597.888-00
Título do Projeto: Estudo da malária aviária em Pinguins-de-Magalhães (Spheniscus magellanicus) recebidos em centros de reabilitação selecionados do litoral brasileiro e mantidos em instituições de cativeiro	
Nome da Instituição : Faculdade de Medicina Veterinária e Zootecnia USP	CNPJ: 63.025.530/0019-33

Cronograma de atividades

#	Descrição da atividade	Início (mês/ano)	Fim (mês/ano)
1	Coleta e envio de amostras biológicas	07/2009	12/2013
2	Coleta e envio de amostras biológicas	01/2014	01/2018

Observações e ressalvas

1	As atividades de campo exercidas por pessoa natural ou jurídica estrangeira, em todo o território nacional, que impliquem o deslocamento de recursos humanos e materiais, tendo por objeto coletar dados, materiais, espécimes biológicos e minerais, peças integrantes da cultura nativa e cultura popular, presente e passada, obtidos por meio de recursos e técnicas que se destinem ao estudo, à difusão ou à pesquisa, estão sujeitas a autorização do Ministério de Ciência e Tecnologia.
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8	Em caso de pesquisa em UNIDADE DE CONSERVAÇÃO, o pesquisador titular desta autorização deverá contactar a administração da unidade a fim de CONFIRMAR AS DATAS das expedições, as condições para realização das coletas e de uso da infra-estrutura da unidade.
9	As atividades contempladas nesta autorização NÃO abrangem espécies brasileiras constante de listas oficiais (de abrangência nacional, estadual ou municipal) de espécies ameaçadas de extinção, sobreexplotadas ou ameaçadas de sobreexplotação.

Outras ressalvas

1	A quantidade de sangue a ser coletada não deve ultrapassar 1% da massa corporal da ave, sendo este quantitativo suficiente para os objetivos pretendidos.
2	A quantidade de sangue a ser coletada não deve ultrapassar 1% da massa corporal da ave, sendo este quantitativo suficiente para os objetivos pretendidos.

Equipe

#	Nome	Função	CPF	Doc. Identidade	Nacionalidade
1	CLAUDIA NIEMEYER	Doutoranda	282.611.748-32	264406576 SSP-SP	Brasileira
2	JULIANA YURI SAVIOLLI	Coleta de amostras biológicas	301.023.498-86	327703994 SAOPAULO-SP	Brasileira

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Nome da Instituição : Faculdade de Medicina Veterinária e Zootecnia USP	CNPJ: 63.025.530/0019-33

3	PATRICIA PEREIRA SERAFINI	Coleta de amostras biológicas	027.472.819-22	66214524 SESP-PR	Brasileira
4	RODOLFO PINHO DA SILVA FILHO	Coleta de amostras biológicas	401.790.010-00	6006891052 SSP-RS-RS	Brasileira
5	Sabrina Epiphanio	Coordenadora adjunta	141.007.908-21	15570072 SSP-SP	Brasileira
6	CLAUDIA CARVALHO DO NASCIMENTO	Coleta de amostras biológicas	269.215.078-31	28220355-2 SSP/SP-SP	Brasileira
7	CRISTIANE KIYOMI MIYAJI KOLESNIKOVAS	Coleta de amostras biológicas	176.142.858-67	15481877 SSP-SP	Brasileira
8	RALPH ERIC THIJL DEL VAL ONORO VANSTREELS	Doutorando	332.714.958-58	44041896-3 SSP-SP	Brasileira
9	VANESSA MARQUES PEDROSO	Coleta de amostras biológicas	004.001.200-03	3061884271 SSP-RS	Brasileira
10	PAULA LIMA CANABARRO	Coleta de amostras biológicas	981.135.420-00	2076352885 SJS-RS	Brasileira
11	ANDRÉA CORRADO ADORNES	Coleta de amostras biológicas	535.371.810-00	2029731111 SSP-RS	Brasileira
12	SILVIA BAINY GASTAL	Coleta de amostras biológicas	018.444.810-77	8080081981 SJS-RS	Brasileira
13	ARYSE MARTINS MELO	Coleta de amostras biológicas	012.549.910-85	2111714693 SSP-RS	Brasileira
14	Cristiane Lassálvia Nascimento	Coleta de amostras biológicas	014.792.837-03	209539938 ssp-SP	Brasileira
15	GUSTAVO HENRIQUE PEREIRA DUTRA	Coleta de amostras biológicas	273.007.978-57	246793260 SSP-SP	Brasileira
16	THIAGO AUGUSTO DO NASCIMENTO	Coleta de amostras biológicas	304.758.368-47	243317372 SSP/SP-SP	Brasileira
17	Raphael Nogueira Ramos	Coleta de amostras biológicas	283.204.358-54	33254836-3 ssp-SP	Brasileira
18	Pryscilla Maracini	Coleta de amostras biológicas	169.546.928-32	243245579 SSP-SP	Brasileira
19	Martha Lima Brandão	Coleta de amostras biológicas	029.282.627-33	101388106 IFP-RJ	Brasileira
20	ÂNGELA LEITZKE CABANA	Coleta de amostras biológicas	006.086.010-39	1073310301 SSP-RS	Brasileira
21	Melissa Orzechowski Xavier	Coleta de amostras biológicas	984.912.530-68	6069748678 SJS-RS	Brasileira
22	RENATA FERREIRA HURTADO	Coleta de amostras biológicas	323.144.298-26	439268898 SSP-SP	Brasileira
23	Juliana Marigo	Pós-doutoranda	255.015.058-94	28136736x SSP-SP	Brasileira
24	KATIA REGINA GROCH	Doutoranda	739.751.419-72	9080491955 SSPPOA-RS	Brasileira
25	Eliana Faquim de Lima Mauro	Coordenadora adjunta	134.270.528-93	21583159-7 SSP-SP	Brasileira
26	Jéssica Domato Ribeiro	Coleta de amostras biológicas	364.651.708-50	430047770 SSP-SP	Brasileira

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Nome da Instituição : Faculdade de Medicina Veterinária e Zootecnia USP	CNPJ: 63.025.530/0019-33

27	VALERIA RUOPPOLO	Coordenadora adjunta e doutoranda	195.315.808-04	21416884-0 SSP-SP	Brasileira
28	ANA CAROLINA EWBANK	Mestranda	317.712.058-73	307903163 SSP-SP	Brasileira
29	marina von atzingen dos reis	Coleta e processamento de amostras biológicas	299.527.228-18	289611970 ssp/sp-SP	Brasileira
30	PEDRO RENATO GONÇALVES FILHO	Coleta de amostras biológicas	027.050.320-02	5101642808 SJS-RS	Brasileira
31	Laura Chrispim Reisfeld	Coleta de amostras biológicas e mestranda	338.691.748-89	460923833 SSP-SP	Brasileira

Locais onde as atividades de campo serão executadas

#	Município	UF	Descrição do local	Tipo
1		RS	Centro de Recuperacao de Animais Marinhos, FURG (Rio Grande)	Fora de UC Federal
2		SC	CETAS do Nucleo de Fauna do IBAMA (Florianopolis)	Fora de UC Federal
3		SP	CETAS do Centro Universitario Monte Serrat (Santos)	Fora de UC Federal
4		SP	Aquario Municipal de Santos (Santos)	Fora de UC Federal
5		SP	Acquamundo Guaruja (Guaruja)	Fora de UC Federal
6		SP	Aquario de Peruibe (Peruibe)	Fora de UC Federal
7		SP	Aquario de Sao Paulo (Sao Paulo)	Fora de UC Federal
8		SP	Fund. Museu de Historia, Pesq. e Arq. do Mar (Sao Sebastiao)	Fora de UC Federal
9		SP	Sabina Escola Parque do Conhecimento (Santo André)	Fora de UC Federal

Atividades X Táxons

#	Atividade	Táxons
1	Coleta/transporte de amostras biológicas ex situ	Spheniscidae
2	Coleta/transporte de amostras biológicas in situ	Spheniscidae

Material e métodos

1	Amostras biológicas (Aves)	Animal encontrado morto ou partes (carcaça)/osso/pele, Ectoparasita, Fezes, Fragmento de tecido/órgão, Regurgitação/conteúdo estomacal, Sangue, Penas, Outras amostras biológicas(Swabs orais e cloacais)
2	Método de captura/coleta (Aves)	Outros métodos de captura/coleta(cativeiro)

Destino do material biológico coletado

#	Nome local destino	Tipo Destino
1	Faculdade de Medicina Veterinária e Zootecnia USP	coleção

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Registro de coleta imprevista de material biológico

De acordo com a Instrução Normativa nº 03/2014, a coleta imprevista de material biológico ou de substrato não contemplado na autorização ou na licença permanente deverá ser anotada na mesma, em campo específico, por ocasião da coleta, devendo esta coleta imprevista ser comunicada por meio do relatório de atividades. O transporte do material biológico ou do substrato deverá ser acompanhado da autorização ou da licença permanente com a devida anotação. O material biológico coletado de forma imprevista, deverá ser destinado à instituição científica e, depositado, preferencialmente, em coleção biológica científica registrada no Cadastro Nacional de Coleções Biológicas (CCBIO).

Táxon*	Qtde.	Tipo de amostra	Qtde.	Data

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* Identificar o espécime no nível taxonômico possível.

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FOLHA DE AVALIAÇÃO

Autor: EWBANK, Ana Carolina

Título: Avaliação morfométrica da hemossiderose e necrose hepática em pinguins-de-Magalhães (*Spheniscus magellanicus*) naturalmente infectados por *Plasmodium* spp.

Dissertação apresentada ao Programa de Pós-Graduação em Patologia Experimental e Comparada da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo para obtenção do título de Mestre em Ciências

Data: _____/_____/_____

Banca Examinadora

Prof. Dr. _____

Instituição: _____ Julgamento: _____

Prof. Dr._____

Instituição: _____ Julgamento: _____

Prof. Dr._____

Instituição: _____ Julgamento: _____

DEDICATÓRIA

Essa tese é dedicada aos animais:
àqueles que mudaram a minha vida
e àqueles cujas vidas eu espero,
de alguma forma, um dia mudar.

AGRADECIMENTOS

Aos meus pais, que assim como em todas as fases da minha vida, me amaram e me apoiaram incondicionalmente durante a realização de mais essa etapa, o meu mais sincero agradecimento e admiração. Essa tese é fruto de todas as vezes em que sonhei alto e vocês me estimularam; todas as vezes em que me senti perdida e vocês me ajudaram a reencontrar o caminho; e por todas as vezes que ao longo dessa jornada, compartilharam comigo os obstáculos e vitórias. Vocês serão para sempre meus maiores fãs, melhores amigos e maiores incentivadores. Amo vocês!

À minha avó Rosa Salero Collalilo, que nos ensinou que não existem limites para o que sonhamos a não ser as barreiras que impômos a nós mesmos. Sua determinação e zêlo para com as pessoas que amava serão para sempre um exemplo a ser seguido.

Ao meu querido Carlos Sacristán, meu grande amor, companheiro na vida pessoal e profissional. Sua determinação, curiosidade infinita e sede de saber me estimulam a sempre dar o meu melhor. Essa tese também é fruto do seu trabalho, paciência e disposição em sempre me ajudar e contribuir para o meu crescimento. Você é o meu revisor mais impiedoso e meu crítico mais exigente, porque sempre acredita no meu potencial e nos meus sonhos. Você faz de mim uma pessoa melhor todos os dias. Te amo pra sempre!

Ao meu orientador, Prof. Dr. José Luiz Catão-Dias, por ter acreditado em mim e me aberto as portas do LAPCOM e da sua sala para termos conversas sempre agradáveis sobre filmes, livros, viagens e claro, de vez em quando também patologia! Obrigada pela generosidade, respeito e paciência, principalmente com os meus projetos faraônicos, textos sem fim e mania de achar que tudo no texto é importante!

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comigo. Obrigada por ser sempre tão presente e disposto a me ajudar e a me ensinar. Esse trabalho não teria sido o mesmo sem a sua colaboração.

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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 este período, animal 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 *Plasmodium-negative*), 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 *Plasmodium-negative* 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 e alterações climáticas levando a anorexia/caquexia e técnicas de manejo e suplementação durante a reabilitação. Necrose hepática foi significativa entre ambos os grupos, o que pode ter ocorrido devido a uma possível relação entre esta patologia e *Plasmodium* spp. (por ex: hipóxia causada por obstrução mecânica da vascularização hepática, vasculite parasitária ou presença de nematódeos gastrointestinais) ou à presença de autólise hepática, levando a quadro histológico semelhante à necrose quando avaliada sob coloração de reticulina. 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 morfométrica 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 (*Plasmodium-negative* 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 *Plasmodium-negative* 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 INH 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 and climatic changes leading to starvation, and husbandry and iron supplementation while under care. Hepatic necrosis was significant between both groups, suggesting a possible relationship between this pathology and *Plasmodium* spp. (e.g., hypoxia due to mechanic obstruction of the hepatic vasculature, parasitic vasculitis leading to hepatic necrosis, or presence of gastrointestinal nematodes) or the presence of hepatic autolysis, leading to histopathologic changes similar to hepatic necrosis whaen evaluated with reticulin staining. 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.

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ANOVA analysis of variance

AST aspartate aminotransferase

BID *bis in die* (twice a day)

CRAM – FURG Centro de Recuperacão de Animais Marinhos da Universidade Federal do Rio Grande

EOD every other day

FLO Associação R3 Animal

GGT gamma-glutamyltransferase

GLDH glutamate dehydrogenase

Hct hematocrit

IM intramuscular

IPRAM Instituto de Pesquisas e Reabilitação de Animais Marinhos

ISD iron storage disease

IV intravenous

PCV packed cell volume

PO *per os* (oral administration)

SID *semel in die* (once a day)

SUMÁRIO

1 INTRODUCTION

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., 1968a,b; 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 (BARSOUM 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 physiopathology 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).

2 LITERATURE REVIEW

2.1 MAGELLANIC PENGUINS: SPECIES DESCRIPTION, BIOLOGY, AND INCIDENCE IN THE BRAZILIAN COAST

Magellanic penguins are migratory upper trophic level predators and the most abundant penguin in temperate areas, widely distributed along the southern coast of South America and nearby islands, both in the Pacific (Chile, south of 29°S) and Atlantic Oceans, with the majority of its population occurring in the southwest Atlantic (located along the southern coasts of Argentina - from Peninsula Valdez (42°04′S, 63°21′W) to Tierra del Fuego (54°54′S, 67°23′W)), and the Falkland/Malvinas Islands (approx. 51°45′S) (BOERSMA; STOKES; YORIO, 1990; GANDINI; FRERE; GANDINI,1996; PINTO; SICILIANO; DI BENEDITTO, 2007; RODRIGUES et al., 2010; BRANDÃO; BRAGA; LUQUE et al., 2011; DA SILVA et al., 2012; RUOPPOLO et al., 2012; STOKES et al., 2014).

During breeding and molting periods (September-April), Magellanic penguins remain in their colonies; while during the rest of the year this species is pelagic, staying at sea (BOERSMA et al., 2013; REZENDE et al., 2013; STOKES et al., 2014). Its population is estimated at 1 to 1.3 million pairs distributed in more than 130 breeding islands, the biggest ones being San Lorenzo and Punta Tombo, in Argentina (SKEWGAR; BOERSMA; SIMEONE, 2014; SILVA-FILHO; RUOPPOLO, 2014). In the last century, Magellanic penguin populations fluctuated considerably, with an overall increase until the 1980s (BOERSMA et al., 1990; WILLIAMS, 1995), followed by a subsequent decrease in some large colonies, where a 22% reduction in the number of breeding pairs has been recorded since 1987 (BOERSMA, 2008). This species is currently classified as ‘‘Near Threatened’’ on the Red List of the International Union for Conservation of Nature (IUCN, 2013), with an overall uncertain population trend (BRANDÃO; BRAGA; LUQUE, 2011; BOERSMA et al., 2013; STOKES et al., 2014).

The life cycle of Magellanic Penguins consists of the following stages: (1) settlement: arrival of the adults of reproductive age to the colonies around September, for territorial disputes and nest construction; (2) incubation: courtship,

copulation and oviposition lasting until mid November; (3) guarding: egg hatching, followed by constant parental warming and protection of the chicks, with parents alternating 2-4 day shifts to feed at sea; (4) nursery and emancipation: chicks are able to self thermoregulation around mid December, allowing parents to leave for gradually longer tours, periodically returning to feed (regurgitated food) to the chick. Once the chick develops their waterproof plumage (in mid February), they become independent, leaving to the sea to feed; (5) post nuptial molt: occurs in adults of both sexes and consists of a complete substitution of all plumage for a brand new one, a phenomenon know as “catastrophic molt”; (6) winter migration: starting in mid April, the last adults and juveniles leave the colonies, until the next September, when birds start returning to the breeding colonies to restart the cycle (BOERSMA et al., 2013; VANSTREELS, 2014).

Penguins are central place foragers while raising chicks, but every year, juvenile and adult Magellanic penguins leave their reproductive colonies and migrate north, following the colder and nutrient rich Falkland/Malvinas current in search of the most abundant resources and food (WILLIAMS; BOERSMA, 1995; FRERE; GANDINI; BOERSMA, 1996; BALDASSIN et al., 2010, 2012; MADER; SANDER; CASA JR, 2010; DA SILVA et al., 2012; REZENDE et al., 2013), such as the Argentine anchovy (*E. anchoita*), their main prey in northern Patagonia (WILLIAMS; BOERSMA, 1995; PÜTZ et al., 2001; BOERSMA et al., 2009). During this journey, birds may reach the continental shelf off the coast of northern Argentina (RUOPPOLO et al., 2012), Uruguay (REZENDE et al., 2013), and southern Brazil (BALDASSIN et al., 2010; GARCÍA-BORBOROGLU et al., 2010; STOKES; BOERSMA; DAVIS, 1998; PUTZ; INGHAM; SMITH, 2008; PUTZ et al., 2007; STOKES et al., 2014) and, rarely, northern Brazil (BOERSMA; STOKES; YORIO, 1990; GARCÍA-BORBOROGLU et al., 2010; DA SILVA et al., 2012; STOKES et al., 2014), as far as Rio de Janeiro (BALDASSIN et al., 2010), with scarce reports in the northeast, in the states of Bahia (ROSS, 2008; BALDASSIN et al., 2010) and Alagoas (GARCÍA-BORBOROGLU et al., 2006). Juveniles tend to travel further north to lower latitudes during the austral winter, reaching Brazilian waters between 20°S and 33°S, the northern limit of the species’ distribution along the Atlantic coast of South America (PINTO; SICILIANO; DI BENEDITTO, 2007). Little is known about the Pacific migration patterns of Magellanic, but winter observations show that many individuals migrate north of their breeding colonies along the coast of Chile and Peru

starting in late March, being reported as far north as Punta San Juan, Peru (SKEWGAR; SIMEONE; BOERSMA, 2009).

2.2 CHALLENGES FACED BY MAGELLANIC PENGUINS DURING THEIR WINTER MIGRATION AND STAY OFF THE BRAZILIAN CONTINENTAL SHELF

Magellanic penguins are increasingly threatened by human activities in coastal areas along their migration path, and although these birds demonstrate some adaptation, environmental disturbances constitute a threat to their population (PÜTZ et al., 2007; BALDASSIN et al., 2012). The increased mortality to which Magellanic penguins are exposed in the South Atlantic Ocean is due to anthropic and climatic factors (PÜTZ et al., 2007; BOERSMA, 2008; GARCÍA-BORBOROGLU et al., 2010; MADER; SANDER; CASA JR, 2010; DA SILVA et al., 2012). Recently, global climate changes (El Niño/La Niña) have lead to increased sea temperatures, consequently affecting the food web and environmental conditions (MÄDER; SANDER; CASA JR, 2010; RODRIGUES et al., 2010; BRANDÃO; BRAGA; LUQUE, 2011; RUOPPOLO et al., 2012; DA SILVA et al., 2012; STOKES et al., 2014). In addition, Magellanic penguins and several other marine species suffer from anthropogenic and non-anthropogenic activities affecting marine health, including: (1) marine pollution: oil spills, chronic oil pollution (GANDINI et al., 1994; GARCÍA-BORBOROGLU et al., 2006, 2008; BOERSMA, 2012), presence of persistent organic pollutants (POPs) and xenobiotic agents, and ingestion of marine debris (BOERSMA; STOKES, 1995; PETRY; FONSECA, 2002; FONSECA; PETRY, 2001; PINTO; SICILIANO; BENEDITTO, 2007); (2) economical activities: unregulated tourism and recreational activities, and commercial fishing (BOERSMA; STOKES, 1995; CARDOSO et al., 2011), overexploitation of fishery resources (BOERSMA, 2008), incidental capture in fisheries (especially bottom trawling and gillnetting) (GANDINI et al., 1999; SIMEONE; BERNAL; MEZA, 1999; YORIO; CAILLE, 1999; TAMINI et al., 2002; PETRY; FONSECA; JOST, 2004; GONZÁLEZ-ZEVALLOS; YORIO, 2006; SKEWGAR; SIMEONE; BOERSMA, 2009; MARINAO et al., 2011; PÜTZ et al., 2011); (3) predation; (4) infection/infestation of parasites against which the species

has no natural defenses; and disease (GANDINI et al., 1994; FOWLER; WINGFIELD; BOERSMA, 1995; STOKES et al., 1998; PETRY; FONSECA, 2002; PETRY; FONSECA; JOST, 2004; MADER; SANDER; CASA JR, 2010; BRANDÃO; BRAGA; LUQUE, 2011; BALDASSIN et al., 2012; RUOPPOLO et al., 2012; STOKES et al., 2014).

