UNIVERSIDADE DE LISBOA

Faculdade de Medicina Veterinária

CHARACTERIZATION OF INFECTION BY MALARIA PARASITES IN PENGUINS HOUSED IN ZOOLOGICAL COLLECTIONS

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To ISIS (International Species Identification System) for providing me, free of charge, the database containing the zoological gardens that have penguin colonies in the entire world.

Abstract

Characterization of infection by malaria parasites in penguins housed in zoological collections

Avian malaria is, if not the main, one of the most important causes of mortality in penguins housed in zoological collections. Knowledge of prevalence in zoos and the control measures applied (diagnostic, treatment and prophylaxis) allows other zoos to increase their colonies protection. In order to evaluate malaria infection in penguin colonies and zoos prophylactic programs, a survey was specifically designed to gather this information from different zoological gardens from Europe, North America and Asia.

Fifteen out of the forty zoos that answered (37,5%) test their colonies for malaria, revealing a global prevalence of 12,5% (5 zoos) of institutions with infected penguins. Diagnostic techniques most currently used are optical microscopy (11), histopathology (10) and observation of clinical signs (9). Twelve zoos use combinated techniques diagnosis protocols. Mixed infections with other blood parasites were reported by one zoo. Significant differences were not presented in clinical and laboratorial signs presented, being lethargy (4) the most prevalent sign. Nine zoos (22,5%) use treatment protocols on detected cases. No significant differences were observed in the protocols being the standard ones for most institutions based on sulfadiazine and pyrimethamine, chloroquine and primaquine. Twenty two zoos (55%) use prophylaxis protocols, being primaquine the most common drug in eight zoos. Seventeen zoos (42,5%) reported that no preventive measures besides preventive therapeutic protocols are used, while reducing the number of potential water catchment containers in order to eliminate the mosquito breeding sites available, was the measure most commonly adopted in 13 zoos (32,5%).

To the author's knowledge, these are the first reports on penguins of anorexia and vomit when using sulfadiazine and pyrimethamine; anorexia when using primaquine and chloroquine and epileptic seizures, sunburns around the eyes and death when using pyrimethamine.

This study shows that many zoological gardens do not have routine control programs for malaria in their penguin colonies. Dissemination of these results allows for a better understanding of this problem, raising awareness and potentially inducing new perspectives on its control.

Key-words: penguin, malaria, diagnosis, treatment, prophylaxis, zoological collections.

Resumo

Caracterização da infeção por agentes de malária em pinguins alojados em coleções zoológicas

A malaria aviária é, se não a principal, uma das causas mais importantes de mortalidade em pinguins alojados em coleções zoológicas. O conhecimento da prevalência em zoológicos e das medidas de controlo aplicadas (diagnóstico, tratamento e profilaxia) permite a outros zoológicos melhorar a proteção das suas colónias. Com o objetivo de avaliar a infeção por agentes de malária em colónias de pinguins e os programas profiláticos dos zoológicos, foi criado um questionário para reunir esta informação em diferentes jardins zoológicos da Europa, América do Norte e Ásia.

Quinze dos quarenta zoológicos que responderam (37,5%) testam as suas colónias relativamente a malária, revelando uma prevalência global de 12,5% (5 zoológicos) de instituições com pinguins infetados. As técnicas de diagnóstico mais frequentemente utilizadas são a microscopia ótica (11 zoos), a histopatologia (10) e a observação de sinais clínicos (9). Doze zoológicos utilizam protocolos de diagnóstico com diferentes técnicas. Infeções mistas com outros parasitas sanguíneos foram referidas por um zoo. Não foram apresentadas diferenças significativas nos sinais clínicos e laboratoriais apresentados, sendo a letargia o mais frequente (4). Nove zoológicos (22,5%) usam protocolos de tratamento em casos detetados. Não foram observadas diferenças significativas nos protocolos utilizados, sendo os padrões para a maioria das instituições baseados em sulfadiazina e pirimetamina, cloroquina e primaquina. Vinte e dois zoológicos (55%) usam protocolos de profilaxia, sendo a primaquina a substância mais comum em oito zoológicos. Dezassete zoológicos responderam que não utilizam medidas preventivas para além dos protocolos terapêuticos preventivos, enquanto que a redução do número de potenciais recipientes de captação de água, de modo a eliminar os locais de reprodução disponíveis para os mosquitos, foi a mais comummente adotada em 13 zoológicos (32,5%).

No conhecimento do autor, estas são as primeiras descrições em pinguins de anorexia e vómito ao administrar sulfadiazina e pirimetamina; de anorexia ao administrar primaquina e cloroquina e de ataques epiléticos, reacções de fotosensibilidade e morte ao administrar pirimetamina.

Este estudo demonstra que muitos jardins zoológicos não têm programas rotineiros de controlo da malária relativamente às suas colónias de pinguins. A divulgação destes resultados permite uma melhor compreensão deste problema, criando sensibilização e, potencialmente, conduzindo a novas perspetivas no seu controlo.

Palavras-chave: pinguim, malária, diagnóstico, terapêutica, profilaxia, coleções zoológicas.

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List of Abbreviations and Symbols

% - Percentage

µl – Microliter

µm – Micrometer

18S – Subunit

a.m. – *ante meridiem*

AAWV - American Association of Wildlife Veterinarians

AAZV – American Association of Zoo Veterinarians

ACZM - American College of Zoological Medicine

AFDPZ - Association Française des Parcs Zoologiques

AIZA - Associación Ibérica de Zoos y Acuarios

ALPZA - Asociación Latinoamericana de Parques Zoológicos y Acuarios

ALT - Alanine aminotransferase

AP - Alkaline phosphatase

ARCA – Área de Recuperación y Conservación de Animales

BIAZA - British Association of Zoos and Aquariums

CR – Critically Endangered

DD – Data Deficient

DNA – Deoxyribonucleic acid

e.g. – *exempli gratia*

EAZA - European Association of Zoos and Aquariums

EAZWV - European Association of Zoo and Wildlife Veterinarians

ECZM - European College of Zoological Medicine

ELISA – Enzyme-linked immunosorbent assay

EN – Endangered

EW – Extinct in the Wild

EWDA - European Wildlife Disease Association

EX – Extinct

FET - Fisher's exact test

FVM – UL – Faculty of Veterinary Medicine – University of Lisbon

GGTP - Gamma-glutamyltranspeptidase

HRP2 - Histidine rich protein 2

ISH – *In-situ* hybridization

ISIS - International Species Identification System

ISSG - Invasive Species Specialist Group

LC – Least Concern

LLP - Lifelong Learning Programme

mg/animal – Milligram per animal

mg/kg – Milligram per kilogram

NE – Not Evaluated

NT – Near Threatened

P. – *Plasmodium*

PCR - Polymerase Chain Reaction

pLDH – *Plasmodium* lactate dehydrogenase

p.m. – post meridiem

RNA – Ribonucleic acid

SID – *Semel in die*

spp. – species

USA – United States of America

VLDL – Very light-density lipoprotein

VU – Vulnerable

WAZA - World Association of Zoos and Aquariums

WDA - Wildlife Disease Association

WHO - World Health Organization

Introduction

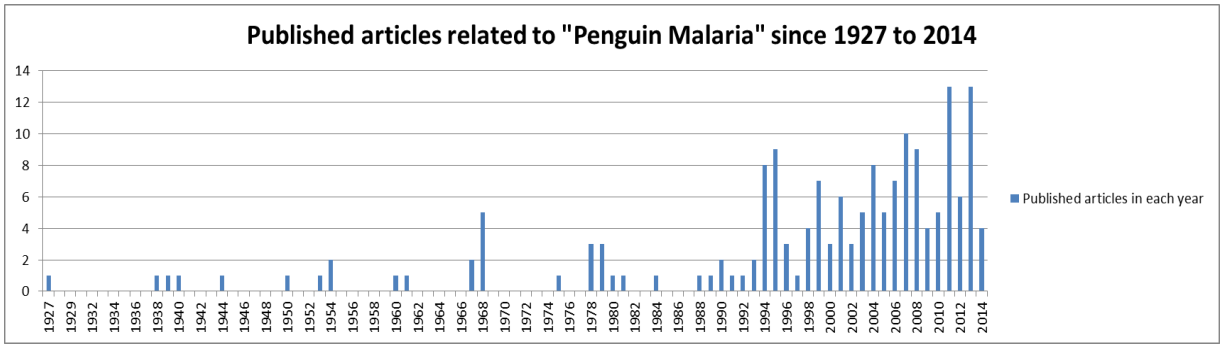
Avian malaria is a widespread disease (Cranfield, 2003, Marzal, 2012), appearing to cause little harm in bird populations where it's prevalent. This is due to the co-evolution process, where parasites try to increase their transmission potential by intensification of infecciosity and birds respond developing effective immune responses to the infection (Huijben, Schaftenaar, Wijsman, Paaijmans & Taaken, 2007). However, animals that come from low temperature, arid and windy habitats (like the penguins) are susceptible individuals for the development of the infection (Valkiūnas, 2005).

Penguins are originally from cold southern regions, where vector's establishment is unlikely (Fordyce & Jones, 1990). Since none or little contact was made in the wild with *Plasmodium* spp., penguins had a limited chance to develop immunity resistance (Jones & Shellam, 1999).

This is, without a doubt, the most popular avian groups in zoological exhibitions (Gailey-Phipps, 1978). With their transportation to zoological gardens worldwide, exposure to the vector enhanced the opportunity for a more intense transmission and an often fatal infection (Jones & Shellam, 1999).

Avian malaria it's a major preoccupation in zoological collections. This disease represents the most important cause of mortality in zoological collections' colonies exhibited outdoor (Alves, 2002; Cranfield, 2003) and a significant cause of death in rehabilitation centers (Cranfield, 2003; Campos & Almosny, 2011), starting to alert the scientific community (Figure 1). Reports in other species kept in zoological collections include deaths in Keas (*Nestor notabilis*) (Bennett, Bishop & Peirce, 1993), Inca terns (*Larosterna inca*) (Bristol Zoo Gardens, 2008) and Atlantic Puffins (*Fratercula arctica*) (Loupal & Kutzer, 1996).

Figure 1 - Distribution in number of articles published over the period 1927-2014 based on a search in Google Scholar® with the terms "penguin malaria". (Original). Similar information about avian and bird malaria articles over the period 1934 – 2011.

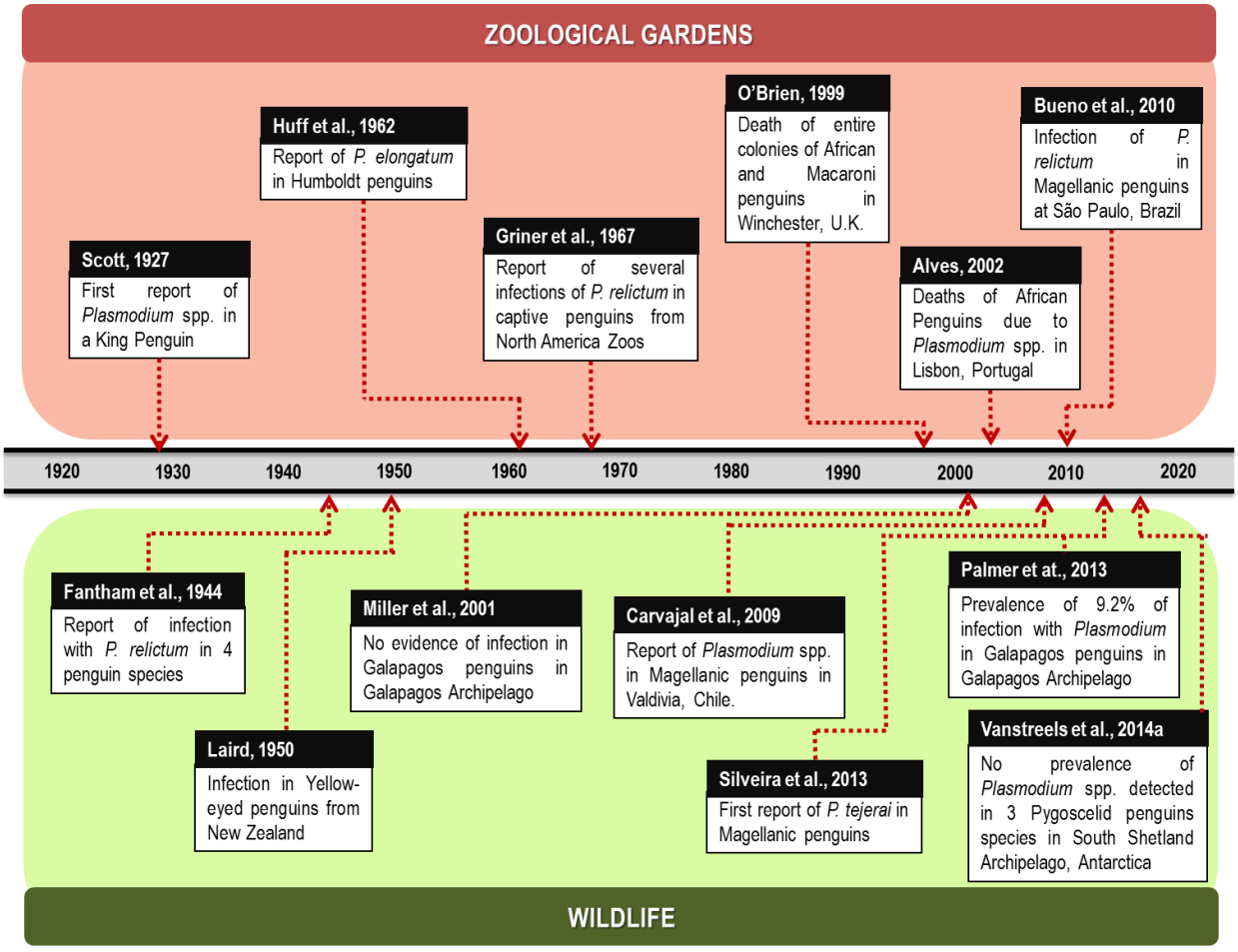


Weissenböck *et al*. (2011) state that a large number of avian individuals kept in zoological gardens from temperate parts of the globe is dying of avian malaria. Jones & Shellam (1999)

alerted for the possibility that infections in captive penguins could be more widespread than suggested by records.

Pathogens are one of the main motors of wildlife populations' extinction (Smith, Sax & Lafferty, 2006) and concerns that malaria poses a conservation threat for wild penguins' colonies is based on rapid mortality in outdoor exposed captive penguins (Vanstreels *et al*., 2014b). It is known that vector migrations associated with climate changes and vector introduction by anthropogenic action in non-endemic habitats represents a risk for endangered species. An example of this problem is the *Plasmodium* spp. prevalence stated in the Galapagos penguin (*Spheniscus mendiculus*) population (Levin, Outlaw, Hernán-Vargas & Parker, 2009; Palmer *et al*., 2013). An example regarding evolution of reports related with penguin malaria is represented in Figure 2.