2.2.1 Climatic changes (El Niño/La Niña)

The term “El Niño” was originally applied to an annual weak warm ocean current that ran southward along the coast of Peru and Ecuador around Christmas time (Referred to as el Niño Jesús, Spanish for “the boy Christ-child”) (TRENBERTH, 1997). The “El Niño” phenomenon occurs when the five-month running mean of Sea Surface Temperature (SST) anomalies is above 0.5 °C for at least six consecutive months (TRENBERTH, 1997; VARGAS; HARRISON; MACDONALD, 2005) off the coast of South America and along the equatorial Pacific in association with a weakening of the trade winds that occur at intervals of 3–7 yr (PERRIMAN et al., 2000; GARCIA et al., 2004; VARGAS; HARRISON; MACDONALD, 2005; LE BOHEC et al., 2008). The atmospheric component tied to El Niño is termed the “Southern Oscillation”, when the atmosphere and ocean collaborate together (TRENBERTH, 1997). ENSO (El Niño–Southern Oscillation) is the dominant mode of coupled atmosphere–ocean variability on interannual timescales (TRENBERTH, 1997; TRENBERTH; STEPANIAK, 2001). El Niño then corresponds to the warm phase of ENSO, while the cold phase; “La Niña” (“the girl” in Spanish), consists of periods during which the 5-month running means of SST is below 0.5 °C for at least six consecutive months (TRENBERTH, 1997; VARGAS; HARRISON; MACDONALD, 2005). The growth rate of zooplankton and larvae fishes is linked to a restricted thermal window; therefore, SST is a good indicator of “El Niño” events (TRENBERTH; STEPANIAK, 2001; VARGAS; HARRISON; MACDONALD, 2005; LE BOHEC et al., 2008). Most “El Niño” begin in the northern spring or perhaps summer, and peak from November to January (TRENBERTH, 1997). Consistently associated with heavy rainfall and flooding on the west coast of South America El Niño last 12 to 18 months, severely disrupting local fish and bird populations (KOVATS et al., 2003).

Meteorological changes induced by “El Niño” events are felt worldwide (GARCIA et al., 2004), with Southern South America (SSA: southern Brazil, Argentina, Chile, Uruguay, and Paraguay) being one of the extratropical regions most affected by “El Niño” (EN) and “La Niña” (LN) events (GRIMM; BARROS; DOYLE, 2000).

Climate fluctuations have important ecological consequences and influence the population dynamics of long-lived organisms. Environmental variables indirectly interfere on the reproductive performance and survival of upper-level predators, such as penguins (e.g., affecting lower levels of the food web) (LE BOHEC et al., 2008). In the case of “El Niño”, this phenomenon possesses important implications for the dynamics of aquatic ecosystems, from coral reefs to upwelling regions (GARCIA et al., 2004), and reduced primary productivity (local environmental conditions, such as temperature, wind, rain, snow, and ocean currents and their interactions) (HAYS, 1986; LE BOHEC et al., 2008). Changes in SST affects the entire food web, by influencing marine productivity and location feeding grounds used by upper trophic-level predators, such as fish, seabirds, and marine mammals, and by decreasing their growth and reproductive success (HAYS, 1986; VARGAS; HARRISON; MACDONALD, 2006; LE BOHEC et al., 2008).

The intensity of “El Niño” is variable. The 1982–1983 and 1997–1998 “El Niño” episodes were comparable in magnitude and were the strongest recorded in the last century, with severe biological effects (GARCIA et al., 2004; VARGAS; HARRISON; MACDONALD, 2006). Recently, the frequency and severity of “El Niño” events appear to have increased, now occurring 2–7 times more frequently than they did 7000–15,000 years ago (VARGAS; HARRISON; MACDONALD, 2006). Climate models suggest that most of the warming observed during the last 50 years is attributable to human activities with an increased El Niño pulse in the last 30 years, which poses a great concern for the conservation of endangered seabird species (VARGAS; HARRISON; MACDONALD, 2006). Predicting the impact of future climate changes on populations and biodiversity is a central issue in the context of global climate warming (LE BOHEC et al., 2008).

“El Niño” and “La Niña” climate perturbations have been shown to affect breeding and mortality of several seabird species, including Galapagos penguins (*Spheniscus mendiculus*) in the Pacific Ocean, Magellanic penguins in Argentina (MADER; SANDER; CASA JR et al., 2010), Humboldt penguins no Peru (HAYS

1986), Galapagos penguins in the Galapagos archipelago (BOERSMA 1978, 1998), Blue Penguins in New Zealand (PERRIMAN et al., 2010).

In ENSO years, warm and nutrient poor waters of the Brazilian current reach the coast of Rio Grande do Sul in early October, dislocating the cold Falkland/Malvinas current further south, decreasing the available food resources and directly contributing with the weakening of the animals on the southern coast of Brazil during that time (FONSECA; PETRY; JOST, 2001; MADER; SANDER; CASA JR et al., 2010). Years with the highest number of carcasses/km at the coast of Rio Grande do Sul, without signs of oil contamination were 1990 and 1997, also years with the highest ENSO indexes since 1983. On the other hand, in years of weak ENSO index (2006 and 2007), a lower number of carcasses was found in these areas (MADER; SANDER; CASA JR, 2010).

The 1982–1983 and 1997–1998 strong “El Niño” events were associated with reductions in the Galapagos penguin population of 77% and 65%, respectively. At the end of the 1998 “El Niño”, survival female penguins were only 80% and males 90% of their average body weight in the absence of this phenomenon. In other South American penguin species, the Humboldt penguins, HAYS (1986) reports an “apparent southward dispersion”, significant decrease of the adult population and failure of the 1982 class of hatchlings in the “El Niño” years of 1982-83. The same author also observed a lack of body fat, empty stomachs, and overall weight-loss affecting mainly juveniles. Survivors were apparently all adults, experienced individuals with larger tissue reserves, more likely to survive a food shortage due to their foraging experience and ability to cover larger feeding areas.

Evidence that weather changes influence the availability of food, by limiting the production and survival of young individuals, as observed in Galapagos penguins, suggests that starvation is the likely cause of the elevated penguin mortality in severe “El Niño” events (VARGAS; HARRISON; MACDONALD, 2006). Female penguins, which have reduced body mass, are probably more likely than males to die during “El Niño” events due to poor body condition. Decreased body condition during incubation, may also force guarding parents to abandon the egg in order to feed themselves or the chicks may hatch and starve before the foraging parent returns with food (YORIO; BOERSMA 1994; BOERSMA; STOKES 1995; GROSCOLAS; ROBIN, 2001; BOERSMA et al., 2009). Climatic perturbations may also explain part

of the variation in onset of breeding, number of clutches and fledging success in blue penguins in New Zealand (PERRIMAN et al., 2000).

Other complications caused by strong El Niño events that may affect the survival of seabird populations include: occurrence along with the critical period of pre-breeding food acquisition, preferred breeding time, or molting; and increased competition with commercial fisheries caused by decreased fish stocks, increasing the occurrence of penguin mortality due to fishing interaction (DARBY; DAWSON, 2000; VARGAS; HARRISON; MACDONALD, 2006).

Under a scenario of increasing greenhouse-gas concentrations, “El Niño-like” conditions could become more frequent (GARCIA et al., 2004). More frequent and intense El Niño episodes have cumulative effects that diminishes the capacity of penguin populations to recover from previous events before the next one occurs, leading to long-term reductions in penguin numbers (VARGAS; HARRISON; MACDONALD, 2006). Estimation of a linear increasing trend of 0.74°C of global surface temperature during the last century and a further warming of 0.2°C per decade for the next 20 years of the Southern Ocean certainly represents a major threat for penguins (LE BOHEC et al., 2008). Long-term monitoring of physical and biological parameters is essential for understanding the effects of El Niño on bird populations, particularly small and/or declining populations (VARGAS; HARRISON; MACDONALD, 2006).

There is evidence of a connection between climate variability and the transmission of mosquito-transmitted diseases. The transmission of vector-borne diseases typically occurs within seasonal patterns, depending on temperature and rainfall variations, which may contribute to the formation of ideal mosquito habitat. A better understanding of the relationship between the ENSO, the climatic anomalies it engenders, and the fluctuations of malaria vector could help prevent or at least decrease, outbreaks in rehabilitation settings (GAGNON; SMOYER-TOMIC; BUSH, 2002).

2.2.2 Interaction with fishing activities

The incidental capture in fisheries by warp cables of trawl nets (GONZÁLEZ-ZEVALLOS; YORIO; CAILLE, 2007; CARDOSO et al., 2011; MARINAO et al., 2014), a range of small scale hook-and-line fisheries (BUGONI et al., 2008b; CARDOSO et al., 2011), gillnet fisheries and fish traps (DARBY; DAWSON, 2000; ZYDELIS et al., 2009;CARDOSO et al., 2011; MARINAO et al., 2014), are probably the main conservation problem affecting seabirds (CARDOSO et al., 2011). Seabird mortality in fishing gear, in both commercial and artisanal fishing operations, is a globally recognized conservation issue, believed to be responsible for declines of approximately 80% of seabird species (PÜTZ et al., 2007; ZYDELIS et al., 2009).

The main focus thus far has been on bycatch of Procellariiform seabirds (albatrosses and petrels) in longline fisheries worldwide, including the southwestern Atlantic Ocean (BUGONI et al., 2008a; ZYDELIS et al., 2009; CARDOSO et al., 2011). However, bycatch of other seabirds, such as penguins, has raised only local or regional attention (ZYDELIS et al., 2009) with only anecdotal reports on penguin mortality due to coastal fishing operations (GANDINI et al., 1999; SIMEONE; BERNAL; MEZA, 1999; DARBY; DAWSON, 2000; PUTZ et al., 2007), regarding the mortality by fisheries as of secondary importance for penguin conservation (BOERSMA, 2008; CARDOSO et al., 2011).

Incidental capture of different species of penguins (e.g. Humboldt, Rockhoper, *Eudyptes chrysocome*, Yellow-eyed, *Megadyptes antipodes*, little penguin, and Magellanic penguin) has been reported in bottom trawl fisheries (GANDINI et al., 1999; YORIO; CAILLE, 1999; GONZÁLEZ-ZEVALLOS; YORIO, 2006; GONZÁLEZ-ZEVALLOS et al., 2007; YORIO et al., 2010), in midwater trawl fisheries (TAMINI et al., 2002), and gill nets in the Atlantic Ocean in Argentina (SCHIAVINI et al., 2005), and in the Pacific Ocean, in Chile and Peru (SIMEONE; BERNAL; MEZA, 1999; DARBY; DAWSON, 2000; MAJLUF et al., 2002; SKEWGAR; SIMEONE; BOERSMA, 2009). Penguins of the genus *Spheniscus* are particularly vulnerable to gill nets because of their foraging method (WILLIAMS, 1995), and probably because they are unable to see the transparent material with which nets are made of (SIMEONE; BERNAL; MEZA, 1999).

Incidental capture rate of Magellanic Penguins may vary according with the year (MARINAO et al., 2014), with hundreds of penguins being killed annually during winter in southern Brazil (PETRY; FONSECA, 2002; CARDOSO et al., 2011), but the mortality indexes show that this species is captured incidentally throughout the year. Target fishing species, total catch, amount of discarded waste, and distances to the penguin colony are factors that also influence on incidental capture (MARINAO et al., 2014). During summer, this species is affected by fisheries adjacent to Argentinean breeding colonies during breeding season, while foraging in relatively coastal areas, probably spatially overlapping with vessels and commuting individuals (CARDOSO et al., 2011; GONZÁLEZ-ZEVALLOS; YORIO; CAILLE, 2011; MARINAO et al., 2014). During their winter northward migration, Magellanic Penguins are affected by bluefish (*Pomatomus saltatrix*) commercial fishing activities, once anchovies are both species' favorite prey (PÜTZ; INGHAM; SMITH et al., 2008; GARCÍA-BORBOROGLU et al., 2010; CARDOSO et al., 2011).

2.2.3 Marine Debri Ingestion/Interaction

Lost and discarded marine debris, particularly items made of persistent synthetic materials, are a major form of marine pollution able to significantly impact wildlife (LAIST, 1997; TOURINHO; DO SUL; FILLMANN, 2009). There are two main ways of interacting with marine debris: entanglement or intentional or accidental ingestion (LAIST, 1997).

On entanglements, loops and openings of various types of debris entangle the animal's appendages or entrap the animals themselves. The types of marine debris most commonly associated with entanglement are lost fishing gear, such as nets, lines, and traps (LAIST, 1997; TASKER et al., 2000). Entanglement in discarded fishing gear such as nets and nylon fishing lines also pose a potential threat and are occasional sources of penguin mortality (PÜTZ et al., 2007). Animals that become entangled may exhaust themselves and drown, have their mobility impaired to a point where they can no longer catch food or avoid predators, become hung up on rocks or other objects, by trailing rope or line, or incur wounds and infections from the

abrasion or constriction of attached debris (LAIST, 1997; SILVA, 2013). For animals unable to free themselves quickly, survival prospects are poor (LAIST, 1997).

During recent decades, the ingestion of marine debris by seabirds, turtles, mammals and fishes has been widely reported as a major threat to marine fauna (LAIST, 1997; TOURINHO; DO SUL; FILLMANN, 2009; BRANDÃO; BRAGA; LUQUE, 2011). The ingestion of solid debris may occur involuntarily, while ingesting food, or voluntarily, after being mistaken by the bird's natural prey (BRANDÃO; BRAGA; LUQUE, 2011; SILVA, 2013). It has been suggested that some of this debris may be ingested secondarily, through ingestion of prey containing plastic particles in the digestive tract (SILVA, 2013). Seabirds are highly affected by debris ingestion, especially plastics, which are able to float, may be non-biodegradable, or only degrade upon exposure to ultraviolet radiation through hundreds of years in the environment (AZARELLO; VAN VLEET, 1987; BRANDÃO; BRAGA; LUQUE, 2011). Some animal behaviors, such as those related to feeding, play, and nest building, may increase the chances of some species to interact with debris (LAIST, 1997). There are a number of physiological and sub-lethal effects related to the ingestion of plastic particles, which could result in partial or total gastrointestinal blockage or internal injury, in species unable to regurgitate, leading to accumulation of solid matter, which may interfere directly with their digestion, reducing the digestive tract functional volume, the feeding stimulus and digestive efficiency, aside from interfering in the satiety feeling, which are long-term major threats, which in migratory animals would ultimately interfere in the pre-migratory energy storage (AZARELLO; VAN VLEET, 1987; TOURINHO; DO SUL; FILLMANN, 2009; SILVA, 2013). The presence of a large number of particles in the stomach may prevent the secretion of gastric enzymes or the movement of food into the small intestine (AZARELLO; VAN VLEET, 1987). Plastic ingestion may also be detrimental to reproduction, since DDT (dichlorodiphenyltrichloroethane), DDE (dichlorodiphenyldichloroethylene), PCB (polychlorinated biphenyl) and other chlorinated hydrocarbon pollutants associated with plastics may lower steroid hormone levels causing delayed ovulation (AZARELLO; VAN VLEET).

Penguins are not widely known to ingest litter and there are currently no available reports of secondary debris ingestion through the ingestion of *E. anchoita* in Magellanic penguins (SCOLARO et al., 1999; BRANDÃO; BRAGA; LUQUE, 2011). However, information on debris ingestion by Magellanic penguins has increased in

recent years (AZEVEDO; SCHILLER, 1991; PINTO, SICILIANO, DI BENEDITTO, 2007; MADER; SANDER; CASA JR, 2010; TOURINHO; DO SUL; FILLMANN, 2010; BRANDÃO; BRAGA; LUQUE, 2011), whereas data on other penguin species remains scarce (BRANDÃO; BRAGA; LUQUE, 2011). Petry, Fonseca e Jost (2004) reported plastic remains in the stomach of beach-washed Magellanic Penguins along the coast of southern Brazil, while Brandão, Braga e Luque (2011) reported that a mean of 35.8% of the Magellanic penguins that arrive to Brazil are affected by debris ingestion. In a recent *post mortem* investigation of 50 Magellanic penguins found during the 2015 mass stranding event observed on the coast of São Paulo state, Southeastern Brazil, fragments of inorganic (plastic, rubber, and ropes) and organic materials (wood and algae) were present in the gastric contents of 24% of the animals (A.C. Ewbank, personal observation). The growing incidence of anthropic material ingestion by Magellanic penguins reflects the pollution of different oceanic regions throughout the species range, and raises concerns regarding its conservation (SILVA, 2013). The migratory pattern of seabirds can give important information related to rates and sources of ingested marine debris (TOURINHO; DO SUL; FILLMANN, 2009).

2.2.4 Oil pollution

Petroleum poses a significant threat to marine wildlife worldwide due to its highly variable toxic effects, in many cases leading to death (FOWLER; WINGFIELD; BOERSMA, 1995; NEWMAN et al., 2000; GARCÍA-BORBOROGLU et al., 2006). Reports of bird mortalities as a result of petroleum product discharges into the marine environment were first recognized in the late 19th and early 20th century (TSENG, 1999; CAMPHUYSEN; HEUBEK, 2001). Petroleum is the most common toxic substance released into the marine environment (GARCÍA-BORBOROGLU et al., 2008), with chronic oil pollution (small but frequent oil discharges from ships, at terminals, or from oily ballast water) accounting for most of its presence in the ocean, suggesting it is a more important problem than generally recognized, responsible for killing many more seabirds than large oil spills (GANDINI et al., 1994; GARCÍA-BORBOROGLU et al., 2006). Oil pollution leaves species more susceptible to local

perturbations and extinction, which may have played a role in the decline of seabird populations (GANDINI et al., 1994). Thus, seabirds, including penguins, may serve as global and highly sensitive monitoring instruments of marine oil pollution (GANDINI et al., 1994; CAMPHUYSEN; HEUBEK, 2001; GARCÍA-BORBOROGLU et al., 2006).

Immediate external effects of oil contamination in birds include rapid penetration of oil into the feathers, leading to a loss of thermal insulation, buoyancy, and flight capabilities, forcing the animal to either become waterlogged and sink or to go ashore, becoming more vulnerable to predators and unable to forage (FRY; LOWESTINE, 1985; JENSSEN, 1994; FOWLER; WINGFIELD; BOERSMA, 1995; TSENG, 1999; BALSEIRO et al., 2005). Without proper insulation, birds become hypothermic and begin to metabolize body fats rapidly to maintain normothermia, which increases basal metabolic rates, leading to starvation (HARTUNG, 1967; VERMEER; VERMEER, 1975; FRY; LOWESTINE, 1985; KHAN; RYAN, 1991; TSENG, 1999). Secondary toxicity from inhalation, ingestion of oil through preening, or through contaminated food may cause stress, suppression of the endocrine and immune systems, and changes in the normal physiological processes (FRY; LOWESTINE, 1985; LEIGHTON, 1986; FOWLER; WINGFIELD; BOERSMA, 1995; TSENG, 1999). Following oil ingestion, decreased growth rate, and impaired osmoregulation have been observed in young birds from a variety of species (JENSSEN, 1994). Other clinical signs include dehydration, exhaustion, significant decrease in body and organ weight, pneumonia, Heinz-body hemolytic anemia, impairment in osmoregulatory abilities and electrolyte regulation, endocrine changes (in adrenocortical function, corticosterone and thyroxin levels), changes in the salt gland, renal diseases, gastrointestinal lesions and disturbances (e.g., anorexia, irritation, hemorrhage, regurgitation, diarrhea, changes in intestinal absorption, altered hepatic enzyme function and disruption of sodium transport mechanisms) and increased parasitism (FRY; LOWESTINE, 1985; PATTEE; FRANSON, 1982; LEIGHTON; PEAKALL; BUTLER, 1983; KHAN ; RYAN, 1991; JENSSEN, 1994; FOWLER; WINGFIELD; BOERSMA, 1995; TSENG, 1999; BALSEIRO et al., 2005). Balseiro et al., 2005 observed that birds suffering from previous weakness, cachectic, and inexperienced youngsters were probably more severely affected by oil contamination.

Upon necropsy, reported findings in seabirds affected by contamination with crude oil or its byproducts include: severe dehydration, exhaustion, weight loss and emaciation with no abdominal and subcutaneous fat, presence of a petroleum smelling black emulsion in the digestive tract, urate deposition in the liver, spleen and kidneys, and liver, kidney and gastrointestinal damage (FRY; LOWESTINE, 1985; LEIGHTON, 1986; KHAN; RYAN, 1991; BALSEIRO et al., 2005). Weight loss is probably associated with malabsorption, impaired liver function and increased metabolic rate (HOLMES, 1984). Hepatic histopathological changes described in oiled contaminated birds include hemosiderosis, hepatocellular dissociation, congestion of the liver, fatty infiltration and degeneration, mild peripheral lymphoplasmacytic hepatitis and significant acute hepatic necrosis, either extensive or characterized by multiple foci (FRY; LOWESTINE, 1985; LEIGHTON, 1986; KHAN; RYAN, 1991). Splenic hemosiderosis (LEIGHTON, 1986), urate deposition in the liver, spleen and kidneys (BALSEIRO et al., 2005), renal focal necrosis and acute renal tubular necrosis (FRY; LOWESTINE, 1985; KHAN; RYAN, 1991), pneumoconiosis (FRY; LOWESTINE, 1985), and intestinal necrosis (KHAN; RYAN, 1991) have also been reported.