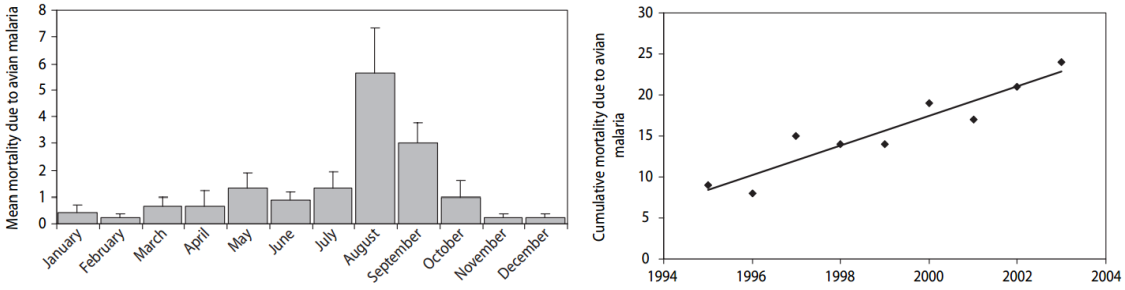
Figure 2 - Schematic representation of a few reports regarding captive and wild penguins' infection with malaria. (Original).



Although different papers report penguin infections in different collections, only Huijben, Schaftenaar, Wijsman, Paaijmans & Taaken (2007) present a study where a survey was conducted in 8 zoological gardens (Belgium and The Netherlands) in order to question the prevalence of avian malaria. This works consists of a period of study between 1995 and

2003. Results showed that all zoos had experienced mortality due to this disease, especially in the penguin colonies. Mortality was experienced all year round, with predominance in the period from August to September and the annual cumulative number of cases of avian malaria increased over the years (Figure 3).

Figure 3 - Monthly distribution (left) and annual cumulative (right) mortality due to avian malaria in bird populations of 8 zoological gardens during the period between 1995 to 2003 stated in the work of Huijben, Schaftenaar, Wijsman, Paaijmans & Taake (2007).



Training Period Activities

During the 6th year of his Integrated Masters in Veterinary Medicine, the author went through a training period of five months in "Oceanogràfic" (Valencia, Spain), since October 1st to February 28th, under the LLP/Erasmus Program.

The training period was supervised by Dr. Daniel Garcia Párraga ("Oceanogràfic") and co-supervised by Prof. Doctor Luís Manuel Madeira de Carvalho (FVM-UL).

"Oceanogràfic" is the largest aquarium in Europe and part of City of the Arts and the Sciences. It has exhibitions of earth's main marine ecosystems (Antarctic, Arctic, Islands, Red Sea, Atlantic Ocean, Temperate and Tropical Seas, Mediterranean and Wetlands) and hosts more than 45.000 specimens of 500 different species (Table 1). "Oceanogràfic" has a conservation and rehabilitation area for marine fauna – A.R.C.A. del Mar – specially designed for the reception and rehabilitation of sea turtles and dolphins.

Table 1 - List of some animal species present at "Oceanogràfic" (Valencia) and their classification according to the IUCN Red List of Threatened Species: NE – Not Evaluated, DD – Data Deficient LC – Least Concern, NT – Near Threatened, VU – Vulnerable, EN – Endangered, CR – Critically Endangered, EW – Extinct in the Wild, EX - Extinct (http://www.iucnredlist.org).

Mammals	Birds	Reptiles	Fish	Invertebrate
Beluga *(Delphinapterus leucas)* **NT**	**Gentoo Penguin** *(Pygoscelis papua papua)* **NT**	**Aldabra Giant Tortoise** *(Dipsochelys dussumieri)* **VU**	**Sand Tiger Shark** *(Carcharias taurus)* **NT**	**Japanese Spider Crab** *(Macrocheira kaempferi)* **NE**
Walrus *(Odobenus rosmarus)* **DD**	**Humboldt Penguin** *(Spheniscus humboldti)* **VU**	**Mediterranean Turtle** *(Mauremys leprosa)* **NE**	**Valencia Toothcarp** *(Valencia hispanica)* **CE**	**Common Octopus** *(Octopus vulgaris)* **NE**
Common Bottlenose dolphin *(Tursiops truncatus)* **LC**	**Chilean Flamingo** *(Phoenicopterus chilensis)* **NT**	**Green Iguana** *(Iguana iguana)* **NE**	**Green Moray** *(Gymnothorax funebris)* **NE**	**Purple Sea Urchin** *(Sphaerechinus granularis)* **NE**
Harbor Seal *(Phoca vitulina)* **LC**	**Great White Pelican** *(Pelecanus onocrotalus)* **LC**	**Red-footed Tortoises** *(Chelonoidis carbonaria)* **NE**	**White Seabream** *(Diplodus sargus sargus)* **NE**	**Jewel Anemone** *(Corynactis viridis)* **NE**
South American Sea Lion *(Otaria flavescens)* **LC**	**Little white egret** *(Egretta garzetta)* **LC**	**Loggerhead Turtle** *(Caretta caretta)* **EN**	**Humphead Wrasse** *(Cheilinus undulatus)* **EN**	**Red Coral** *(Corallium rubrum)* **NE**

In Valencia, the author followed the routine activities performed by the veterinary staff (prophylactic measures regarding the collection, rounds to get updated informations about the animals' behaviour, literature research regarding new cases of diseased animals). The author, as the other interns, had a weekly rotative task position that could vary from preparing the medication for the animals in treatment, being responsible for all the necropsies and laboratory tasks or helping in ARCA activities. Apart from that, the author had

the opportunity to visit “La Granja del Saler Wildlife Rehabilitation Center” and watch ecography and surgery interventions on wild animals, as well as the Education and Investigation Department of the “Oceanogràfic”, where Dr. Susana Ortiz explained the educational program and activities being held on this institution. The amount of hours spent in each activity is stated in Table 2.

Table 2 - Hours spended in each activity during the training period.

Activity	Hours
Prophylatic measures and routine rounds	241
Laboratory	315
Necropsy	105
Clinical activities	42
A.R.C.A. activities	147
Training sessions	2
Nocturnal watches	8
Research activities	20
Total	880

Regarding the activities performed, the author had the opportunity to do:

- Prophylatic measures – perform the physical exam on cetaceans, reptiles, fish and birds; collect blowhole samples from bottlenose dolphins (*Tursiops truncatus*) and belugas (*Delphinapterus leucas*); collect blood from loggerhead turtles and aquatic birds; prepare imunostimulants to be added to fish feeding;
- Laboratory exams – process blood samples for hematology, biochemistry and coagulation analysis; perform blood smears and observe them under the microscope for evaluation and differencial leucocyte count; perform urianalysis, gastric content analysis and fecal analysis; perform parasitological, microbiological and fungal analysis/cultures;
- Necropsy procedures – conduct the necropsy of mammals, fish, aquatic birds and reptiles;
- Imaging Diagnosis – perform x-rays on loggerhead turtles and birds, ecographic exam on belugas (*Delphinapterus leucas*) and loggerhead turtles (*Caretta caretta*);
- Surgery techniques – perform a exploratory laparoscopy for sex determination in loggerhead turtles, assisting on branchial repair surgery on an Yellow-edged morayeel (*Gymnothorax flavimarginatus*), aid on the penile prolapse of a Mediterranean Turtle (*Mauremys leprosa*);

- Medical training behaviours – help in the blood collection training of harbor seals (*Phoca vitulina*) and abdominal ultrasound training of South American sea lion females (*Otaria flavescens*);
- Research activities – assistance in two research projects: use of thermography exam on walruses (Odobenus rosmarus) to evaluate internal temperature; and determination of the breeding pattern and gas composition in loggerhead turtle;
- Others – taking daily rectal temperature and intermammary distance in a pregnant bottlenose dolphin, administration of injectable drugs to reptiles and birds, perform daily physical therapy on a loggerhead turtle recovering from a reconstruction surgery of a fractured humerus (Figure 4).

Figure 4 - Physical therapy on a logerhead turtle recovering from fractered humerus. (Original)

1. Etiology

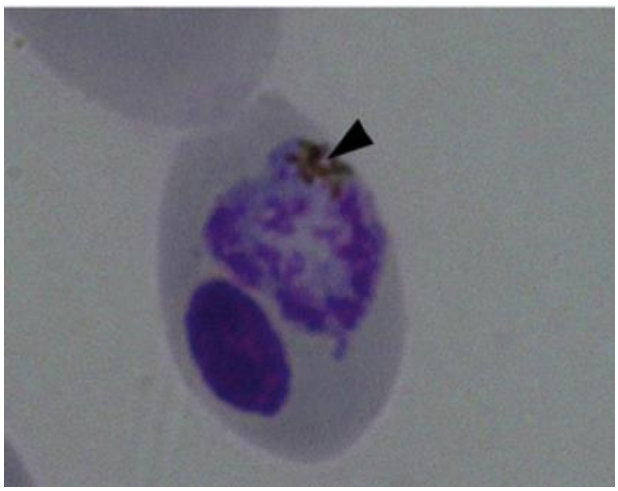
1.1. Taxonomy

Hemosporidians, of the *Sporozoa* class (Marzal, 2012), represent a group of obligatory heteroxen protists that use dipteran hematofagous insects as vectors. They have a worldwide distribution, except for Antarctic (Campos, 2011).

Because life cycles of genera *Plasmodium*, *Haemoproteus* and *Leucocytozoon* are similar, controversial views regarding the classification as "malaria parasites" have been generated. The use of genetic molecular techniques helped to establish the phylogeny of the group and due to the fact that both parasites provoque anaemia and deposition of haemozoin, it has been advocated that *Haemoproteus* should be considered among the malaria parasites because of their genetical proximity. However, as they differ in important aspects (vectors, life cycles and epidemiology), the more traditional view accepts only *Plasmodium* as the true malaria parasites (Pérez-Tris *et al*., 2005; Valkiūnas *et al*., 2005; Huijben, Schaftenaar, Wijsman, Paaijmans & Taaken, 2007; Marzal, 2012).

The Plasmodiidae family contains only one genus - *Plasmodium* (Marchiafava & Celli, 1885). Its members go through merogony in vertebrate hosts tissue and erythrocytes, producing malarial pigments (haemozoin) (Campos, 2011) (Figure 5). Avian malaria parasites are distributed in five subgenera: *Bennettinia*, *Giovannolaia, Haemamoeba*, *Huffia* and *Novyella* (Bennett, Bishop & Peirce, 1993; Campos, 2011). Zoonotic risk does not exist from avian malaria species (American Association of Zoo Veterinarians [AAZV], 2013).

Figure 5 - *Plasmodium tejerai* erythrocytic meront showing hemozoin pigment (black arrow) (Courtesy of Dr. Érika M. Braga, Departamento de Parasitologia, Instituto de Ciências Biológicas - Universidade Federal de Minas Gerais, Brazil).



In captive penguins, four malaria parasites have been recorded: *Plasmodium relictum* (Cranfield, 2003), *P. elongatum* (Alves, 2002), *P. juxtanucleare* (Grim *et al*., 2003; Weissenböck *et al*., 2011) and *P. tejerai* (Silveira *et al*., 2013). A case report of a King penguin (*Aptenodytes patagonicus*) infected with *P. cathemerium* has been questioned because of the lack of methods and criteria for species identification and also because the great morphological similarity of this parasite and *P. relictum* (Vanstreels *et al*., 2014b). Reports of other blood parasites than *Plasmodium* spp. in penguins are stated in Table 3.

Table 3 – Literature references to blood parasites infections other than *Plasmodium* spp. in penguins.

Reference	Parasite	Species	Location
Fallis, Bisset & Allison, 1976	*Leucocytozoon tawaki*	Fiordland crested penguin (*Eudyptes pachyrhynchus)*	Kaikoura, New Zealand
Jones & Woehler, 1989	*Trypanosoma eudyptulae*	Little Penguin (*Eudyptula minor*)	Tasmania, Australia
Earle, Huchzermeyer, Bennett & Brassy, 1993	*Babesia peircei*	Black-footed Penguin (*Spheniscus demersus*)	Cape Town, South Africa
Sano *et al*., 2005	*Dirofilaria immitis*	Humboldt penguin (*Spheniscus humboldti*)	Akita, Japan
Cannell *et al*., 2013	*Haemoproteus* spp.	Little Penguin (*Eudyptula minor*)	Penguin Island, Australia

1.2. Vectors

As mentioned before, blood-sucking insects from order *Diptera*, specially family Culicidae, are the vectors of malaria parasites of penguins. Only the females feed on blood and, consequently, participate in spreading the infection (Valkiunas, 2005).

Avian malaria has a wide geographical distribution, implying a wide variety of vector species (Valkiunas, 2005). The most documented vectors are *Culex* spp. (Huijben, Schaftenaar, Wijsman, Paaijmans & Taaken, 2007).

Plasmodium relictum is known to complete its life cycle in 29 species of the *Culicidae* family (Valkiunas, 2005), including the genera *Aedes* (*Aedes aegypti*, *A. communis*, *A. concolor, A. dorsalis*, *A. mariae* and *A. vexans*), *Anopheles* (*Anopheles albimanus*, *A. crucians*, *A. freeborni*, *A. quadrimaculatus* and *A. subpictus*), *Culex* (*Culex apicalis*, *C. bitaeniorhynchus*, *C. fuscanus*, *C. gelidus*, *C. hortensis*, *C. pipiens*, *C. quinquefasciatus*, *C. salinarius*, *C. stigmatosoma*, *C. tarsalis*, *C. territans*, *C. theileri* and *C. whitmorei*), *Culiseta* (*Culiseta annulata* and *C. longiareolata*) (Valkiunas, 2005; Huijben, Schaftenaar, Wijsman, Paaijmans

& Taaken, 2007), *Armigeres* and *Mansonia* (Valkiunas, 2005) and *Aedeomya* (Goswami & Swamy, 2013).

P. juxtanucleare is more selective regarding vectors, since sporogony has only been successful in members of the genus *Culex* (*C. annulus, C. gelidus, C. pipiens fatigans, C. p. pallens, C. pseudovishnui, C. sitiens* and *C. tritaeniorhynchus*) (Valkiunas, 2005).