Even at low levels of oil fouling, seabirds may suffer both physical and physiological effects leading to decreased reproductive success (FRY; LOWESTINE, 1985; GANDINIet al., 1994; FOWLER; WINGFIELD; BOERSMA, 1995), as documented in penguins (GARCÍA-BORBOROGLU et al., 2006) seabirds (LEIGHTON, 1986), and waterfowl (HARTUNG; HUNT, 1966).

Among penguins, petroleum is known to be important in the decline of the African penguin (*Spheniscus demersus*; FROST; SIGFRIED; COOPER, 1976) (GANDINI et al., 1994). In South America, several hundred live and dead oiled penguins are found ashore every year (GANDINI et al., 1994; PETRY; FONSECA, 2002; GARCÍA-BORBOROGLU et al., 2006; RODRIGUES et al., 2010; RUOPPOLO et al., 2012). For decades, oil pollution has killed Magellanic penguins along the coast of Argentina (PERKINS, 1983; GANDINI et al., 1994; Fowler et al., 1995; GARCÍA-BORBOROGLU et al., 2006), where oil pollution dramatically increased in the mid 1990s, coinciding with exponential growth of oil exportations (GARCÍA-BORBOROGLU et al., 2006). Oil pollution may be particularly hazardous for this species during their north migration, once their routes between Argentina and Brazil, and their territory off the Brazilian continental shelf may overlap with areas of intense

oil tankers and petroleum development (BOERSMA et al., 1990; GANDINI et al., 1994; STOKES; BOERSMA, 1998; PÜTZ; INGHAM; SMITH et al., 2008; GARCÍA-BORBOROGLU et al., 2006; MADER; SANDER; CASA JR, 2010). Once in Brazil, penguins may also be affected by oil pollution (PETRY; FONSECA, 2002; PETRY; FONSECA; JOST, 2004).

Any decrease in adult survival can lead to a decline in population of long-lived seabirds such as penguins, which have low and variable reproductive rates (FOWLER; WINGFIELD; BOERSMA, 1995; GARCÍA-BORBOROGLU et al., 2006). It has been suggested that oil pollution may be a more important mortality factor for adults than juvenile Magellanic Penguins and that female penguins appear to be more seriously affected than males (FOWLER; WINGFIELD; BOERSMA, 1995), which could contribute to the long-term decline of this species along the coast of Argentina (GANDINI et al., 1994; BOERSMA, 1997; GARCÍA-BORBOROGLU et al., 2006). Many reproductive complications have been observed in adult birds, chicks exposed to oil or fed contaminated food, and in chicks hatched from eggs either of exposed birds or exposed to oil (FRY; LOWESTINE, 1985). Suppression of reproductive hormonal levels, impairment of egg formation, cessation or delay in egg laying, slow embryonic growth, embryonic death, teratogenic malformations, decreased hatchability and nest abandonment have been observed in seabird populations (FRY; LOWESTINE, 1985; JENSSEN, 1994; FOWLER; WINGFIELD; BOERSMA, 1995; TSENG, 1999).

2.2.5 Fasting *versus* Starvation

From changes in protein utilization, it is possible to characterize three periods during long-term fasting in birds, for instance, in king penguin *Aptenodytes patugonicus*, emperor penguin (*A. forsteri*) and domestic geese *Anser anser domesticus*, and in mammals (rats): it decreases in phase I (rapid adaptation period), is maintained at a low value during phase II (long period of economy) and further increases in phase III (critical period) (LE MAHO, 1983; ROBIN et al., 1987; ROBIN et al., 1988; CHEREL et al., 1988; BOISMENU; GAUTHIER; LAROCHLLE, 1992; LINDGÅRD et al., 1992; WILLIAMS et al., 1992).

Phase I is the short initial adaptation period to long-term fasting (LE MAHO, 1983; CHEREL et al., 1988; WILLIAMS et al., 1992; GROSCOLAS; ROBIN, 2001). This period is marked by a reduction in the rates of daily body mass loss (DBML), reduced nitrogen excretion, marked increase in fat mobilization with a sharp increase in the plasma concentration of free fatty acids and a corresponding decrease in protein utilization and uric acid and alanine (LE MAHO, 1983; CHEREL; LE MAHO, 1985; CHEREL et al., 1988; GROSCOLAS; LELOUP, 1988; LINDÅRDet al., 1992; WILLIAMS et al., 1992; GROSCOLAS; ROBIN, 2001).

Phase II is a period of economy, and also the longest period, lasting as long as 3-4 mo in Emperor penguin and chicks of *Aptenodytes patagonicus* (CHEREL et al., 1988). This phase is characterized by protein and lipid utilization in constant proportions; through protein sparing, low and steady daily body mass loss (DBML), low metabolism, low levels of plasma uric acid, and increased plasma; hydroxybutyrate (CHEREL et al., 1988; BOISMENU; GAUTHIER; LAROCHLLE, 1992; WILLIAMS et al., 1992; HANDRICH; NICOLAS; LE MAHO, 1993; GROSCOLAS; ROBIN, 2001). In this phase, 94% of the energy expenditure derives from lipids (ROBIN et al., 1988; WILLIAMS et al., 1992).

One good criterion for adaptation to prolonged starvation is the ability to conserve protein during phase II (CHEREL; LE MAHO 1985; HANDRICH; NICOLAS; LE MAHO, 1993), as king penguin chicks, for instance, which can spend up to 4 months in this phase before entering phase III (LE MAHO et al., 1988). This energy and protein saving strategy lasts as long as the bird has sufficient fat reserves, and ceases when adiposity (fat mass/body mass) reaches a threshold lower than 5-10% (ROBIN et al., 1988; CHEREL; GROSCOLAS, 1999; GROSCOLAS; ROBIN, 2001). There is, however, an still unknown minimum limit of protein utilization during starvation, which represents 3 to 5% (HANDRICH; NICOLAS; LE MAHO, 1993), or even 3-7% (CHEREL et al., 1992) of the total energy expenditure (ROBIN et al 1988).Phase III is critical because it involves a progressive increase in protein breakdown, and it is depletion of protein, rather than fat, which limits survival during starvation (CHEREL; LE MAHO, 1985; CHEREL, et al., 1988; LE MAHO et al., 1988; ROBIN et al., 1988; BOISMENU; GAUTHIER; LAROCHLLE, 1992; CHEREL et al., 1992; LINDGÅRD et al., 1992; WILLIAMS et al., 1992). Consequences of the increased protein catabolism related to fat stores reaching a lower threshold level include rising rates of N_2 excretion and increasing daily body mass loss (DBML) (LE

MAHO, 1983; GROSCOLAS; LELOUP, 1988; BOISMENU; GAUTHIER; LAROCHLLE, 1992; GROSCOLAS; ROBIN, 2001). Since the energy density of protein-yielding tissues (e.g., muscles) is approximately nine times lower than that of adipose tissue, this explains the higher DBML and is partly explained by an increase in locomotor activity and thus in energy expenditure as demonstrated in rats, penguins and geese (LINDGÅRD et al., 1992; GROSCOLAS; ROBIN, 2001). Moreover, the utilization of protein is associated with much more important loss of water (GROSCOLAS; LELOUP, 1988; CHEREL; LELOUP; LE MAHO*,* 1988). Phase III is also characterized by increased plasma concentrations of uric acid, urea, and alanine, and a drop in plasma P-hydroxybutyrate (CHEREL; LE MAHO, 1985; CHEREL et al., 1988). However, from data on penguins, the increase in protein utilization characterizing entrance into phase III should not be considered as pathological, because it is not directly caused by total exhaustion of lipid reserves (ROBIN et al., 1987; ROBIN et al., 1988). Penguins regularly enter phase III under natural conditions, a stage that is reversible as long as there are still significant fat stores available and animals refeed just before total depletion of these reserves (ROBIN et al., 1987; CHEREL et al., 1988; BOISMENU; GAUTHIER; LAROCHELLE, 1992; CHEREL et al., 1992). Studies have shown that feeding behavior is triggered by a rapid but still reversible increase in the rate of DMBL and in muscle-protein utilization (HANDRICH; NICOLAS; LE MAHO, 1993).

2.3 FINDINGS IN MAGELLANIC PENGUINS STRANDED AT THE BRAZILIAN COAST

The presence of penguins off the southern and southeastern Brazilian coasts during the fall and winter is a reoccurring natural phenomenon, as shown from archeological remains (BALDASSIN et al., 2010; BALDASSIN et al., 2012). Strandings of large numbers of penguins on the Brazilian coast have been described as early as 1927, while isolated individuals have been reported since the 1500s in Vitória, Espírito Santo State (CARDOSO et al., 2011). Most of these birds are juveniles, with a thin or missing fat layer, severe emaciation and empty stomachs (MADER; SANDER; CASA JR, 2010; CARDOSO et al., 2011). Most of the penguins arriving on the Brazilian coast are first-year juveniles (97%) (PINTO; SICILIANO; DI

BENEDITTO, 2007; GARCÍA-BORBOROGLU et al., 2010; MADER; SANDER; CASA JR, 2010; BRANDÃO; BRAGA; LUQUE, 2011; CARDOSO et al., 2011), that probably due to inexperience, get lost from the migratory groups and face stressful situations, getting more vulnerable to anthropogenic impacts (STONEHOUSE, 1975; MADER; SANDER; CASA JR, 2010). There are differences in geographic distribution between age classes; juveniles are more frequently found in Brazil, while adults stay in Uruguay and Argentina (GARCÍA-BORBOROGLU et al., 2006; MADER; SANDER; CASA JR, 2010). Some seabirds disperse more widely in their first year before returning to breeding colonies, which could explain the migratory range of this age class in the Atlantic (MADER; SANDER; CASA JR, 2010). Recent studies show that the high incidence of stranded Magellanic penguins observed in the Brazilian coast is recurrent (VANSTREELS et al., 2011; REIS et al., 2011; NUNES et al., 2015).

2.4 IRON METABOLISM IN BIRDS

Iron is a redox metal that can exist either as ferrous (Fe^{2+}) (more easily absorbed) or ferric (Fe^{3+}) iron (CRISSEY et al., 2000; HELMICK; KENDRICK; DIERENFELD*,* 2011; KLASING; DIERENFELD; KOUTSOS*,* 2012). Well over half of the iron present in the body is in the form of heme, the oxygen-binding site in hemoglobin and myoglobin, both responsible for transporting oxygen to tissues in all vertebrates (ROSSER; GEORGE, 1985; SHEPPARD; DIERENFELD, 2002; KLASING; DIERENFELD; KOUTSOS*,* 2012; PEREIRAet al., 2014). The remaining iron present in the body constitutes cytochromes, several enzyme systems and iron-sulfur proteins (ROSSER; GEORGE, 1985; SHEPPARD; DIERENFELD, 2002). Iron is also stored primarily as 2 nonheme compounds, ferritin and hemosiderin, both found throughout the body, but especially in the liver and spleen. The iron in enzyme systems occurs frequently in kidneys, heart, lungs, and especially the liver of wild and domestic birds from several orders and families worldwide (WILSON, 1994; CORK; ALLEY; STOCKDALE, 1995; MATHESON et al., 2007; PEREIRA et al., 2010).

Iron is essential for plants and animals as a cofactor for enzymes that catalyze oxidation–reduction reactions, especially those involved in aerobic metabolism

(KLASING; DIERENFELD; KOUTSOS, 2012). As iron levels increase, free oxygen radicals are released, damaging cell membranes (e.g., lysosomes) and proteins, oxidizing lipids and nucleic acids, and releasing ionic iron (SHEPPARD; DIERENFELD, 2002; HELMICK; KENDRICK; DIERENFELD, 2011; KLASING; DIERENFELD; KOUTSOS, 2012). Damaged cells may die and be replaced by fibrosis (SHEPPARD; DIERENFELD, 2002). The organs most commonly affected are the liver, heart, and spleen (SHEPPARD; DIERENFELD, 2002). Thus, animals maintain iron in a tightly chelated form when it is in cells or body fluids (KLASING; DIERENFELD; KOUTSOS, 2012).

In mammals and birds, iron homeostasis is a multistep process regulated by duodenal and small intestinal absorption of dietary iron (SHEPPARD; DIERENFELD, 2002; HELMICK; KENDRICK; DIERENFELD, 2011; KLASING; DIERENFELD; KOUTSOS, 2012). 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 diet components (CRISSEY et al., 2000; SHEPPARD; DIERENFELD, 2002; PEREIRA, 2014). The increase of circulating iron may increase absorption of iron by the liver, resulting in iron hepatocyte accumulation in the form of cytosolic ferritin and a striking accumulation of ferritin and hemosiderin within lysosomes (WARD et al., 1988; DIERENFELD; PINIS; SHEPPARD, 1994; SHEPPARD; DIERENFELD, 2002). Excessive iron accumulation damages hepatocytes, often leading to fibrosis (SHEPPARD; DIERENFELD, 2002).

In chickens (*Gallus domesticus*) infused with iron dextran, iron is initially taken up to the greatest extent by phagocytes, and over time iron is apparently transferred to hepatocytes, where iron is initially stored in a complex with ferritin, free in the cytosol and not visualized with routine histological staining methods. As the amount of iron in the liver increases, greater amounts seem to be present as cytosolic hemosiderin (PEREIRA et al., 2010; KLASING; DIERENFELD; KOUTSOS, 2012). Hemosiderin is predominantly present in the liver, but also in the spleen, bone marrow, and reticulocytes of birds (SHEPPARD; DIERENFELD, 2002; MATHESON et al., 2007). In chickens, humans, and laboratory rodents, iron excretion occurs only by accidental loss via sloughing of cells, hair, feathers, and small amounts in urine (KLASING; DIERENFELD; KOUTSOS, 2012).

2.4.1 Iron overload syndrome or iron storage disease (ISD)

Iron overload syndrome or iron storage disease (ISD) is defined as a pathologic process of multifactorial origin, characterized by intracellular accumulations of iron in liver and other tissues (mainly kidney, heart and lung) which could be present as; (1) hemosiderosis – a the pathological process of iron accumulation in hepatic and splenic sinusoidal macrophages, as well as parenchymal cells such as hepatocytes, without significant architectural, cellular, or functional alterations; or (2) hemochromatosis - defined as the excessive accumulation of iron accompanied by functional and/or morphological consequences leading to pathologic and symptomatic changes attributed to iron toxicosis (CRISSEY et al., 2000; OSOFSKY et al., 2001; WEST*;* GARNER; TALCOTT, 2001; MATHESONet al., 2007; PEREIRA et al., 2010; HELMICK; KENDRICK; DIERENFELD*,* 2011).

Although some diseases in mammals may cause ISD, it is more commonly observed in reptiles and some groups of birds (CRISSEY et al., 2000). ISD has been described, disease associated or not, in several families or order of birds: *Sturnidae*, *Paradiseadae*, *Ramphastidae*, *Corvidae*, *Cotingidae*, *Ptylonorhynchidae*, *Bucerotidae*, *Psitacidae, Passeriformes*, *Anseranatidae* and *Strigiformes* (WARD et al., 1988, 1991; WEST*;* GARNER; TALCOTT, 2001; MATHESON et al., 2007; PEREIRA et al., 2010; HELMICK; KENDRICK; DIERENFELD*,* 2011). Hepatic hemosiderosis varies considerably within species, an indication that multiple factors may apply (SHEPPARD; DIERENFELD, 2002). The pathophysiology of ISD is not well described in the avian patient, but two alternatives have been suggested: increased dietary iron or an increased absorption of normal or low concentrations of dietary iron (WEST*;* GARNER; TALCOTT, 2001; MATHESON et al., 2007). Frugivorous and, to a lesser extent, insectivorous species are likely to be susceptible to ISD, while more granivorous or omnivorous species are likely to be resistant (KLASING; DIERENFELD; KOUTSOS*,* 2012). Based on this observation, Klasing, Dierenfeld and Koutsos (2012), suggest that the cause of ISD might be the convergent evolution driven by the common nutritional characteristics of fruits and insects (e.g., low iron content).

There is little information available on the normal iron metabolism of different avian species and normal hepatic mechanisms of iron metabolization are incompletely understood (CORK; ALLEY; STOCKDALE, 1995). Although there are no conclusive evidence that the presence of stainable iron in the liver of birds has any clinical significance, the presence of hemosiderin has been linked to the presence of concurrent infections, neoplastic and parasitic diseases, anemia, hepatopathies, intoxications, starvation, trauma and the normal physiological cycle, such as age and seasonal events (e.g., egg production and egg laying, migration and molting) (CORK; ALLEY; STOCKDALE, 1995; MATHESON et al., 2007; PEREIRA et al., 2010; HELMICK; KENDRICK; DIERENFELD*,* 2011; KLASING; DIERENFELD; KOUTSOS*,* 2012). Some studies suggest a positive correlation between hepatic hemosiderosis with age and time in captivity (TAYLOR 1984; LOWENSTINE, 1986; CRISSEY et al., 2000; METE et al., 2005; PEREIRA et al., 2010; KLASING; DIERENFELD; KOUTSOS*,* 2012), while others suggest there is no correlation (WARD et al., 1988). Clinical signs are often unspecific and usually noted in the final stages of the disease, and may include cardiac and hepatic failure leading to ascites, dyspnea and apathy (in severe cases). However, most commonly observed clinical signs include cardio, hepato and splenomegalies, and a yellow to brown hepatic discoloration (PEREIRA et al., 2014).

A similarity has been suggested between avian idiopathic or primary hemochromatosis to human hemochromatosis, which is caused by a homozygous gene mutation in the HLA locus of chromosome 6, that allows for excessive gastrointestinal (GI) iron absorption from iron-balanced diets (WARD et al., 1988; DIERENFELD*;* PINIS; SHEPPARD, 1994; WEST*;* GARNER; TALCOTT, 2001; SHEPPARD; DIERENFELD, 2002; PEREIRA et al., 2010; HELMICK; KENDRICK; DIERENFELD*,* 2011; KLASING; DIERENFELD; KOUTSOS*,* 2012). However, Dierenfeld, Pinis and Sheppard (1994) suggested that susceptible avian species may be genetically predisposed, more specifically mynahs birds, due to their histopathologic similarities with human hemochromatosis. Clinical signs associated with hemochromatosis are attributed to declining hepatic function, including chronic wasting, abdominal distention, hepatomegaly, coelomic effusion, dyspnea and ascites. Birds may not demonstrate any clinical signs and simply be found dead (WILSON, 1994; MATHESON et al., 2007).

Antemortem identification of hemosiderosis in the avian patient is a diagnostic challenge, with several reports on image analysis (radiographs, magnetic resonance imaging (MRI) and ultrasound imaging) and blood tests (serum iron, total iron binding capacity, serum transferrin concentrations, transferrin saturation, serum ferritin measurements, and iron saturation) presenting inconsistent results (CORK; ALLEY; STOCKDALE, 1995; CRISSEY et al., 2000; MATHESON et al., 2007; HELMICK; KENDRICK; DIERENFELD*,* 2011; KLASING; DIERENFELD; KOUTSOS*,* 2012). The definitive diagnostic test for ISD is liver biopsy with histologic examination of the tissue, iron-specific stains, and colorometric assay of iron content (MATHESON et al., 2007; HELMICK; KENDRICK; DIERENFELD*,* 2011; KLASING; DIERENFELD; KOUTSOS*,* 2012). In physiological conditions, intracellular iron may be stored as ferritin, free in the cytosol and not visualized with routine histological staining methods, or as hemosiderin, which is detected as brown or blue granules, on hematoxylin and eosin (HE) and Prussian blue (PB) techniques, respectively (PEREIRA et al., 2010). In general, excess iron from the diet is predominately stored in hepatocytes (e.g., Mynah birds), whereas iron storage resulting from excessive hemolysis, septicemia, and starvation is stored in phagocytes, while concurrent infections have shown increased storage in phagocytes relative to hepatocytes (KLASING; DIERENFELD; KOUTSOS*,* 2012). Intraspecies variation in storage location may indicate a range of etiological factors, or the presence of concurrent diseases. (LOWENSTINE; PETRAK, 1980; CORK; ALLEY; STOCKDALE, 1995). Treatment consists of phlebotomy (forcing the body to use stored iron), iron chelators (i.e., desferoxamine) and appropriate diet levels of iron (SHEPPARD; DIERENFELD, 2002; PEREIRA et al., 2010; HELMICK; KENDRICK; DIERENFELD*,* 2011; PEREIRA et al., 2014).

2.5 AVIAN MALARIA

This section will briefly explain the etiology, epidemiology, life cycle, pathology, clinical signs, lesions, diagnosis, treatment and prevention of avian malaria in penguins, with emphasis on Magellanic penguins.

2.5.1 Etiology

Avian haemosporidian blood parasites (Sporozoa: Haemosporida) comprise the genera of *Haemoproteus*, *Plasmodium* and *Leucocytozoon*, divided in four families: *Haemoproteidae* (*Haemoproteus*, 132 species), *Plasmodiidae* (*Plasmodium*, 38 species), *Garniidae* (*Fallisia*, 1 species) and *Leucocitozoidae* (*Leucocytozoon*, 35 species). These cosmopolitan groups of obligate heteroxenous protists parasitize amphibians, reptiles, birds and mammals. In birds, they are responsible for severe disease in domestic, zoo and wild populations, belonging to many families and orders around the world, except Antarctica (VALKIUNAS, 2005; BRAGA et al., 2011; SILVEIRA et al., 2013). Avian haemosporidian develop in two groups of hosts: the vertebrates (birds), which are intermediate hosts, and the vectors (blood-sucking dipterans, *Insecta*), the final (definitive) hosts in which the sexual phase takes place (DINHOPL et al., 2001; VALKIUNAS, 2005; BRAGA et al., 2011; SILVEIRA et al., 2013).