Some species are susceptible to *P. elongatum,* such as *Culex pipiens*, *Culex quinquefasciatus*, *Culex restuans*, *Culex salinarius*, *Culex tarsalis*, *Culex territans* and *Aedes triseriatus* (Huff, 1965). Beier & Trips (1981) reported that *Culex pipiens* and *Culex restuans* were responsible for causing malaria to African penguins (*Spheniscus demersus*) at the Baltimore Zoo.

P. tejerai vectors have not been reported (Valkiunas, 2005).

1.3. Life Cycle & Morphology

Plasmodium spp. are obligate heteroxenous parasites, which develop in two kinds of hosts: a intermediate host (e.g. the penguin) and a vector (blood-sucking dipterans), the definitive host (Valkiūnas, 2005; Campos, 2011). Different species of avian malaria parasites have similar life cycles (Huijben, Schaftenaar, Wijsman, Paaijmans & Taaken, 2007). The life cycle of malaria parasites of penguins is represented in Figure 6.

When the females of dipterans are feeding on the blood of the penguin, the saliva that is injected in the bird's blood stream contains enzymes that increase blood uptake and prevent coagulation, as well as, sporozoites that are picked up by macrophages and reticuloendothelial cells (Invasive Species Specialist Group [ISSG], 2005; Huijben, Schaftenaar, Wijsman, Paaijmans & Taaken, 2007). Most vectors feed on exposed skin (like the areas surrounding the eyes, the legs, the feet and the beak) (Alves, 2002).

The sporozoites turn into schizonts or exoerythrocytic meronts as they undergo asexual division in cells of mesodermal origin (endothelial cells of capillaries and the cells of hemopoietic and lymphoid macrophage systems) (Figure 6, number 1) (Alves, 2002; Valkiūnas, 2005; Campos, 2011). The meront will suffer multiple divisions to form uninuclear merozoites. These stages serve as distribution of the parasite within the organism of the penguin and there can be several cycles of exoerythrocytic merogony, resulting in a major growth of the parasite population. Merozoites can either undergo new cycles of exoerythrocytic merogony or initiate the formation of gametocytes (sexual stages), which occur mainly in mature erythrocytes. The erythrocytic meronts develop in cells of the erythrocytic series (Valkiūnas, 2005).

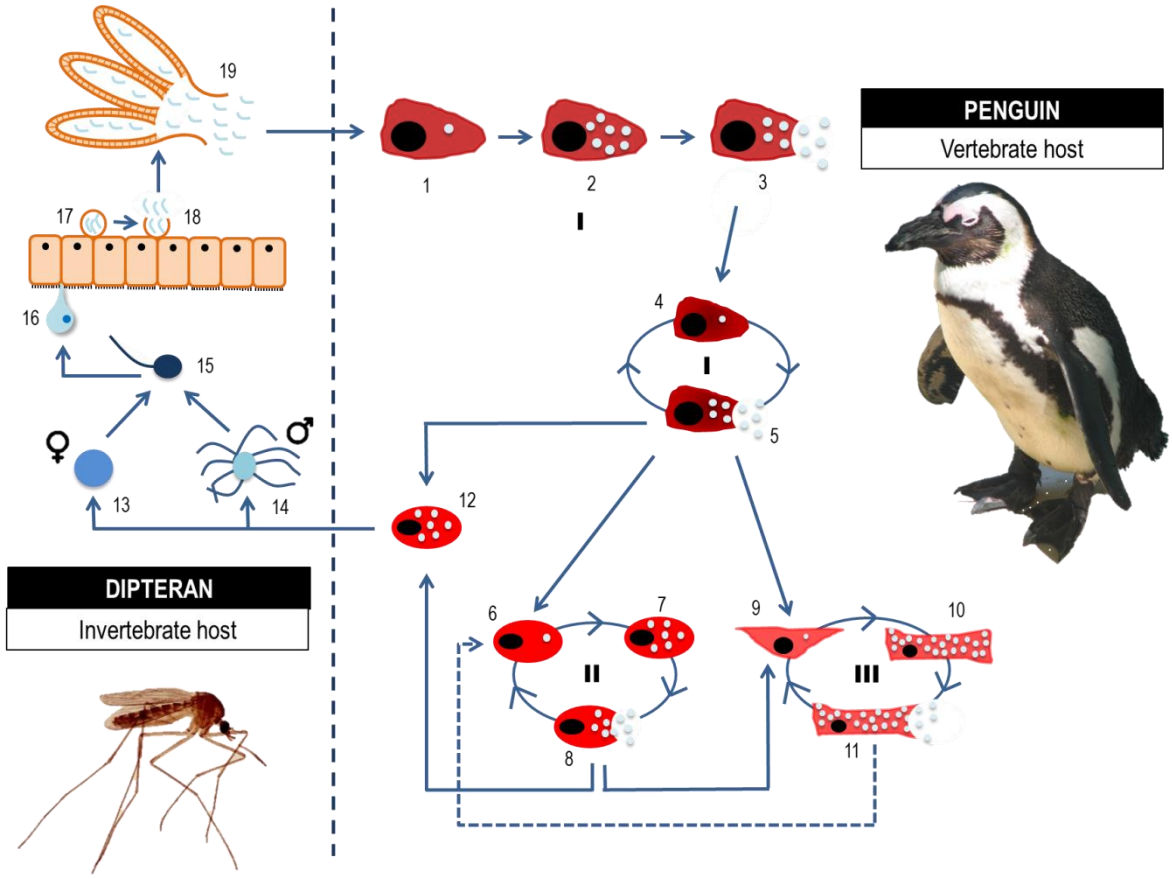
Gametocytes include macrogametocytes, that origin one macrogamete (Figure 6, number 13) each, and microgametocytes, originating eight microgametes after exoflagelation in the vector (Figure 6, number 14) (Huijben, Schaftenaar, Wijsman, Paaijmans & Taaken, 2007;

Campos, 2011). It's possible to distinguish the sexual stages. Macrogametocytes, usually, present a more intense staining of the cytoplasm and a dense nucleus with clear outline (Valkiunas, 2005). These stages stay inside erythrocytes and do not continue their development until being consumed by the dipteran (Huijben, Schaftenaar, Wijsman, Paaijmans & Taaken, 2007). After the vector feeds, the gametocytes initiate gametogenesis in the midgut. The major stimulus to this process is the change of oxygen and carbon dioxide concentration that occurs from the transference of blood from the penguin to the vector. When the fertilization occurs in the extracellular space (Figure 6, number 15), within 24 hours the ookinete is formed (Figure 6, number 16) (Huijben, Schaftenaar, Wijsman, Paaijmans & Taaken, 2007). It possesses mobility and penetrates through the epithelial layer of the midgut. This is the only stage of the parasite that is diploid (Alves, 2002; Valkiūnas, 2005), but meiosis occurs in the initial stage of its development (Valkiūnas, 2005). When it is under the basal lamina, it rounds up and develops into an oocyst (Figure 6, numbers 17 and 18). During its development, the sporogony takes place and the sporozoites are formed. When mature, they migrate into the haemocoele and then to the salivary glands (Figure 6, number 19) (Valkiūnas, 2005), where they will be injected later, during the dipteran's blood meal. The time for parasite development in the vector is approximately seven days (Huijben, Schaftenaar, Wijsman, Paaijmans & Taaken, 2007).