The definition of the term “avian malaria” in this study will refer only to the infection by the *Plasmodium* genus, in accordance with the definition of Ricklefs and Fallon (2002) and Perez-Tris et al., (2005). Some previous studies include infections by *Haemoproteus*, *Leucocytozoon* and *Fallisis* into this term, due to their taxonomic proximity and shared epidemiological characteristics. In our understanding, although further studies are still needed to clarify the correct indication in avian species, the term “avian malaria” has a useful working concept.

Avian infecting *Plasmodium* are divided into five subgenera: *Haemamoeba* (10 spp.), *Giovannolaia* (15 spp.), *Novyella* (9 spp.), *Huffia* (3 spp.) and *Bennettinia* (1 spp.). The *Plasmodium* genus presents moderate species diversity (VALKIUNAS, 1997; BRAGA et al., 2011; VANSTREELS; PARSON, 2015). *Culicidae* mosquitoes of the genus *Culex* are believed to be the most common vectors of avian *Plasmodium,* and less commonly *Mansonia, Culiseta* e *Aedeomyia;* but laboratory studies have shown that *Mansonia* spp.*, Aedes* spp.*,* and *Armigeres* spp. may also be competent vectors (LAPOINTE; ATKINSON; SAMUEL, 2012; VANSTREELS; PARSON, 2015). *Plasmodium* spp. has been reported in several bird species (STRANDBERG; STOSKOPF; CRAFT, 1981; MURATA et al., 2008; BELO et al., 2009; BRAGA et al.,

2011; CHAGAS et al., 2013; THURBER et al., 2014; TOSTES, 2015; SCAGLIONE et al., 2016), but to remain within the scope of this study, we will focus mainly on avian malaria in penguins.

Malaria in penguins is mostly caused by *P. (Haemamoeba) relictum* and *P. (Huffia) elongatum* (FLEISCHMAN et al., 1968a,b; BAK; PARK; LIM, 1984; FIX et al., 1988; CRANFIELD et al., 1994; VANSTREELS et al., 2014, 2015), but also by *P. (Haemamoeba) tejerai* (SILVEIRA et al., 2013; VANSTREELS et al., 2014, 2015), *P. (Haemamoeba) cathemerium* (VANSTREELS et al., 2014, 2015), *P. (*Bennettinia*) juxtanucleare* (GRIM et al., 2003; VANSTREELSet al., 2014, 2015), *P. (Novyella) nucleophilum* (VANSTREELS et al., 2014, 2015), *P. (Huffia) elongatum* (VANSTREELS et al., 2015), and 5 unidentified lineages that correspond to *Plasmodium* spp. not yet reported in penguins (lineages B, C, E, F and J) (VANSTREELS et al., 2015).

2.5.2 Epidemiology and life cycle

Plasmodium esporozoites are inoculated by the infected vector during feeding and invade endothelial and mononuclear fagocytic cells of several tissues, including spleen and skin, originating criptozites, which will reproduce asexually and burst, releasing great numbers of merozoites. This is the initial phase, also known as “primary exoerytrocytic merogonia”. Merozoites reach the blood stream to infect other endothelial and phagocytic mononuclear cells – pulmonary, splenic, hepatic and bone marrow cells, mainly – in which metacryptozoites (also named tissue merontes or tissue esquizontes), developed by asexual reproduction, burst releasing several merozoite forms. This phase is called “secondary exoerytrocitic merogony”. After this phase, merontes are able to follow four different pathways: (1) return to the secondary exoerytrocytic merogony phase; (2) erythrocytic merogony: invasion of hematopoietic cells (specially redblood cells), originating erythrocytic merontes (also called erythrocytic esquizontes) and releasing some of these forms; (3) terciary erythrocytic merogony: invasion of endothelial and phagotic mononuclear cells from a variety of tissues, where they will be able to remain for longer periods, until rupturing and releasing great number of merozoites; or (4) gametogonia: invasion of blood

cells (typically erythrocytes) and development of throphozoites, which will untimatelly turn into magagametocytes and microgametocytes (sexual forms of the parasite). Once in the midgut of a mosquito vector, gametocytes undergo gametogenesis to form true gametes. Gametocytes of all species of avian *Plasmodium* remain within erythrocytes until ingested by an invertebrate hematophagous, when they are released and become macrogamets and microgamets. One microgamete will fertilize a macrogamete, and within 24 hours a motile zygote develops that is capable of penetrating the midgut wall to begin development as an oocyst – this is the only sexual reproduction phase. Now in the esporogony phase, the zygotous is a mobile oocinet that lodges within the intestinal mucosa of the lamina propria, developing into an oocyst, responsible for the production of a great number of esporozoites by asexual reproduction. When matured, the oocysts bursts, releasing esporozoites into the invertebrate's coelomic cavity, which actively penetrate the vector's salivary glands, where they will remain for several weeks until being inoculated into an intermediate host and restarting the cycle (LAPOINTE; ATKINSON; SAMUEL, 2012; VANSTREELS; PARSONS, 2014).

The host-specificities of *Plasmodium* species are variable, occurring in many avian species and families, but primarily affecting passerine birds. Nevertheless, *P. relictum,* for example, has very low specificity and are known to infect a great number of bird species, already identified in over 400 avian species from 11 orders. This ability in alternating hosts must be taken into account when considering management and disease prevention of captive and wild populations (JONES; SHELLAM, 1999; LAPOINTE; ATKINSON; SAMUEL, 2012; VANSTREELS; PARSON, 2015).

According to Sijbranda et al. (2016), the establishment of avian *Plasmodium* lineages in an ecosystem and their consequent prevalence of infection in bird species depend on (1) the avian hosts' susceptibility and tolerance to infection; (2) the presence and transmission efficiency of arthropod vectors; (3) the virulence of *Plasmodium* spp.; (4) the spatial and temporal distribution of host and vector; and (5) climate.

Morbidity and mortality can be severe, especially in immunologically naïve and susceptible hosts, or when concurrent infections with other infectious agents (e.g., avipoxvirus) or intestinal parasites are present (SIJBRANDAet al., 2016). A few avian groups are considered highly susceptible and may develop severe disease, such as penguins (*Sphenisciformes*) and some native Hawaiian species, the honeycreepers

(*Passeriformes*: *Drepanidinae*) (VAN RIPER III et al., 1986; FIX et al., 1988; JONES; SHELLAM, 1999; ATKINSON; DUSEK; LEASE, 2001; GRIM et al., 2003; ATKINSON, 2008; LEVIN et al., 2011; LAPOINTE; ATKINSON; SAMUEL, 2012; VANSTREELS; PARSON, 2015; VANSTREELS et al., 2015).

Island species, due to their geographic isolation, are thought to be especially susceptible to introduced diseases (VAN RIPER et al., 1986; ATKINSON et al., 2000), which can severely impact the health of small populations and have been the cause of species extinctions, as seen after the introduction of *P. relictum* to the Hawaiian Islands in the XX century, leading to the extinction, population decline, and restricted distribution of Hawaiian bird species (VAN RIPER et al., 1986; ATKINSON et al., 1995, 2000; WIKELSKI et al., 2004; LAPOINTE; ATKINSON; SAMUEL, 2012). *Plasmodium* has also been identified in New Zealand in introduced European, native and endemic species (SIJBRANDA et al., 2016).

The fact that these birds have not coevolved, and therefore, have not adapted evolutionarily and physiologically with haemosporidians, is suggested as the cause for their inappropriate immunological responses and low natural resistance to these parasites (VALKIUNAS, 2005; VANSTREELS et al., 2015).

In wild captive and penguins, avian malaria is one of the most significant diseases, (FIX et al., 1988; CRANFIELD, 1990; GRACZYK et al., 1995b; GRIM et al., 2003; BUENO et al., 2010; SILVEIRA et al., 2013; VANSTREELS et al., 2015), and also poses an obstacle to successful rehabilitation of wild penguin populations, some of them considered by the IUCN Red List as near threatened (Magellanic penguin), and threatened (Galapagos penguin, Humboldt penguin and yellow-crowned penguin) (IUCN Red List website: http://www.iucnredlist.org/search, accessed in May 15th, 2016). *Plasmodium* spp. infection was first reported in 1927, with the observation of several stages of *P. praecox* (*P. relictum*) in the peripheral blood of a king penguin at the London Zoological Society's Gardens (SCOTT 1927; FANTHAM; PORTER, 1944; PENRITH et al., 1994; LECLERC et al., 2014), and since then in African black-footed (FANTHAM; PORTER, 1944; FLEISCHMAN et al., 1968 a,b; STOSKOPF; BEIER, 1979; CRANFIELD et al., 1990; BROSSY 1992, 1993; GRACZYK et al., 1994c; GRACZYK, STOSKOPF; BEIER 1995; LOMBARD; BROSSY; BLACKBEARD, 1999; DUIGNAN, 2001; LECLERC et al., 2014), Magellanic (FIX et al., 1988; LECLERC et al., 2014; VANSTREELS et al., 2014; VANSTREELS; PARSONS, 2014; VANSTREELS et al., 2015), Galapagos (LEVIN et

al., 2009; PALMER et al., 2013); yellow-crowned (*Eudyptes antipodes*) (FANTHAM; PORTER, 1944); southern rockhopper (*E. chrysocome*) (FANTHAM; PORTER, 1944), Humboldt (*S. humboldti*) (BAK et al., 1984; CARVAJAL; ALVARADO, 2009), Macaroni penguin (*E. chrysolophus*), little (*Eudyptula minor*), Chinstrap (*Pygoscelis antarctica*), Gentoo (*Pygoscelis papua*) (JONES; SHELLAM, 1999), Fiordland crested (*E. pachyrhynchus*), and Yellow-eyed Penguins (LAIRD, 1950).

In these species, avian malaria causes rapid and severe outbreaks often associated with high morbidity and mortality (up to 50–80%) within a few weeks (FIX et al., 1988; LEVIN, 2009; BUENO et al., 2010; VANSTREELS et al., 2014). High mortality levels usually occur during periods of mosquito abundance (spring and summer), but seasonality regarding the detection of *Plasmodium* in penguins has been previously reported (FIX et al., 1988; PARSONS; UNDERHILLL, 2005; LECLERC et al., 2014; VANSTREELS et al., 2014; VANSTREELS et al., 2015).

Penguin chicks and juveniles are more susceptible, usually presenting high-grade infections (GRIM et al., 2003; PARSONS; UNDERHILLL, 2005), but any naive penguin can contract the disease (GRIM et al., 2003; DINHOPL et al., 2011). Females can pass antibodies to their chicks, allowing for short-term protection from *Plasmodium* spp. infection, and as soon as these antibodies are no longer detectable, then juveniles become susceptible to infection; approximately 10 weeks of age in captive African black-footed penguin (GRACZYK et al., 1994e).

In the *Spheniscidae* family*, Plasmodium* spp. have been recorded in wildlife rehabilitation centers (BROSSY, 1992; GRIM et al., 2003; PARSONS; UNDERHILLL, 2005), in the wild (GRACZYK et al., 1995a,b; LEVIN, 2009; PALMER et al., 2013), and in captive penguin colonies (GRINER; SHERIDAN, 1967; FLEISCHIMAN et al., 1968a,b; FIX et al., 1988; GRACZYK et al., 1994c,e; McCONKEY et al., 1996). In Brazil, *Plasmodium* spp. infections have mostly been reported in Magellanic penguins undergoing rehabilitation (SILVEIRA et al., 2013; VANSTREELS et al., 2014; VANSTREELS et al., 2015), and only once in an outbreak at the São Paulo Zoo (BUENO et al., 2010). The Galapagos penguin vulnerability to infectious diseases, for example, is likely threatening for the species, due to its small population size, low genetic diversity, and very low variation in the major histocompatibility complex (MHC) and environmental stresses such as El Niño events (WIKELSKI et al., 2004; VARGAS et al., 2006; LEVIN et al., 2013).

Braga et al. (2011) suggested that the increased susceptibility of penguins to malaria could be due to the fact that most penguin species spend majority of their life in habitats that usually cannot sustain active malaria transmission (cold environments and/or the absence of susceptible mosquito vectors).

2.5.3 Pathogenicity

After inoculation of sporozoites into the vertebrate host, there is a pre-patent and asymptomatic phase lasting 5 to 10 days that anticipate the appearance of the first blood parasitic forms. Afterwards, birds typically undergo an acute phase of infection with steadily increasing parasitemia until reaching a peak (the “crisis”) around the 21st day of the post-infection, approximately 6–12 days after initial parasitemia. This is the onset of clinical signs and symptoms, which can progress very rapidly and variable intensity, based on host immunity, seasonal photoperiod, and reproductive hormones (BRAGA et al., 2011). The crisis last for approximately seven days, followed by a rapid decline into chronic levels, due to strong antibody and cell-mediated responses to the parasites (LAPOINTE; ATKINSON; SAMUEL, 2012; VANSTREELS; PARSONS, 2014). The chronic stage is subclinical in most cases, recrudescence and relapses may occur (CRANFIELD et al., 1990; ATKINSON; VAN RIPER, 1991; PALMER et al., 2013), typically less severe than the prime infection because of acquired immunity to the parasite (PALMER et al., 2013), but relapses might lead to severe disease under stressful conditions (e.g., seasonal changes or the occurrence of concurrent infections (CRANFIELD et al., 1994; GRACZYK et al., 1994c; BRAGA et al., 2011).

The pathogenicity of most avian species of *Plasmodium* is poorly understood, ranging from asymptomatic and sub lethal effects on host fitness (e.g., mate selection, reproductive success, and immune response) (KNOWLES PALINAUSKAS; SHELDON, 2009; LAPOINTE; ATKINSON; SAMUEL, 2012; VANSTREELS; PARSON, 2015), being a strong selective force operating within natural populations (BRAGA et al., 2011), to population decline and extinction (VAN RIPER III et al., 1986; ATKINSON et al., 2000; BEADELL et al., 2006). The severity of pathologic effects caused by avian malaria infections varies according with *Plasmodium* and

avian species (SIJBRANDA et al., 2016). According to Braga et al. (2011), pathogenicity-related variables, such as *Plasmodium* spp., should be considered, in particular the differences in virulence for different lineages, and the differences in susceptibility between different species of avian hosts and even individuals within the same species. Vanstreels et al. (2015) observed that even though penguins are susceptible to infection by a variety of *Plasmodium* lineages, *P. tejerai*, *P. cathemerium* and *P. relictum*, all member of the *Plasmodium* subgenus *Haemamoeba*, seemed to be more pathogenic to these birds than other subgenera of *Plasmodium*. Magellanic penguins diagnosed with *P. tejerai* presented very particular necropsy and histopathologic signs, suggesting that this lineage may be a highly pathogenic parasite for penguins (VANSTREELS et al., 2014).

Chronic infections persist for a lifetime at extremely low intensities, with circulating parasites and persistent exoerythrocytic meronts serving as a source for recrudescing infection (LAPOINTE; ATKINSON; SAMUEL, 2012). Cranfield et al. (1994), suggested that most likely, the infected erythrocytes of African penguins persisted in deep vascular sites or dormant sporozoites and pre-erythrocytic forms of malaria parasites survived in the endothelial tissues. In temperate climates, a recrudescence, or spring relapse, occurs during the breeding season when increased corticosteroid level suppresses the host's immune system (LAPOINTE; ATKINSON; SAMUEL, 2012).

Intravascular hemolysis and the phagocytosis of parasitized cells leads to hemosiderin buildup in visceral macrophages, and to hemosiderosis (specially hepatic) in severe cases. Merontes in tissue endothelial cells and macrophages lead to vasculitis and diffuse parenchymal inflammation. Congestion, edema and hypercellularity lead to splenomegaly and hepatomegaly, which are very frequent malarial findings. Other changes include: splenic hematopoiesis, hemosiderosis, ductal hyperplasia, varied levels of acute and chronic inflammation, hypoxia, hemorrhage, increased vascular permeability and edema, ultimately leading to multifocal degeneration and necrosis a variety of organs, such as heart, liver, spleen, lungs, kidneys, and skeletal muscles, among others (FIX et al., 1988; VALKIUNAS, 2005; VANSTREELS et al., 2014; VANSTREELS et al., 2015). In penguins, intense pulmonary parenchymal inflammation, congestion and edema cause severe respiratory complications, that ultimately may lead to respiratory insufficiency and

death. Hypoperfusion and shock are caused by pericardic perfusion due to vasculitis, reduced blood pressure, compromised blood coagulation, and electrolytic unbalance.

2.5.4 Clinical signs

Usually, sudden death occurs before the observation of any *ante mortem* clinical signs. When present, clinical signs are unspecific and include: ruffed feathers, lethargy, anorexia, depression, mucosal pallor, dyspnea, diarrhea, regurgitation, vomiting, hyper or hypothermia, and green droppings, and neurological signs, such as convulsion and paralysis have bee reported. Reddened mosquito bites may be observed in apterygial areas, around the beak and on the eyelids (FLEISHMAN et al., 1968a; STOSKOPF; BEIER, 1979; BAK et al., 1984; FIX et al., 1988; ATKINSON et al., 1995; GRIM et al., 2003; GRIM et al., 2004; VALKIUNAS, 2005; DINHOPL et al., 2011; VANSTREELS; PARSONS, 2014; PARSONS; UNDERHILL, 2015). According to Vanstreels and Parsons (2015), possible clinical pathologic findings include regenerative anemia, lymphocytosis, elevated plasmatic protein, reduced albumin and a2-globulins, elevated g1 and 2 globulins, elevated AST, GLDH, GGT and decreased creatinin.

2.5.5 Lesions

Most common necropsy and histopathologic findings in penguins include: marked splenomegaly and hepatomegaly, vascular collapse, the presence of thrombus and thromboembolism, generalized congestion, intense splenic and pulmonary congestion and edema, hepatic and muscular congestion and dilation of the major veins and arteries, and hidropericardium. Presence of exoerythrocytic schizonts in the reticulo-endothelial system (BAK et al., 1984; FIX, 1988; DINHOPL et al., 2011; VANSTREELS; PARSONS, 2014).

2.5.6 Diagnosis

Morphologic detection of the parasite in blood smears is the most commonly used diagnostic method for avian malaria, even though presenting low sensitivity and requiring extensive training. Histopathology allows post mortem confirmation of an avian malaria diagnosis through the observation of tissue meronts in macrophages, miofibroblasts and endothelial cells of various tissues, such as spleen, liver, cardiac muscles, kidneys and bone marrow (VANSTREELS; PARSONS, 2014). Many *Plasmodium* spp. cannot be identified solely by morphologic characteristics of the intracellular stages, requiring molecular diagnostics (GRIM et al., 2003). Polymerase chain reaction (PCR) can accurately identify *Plasmodium* spp. based on the Small subunit ribosomal ribonucleic acid (SSU rRNA) in blood (live birds) and tissue samples of dead birds (spleen, liver, lungs and kidneys) (GRIM et al., 2004; GRACZYK et al., 2003e; VANSTREELS; PARSONS, 2014).A combination of molecular biology techniques (PCR) and traditional parasitology (microscopy) remarkably improve the parasites identification and detection of parasitemia in wild birds (VALKIUNAS 2011; SILVEIRA et al., 2013).

2.5.7 Treatment

Avian malaria treatment is preconized in susceptible species, debilitated birds, high parasitemia and/or evident clinical signs. Treatment usually combines the antiprotozoal effect of cloroquine on the circulating stages and of primaquine on the tissue stages. However, treatment does not eliminate the quiescent tissue forms of the parasite, so stressful events or corticoid treatment may lead to recrudescence in infected penguins, although less severe than the prime infection (VANSTREELS; PARSONS, 2014). Cranfield et al. (1994) described decreased parasitaemia in penguins infected with *P. elongatum* and *P. relictum* treated with chloroquine, but poor efficacy has been suggested in penguin species after implementing treatment and maintenance of high mortality rates (FIX et al., 1988; BUENO et al., 2010).

2.5.8 Prevention

Preventive strategies include: (1) mechanical barriers: anti-mosquito screens, fans, repellents; (2) prophylactic treatment with oral medications during spring and summer, that although able to reduce the frequency and gravity of avian malaria, does not prevent episodes of morbidity and mortality; (3) early diagnosis, quarantine and treatment of malaric birds; (4) haemoparasite monitoring through blood screening, especially in penguins under rehabilitation (VANSTREELS et al., 2014; VANSTREELS; PARSONS, 2014); and (5) vaccination (GRIM et al., 2004; VANSTREELS; PARSONS, 2014). Wild free-ranging birds inhabiting zoo areas have been suggested by Cranfield et al., (1990) and Beier (1981) at the Baltimore Zoo (United States), and by Hugh-Jones (1999) at Marwell Zoo (United Kingdom) as possible reservoirs for the *Plasmodium* infection in penguins, and this theory was recently confirmed Leclercet al. (2014) with the aid of molecular diagnostics. Vanstreelset al. (2015), suggested that birds living in the surrounding forested areas of a rehabilitation center in Santa Catarina, Brazil, could also serve as *Plasmodium* spp. reservoirs. Controlling the free-ranging wild avian fauna living around zoo and rehabilitation areas would also greatly contribute with the control of malaria dissemination, once these birds serve as possible reservoirs for *Plasmodium* spp. Vanstreels et al. (2015) suggested narrowing the malaria- prevention efforts of rehabilitation facilities in Brazil, to a relatively lower number of Magellanic penguin specimens, under care under October and April, period of higher vector exposure, in order to improve prevention and early diagnosis.