The development of the parasite in the penguin is divided into exoerythrocytic merogony (including primary or preerythrocytic [Figure 6, roman number I] and secondary or posterythrocytic [Figure 6, roman number III]), erythrocytic merogony (Figure 6, roman number II) and formation of gametocytes (Valkiūnas, 2005). In the primary exoerythrocytic merogony, two generations of meronts are formed. The firsts are called cryptozoites (Figure 6, numbers 2 and 3) and develop mostly on reticular cells of many tissues (eg. skin) and organs (e.g. spleen). These stages can not infect erythrocytes so they need to go through another cycle of merogony in macrophages (Figure 6, number 4) to generate metacryptozoites (Figure 6, number 5), which contain a greater quantity of merozoites. This process will take up to 36-48 hours (Valkiūnas, 2005; Huijben, Schaftenaar, Wijsman, Paaijmans & Taaken, 2007). The resulting merozoites can either invade erythrocytes (Figure 6, number 6) and produce asexual stages or gametocytes (Figure 6, number 12); stay in the primary exoerythrocytic cycle and infect new macrophages again or can initiate the secondary exoerythrocytic merogony (Figure 6, number 9), including several generations of meronts – the phanerozoites (Figure 6, numbers 10 and 11). The parasites that penetrate erythrocytes originate growing non-fissionable parasites, called trophozoites, depositing granules of malarial pigment (haemozoin) (Valkiūnas, 2005; Huijben, Schaftenaar, Wijsman, Paaijmans & Taaken, 2007). This occurs because the erythrocyte's cytoplasm and hemoglobin are digested by the parasite and the heme group is stored as an insoluble pigment (Marzal, 2012). The pigment granules, which commonly group together in mature

meronts, refract light heavily, permitting their visualization under the light microscope (Valkiūnas, 2005).

Figure 6 - Schematic representation of the life cycle of penguin malaria parasites (taking *Plasmodium relictum* as example). (Adapted from Valkiūnas, 2005; Huijben, Schaftenaar, Wijsman, Paaijmans & Taaken, 2007). I – primary (preerythrocytic) exoerythrocytic merogony; II – erythrocytic merogony; III – secondary (posterythrocytic) exoerythrocytic merogony; 1 – sporozoite in reticuloendothelial cell; 2, 3 – cryptozoites; 4 – merozoite in macrophage; 5 – metacryptozoites; 6 – merozoites in erythrocytes; 7, 8 – erythrocytic meronts; 9 - merozoite in endothelial cell of capillaries; 10, 11 – phanerozoites; 12 – gametocytes; 13 - macrogamete; 14 - exoflagelation of microgametes; 15 – fertilization of macrogamete; 16 – ookinete; 17, 18 – sporogony; 19 - sporozoites in the salivary glands of vector. Source of dipteran: "Culex-female" by Alan R Walker - Own work. Licensed under Creative Commons Attribution-Share Alike 3.0 via Wikimedia Commons - http://commons.wikimedia.org/wiki/File:Culex-female.jpg#mediaviewer/File:Culex-female.jpg.



The erythrocytic cycles continue until the penguin dies or the immune system suppresses the parasites development (Huijben, Schaftenaar, Wijsman, Paaijmans & Taaken, 2007).

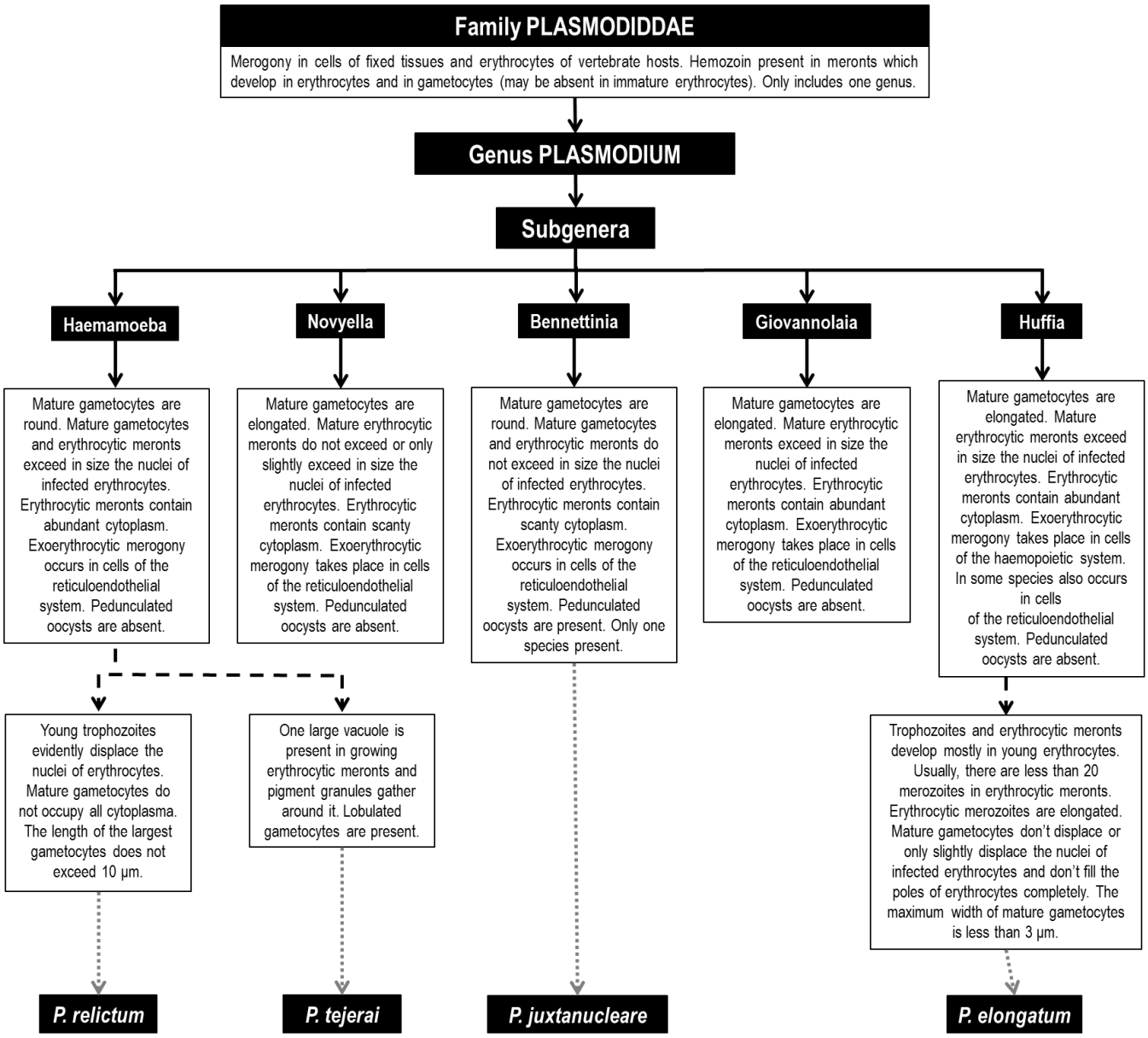
The parasites that initiate secondary exoerythrocytic merogony invade endothelial cells of different organs (e.g. liver and brain), where the phanerozoites develop (Huijben, Schaftenaar, Wijsman, Paaijmans & Taaken, 2007). The rising of the first generation of phanerozoites overlaps with the increase of parasitemia. These stages, together with the erythrocytic meronts, maintain parasitemia during the chronic period of infection. Apart from that, phanerozoites are responsible for the recrudescence situations (Valkiūnas, 2005).

It is possible to detect distinct sizes of merozoites. In a work developed by Fix, Waterhouse, Greiner & Stoskopf (1988), macro and micromerozoites were detected. Also, merozoites

inside meronts obtained from heart and lung of the penguins had different apical complex, presenting tear-shaped rhoptries (secretory organelle).

The different morphological characteristics of *Plasmodium* spp. stages allow differentiating the four species recorded in penguins (Figure 7).

Figure 7 - Criteria for the morphological differentiation of the four malaria parasites recorded in penguins. (Adapted from Valkiūnas, 2005).

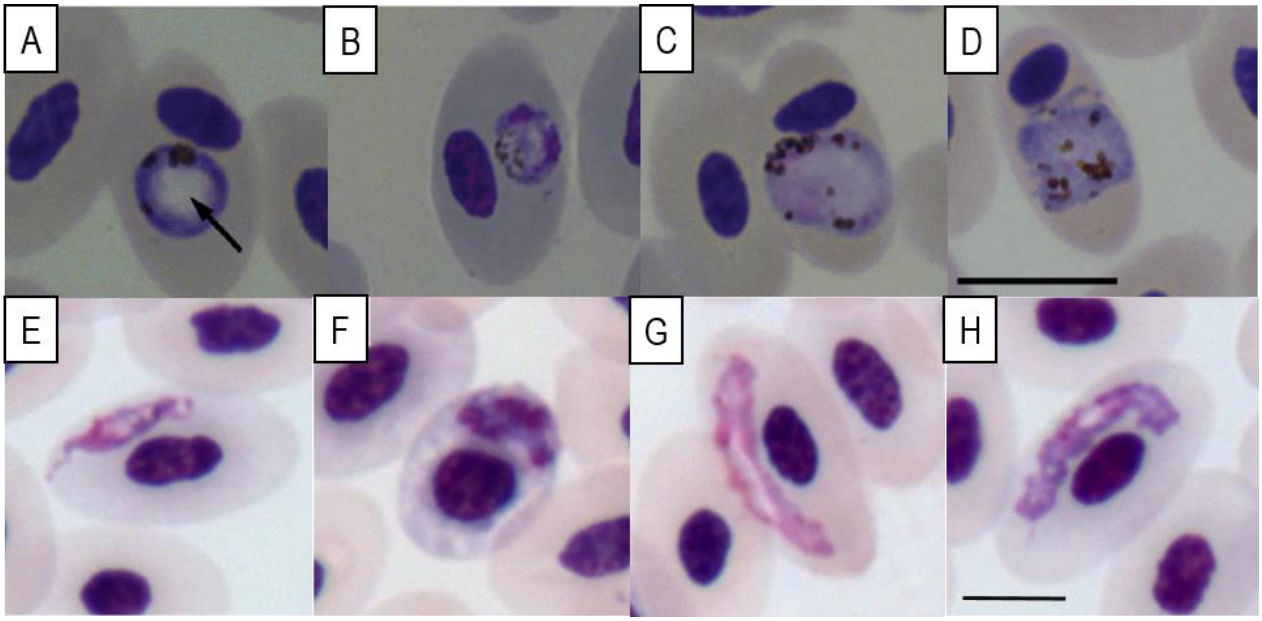


The infection in penguins include five periods: prepatent, since the inoculation of sporozoites occur until the maturation of the first generation of metacryptozoites (five days for *Plasmodium relictum*) (Valkiūnas, 2005; Huijben, Schaftenaar, Wijsman, Paaijmans & Taaken, 2007); acute, when the parasites appear in circulation and a rapid proliferation of the infection occurs; crisis, when the parasitaemia reaches the peak; chronic, characterized by

the absence of symptoms and only few parasites are found in the blood (duration varies considerably); and latent, occurring when the immune system controls the infection and parasites disappear completely from the peripheral blood, persisting in internal organs. Recrudescence can occur when fragilities on the immune system compromise the control of the infection (e.g. molt) and parasitaemia is established (Valkiūnas, 2005). Some species of the subgenera *Haemamoeba*, such as *P. relictum*, cause a rapid growth of primary parasitemia followed by a fast crisis and an enduring low chronic parasitaemia (Zehtindjiev *et al*., 2008).

Some examples of different stages of *Plasmodium* species reported in penguins are presented in Figure 8.

Figure 8 - Different development stages of: *Plasmodium tejerai*: A – trophozoite, B - erythrocytic meront, C – microgametocyte, D – macrogametocyte (Bar - 10 µm, arrow – vacuole) (Courtesy of Dr. Érika M. Braga, Departamento de Parasitologia, Instituto de Ciências Biológicas - Universidade Federal de Minas Gerais, Brazil); *Plasmodium elongatum*: E – trophozoite, F - meront, G - microgametocyte, H – macrogametocyte (Bar – 5 µm) (Courtesy of Dr. Ralph Vanstreels, Laboratório de Patologia Comparada de Animais Selvagens, Departamento de Patologia, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, Brazil).



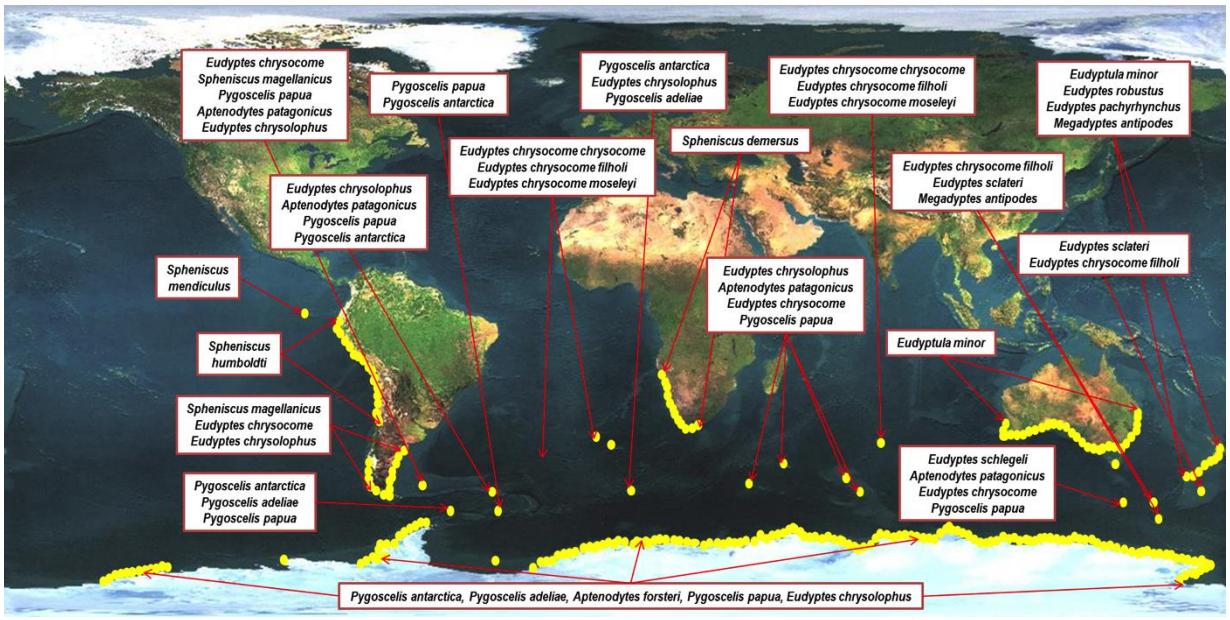
2. Epidemiology

A better knowledge of penguin malaria's epidemiology allows a proper husbandry of these animals when in captivity (Beier & Stoskopf, 1980).