Avian malaria has the potential to exert major effects on native biodiversity, likely to manifest in the near future due to ongoing climatic and land-use disturbance, through their influence on mosquito distribution and abundance; key determinants of both the pattern of parasite occurrence and the future dynamics of parasite emergence (TOMPKINS; GLEESON, 2006).

3 OBJECTIVES

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.

4 MATERIAL AND METHODS

This section will describe the origin and selection of the Magellanic penguins evaluated in this study.

4.1 SAMPLES

All the animals included in the present study (*Plasmodium-negative* and positive groups) were part of the research study: “Investigation of avian malaria and other blood parasites in penguins along the Atlantic coast of South America”, by Dr. Ralph Eric Thijl Vanstreels, DVM, PhD, as part of his doctorate degree at the Programa de Pós-Graduação em Patologia Experimental e Comparada do Departamento de Patologia (VPT), Faculdade de Medicina Veterinária e Zootecnia (FMVZ), Universidade de São Paulo (USP), São Paulo, Brazil, linked to the Thematic Project FAPESP 2010/51801-5, “Avian Malaria in penguins from Brazil: An epidemiologic and pathologic study of an infirmity with potential risk to the conservation of avifauna”.

Samples from 3 rehabilitation centers along the southeastern coast of Brazil were collected between 1999 and 2013: Centro de Reabilitação de Animais Marinhos – Universidade Federal do Rio Grande (CRAM-FURG) – Rio Grande, Rio Grande do Sul; Associação R3 Animal – Florianópolis, Santa Catarina; e Instituto de Pesquisas e Reabilitação de Animais Marinhos (IPRAM) – Cariacica, Espírito Santo.

4.2 *PLASMODIUM*-POSITIVE GROUP

This group comprised a total of 21 animals naturally infected by *Plasmodium* spp. and diagnosed by Vanstreels (2014) based on morphologic (blood smears, histopathology) and molecular techniques (nested polymerase chain reaction and genetic sequencing). Subsequently, three new positive specimens, identified with the aid of the same diagnostic methods, were incorporated to the group, now with 24

diagnosed individuals. *Plasmodium* spp. lineages were characterized afterwards by blood smears and phylogenetic analysis of the *cyt-b* mitocondrial gene (VANSTREELS, 2014).

4.3 *PLASMODIUM*-NEGATIVE GROUP

Nine *Plasmodium-negative* animals were selected based on individual history and a negative diagnosis for *Plasmodium* spp. Each animal's history was evaluated according with the following criteria: institution, age (juvenile or adult), sex (male, female or undetermined), presence or absence of oil contamination upon admission at the rehabilitation center, total period of stay in the rehabilitation center and period of stay in the rehabilitation center during summer (March 1st through October 31st). *Plasmodium* spp. animals were those that were negative for tPCR (nested polymerase chain reaction and genetic sequencing) and blood smear, that died without presenting any clinical signs of concurrent infections (e.g., aspergillosis, avian poxvirus), and that remained in captivity for similar periods as the positive animals comprising the positive group, and under the same infrastructure and management conditions.

4.4 SAMPLE PROCESSING

This section will discuss the technique used to capture microscopic images, histologic procedures.

4.4.1 Histochemistry

Paraffin embedded liver fragments were identified and sectioned at 5μm with the aid of a rotary microtome and stained with hematoxiline-eosine, Perls and reticuline, as described by Luna (1968).

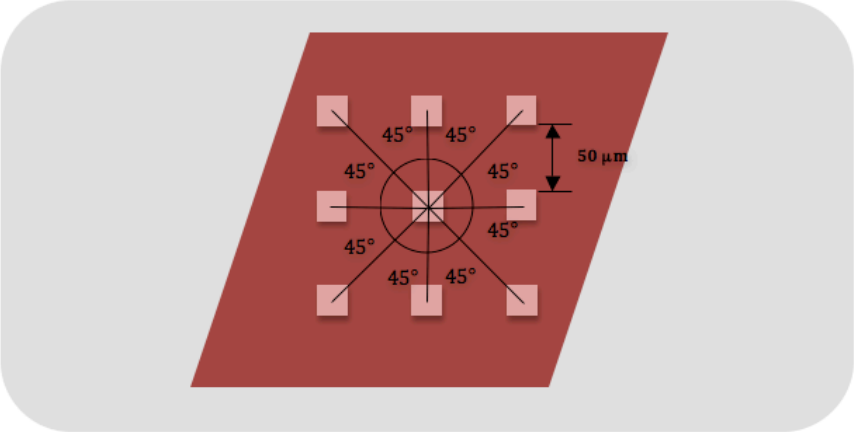
4.4.1.1 Histologic evaluation

This section will discuss the evaluation of the IHH and IHN.

4.4.1.1.1 Index of Hepatic Hemosiderosis (IHH) and the Index of Hepatic Necrosis (IHN)

Slides were analyzed under a microscope (Trinocular Olympus H BX 50, Olympus, Melville, NY) equipped with a digital camera (Evolution MPH, 5.1 megapixels, Media Cybernetics, Silver Spring, MD), a computerized image analyses software (Image ProPlus, version 5.1.2.59, Media Cybernetics), and a 19" monitor (L1950HQ-SN model, LG Electronics, São Paulo, Brazil). The center of the slide, determined as the intersection of the two longest axis of the hepatic fragment, was photographed and used as the reference field. Another eight images were captured as TIF files, at 50μm of this point, and 45° intervals, under the same lighting conditions and 20X magnification (Figure 1). Hemosiderin deposits and areas of necrosis were semi-automatically delineated, respectively, on the Perls and reticuline stained slides, starting at the lowest blue intensity to the most intense, to avoid the inclusion of artifacts and the background. In order to standardize the technique, a maximum 50% zoon was stipulated. The parameter obtained in each microscopic field was the percentage of the areas of hemosiderin deposition or necrosis. The index of hepatic hemosiderosis (IHH) and index of hepatic necrosis (IHN) were obtained by the mean values of nine microscopic fields.

Figure 1 - Schematic drawing of the Image Pro Plus (IPP) Software capture technique. The center of the slide was determined as the intersection of the two longest axis of the hepatic fragment (red). Further captures were taken at 50μm and 45° intervals from this point (pink)

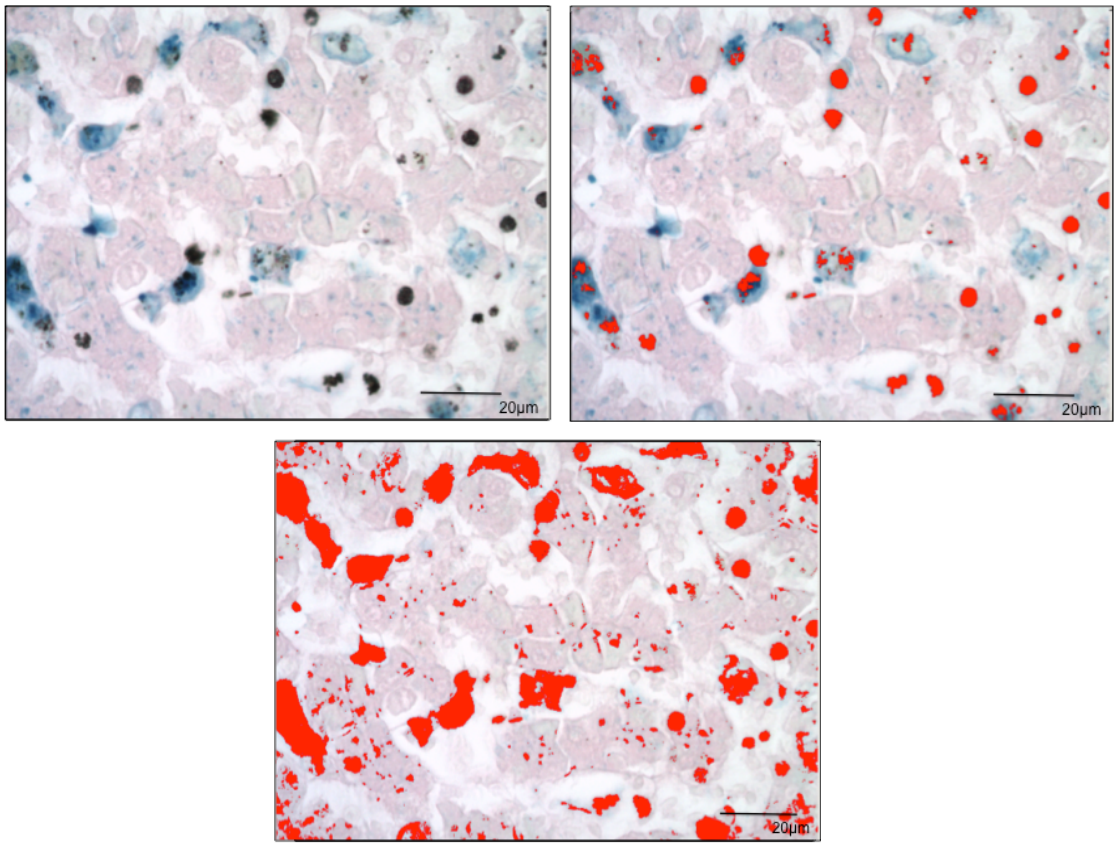


Source: Personal archive.

4.4.1.1.2 Presence of formolic pigment in Perls stained slides

Once some of the evaluated Perls stained slides presented formolic pigment artifacts, we adopted a duplicate caption method (“mask”), consisting of the following: first, the original image was capture (Photograph A), followed by delineation of all areas of artifact and formolic pigment deposition (Photograph B), the values obtained by the IPP Software evaluation were included into a Microsoft Excel spreadsheet. A copy of the original image (duplicate/mask), already presenting the undesirable areas (artifacts and formolic pigment) marked (Photograph B), was than reevaluated, and only the areas presenting hemosiderin deposition were delineated (Photograph C). This photograph was saved as: institution abbreviation-animal number.II. The true area of hemosiderosis deposition was the remaining area obtained by the difference between the IPP mask of the duplicate image (Photograph C) and the IPP mask of the original image, with only the areas of formolic pigment deposition marked (Photograph B). These values were evaluated based on the Microsoft Excel spreadsheet, ultimately providing the true values of the areas presenting hemosiderosis deposition, once areas with formolic pigment and artifacts were removed.

Figure 2 - An example of IPP image captures and the “masks” used to determine the IHH. Photomicrograph of liver, Spheniscus magellanicus, CRAM 2125, Perls, 400X: **(A)** original photomicrograph, **(B)** delineated areas of artifact and formolic pigment deposition, **(C)** delineated areas of artifact, formolic pigment and hemosiderin deposition.



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4.5 STATISTICAL ANALYSIS

Positive animals were divided into groups according with their ‘*Plasmodium* subgenera’, classified based on genetic proximity, according with the phylogenetic tree described by Vanstreels (2014): *Haemamoeba* (*P. cathemerium* e *P. tejerai*) genus, *Ruffia* (*P. elongatum*) genus, Lineages (*Plasmodium* D, E, G e H lineages) and *Plasmodium* (*Plasmodium* spp.), the latter included unidentified *Plasmodium* lineages. Statistical evaluation was performed with by Student’s t and One-way ANOVA tests.

5 RESULTS

Among the positive animals, females had IHH values (17.53 ± 12.95%) markedly higher than the males' (7.20 ± 4.25%; p=0.041), even though no significant differences between both values were observed. Significant differences were not detected between IHH and INH regarding institution, age, sex, oil contamination, and *Plasmodium* lineages/species (p>0.05; Table 1). Table 2 shows the IHH and IHN, according with the presence of infection and type of *Plasmodium*.

Figure 3 - Photomicrograph of liver, *Spheniscus magellanicus*, CRAM 1229, Perls, 400X. Areas of hemosiderin deposition (red arrows).

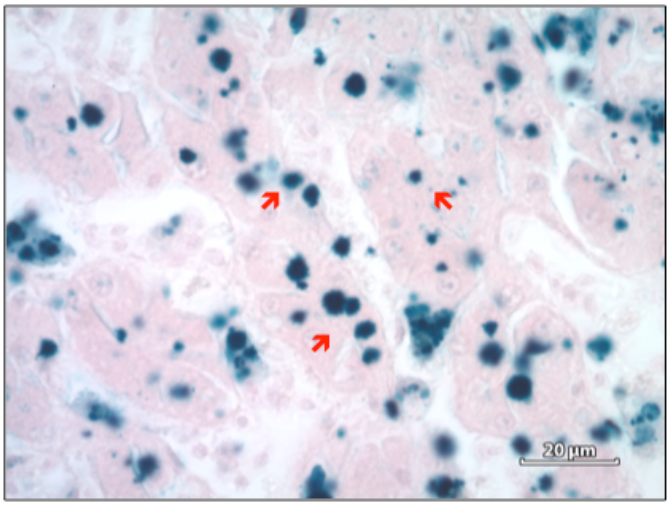

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Figure 4 - Photomicrograph of liver, *Spheniscus magellanicus*, IPRAM 145, reticulin, 400X. Reticulin deposition on reticular fibers (blue arrows).

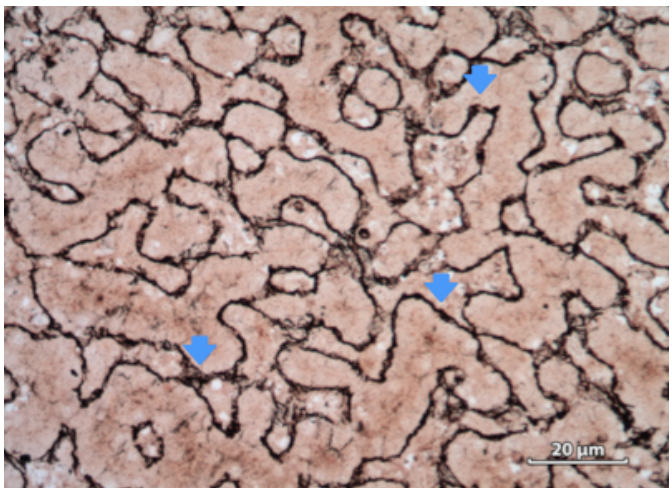

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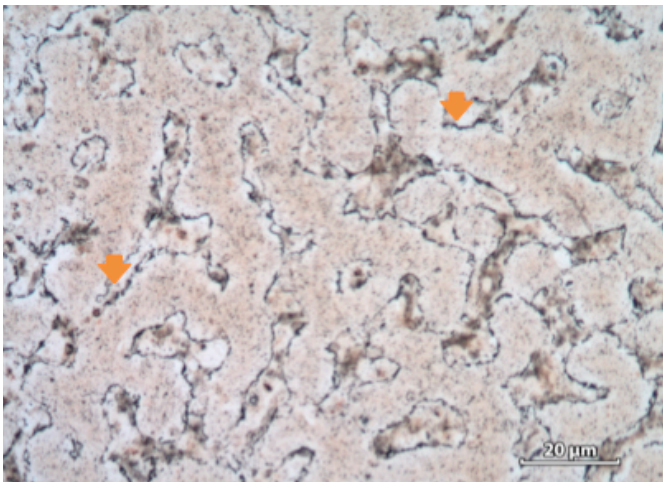
Figure 5 - Photomicrograph of liver, *Spheniscus magellanicus*, FLO 290, reticulin, 400X. Reticulin deposition on reticular fibers (orange arrows).



Source: Personal archive

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) ($p>0.05$).

In the *Plasmodium-negative* group, the IHN was significantly higher than the IHN of the positive group ($p<0.001$), however, no difference was detected between the IHH of both groups.

Table 1 - Values for p and r obtained from the comparisons between analyzed parameters

	IHH		IHN	
Comparisons	**P Value**	**r Value**	**P Value**	**r Value**
Positive Group X *Plasmodium-negative* Group	P=0.2331		P=0.001	
Adult X Juvenile	P=0.9433		P=0.5455	
Male X Female	P=0.0410		P=0.7282	
Presence X Abscense of oil	P=0.2957		P=0.3524	
Institutions	P=0.8742		P=0.7797	
Total period of stay in the rehabilitation center	P=0.6796	r=-0.2254	P=0.0978	r=-0.3620
Period of stay in the rehabilitation center during summer	P=0.6782	r=-0.09145	P=0.7953	r=0.05725
Plasmodium lineages/species	P=0.6553		P=0.9449	
With X Without concurrent diseases	P=0.3407		p=0.1575	
With X Without Avian Poxvirus	P=0.5754		p=0.6677	
With X Without Pododermatitis	P=0.9225		0.4426	
In X Not in molt	P=0.4551		p=0.4831	

Table 2 - IHH and IHN values of the animals naturally infected by Plasmodium spp. ("positive"), distributed according with the animal number (ID) and involved *Plasmodium* species/lineage (N = 24)

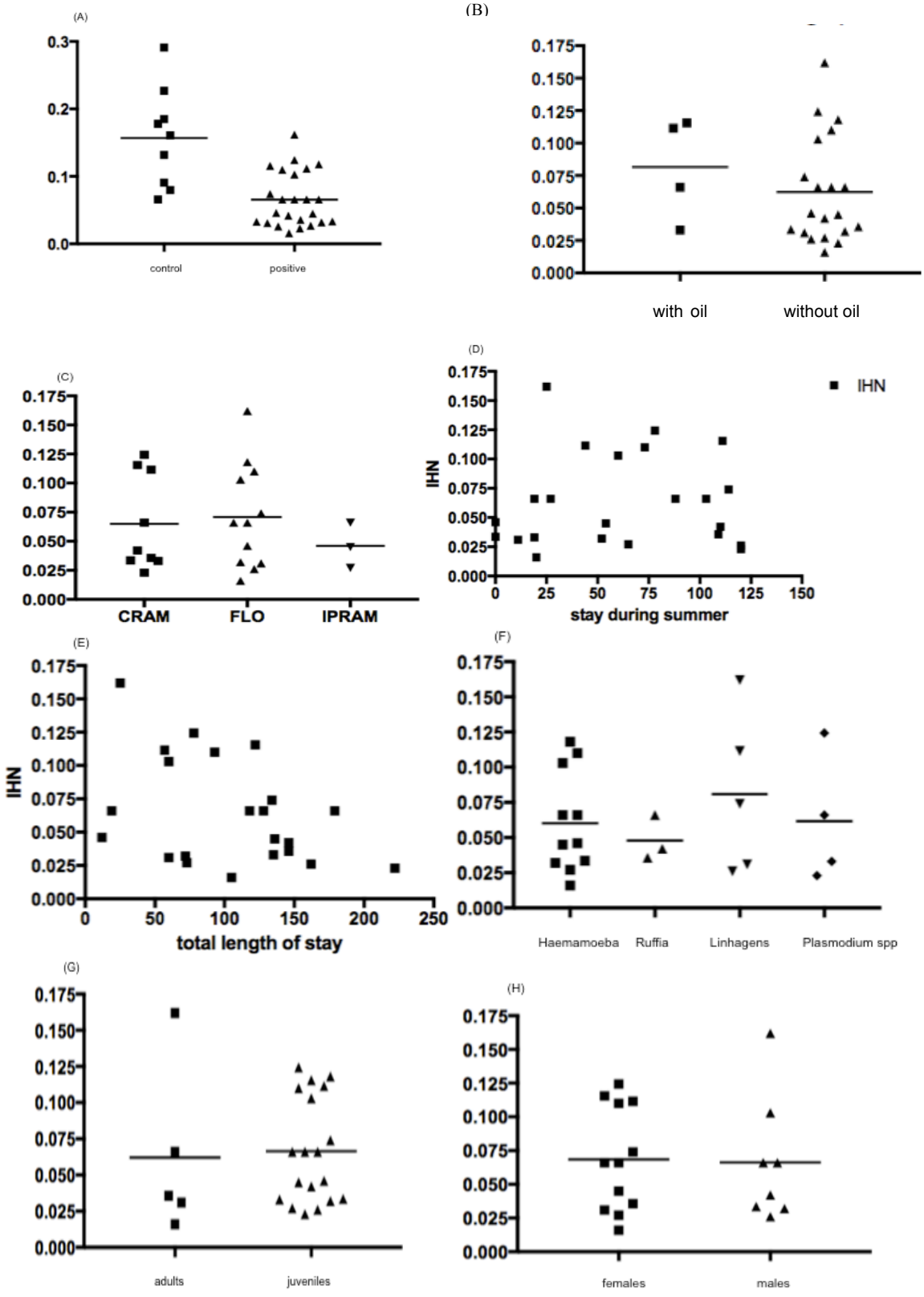
Animal Number (ID)	Species/lineage	IHH (%)	IHN (%)
IPRAM-ES114	*P. elongatum*	0.228	0.066
IPRAM-ES167	*P. cathemerium*	0.004	0.045
IPRAM-RJ128	*P. cathemerium*	0.148	0.027
FLO-IF506	*P. tejerai*	0.108	0.066
FLO-IF584	*P. tejerai*	0.015	0.118
FLO-IF593	*P. tejerai*	0.382	0.046
FLO-R0040	*P. tejerai*	0.319	0.016
FLO-R0091	*P. tejerai*	0.094	0.066
FLO-R0093	*Plasmodium* sp. lineage E	0.062	0.031
FLO-R0263	*Plasmodium* sp. lineage G	0.048	0.026
FLO-R0268	*Plasmodium* sp. lineage G	0.454	0.074
FLO-R0272	*P. cathemerium*	0.058	0.103
FLO-R0282	*Plasmodium* sp. lineage H	0.146	0.162
FLO-R0284	*P. cathemerium*	0.183	0.110
FLO-R0290	*P. cathemerium*	0.042	0.032
CRAM-0580	*Plasmodium* sp.	0.107	0.023
CRAM-1229	*Plasmodium* sp.	0.128	0.124
CRAM-0628	*Plasmodium* sp.	0.083	0.033
CRAM-0630	*Plasmodium* sp.	0.138	0.066
CRAM-2125	*Plasmodium* sp. lineage D	0.150	0.112
CRAM-2127	*P. nucleophilum*	0.276	0.116
CRAM-2876	*P. cathemerium*	0.011	0.033
CRAM-2911	*P. elongatum*	0.014	0.036
CRAM-2912	*P. elongatum*	0.069	0.042

Table 3 - IHH and IHN values (%) of the animals confirmed negative for *Plasmodium* spp. ("*Plasmodium-negative*"), listed according with the evaluated specimens (N = 9)

Animal	IHH (%)	IHN (%)
IPRAM-111	0.009	0.132
IPRAM-144	0.181	0.185
IPRAM-145	0.116	0.178
IPRAM-197	0.029	0.161
FLO-F1244	0.009	0.291
FLO-F1246	0.056	0.227
CRAM-2096	0.134	0.066
CRAM-2098	0.065	0.091
CRAM-2128	0.125	0.080

Source: Personal archive

Figure 7 - Graphics showing the results of the Student's t and One-Way ANOVA statistical analysis: (A) *Plasmodium-negative* group *vs* positive group;(B) oiled birds *vs* birds without oil;(C) institutions (CRAM *vs* IPRAM *vs* FLO);(D) stay during summer (IHN) ;(E) total length of stay (IHN);(F) *Plasmodium* lineages/species;(G) adults *vs* juveniles;(H) females *vs* males



6 DISCUSSION

The present study evaluated the hepatic hemosiderosis and necrosis presented by Magellanic penguins naturally infected by *Plasmodium* spp., that died while under care in rehabilitation centers along the southern and southeastern coasts of Brazil.

6.1 INDEX OF HEPATIC HEMOSIDEROSIS (IHH)

Despite previous associations with hemosiderosis (GOTTDENKER et al., 2008; VANSTREELS et al., 2015; GRILO et al., 2016), it was not possible to statistically correlate the presence of *Plasmodium* spp., with the lineage or species, the presence of moderate to severe hepatic hemosiderosis has been observed in the Magellanic penguins belonging to the positive group. Therefore, the hemosiderosis observed in the analyzed animals is possibly related to one or more factors to which these animals are exposed during their life cycle and/or stay in rehabilitation centers.

6.2.1 Breeding and Molting Phases: a brief description

This section will be divided in two parts: (1) the “Breeding Phase”, comprising the egg laying, incubation and chick rearing stages; and (2) “Molting Phase”, referring to the pre-molt and molt. The goal is to discuss the energetic burden imposed by physiologic fasting in Magellanic penguins during breeding and molting, and how it influences this species’ annual life cycle. Once there are no studies specifically on Magellanic penguins, this section will focus on penguin species in general, but mainly on middle-sized ones, and take a closer view on how the different phases of the Magellanic penguins’ life cycles dictate the conditions in which these birds arrive on our shore. And finally, we will emphasize on the possible link between such physiological demands, migration and the initial phase of their rehabilitation, and how they may influence the presence of the hepatic hemosiderosis observed in the evaluated birds.

6.2.1.1 “Breeding Phase”

Wild birds may fast during fluctuations on food availability (e.g., winter) or even in the presence of food, when feeding competes with other more important activities for the individual’s or the species’ survival, such as reproduction, incubation, migration and molt, requiring birds to accumulate great amounts of fat during the period immediately prior to spontaneous fasting (ROBIN et al., 1987; LINDGÅRD et al., 1992; WILLIAMS et al., 1992; GROSCOLAS; ROBIN, 2001; BOISMENU; GAUTHIER; LAROCHLLE, 2002). During fasting, muscle mass is relatively maintained on the expenses of fat stores (MAITRA; KUMAR, 2008). Prolonged fasting during the breeding period, especially during oviposition and incubation, have been described in red jungle fowls (*Gallus gallus*), petrels and albatrosses (GROSCOLAS; ROBIN, 2001), and several waterfowl species, such as the common eider (*Somateria mollissima*) (KORSCHGEN, 1977) and the domestic geese (*Anser anser*) (BENEDICT; LEE, 1937; LE MAHO, 1983; ROBIN et al., 1987; GROSCOLAS; ROBIN, 2001).

Among birds, penguins endure the longest fasting periods (LE MAHO, 1983), providing enough studies on breeding and molting fasts (JHONSON; WEST, 1973; WILLIAMS et al., 1977; CROXALL, 1982; BROWN, 1984; CHEREL; LE MAHO, 1985; CHEREL et al., 1988; GROSCOLAS; LELOUP, 1989; GROSCOLAS et al., 1990; CHEREL et al., 1992; WILLIAM et al., 1992; GROSCOLAS; ROBIN, 2001). Spontaneous fasting during reproduction and molt is a major characteristic of their annual life cycle (GROSCOLAS; ROBIN, 2001). Although physiologically well adapted to fasting, these birds must have sufficient fat reserves and good muscular condition to endure it, once during this period muscle proteins provide blood glucose for maintenance of the central nervous system, and glucogenic amino acids for oxaloacetate formation (WILLIAMS et al., 1977). Pre-breeding fast, also called “incubation fast”, is based on the accumulation of fat. In Magellanic penguins, this phase starts as soon as birds arrive from migration grounds to the breeding sites. From this point on until the chicks emancipate and become independent, parents will alternate periods of fast and feeding in order to care for the youngs (more details of the Magellanic penguin life cycle are described on item 2). The breeding phase is even more burdensome for reproductively active females, due to egg formation, oviposition and incubation. According to Croxall (1982), the daily energy cost of incubation in penguins is about 1.3-1.4 times the estimated cost of basal metabolism.

Domestic birds are known to sustain great iron losses (up to 1mg Fe/egg) during oviposition and regular molts (CRISSEY et al., 2000). First, there is an initial transitory decrease in hematrocrit, hemoglobin values and the female’s hepatic iron store at the beginning of oviposition, followed by a sharp increase in plasmatic iron concentrations, which remains elevated during the whole egg production phase. It has been suggested that laying females compensate this physiological iron demand by increasing their intestinal iron absorption efficiency. Conversely, female common eiders (*Somateria mollisima*) have showed an increase in liver iron concentration after 2-3 weeks into incubation fasting (NORAMBUENA; BOZINOVICI, 2009). The others argued that possible causes included increased cellular replacement rate, endogenous iron release to the blood stream or liver size reduction, which could increase the hepatic iron concentration. Elevated hemolysis related to the rate of iron reuse has been reported in cachexia cases in herring gulls and Atlantic puffins that ingested Prudhoe Bay crude oil (LEIGHTON, 1986), and could be considered in the birds with hemosiderosis evaluated in this study.

Our reduced number of animals, especially of sexually mature ones, did not allow the comparison between distinct annual seasons. The number of adult females analyzed in this study was insufficient to establish the relationship between the IHH of females in reproductive age and juveniles, or between adult females and males (adults and juveniles), in an attempt to investigate if mature females potentially in the post-reproductive period, would have higher IHH values.

6.2.1.2 “Molting Phase”

Penguins present the so-called “catastrophic molt”; in which they change all their feathers simultaneously and not gradually, as most bird species (SILVA FILHO; RUOPPOLO, 2014). Both adult and juvenile Magellanic penguins go through molting, a highly energy demanding process, immediately prior to their winter migration (NORAMBUENA; BOZINOVICI, 2009), and soon after the breeding season, therefore also called: “post nuptial molt” (VANSTREELS, 2014). During this annual event, penguins entirely replace their whole plumage, being forced them to stay ashore due to the reduction in thermal insulation that precludes feeding (GROSCOLAS; ROBIN, 2001). In order to survive the fasting imposed by molting, penguins become hyperphagic during the pre-molt period (OTSUKA; MACHIDA; WADA, 2004; SILVA FILHO; RUOPPOLO, 2014); juveniles rely on energy reserves supplied by their parents, and adults have to replace all the energy spent during breeding (mainly lipids), while storing fat and protein (this latter item primarily for feather conversion) (WILLIAMS et al., 1977; GHEBREMESKEL et al., 1989; GROSCOLAS; ROBIN, 2001). During this phase there is an increase in their body mass (40 a 70%) in fat, which will be lost throughout the molting period (SILVA FILHO; RUOPPOLO, 2014). Ghebremeskel et al. (1989) calculated in 20% this pre-molt nutrient increase required in tropical regions, the territorial range of Magellanic penguins. Molt fasts range from 2-5 weeks, depending on the penguin species (GHEBREMESKEL et al., 1989; GROSCOLAS; ROBIN, 2001). This period is marked by great energy expenditure and penguins must rely on body stores for feather replacement and increased thermogenesis, maintenance of basic physiological functions, feeding in an accelerated rate and migrating between feeding and molting

sites. (GHEBREMESKEL et al., 1989; WILLIAMS et al., 1992; NORAMBUENA; BOZINOVICI, 2009).

According to Croxall (1982), the daily molt costs in penguins are about twice the basal metabolism. There are no descriptions regarding the losses of Magellanic penguins during breeding fast, but for discussion purposes, the values reported for the molt-fast in black-footed penguins could be extrapolated, once both species belong to the same genus: 45.1% water, 56% fat, 42.5% protein and 52.6% energy (COOPER, 1978). Although data on other penguin species are helpful, it is important to emphasize that Magellanic penguins have a few particular characteristics that must be considered when using other species' data to estimate the cost of their breeding and molting fasts, such as equal gender responsibilities/duties during egg incubation and chick rearing, and their markedly migratory behavior among all other penguin species.

Iron content has a seasonal pattern, increasing from the post-breeding low to reach its peak near the end of the molt period (OSBORN, 1979). In a study by Ghebremeskel et al. (1989), mean post-molt plasma iron declined 74.3% in Magellanic penguins, possibly due to plasma transferrin depletion, while the analysis of hemoglobin showed a decrease of 19.2%. Anseriformes (CORK; ALLEY; STOCKDALE, 1995) and Passeriformes of the *Sturnidae* family, such as the common starling (*Sturnus vulgaris*) (WARD et al., 1988), showed an association between the hepatic level of hemosiderosis and the period of post nuptial molt prior to migration, presenting increased amounts of positive iron staining primarily located within lysossomic organeles of parenchymal hepatic cells (WARD et al., 1988; CORK; ALLEY; STOCKDALE, 1995). Such findings are possibly promoted by the increased hematopoietic activity associated with changes in the thyroid hormone levels involved in the molting process (CORK; ALLEY; STOCKDALE, 1995; CORK, 2000), increased metabolic iron needs during the summer months or iron storage for migratory needs (WARD et al., 1988).

Considering the Magellanic penguins analyzed in this study, it is possible that the period elapsed between the postnuptial molt (February – March) and their death, could have been insufficient to observe changes in iron storage. Increased iron deposition has been reported in some severe cases of prolonged malnutrition in obese humans subjected to a variety of fasting periods (ROZENTAL et al., 1967), in the liver and spleen of starvation victims from concentration camps (UEHLINGER,

1948), in the liver of rats subjected to food restriction (SCHARTZ; TORNABEN; BOXILL, 1973), in the plasma and several organs of rats subjected to anorexia and fed a protein depleted diet (CONRAD et al., 1967), and in malnourished black-necked swans (*Cygnus melanocoryphus*) (NORAMBUENA; BOZINOVICI, 2009). Rozental et al. (1967) and Uehlinger (1948) speculated that the increased iron deposition in hepatic (parenchymal and Kupffer cells) and splanchnic tissue was caused by anemia, which generally accompanies cases of chronic anorexia. Normocytic normochromic anemia reports in humans during anorexic periods may be related to an enzyme system deficiency needed to release ferritin iron and consequently to plasma, not allowing the use of iron during anorexia (ROZENTAL et al., 1967).

6.2.1.3 Breeding and Molting Phases: Physiological Fasting

Breeding and molt fasting are regular phases during the life cycle of penguins, including Magellanic penguins. These birds must be able to efficiently store energy in order to keep their basic physiological needs while breeding and molting successfully. Once molt is over, penguins rely on these same energy reserves to migrate to their feeding grounds (GHEBREMESKEL et al., 1989). Fasting is a natural process in the life cycle of penguins, while starvation is a consequence of one or all of the following possibilities: inability to obtain food, to feed, to metabolize food, or a combination of one or more of these factors. Therefore, there are two main possibilities to justify the debilitated nutritional state in which Magellanic penguins arrive on the Brazilian coast: (1) either these birds were unable to successfully go through the above mentioned pre-migratory phases of intermittent energy storage, fasting and increased energy demands while on breeding sites (e.g., food shortage, abnormal climatic events, anthropogenic activities, or diseases), forcing them to migrate without meeting the adequate requirements for such a demanding period; or (2) the threats these birds face during migration, are enough to partially compromise even those birds in optimal conditions or push those others already in risk, over the edge.

The hemosiderosis observed in the *Plasmodium-negative* and positive penguins could possibly be a direct consequence of starvation caused by the above mentioned factors, once it has been linked to this condition by several authors (CORK; ALLEY; STOCKDALE, 1995; MATHESON et al., 2007; PEREIRA et al., 2010; HELMICK; KENDRICK; DIERENFELD*,* 2011; KLASING; DIERENFELD; KOUTSOS*,* 2012;). Nevertheless, another consideration must be inferred regarding the feeding of these animals. Starvation and refeeding produce marked effects on the metabolism of lipids, carbohydrates and proteins in birds and mammals, however, little is known regarding the effect of starvation and refeeding on the status of trace elements of these animals (RICHARDS et al., 1989). The same author found that hepatic iron concentration and total organ content increased 2.1-fold in starving 2-week-old turkey poults. The reasons for this increase are unknown, although some possible explanations include: increased hepatic blood entrapment due to red blood cells redistribution as a result of catecholamine-stimulated contraction of the splanchnic capsule, and a conservation mechanism involving ferritin or more efficient utilization of dietary iron. In the same study, the longer the previous period of starvation, the lower were the final liver metal concentrations after refeeding. However, in a study with rats, repeated cycles of starvation and refeeding produced hepatic siderosis, even more pronounced if the rats were re-fed a diet high in iron, which is not the case of our birds, but could be possible due to iron supplementation (RICHTER, 1974).

6.2.2 Migratory factors that could potentially influence the IHH

Magellanic penguins are exposed to several adversities during their winter migration from the reproductive colonies to the Brazilian continental shelf, such as climate changes (FONSECA PETRY; JOST, 2001; MADER; SANDER; CASA JR, 2010), a diversity of pathologic processes, including parasitosis, anorexia/cachexia, trauma (BALDASSIN et al., 2012), oil contamination (PETRY; FONSECA, 2002; PETRY; FONSECA; JOST, 2004; BALDASSIN et al., 2012; DA SILVA et al., 2012), commercial fishing interactions (PETRY; FONSECA, 2002; MADER; SANDER; CASA JR, 2010; CARDOSO et al., 2011), and marine debri (PETRY; FONSECA;

JOST, 2004; PINTO; SICILIANO; DI BENEDITTO, 2007; TOURINHO; DO SUL; FILLMANN, 2009; MADER; SANDER; CASA JR, 2010; BRANDÃO; BRAGA; LUQUE, 2011; DA SILVA, 2013). As mentioned by Vanstreels et al. (2011), juvenile inexperience in locating and apprehending food could lead to malnutrition and aggravate the already existing challenges faced during migration.

6.2.2.1 Anthropogenic factors: commercial fishing interaction and pollution

Commercial fisheries interact with seabirds in many different ways, the most common being competition for food resources (OLMOS, 1997; PÜTZ et al., 2011; WAGNER; BOERSMA, 2011), depletion of marine fish populations due to overfishing (WAGNER; BOERSMA, 2011), incidental mortality (CARDOSO et al., 2011; NEVES et al., 2007; BUGONI et al., 2008a; MARINAO et al., 2014), the use of fisheries waste as a food resource (OLMOS, 1997), and traumatic injuries that may compromise their locomotor and/or foraging skills, and unable predator avoidance. Marine debris can significantly impact wildlife (TOURINHO; DO SUL; FILLMANN, 2009) through (1) entanglement, that may lead to injuries and/or exhaustion and consequently death, or inability and/or difficulty in foraging and predator avoidance; (2) accidental ingestion (while ingesting captured prey or prey with gastrointestinal plastic particles); or (3) intentional ingestion (by mistaking debris with prey) (LAIST, 1997; BRANDÃO; BRAGA; LUQUE, 2011; DA SILVA, 2013). Consumption of plastic particles could result in partial or total gastrointestinal blockage or internal injury, reduction of the gastrointestinal tract functional volume, feeding stimuli and digestive efficiency, aside from interfering in the satiety feeling, or even in the individual's ability to reproduce (AZARELLO; VANVLEET, 1987; TOURINHO; DO SUL; FILLMANN, 2009; DA SILVA, 2013). Penguins are not widely known to ingest litter, however, data regarding marine debris ingestion by Magellanic penguins has recently become more frequent (PETRY; FONSECA; JOST, 2004; PINTO; SICILIANO; DI BENEDITTO, 2007; TOURINHO; DO SUL; FILLMANN, 2009; MADER; SANDER; CASA JR, 2010), with a reported mean of 35.8% of the Magellanic penguins stranded in Brazil showing some kind of consequence of debris ingestion (BRANDÃO; BRAGA; LUQUE, 2011). Interaction with commercial fishing

activities and marine debris also contribute to the occurrence of trauma and/or inability to feed, leading to anorexia and cachexia.

Petroleum toxicity in seabirds has been associated with hemosiderosis (KHAN; RYAN, 1991; KHAN; NAG, 1993) after accidental oil exposure in Common murres (*Uria aalge*) (FRY; LOWESTINE, 1985; KHAN; RYAN, 1991; KHAN; NAG, 1993; BALSEIRO et al., 2005), razorbills (*Alca torda*), and puffins (*Fratercula arctica*) (BALSEIRO et al., 2005), thick-billed murres (*U. lomvia*), and an oldsquaw (*Clangula hyemalis*) (KHAN; NAG, 1993), wild white winged scoter (*Melanitta fusca*), American coots (*Fulica americana*), and in waterfowl (SNYDERet al., 1973), and experimental oil exposure in Cassin's Auklets *(Ptychoramphus aleuticus*) *(Ptychoramphus aleuticus*) (FRY; LOWESTINE, 1985), nestling herring gulls (*Larus argentatus*)and Atlantic puffins (*Fratercula arctica*) (LEIGHTON, 1986) and American kestrels (*Falco sparverius*) (PATTEE; FRANSON, 1982). However, hemosiderosis is not a consistent finding in oiled birds (FRY; LOWESTINE, 1985; NEWMAN et al., 2000).

Peripheral red blood cells of birds are known to be compromised after crude oil ingestion (LEIGHTON; PEAKALL; BUTLER, 1983; YAMATO; GOTO; MAEDE, 1996). Oil ingestion may irritate the gastrointestinal mucosa, and subsequently cause hemorrhage (TSENG, 1999) and oxidative damage of hemoglobin and red blood cells, leading to the formation of Heinz bodies and hemolytic anemia (LEIGHTON; PEAKALL; BUTLER, 1983; PATTEE; FRANSON, 1984; LEIGHTON, 1986; KHAN; RYAN, 1991; KHAN; NAG, 1993; JENSSEN, 1994; FOWLER; WINGFIELD; BOERSMA, 1995; BRIGGS; YOSHIDA; GERSHWIN, 1996; TSENG, 1999; NEWMANet al., 2000; BALSEIRO et al., 2005; RODRIGUES et al., 2010), and consequently increased hemosiderin deposition in liver, spleen, and kidneys (LEIGHTON; PEAKALL; BUTLER, 1983; LEIGHTON, 1986; YAMATO; GOTO; MAEDE, 1996; BALSEIRO et al., 2005). The formation of Heinz bodies has also been reported in several wild bird species (LEIGHTON; PEAKALL; BUTLER, 1983; JESSUP; LEIGHTON, 1996; YAMATO; GOTO; MAEDE, 1996). The binding of Heinz bodies to cell membranes may affect fluidity and permeability to the point of intravascular or extravascular hemolysis. (LEIGHTON, 1986). A petroleum dose-dependent relationship with the intensity of hemosiderosis has been reported in common murres (KHAN; NAG, 1993). KHAN; NAG (1993) observed that, hemosiderosis generally appeared to be greater in birds that were heavily oiled and emaciated than in lightly oiled birds that showed evidence of slight weight loss,

suggesting that hemosiderosis could be a useful indicator of hemorrhage/anemia and, to some extent, the severity of the exposure. Petroleum poisoning may also cause intestinal mal-absorption and metabolic abnormalities, both contributive to weight loss (BRIGGS; YOSHIDA; GERSHWIN, 1996). Furthermore, birds presenting a previous history of weakness, cachexia and young inexperienced animals are possibly more severely affected by oil contamination (BALSEIRO et al., 2005). Oil ingestion in young birds leads to decreased growth rates in a variety of species, compromising osmoregulation and interfering with intestinal absorption (KHAN; RYAN, 1991; JENSSEN, 1994). However, significant differences were not observed between juveniles with or without oil. In the present study, comparison between IHH and IHN in adult birds with or without oil could not be analyzed due to the reduced number of adult specimens.

Oil contamination is one of the main causes of Magellanic penguin admissions in Brazilian rehabilitation centers (PETRY; FONSECA, 2002; SILVA FILHO; RUOPPOLO, 2014). Despite presenting variable degrees of oil contamination upon admission, our birds did not present a significant correlation between oil and IHH, possibly because at the time, the sustained levels of oil contamination were bellow the detection levels. Oil pollution may be particularly hazardous for Magellanic Penguins during their north migration (from late January to early April) (GANDINI et al., 1994), once their Atlantic Ocean route overlaps with heavy maritime traffic and petroleum development (STOKES; BOERSMA, 1998; PÜTZ; INGHAM; SMITH et al., 2008; GARCÍA-BORBOROGLU et al., 2006) and their final destination – the Brazilian continental shelf - has recently shown to be affected by oil pollution (PETRY; FONSECA, 2002; PETRY; FONSEA; JOST, 2004; GARCÍA-BORBOROGLU et al., 2006). Chronic petroleum pollution (small but frequent oil discharges from ships, at terminals, or from oily ballast water) accounts for most petroleum pollution in the ocean, and may be a more important problem than generally recognized, killing many more seabirds than large oil spills (GANDINI et al., 1994; GARCÍA-BORBOROGLU et al., 2006).

6.2.2.2 Climate Changes

Seabirds are sensitive indicators of changes in marine ecosystems and might integrate and/or amplify the effects of climate forcing on lower levels in food chains (LE BOHEC et al., 2008). Studies have shown that climatic phenomena such as “El Niño” (also known as El Niño – Southern Oscillation - ENSO) and “La Niña” affect breeding and mortality of several seabird species, including the Galapagos penguin (VARGAS et al., 2006), Magellanic penguin (MADER; SANDER; CASA JR, 2010; PERRIMAN et al., 2010), Humboldt penguin (BOERSMA 1978,1998; HAYS, 1986; PERRIMAN et al., 2010), and blue penguin (PERRIMAN et al., 2010). In ENSO years, warm and nutrient-poor waters of the Brazilian current reach the coast of Rio Grande do Sul in October, dislocating the cold Falklands/Malvinas current south, decreasing food resources availability and weakening the Magellanic penguins present in the southern areas of our coast (MADER; SANDER; CASA JR, 2010; FONSECA; PETRY; JOST, 2001). For discussion purposes, the year in which the penguins evaluated in this study were admitted into rehabilitation will also be considered as the year of their arrival in Brazil, which are: 2000, 2001, 2005, and 2008 through 2014. To assess whether ocean climate may have influenced our penguin arrival rate, we compared these values with the Multivariate El Niño-Southern Oscillation (ENSO) Index (MEI) (www.esrl.noaa.gov/psd/enso/mei), which classifies ENSO years (2002, 2004, 2006, 2008 and 2010) and non-ENSO years (2000, 2001, 2003, 2005, 2007 and 2009). One may suggest that the animals that arrived in Brazil in 2008 and 2010, possibly suffered the influence of climate changes. Our arguments corroborate with García-Borboroglu et al. (2010) that reported an increased number of Magellanic penguins – a total of 3371 - admitted by 12 rehabilitation centers along the coast of Brazil. Most of these penguins were not oiled and many presented low body weight and poor body condition, suggesting the lack of food as a possible cause for their aberrant north migration along the coast of Brazil. Dantas et al. (2013) registered a total of 5404 Magellanic Penguins logged at seven Brazilian rehabilitation centers during 2000–2010, with the majority of penguins (60%) recorded in 2008, an atypical year. The author also reports the northward extension of the species’ winter range, registered for the first time in 2008, and annually ever since. We agree with Dantas et al. (2013) and García-Borboroglu

et al. (2010) regarding the suggestion of decreased availability of specific prey and changes in oceanic currents, especially during ENSO, being the factors behind the poor overall conditions and hepatic hemosiderosis the Magellanic penguins arriving in 2008 and 2010 presented.

6.2.3 Stay in Rehabilitation Centers

Aside from all the above-mentioned challenges to which the evaluated birds have been exposed, a common factor is the period of time spent under rehabilitation. Management of penguin species in captivity may vary slightly from one institution to another, but overall guidelines are available (AZA Taxon Advisory Group, 2014; FOWLER; FOWLER, 2001; SILVA-FILHO; RUOPPOLO, 2014; WALLACE; WALSH 2014).

Magellanic penguins generally arrive to our shore debilitated and malnourished, in poor body conditions due to starvation, which is the result of loss of body fat and non-fat mass due to inadequate intake of protein and energy (XAVIER et al., 2007; CAMPOS et al., 2013). If lasting for days or even weeks, it leads to emaciation, also observed in these animals (RUOPPOLO et al., 2012). Other frequent findings include dehydration, hypothermia, skin lesions, trauma, oil, and infectious or non-infectious diseases, which may interfere with the rehabilitation process (XAVIER et al., 2007; SERAFINI et al., 2010; CAMPOS et al., 2013). These birds are sent to rehabilitation centers or treated by emergency response teams, to be rehabilitated, banded and released back to their environment after meeting specific health criteria (RUOPPOLO et al., 2004; GARCÍA-BORBOROGLU et al., 2006; RUOPPOLO et al., 2012).

However, although gregarious (SILVA-FILHO; RUOPPOLO, 2014), the high concentration of debilitated birds, under the stress of the previous journey and handling for medical treatment and rehabilitation, favors the development of secondary diseases. According to Vanstreels (2014), there are several infectious pathogens and diseases that may constitute relevant threats to penguin conservation, and impair efforts to rehabilitate these birds, such as avian malaria and other hemoparasitosis, aspergillosis, avianpox and chlamidiosis. Non-infectious

diseases including keel wounds and bumblefoot may also delay their rehabilitation. Endo and ectoparasitosis should also be considered once these animals are under stress and in some cases, sustaining concomitant infirmities, which could further suppress the immune system and potentiate parasitosis. In birds, stress also increases the transferritin level and iron absorption, predominantly in Kupffer cells (CORK; ALLEY; STOCKDALE, 1995; SHEPPARD; DIERENFELD, 2002).

6.2.3.1 Concomitant infections observed in the evaluated animals

Iron availability is a determinant factor in the outcome of host-pathogen interactions (CORK; ALLEY; STOCKDALE, 1995). In a retrospective study of 180 birds of 40 different species, belonging to six different orders, Cork, Alley and Stockdale (1995) observed that infectious diseases were considered the *causa mortis* of 79 specimens, the majority with significant hepatocyte iron levels (50%). In the same study, the author observed that psittacines presented a significantly higher amount of stained iron in specimens presenting infectious. In 75% of the animals, independently of species, birds that presented hepatic hemosiderosis, also sustained concomitant parasitic and microbial infections.

According to Sheppard and Dierenfeld (2002), pathogen growth often depends on iron, and its mobilization is the initial step that homeothermic species (mammals and birds) use to fight parasitic or bacterial infections. It has been suggested that ISD could predispose patients to bacterial infections by increasing iron availability for pathogen growth according with transferrin saturation (HELMICK; KENDRICK; DIERENFELD, 2011), increasing tissue transport, specially hepatic, of circulating iron (SHEPPARD; DIERENFELD, 2002).

In our work, although the animals presenting concurrent diseases (box I) were not statistically significant, the presence of hemosiderin has been linked to concurrent infections and parasitic diseases by several authors (GRINER; SHERIDAN, 1967; FIX et al., 1988; CORK; ALLEY; STOCKDALE, 1995; MATHESON et al., 2007; PEREIRA et al., 2010; HELMICK; KENDRICK; DIERENFELD, 2011; KLASING; DIERENFELD; KOUTSOS, 2012; NEYMEIER et al., 2014; VANSTREELS et al., 2015). One could suggest that the small number of

animals with secondary diseases possibly affected our results. Further and complementary studies are necessary to clarify this possibility in naturally infected malaric penguins.

6.2.3.2 Age and time in captivity

In spite of the important metabolic phases associated with the distinct stages of Magellanic penguin's life cycle, we did not find significant differences between IHH and the age groups (juvenile and adult). Juveniles go through a period of intense learning on how to locate, identify and catch prey, initially relying on the energetic stores acquired while being fed by their parents. Nevertheless, aside from the previously mentioned factors to which these birds are exposed during migration, inexperience may lead to malnutrition and could be an aggravating factor to the challenges already faced by these birds during this life age. Based on a combination of one or more of the above mentioned factors, it is estimated that approximately 58% of the recently fledged Magellanic penguins die during their first pelagic migration, aside from the high mortality rates of pre-breeding birds (SCOLATO, 1987; RODRIGUES et al., 2010).

The occurrence of hemosiderosis in relation to age and time in captivity has been the subject of some debate (WARD et al., 1988; DIERENFELD*;* PINIS; SHEPPARD, 1994; CRISSEY et al., 2000; PEREIRA et al., 2010; KLASING; DIERENFELD; KOUTSOS*,* 2012). Total liver iron has not been correlated with age, but its correlation with captivity is controversy. According to some authors, the length of time in captivity may (DIERENFELD*;* PINIS; SHEPPARD, 1994; CRISSEY et al., 2000) or may not correlate with the degree of iron storage (WARD et al., 1988).

Penguins can be aged by plumage, and still for a brief period of their lives; from the chick until the fledgling stages (plumage/downy covers), when they acquire their yearling plumage (during the first week of February according to Scolaro 1987), up to when they molt into the adult feather pattern (January-February) (SILVA et al., 2014). After acquiring the adult plumage, reproductive activity might be helpful, once females begin breeding in the fourth year whereas males begin in the fifth year. However, these observations may be very hard in the field and more helpful in the

case of captive birds. Once, the studied subjects were only classified either as adults or juveniles, which is very vague considering the real age of the adults, it is challenging to determine if age could have played a role in the accumulation of hepatic iron in these birds. It would be interesting to see if in future studies, specially those of captive collections with complete data records of the specimens, if there is any significant statistical data regarding age and hemosiderosis.

It is believed that diet is the link between the amount of time in captivity and hepatic iron storage. High levels of iron in the diet in captivity are considered the major determining factor in the development of iron overload, even in non-susceptible birds, possibly due to increased iron absorption (NILSSEN; BORCH-IOHNSEN, 1991; DIERENFELD*;* PINIS; SHEPPARD, 1994; METE et al., 2003). The prevailing theory is that species susceptible to iron storage disease evolved on diets that are low in bioavailable iron, primarily on fruits and insects, developing physiological mechanisms to extract dietary iron very efficiently (SHEPPARD; DIERENFELD, 2002; KLASING; DIERENFELD; KOUTSOS*,* 2012). This leads to inadequate down-regulation in iron absorption when provided diets that have high bioavailable iron content, leading to excessive dietary iron intake (CRISSEY et al., 2000; SHEPPARD; DIERENFELD, 2002; MATHESON et al., 2007; KLASING; DIERENFELD; KOUTSOS*,* 2012).

Information on the diet of the Magellanic penguin has been studied mainly in nesting areas during breeding, when fish are the most abundant prey, but little information is available on the species' diet outside of the breeding season, during their north migration (FONSECA; PETRY; JOST, 2001; PINTO, SICILIANO; DI BENEDITTO, 2007). It is believed the Magellanic Penguin diet is basically comprised of small, pelagic schooling fish, cephalopods and crustaceans (SIMEONE; BERNAL; MEZA, 1999; PINTO, SICILIANO; DI BENEDITTO, 2007; BOERSMA et al., 2009). One of the Magellanic penguin's main prey in Argentina are anchovies, *Engraulis anchoita*, also found in big fish schools in the southern coast of Brazil (MADER; SANDER; CASA JR, 2010; MICHELS-SOUZA et al., 2010; REZENDE et al., 2013). Studies carried out in Brazil report a greater frequency of traces of cephalopods in the stomachs of stranded juveniles (FONSECA; PETRY; JOST, 2001; PINTO, SICILIANO; DI BENEDITTO, 2007; BALDASSIN et al., 2010; MADER; SANDER; CASA JR, 2010; REZENDE et al., 2013).

There are insufficient data from research with penguins to set nutrient requirements with certainty, but the minimum amount of iron concentrations suggested for adult penguin diets are 80mg/kg (CRANFIELD; GRACZYK; BEALL 2003; CRISSEY et al., 2012). However, these levels should be considered tentative until more specific nutrient requirements for penguins are defined (CRISSEY et al., 2012). The literature available on the content of iron in fish is also scarce. Once deemed ready to eat solid food and normally hydrated, all three rehabilitation centers evaluated in this study fed the birds with a diet based on thawed whole fish. R3 and IPRAM fed their penguins almost exclusively on Brazilian sardines (*Sardinella brasiliensis*), a potential prey species of Magellanic penguins (SILVA et al., 2014). CRAM had a more varied diet based on donations, but also exclusively on fish, mainly banded croaker (*Paralonchurus brasiliensis*), stripped weakfish (Cy*noscion guatucupa*), acoupa weakfish (*Cynoscion acoupa*), Argentine croaker *(Umbrina canosai*) and whitemouth croaker (*Micropogonias furnieri*). Most fish species are valuable sources of major and trace minerals (CRISSEY et al., 2012). Seafoods, darker flesh fish especially, are good sources of iron, supplying 1–2 mg/100 g muscle (TURHAN; USTUN; ALTUNKAYNAK et al., 2004). The same author found the total iron contents in raw anchovy muscles to be 38.4 μg/g, while Crissey et al. (2012) reports 98-2060 ppm (evaluated in only 10 specimens). Chaijan et al. (2005) found that sardines (*S. gibbosa*) had higher heme iron content in dark than in ordinary muscle. Heme pigments, present mostly in dark muscles, are the major source of iron in sardine, corresponding to 9.16 mg/100 g of heme iron content, while ordinary muscles had 3.36 mg/100 g.

Although information on the iron content in seafood is scarce and usually based on human consumption (muscles) and not whole fish, there is no clear and unequivocal evidence that the hepatic hemosiderosis observed on the birds in our study is of dietary origin. Furthermore, prolonged freezing or inadequate storage can lead to a loss of fat-soluble vitamins through fat degradation and rancidity (NICHOLS et al., 1983) and degradation of muscle proteins possibly through denaturation of heme pigment or other iron-containing proteins, resulting in the release of iron. Therefore, it is possible to suggest that the current thawing process described for all three institutions may have compromised the nutritional values of the offered fish, and could potentially interfere in the available iron content.

6.2.3.3 Iron supplementation as a tentative treatment for anemia

Iron is an important nutrient for host requirements and for the metabolism of invading pathogens (KABYEMELA et al., 2008). Iron is thought to inhibit absorption of zinc, with the potential of compromising the immune response to infection. Free iron is essential for multiplication of bacteria, including *Escherichia coli*, *Mycobacteria* sp., *Pasteurella* sp*., Shigella* sp., and *Staphylococcus* spp., and also for parasites like plasmodia (SAZAWAL et al., 2006). *Plasmodium falciparum* in humans, for example, relies on non-heme iron for its asexual growth in mature erythrocytes (SANCHEZ-LÓPEZ; HALDAR, 1992). However, different organisms interact differently with iron in their hosts (ROSENTHAL; MESHNICK, 1996). Identifying the source of iron used by parasites and characterization of mechanisms influencing iron metabolism, have important therapeutic implications (SANCHEZ-LÓPEZ; HALDAR, 1992). In humans from areas with endemic iron deficiency anemia and malaria, some studies show increased parasitaemia, while other conflicting studies question on whether individuals treated with iron have a higher risk of developing malaria (OPPENHEIMER, 2001; KABYEMELA et al., 2008; GWAMAKA et al., 2012), or not (MENENDEZ; FLEMING; ALONSO, 2000; MEBRAHTU et al., 2004; IANNOTTI et al., 2006). Increased malaria morbidity and mortality among iron-supplemented children in areas of malaria endemicity in Africa have been reported by Gwamaka et al (2012) and Kabyemela et al. (2008).

Limited metabolically active iron in pathogen-invaded cells inhibits pathogen growth (SAZAWAL et al., 2006). According to Kabyemela et al. (2008), there are a few proposed mechanisms to explain how the risk of malaria might increase with iron supplementation and decrease with iron deficiency: (1) using defensive strategies, such as iron-binding proteins that withhold iron (hypoferremia), in order to reduce the amount of iron available for parasites and other organisms (also known as nutritional immunity); (2) inhibiting the expression of inducible nitric oxide synthase (iNOS), which down-regulates the formation of nitric oxide in macrophages, apparently a critical component in the defense against *P. falciparum.* Likewise, iron deficiency may amplify iNOS- mediated defenses against this pathogen; and (3) through stimulation of erythropoiesis and production of reticulocytes, iron supplementation might

increase susceptibility to parasite species that preferentially infect young red blood cells, such as *P. falciparum*.

All three rehabilitation centers use iron supplementation as shown on Table 1. There are no such studies available for birds, nor was possible to verify such hypothesis through the evaluation of IHH or IHN, but considering how relevant hemoparasitosis, especially malaria, are for the captive/under rehabilitation penguins, further studies regarding the influence of iron status on the risk of worsening malaria are necessary on the decision making of whether iron supplementation would be beneficial or detrimental for the patient. According to the financial possibilities and personnel experience of each rehabilitation center, a simple blood smear performed upon admission could be very informative, or even molecular diagnostics prior to iron supplementation. Until properly understood, the relationship between iron status and malaria is a continuum of risk. These hypotheses warrant additional interventional studies to ascertain the benefits and more information on the risks of iron supplementation for birds kept in malaria-endemic regions are needed.

Box 1 - Iron supplementation sources, iron concentration, doses and criteria for iron supplementation in the evaluated rehabilitation centers

Institution	Iron Source	[Fe]	Dose/Via	Use criteria
CRAM	Hemolitan® Ferro	4500 mg	PO 0,2mg/kg/IM	Uncomplicated cases Severe cases
FLO (R3)	Ferrodex®	100mg/ml	1ml/IM – single dose	After 2-3 days of unresponsive anemia (Hct <32% with hypochromic anemia)
IPRAM	Hemolitan®	4500 mg	1ml PO	Debilitated birds upon admission

Source: CRAM, IPRAM, FLO (R3 Animal).

6.2.3.4 Vitamin supplementation

The recommendations before supplementing the diet of a wild animal are to consider the species nutritional requirements and compensate particular variations that a debilitated individual may present. Iron bioavailability can be influenced by dietary components (HELMICK; KENDRICK; DIERENFELD*,* 2011). Iron absorption decreases with high dietary levels of manganese, copper, cobalt, cadmium, and zinc by competition for binding sites (CRISSEY et al., 2000; SHEPPARD; DIERENFELD, 2002). Copper and iron exist in multiple interdependent valence states within the body, which determine their relative bioavailability (CRISSEY et al., 2000). Protein concentrations of copper do not appear to change year round, while zinc protein concentrations are only slightly elevated after the start of molt (OSBORN, 1979). Zinc, copper and iron are essential for avian embryonic growth and development and must be deposited in the egg at the time of its formation. Egg production constitutes a major loss of trace elements by the laying hen; approximately 0.7 mg zinc, 0.15 mg copper and between 1mg to 1.9 mg iron in an average turkey egg, placing a significant demand on the tissue metal stores of the hen during egg production (RICHARDS, 1989; CRISSEY et al., 2000). Tannins (polyphenols that reduce absorption) or ascorbic acid, that supposedly enhances absorption, have also been reported (CRISSEY et al., 2000; HELMICK; KENDRICK; DIERENFELD*,* 2011).

All three centers administered supplements to the rehabilitating Magellanic penguins, as show in Table 2. Proper nutrition is a critical determinant in the successful conservation of penguins, either of captive or wild/under rehabilitation populations. There are insufficient data from research with penguins to set nutrient requirements with certainty; therefore, requirements often are based on those of domestic birds (CRISSEY; MCGILL; SIMEONE, 1998; CRISSEY, 2005). Limited references for nutrient ranges are available for unspecified penguin species in captivity (WALLACE; WALSH, 2005a,b; AZA Taxon Advisory Group. 2014) and for little penguins (WILLIAMS, 2009).

Penguin foods are perishable and particularly susceptible to loss of thiamin and vitamin E. Foods for marine animals should be supplemented with 100 IU of vitamin E/kg of diet on a wet basis or approximately 400 IU/kg DM, because prolonged storage can cause depletion of this element. Essential mineral

concentrations in fish appear to be sufficient, although a few fish have relatively low concentrations of copper and manganese (CRISSEY; MCGILL; SIMEONE, 1998; CRISSEY, 2005).

According to Powers et al. (1991), there is considerable evidence from studies in both animals and humans that poor riboflavin status can disturb iron economy. The same author showed that Fe absorption was impaired, and daily Fe loss was increased in riboflavin deficient rats, and concluded that riboflavin deficiency leads to impaired Fe absorption and increased Fe loss from the gastrointestinal tract.

Although qualitative information on feeding habits is available for most penguin species, information on consumed quantities of specific foods, seasonal patterns in diet composition, age, sex, health status and physiological changes (e.g., molt, breeding) and individual variations, should all be considered before starting a supplementation regimen.

Box 2 - Other supplement sources

Institution	Supplements	Dosage/Route	Comments
CRAM	Vitamin B1 (Tiamine)	75 mg PO	Used until 2011
	pantotemic acid 1.5mg, vit C 25 mg, vit A 650 UI, riboflavine 1.5 mg, thiamine 20 mg, vit. B6 1.5mg, vit. E 2 mg, folic acid 50 mcg	1 capsule/PO 3X/week	Since 2011. Only after ingesting solid food (whole fish), until being released.
	Mercepton®	0.5 ml/kg PO SID	For the duration of oral fluid therapy (usually 2-3 weeks)
	Ponteforte®	0.5 ml/kg PO SID	For the duration of oral fluid therapy (usually 2-3 weeks)
FLO (R3)	Potenay® Gold B12	1 ml/PO – single dose	Only for anemic birds (Hct <32% with hypochromic anemia)
	Tiamina 75mg	1cps EOD	Only after ingesting solid food (whole fish), until being released
IPRAM	Glicopam®	1ml PO BID	For the duration of the PO fluid therapy
	Vitamine A, E, C, B1, Riboflavine, B6, biotin, pantotemic acid, folic acid	1 capsule/PO 3X/week	Only after ingesting solid food (whole fish), until being released

Source: CRAM, IPRAM, FLO (R3 Animal).

6.2.4 Increased hemosiderosis prevalence in females in comparison with males

According to Widdonwson and Mccance (1948), the metabolism of iron is controlled, to some extent at any rate, by sex hormones. The liver of sexually mature female rats, mice, hens and eels have been shown to have a greater concentration of iron than the livers of males at a corresponding stage of development (WIDDONWSON; MCCANCE 1948). Such finding disagrees with the ones observed by Cubas, (2007) in toucans (Ramphastidae family) and Pereira et al. (2010) in red-spectacled amazons (*Amazona pretrei*). Although not statistically significant, our findings demonstrated difference between sex and IHH, with higher values in females than in males. With the onset of sexual development, female rats have been found to accumulate hepatic iron at a much greater rate than males, but the percentage of iron rapidly reduced the in the liver of female rats during pregnancy and with a comparatively slow reaccumulation (WIDDONWSON; MCCANCE, 1948).

There are two possible arguments to explain higher female IHH levels found in our study: recent egg laying phase of the female adults (as previously discussed), which could increase mean IHH, or the higher number of females analyzed in this study, reflecting what is commonly observed in the Brazilian coast, as reported by Vanstreelset al. (2011), Reis et al. (2011) and Nunes et al. (2015).

6.3 INDEX OF HEPATIC NECROSIS (IHN)

Centrilobular hepatic necrosis of ischemic origin has been reported in *Macaca mulatta* experimentally infected with *Plasmodium* spp.*,* and in severe, complicated *P. falsiparum* infections in humans, in both cases caused by a major reduction in splanchnic flow induced by heavy *P. falciparum* parasitemia (COOK, 1994). The relationship between the degree of parasitemia and the presence of necrosis was not evaluated in our study; however, Clark et al. (1987) observed midzonal coagulative hepatic necrosis only on *P. vinckei* infected mice presenting parasitemia above 70%. Based on these findings, a suggestion could be made that the hepatic necrosis found

in the malaric penguins could also have been caused by hypoxia secondary to mechanical obstruction of the hepatic vascular system, or by parasitic vasculitis.

The presence of concomitant gastrointestinal nematode infections (Box 1) – specimens CRAM 0628 and FLO-R0282 – may have also contributed with the presence of hepatic necrosis. Experimental helminthic-malaria co-infection models suggest that helminthes might play a role in altering the immune response and progression of malaria, leading to a sharp increase in mice mortality, apparently mediated by cytokine pro-inflammatory pathways involving IL-23/IL-17 e IFN-γ (HELMBY, 2009). The same pathways may have also been triggered as an inflammatory response to merontes, leading to parenchymal liver damage, as seen in the presence of nematodes. Further studies are needed to clarify the mechanisms of this hypothesis.

Hepatic necrosis can be evaluated through analysis of the reticulin framework, recognized as zones of loosely gathered reticulin fibers. However, autolysis may have influenced our findings, once the difference between necrotic and autolysed reticulin fibers is not possible based only on reticulin patterns. Autolysis may have been established through different ways, such as delayed necropsy procedure, and inappropriate sample handling and processing. Therefore, the IHN may have not only included the hepatic necrosis, but also hepatic autolysis present in these penguins, both *Plasmodium*-positive and -negative.

Our results did not show a significant relationship between IHN and age groups, sex, presence or absence of oiled feathers, rehabilitation facilities, total length of time in captivity, length of time in captivity during the summer months or between lineages/species of *Plasmodium* ($p>0.05$). To our knowledge, this is the first study to evaluate the occurrence of necrosis in penguins naturally infected by *Plasmodium*, and consequently, there are no other studies to use as comparative references, which makes further discussion more challenging. Nevertheless, in mammals, significant hepatic necrosis was higher in female Egyptian fruit bats (*Rousettus aegyptiacus*) from the Metropolitan Toronto Zoo, Canada, even when males presented superior or comparable hepatic iron levels (FARINA et al., 2005). The explanation for these findings was not clear and due to the small sample size, such difference was not considered statistically significant by the authors.

Several necrotic patters have been described in birds contaminated by petroleum (FRY; LOWESTINE, 1985; LEIGHTON, 1986; KHAN; RYAN, 1991).

Although some of our birds, both from the positive and *Plasmodium-negative* groups had variable amounts of oil upon admission into the rehabilitation centers, no significant difference was observed on their IHN. This could have been caused by exposure to a small amount of oil, acute oil exposure instead of chronic, fast and efficient cleansing of feathers/treatment for oil ingestion or brief period between oil contamination and necropsy.

7 FINAL CONSIDERATIONS

Magellanic penguins are a frequent subject of biology, ecology and veterinary researches. However, more detailed studies are still needed, not only in an attempt to clarify the details of its annual life cycle and the challenges they face during their annual winter migration, but most importantly, to better understand their behavior and threats to which they are exposed while in Brazilian territory. Avian malaria is one of the most significant threats to the survival of Magellanic penguins during their stay in rehabilitation centers. Understanding the clinical development and contributing factors to the presence and development of this disease are of great importance to its successful rehabilitation and conservation, aside from enabling further understanding on its physiopathological mechanisms.

Our findings indicate that the presence of *Plasmodium* did not interfere with the IHH, but possibly was involved in the process of hepatic necrosis showed by the IHN. Even though avian malaria is able to interfere with the iron metabolism of birds (GOTTDENKER et al., 2008; VANSTREELS et al., 2015; GRILO et al., 2016), in weakened rehabilitation animals, under crowding and stressful situations, other factors, such as concomitant disease, iron supplementation, diet, nutritional status and stage of the annual life cycle, should also be considered.

Further studies are needed to establish the normal iron reference range and nutritional requirements for apparently healthy, wild Magellanic penguins in their colonies, assessing iron levels throughout the different stages of their life cycle, according to sex and age. Field necropsies followed by histopathological evaluation (Perls staining) would provide specific information regarding physiological and pathological hepatic iron deposition.

Data from field studies on Magellanic penguins are more abundant during the period in which these birds are onshore (either breeding or molting), but the study of stranded birds represents an unique opportunity of evaluating the physiological and pathological changes through which these birds are exposed to while during migration. Complete physical exams, biometrics, sexing and comprehensive blood and biochemistry profiles, would allow a good database for comparison with existing data from breeding colonies and captive institutions, but first and foremost, would provide valuable insight on the condition in which these animals arrive to our shore.

This kind of information would ultimately maximize rehabilitation efforts and available resources, and contribute to the discussion of whether the increased number of stranded juveniles reflects just a natural population *Plasmodium-negative* or if the threats faced by these birds during migration are not just “contributing” to these birds mortality, but imposing a real threat to these species’ conservation.

In order to further evaluate the subject of hemosiderosis in captive Magellanic penguins (with and without malaria), further practical challenges would also have to be addressed depending upon the availability of each rehabilitations center’s budget, trained personnel and facilities. We will take the opportunity to contribute with the continuous learning and improvement of the rehabilitation of penguins, by briefly analyzing and discussing the admission protocol basis mostly used in rehabilitation centers caring for these birds.

7.1 PHYSICAL EXAMINATION

Identifying the nutritional status of these birds upon admission allows a more precise and effective treatment, and ultimately maximizes rehabilitation efforts for those animals that still are in a reversible starvation stage. Such assessment requires evaluation of the animal’s body score (1-5), body mass (kg), hydration status, complete blood cell count (blood smear), hematocrit or PCV (packed cell volume), total proteins, and selected basic biochemical parameters, to determine anemia and depending on the findings, the stage of starvation upon admission. There are many sources of reference ranges in Magellanic penguins for the above mentioned parameters (AZA Taxon Advisory Group, 2014; CORAIOLA et al., 2014; SILVA-FILHO; RUOPPOLO, 2014; MARTINS et al., 2015), and altered parameters found in studies of starvation in penguins (JHONSON; WEST, 1973; WILLIAMS et al., 1977; CROXALL, 1982; BROWN, 1984; CHEREL et al., 1988; GROSCOLAS et al., 1988; CHEREL et al., 1988; CHEREL et al., 1992; WILLIAM et al., 1992; GROSCOLAS; ROBIN, 2001).

It is important to consider that dehydrated birds will have increased hematocrit (Hct) values due to hemoconcentration (MCCUE, 2010). To avoid misinterpretation, it is important to provide maintenance fluid therapy plus the rehydration rate required

according to physical findings (sunken eyes, yellowish dry skin, tacky mucosas), and repeats the exam within 48 hours. If such findings are due to dehydration, normal values should be observed on the next laboratory results. Mccue (2010) suggested that during starvation, changes in hemoglobin concentration generally parallel changes in hematocrit, and that reduced hematocrit and hemoblobin concentrations may be attributed to starvation-induced depression in erythropoiesis.

If PCV values are below 20% upon admission and decreases or do not stabilize in 48 hours, a blood transfusion may be necessary. According to Wallace and Walsh (2014), in malarial birds with a stable PCV in the teens, blood transfusion apparently shortens the convalescing time until cloroquine or primaquine treatment takes effect. Guidelines for penguin blood transfusions are described elsewhere (WALLACE; WALSH, 2005a,b). Complete blood cell counts indicate the morphology of cells and are good indicators of hemoparasites and anemia. Other basic parameters that should be evaluated in order to determine the starvation phase upon admission are listed on Table 2.

In our opinion, iron supplementation should be avoided at all times, based on the following arguments: (1) even if asymptomatic, these animals may sustain infectious, bacterial, viral or fungal pathogens; (2) considering how challenging it is to *Plasmodium-negative* malaria vectors, how susceptible penguins are to this disease, its incidence in rehabilitation centers worldwide, and the contradictory studies on human malaria, the risk of iron supplementation would surpass the possible benefits; (3) there are no established nutritional iron requirements for penguins, reference iron levels on healthy birds, or recommended iron supplementation doses for penguins; (4) iron supplements are manufactured based on dogs, cats and mostly poultry needs, which does not necessarily reflect the needs of penguins; (5) IM injections of iron are extremely painful and may case local necrosis on injection sites.

Box 3 - Basic parameters used to classify and evaluate the starvation phase of penguins

Phase I	Phase II	Phase III
•increased free fatty acids •decreased uric acid and alanine • plasma iron concentration •white cream colored droppings	•low levels of plasma uric acid •increased P-hydroxybutyrate. • plasma iron concentration •greenish and increasingly translucid droppings (reflecting body-protein utilization)	•increased uric acid •increased urea •increased alanine •decreased P-hydroxybutyrate. • decreased plasma protein, especially albumin • plasma iron concentration • increase in plasma free fatty acids at the beginning of phase III, followed by a decrease infatty acids • decreased triglycerides and ketone bodies •milk like brownish colored droppings (which can be attributed to a higher uric-acid content in excreta caused by the rise in nitrogen excretion)

Source: (GROSCOLAS, 1982a, 1986 and 1990; LE MAHO, 1983; CHEREL; LE MAHO, 1985; ROBIN et al. 1987; CHEREL et al. 1988; CHEREL; LELOUP; LE MAHO, 1988; ROBIN et al. 1988; GROSCOLAS; ROBIN 2001;).

7.2 PENGUINS DIAGNOSED WITH MALARIA

As discussed earlier, penguins are very susceptible to avian malaria, a disease that potentially threatens their rehabilitation. Diagnostics and treatment of avian malaria in penguins are beyond the scope of this discussion. However, in attempt to evaluate the role of iron in this disease, and monitor the possible development of hepatic hemosiderosis and necrosis in positive birds, further diagnostic testing is necessary. Ideal diagnostic possibilities include ultrasound (US), US-guided biopsy and routine hepatic biochemical profiles and plasma iron concentration. Nevertheless, such diagnostic are not compatible with the economical means of most rehabilitation facilities, especially in a daily routine, and in the case

the US, the bird's condition would be the main determinant for the performance of the exam. Therefore, our suggestion for realistic and objective diagnostics, aiming for the patient's welfare and highest chances of rehabilitation, include: (1) CBC; (2) plasma iron concentration; (3) basic hepatic biochemical profile; and (4) in case of death, *post mortem* examination within 4 hours, and sample collection and processing as described below.

7.3 POST MORTEM EXAMINATION

Perform through necropsy examination, associated with complete history of those animals that die while under care. When working with wild free ranging animals, clinical history is uncertain and deductive most of the time, therefore, any external signs observed during visual inspection, history provided by the animal's rescuer, and careful data collection and maintenance during rehabilitation are of uttermost importance in the evaluation of necropsy findings. Appropriate data collection and keeping, and complementary diagnostics (regarding hemosiderosis, the use of Perls stain on histopathologic material) are also important on the elaboration of the *causa mortis*. And finally, all findings should be evaluated as a whole, providing a view of the "population" under rehabilitation.

7.4 DIET IN CAPTIVITY

Further studies are needed to establish the iron nutritional requirements of Magellanic penguins and iron contents of seafood provided to these animals while in captivity. Ideally, such diet should resemble, as close as possible, their diet in the wild. Although there are several studies regarding their diet on breeding sites, such information on wintering grounds are still scarce and contradictory.

Hepatic hemosiderosis in captive birds has been extensively studied, but still holds several questions to be answered, especially regarding species not know as "susceptible", such as penguins. Iron is a vital element to most life form, including

birds, species in which iron metabolism is still poorly understood. Despite the challenges previously discussed here, regarding the study of free ranging wild Magellanic penguins and the many variables regarding hepatic hemosiderosis in birds, the adoption of the above mentioned suggestions applied to the rehabilitation of penguins, would further contribute to this subject, both in *Plasmodium* sp. - infected and non-infected Magellanic penguins.

8 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 finding s 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.

In view of all these issues discussed in this study and the great rehabilitation effort by rehabilitation centers involved in Magellanic penguins care along the coast of Brazil, further studies are still needed to: (1) identify all the *Plasmodium* lineages affecting these birds while under rehabilitation, in an attempt to understand the diversity, prevalence and distribution patterns of *Plasmodium*; (2) identify possible wild and free-ranging reservoirs living in close proximity with these birds while under treatment; (3) strive to implement efficient preventive and monitoring measures of captive populations; (4) decrease the vector incidence in rehabilitation facilities; and (5) investigate the (still remote) possibility of wild birds getting infected in their colonies or during migration, prior to their arrival to winter grounds.

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, on this latter subject, suggesting studies on the plasma iron levels of Magellanic penguins in their colonies in an attempt to determine their normal iron concentration range, as well as evaluate captive specimens without a history of iron supplementation (both *Plasmodium*-positive and *Plasmodium*-negative). We also emphasize 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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APPENDIX A

Annex I. Box 4: Individual history of the positive and *Plasmodium-negative* animals based on IHH, age group, presence of oil contamination, sex, total stay in the rehabilitation center, stay in the rehablitation center during summer, year of admission, weight on admission and concurrent diseases. ND: not determined. Blank cells: information not available. NCD: no concurrent disease.

Positive Animals												
Animal number (ID)	IHH	IHN	*Plasmodium* species	Family (Group)	Age group	Oil	Sex	Total Period	Summer Period	Year of admission	Weight on admission (gr)	Concurrent Diseases
IPRAM-ES114	0,228	0,066	P. elongatum	*Rufia*	Juvenile	No	Female	180	89	2012	2.260	NCD
IPRAM-ES167	0,004	0,045	P. cathemerium	*Hemamoeba*	Juvenile	No	Female	137	55	2012	1 915	NCD
IPRAM-RJ128	0,148	0,027	P. cathemerium	*Hemamoeba*	Juvenile	No	Female	74	66	2012	2.050	NCD
FLO-IF506	0,108	0,066	P. tejerai	*Hemamoeba*	Juvenile	No	Male	129	129	2008	2.400	NCD
FLO-IF584	0,015	0,118	P. tejerai	*Hemamoeba*	Juvenile	No	ND	125	125	2008	2.600	NCD
FLO-IF593	0,382	0,046	P. tejerai	*Hemamoeba*	Juvenile	No	ND	13	13	2009	3.450	Molting
FLO-R0040	0,319	0,016	P. tejerai	*Hemamoeba*	Adulto	No	Female	106	21	2010	3.300	NCD
FLO-R0091	0,094	0,066	P. tejerai	*Hemamoeba*	Juvenile	No	Male	20	20	2011	2.100	NCD
FLO-R0093	0,062	0,031	Plasmodium sp. linhagem E	Linhagens	Adulto	No	Female	61	26	2011	3.220	NCD

FLO-R0263	0,048	0,026	Plasmodium sp. linhagem G	Linhagens	Juvenile	No	Male	163	143	2012	3.060	Avian Poxvirus Molting 2nd degree Bumblefoot
FLO-R0268	0,454	0,074	Plasmodium sp. linhagem G	Linhagens	Juvenile	No	Female	135	135	2012	2.700	Avian Poxvirus Abnormal molting Bilateral 1st degree Bumblefoot
FLO-R0272	0,058	0,103	P. cathemerium	*Hemamoeba*	Juvenile	No	Male	61	61	2012	3.240	Avian Poxvirus Bilateral 3rd degree Bumblefoot Abnormal molting
FLO-R0282	0,146	0,162	Plasmodium sp. linhagem H	Linhagens	Adulto	No	Male	26	26	2012	3.260	Molting Bilateral 1st degree Bumblefoot Nematodes in the oral cavity and gastrointestinal tract
FLO-R0284	0,183	0,11	P. cathemerium	*Hemamoeba*	Juvenile	No	Female	94	94	2012	2.560	Abnormal molting 1st degree Bumblefoot
FLO-R0290	0,042	0,032	P. cathemerium	*Hemamoeba*	Juvenile	No	Male	73	73	2013	-	Avian Poxvirus Abnormal molting Non classified Bumblefoot
CRAM-0580	0,107	0,023	Plasmodium sp.	*Plasmodium*	Juvenile	No	ND	223	152	2000	3.890	NCD
CRAM-1229	0,128	0,124	Plasmodium sp.	*Plasmodium*	Juvenile	No	Female	79	79	2005	2.910	Aspergilosis
CRAM-	0,083	0,033	Plasmodium sp.	*Plasmodium*	Juvenile	Yes	ND	136	20	2001	2.645	Nematode in stomach

0628												
CRAM-0630	0,138	0,066	Plasmodium sp.	*Plasmodium*	Adulto	Yes	Female	119	28	2001	2.750	NCD
CRAM-2125	0,15	0,112	Plasmodium sp. linhagem D	Linhagens	Juvenile	Yes	Female	58	45	2009	2.810	Aspergilosis
CRAM-2127	0,276	0,116	P. nucleophilum	Linhagens	Juvenile	Yes	Female	123	112	2009	2.802	NCD
CRAM 2876	0,011	0,033	P. cathemerium	*Hemamoeba*	Juvenile	No	Male	-	-	2014	-	NCD
CRAM 2911	0,014	0,036	P. elongatum	*Rufia*	Adulto	No	Female	150	37	2013	-	NCD
CRAM 2912	0,069	0,042	P. elongatum	*Rufia*	Adulto	No	Male	151	37	2013	-	NCD

Animais *Plasmodium-negativee*										
Animal number (ID)	IHH	IHN	Age group	Oil	Sex	Total Period	Summer Period	Year of admission	Weight on admission (gr)	Concurrent Diseases
IPRAM-ES111	0,009	0,132	Juvenile	Yes	Female	151	58	2012	1. 750	NCD
IPRAM-ES144	0,181	0,185	Juvenile	Yes	Female	143	57	2012	1.865	NCD
IPRAM-ES145	0,116	0,178	Juvenile	Yes	Female	161	75	2012	2.015	NCD
IPRAM-ES197	0,029	0,161	Juvenile	Yes	Female	130	52	2012	2.250	NCD
FLO-F1244	0,009	0,291	Juvenile	Yes	ND	62	0	2009	-	NCD
FLO-F1246	0,056	0,227	Adulto	Yes	ND	62	0	2009	-	NCD
CRAM-2096	0,134	0,066	Juvenile	Yes	Male	77	0	2009	2248	NCD
CRAM-2098	0,065	0,091	Juvenile	Yes	Male	89	0	2009	2268	NCD
CRAM-2128	0,125	0,08	Juvenile	Sim	Male	65	53	2009	3110	NCD