The reason why penguins are so susceptible to malaria is due to their lack of interaction with the parasite in their habitats, with the consequent lacking of evolutionary adaptation. In the wild, penguins inhabit low temperature climates or coastal rockeries and islands where there is a lack of fresh water and the presence of vigorous wind currents. These conditions make the prevalence of mosquitoes null or very low (Cranfield, 2003; Weissenböck *et al*., 2011).

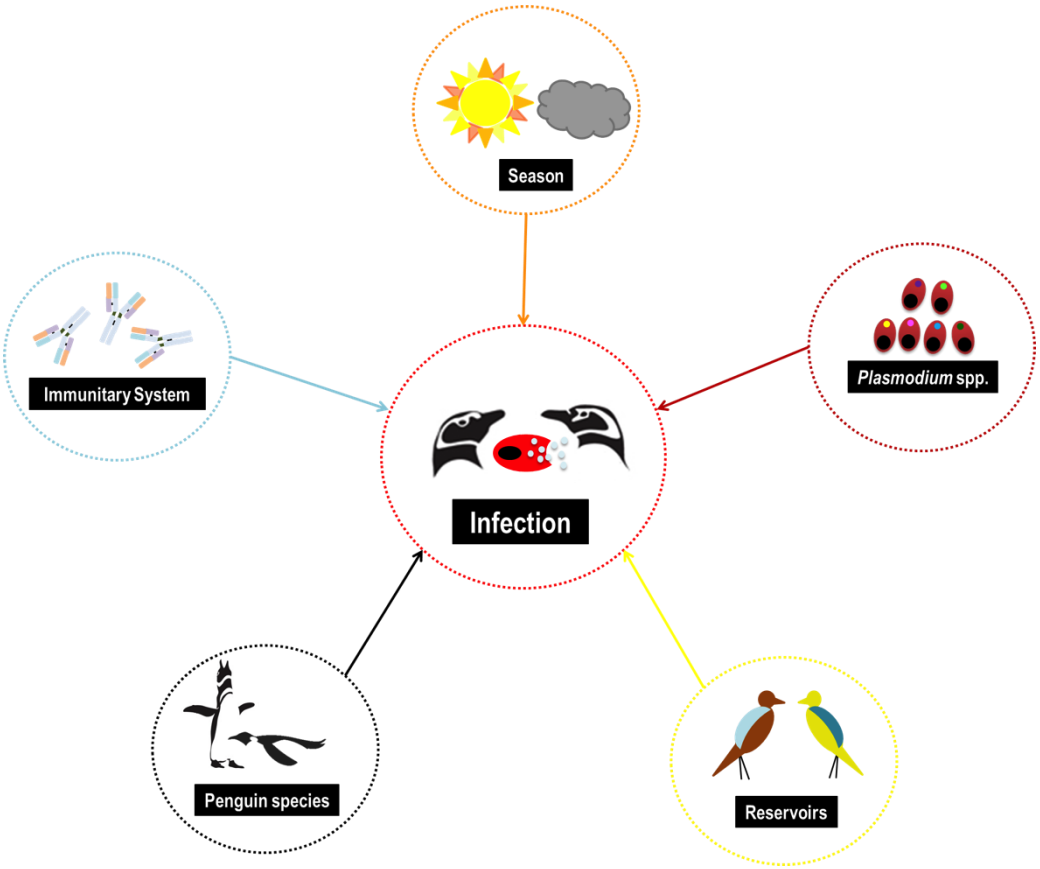
On the other hand, *Plasmodium* spp. have a wide distribution. One theory for this fact was the carrying of parasites by migratory birds, introducing *Plasmodium* spp. where the cycle of transmission did not occur before (Alves, 2002; Huijben, Schaftenaar, Wijsman, Paaijmans & Taaken, 2007). This fact makes it an important reminder to the introduction of penguins in climates where the malaria cycle occurs. The Figure 9 shows the world distribution of the different penguin species in the wild.

Figure 9 – Occurrence of the different wild penguin species in the world. (Adapted from http://www.penguins.cl/penguins-region.htm). Legend: *Aptenodytes forsteri* - Emperor penguin; *Aptenodytes patagonicus* - King penguin; *Eudyptes chrysocome chrysocome* - Southern Rockhopper penguin; *Eudyptes chrysocome filholi* - Eastern Rockhopper penguin; *Eudyptes chrysocome moseleyi* - Northern Rockhopper penguin; *Eudyptes chrysolophius* - Macaroni penguin; *Eudyptes pachyrhynchus* - Fiordland crested penguin; *Eudyptes robustusi* - Snares Island penguin; *Eudyptes schllegeli* - Royal penguin; *Eudyptes sclateri* - Erect-crested penguin; *Eudyptula minor* - Little (Blue or Fairy) penguin; *Megadyptes antipodes* - Yellow-eyed penguins; *Pygoscelis adeliae* - Adelie penguin; *Pygoscelis antarctica* - Chinstrap penguin; *Pygoscelis papua* - Gentoo penguin; *Spheniscus demersus* - African penguin; *Spheniscus humboldti* - Humboldt penguin; *Spheniscus magellanicus* - Magellanic penguin; *Spheniscus mendiculus* - Galapagos penguin.



In advance, some factors related with the epidemiology of malaria infection in penguins are discussed (Figure 10).

Figure 10 - Schematic representation of the risk factors associated with malaria infection in penguins kept in zoological collections (Original) (Penguin Images Source: http://www.arup.com/News/Events_and_exhibitions/Penguin_Pool.aspx).

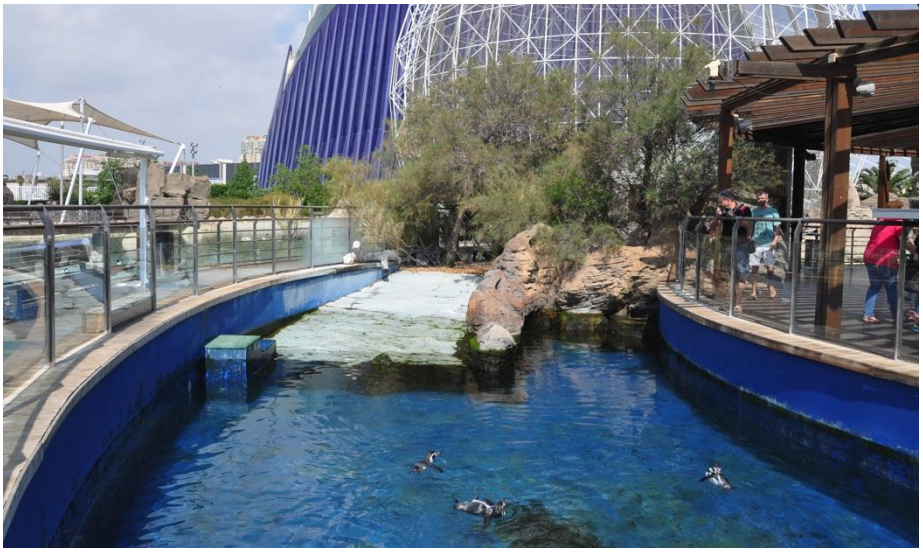


2.1 Season

Malaria is a seasonal disease, with a life cycle much depending on the availability of vectors (Alves, 2002). In penguins kept in captivity in outdoor exhibitions (Figure 11), mortality is usually reported in single summer or fall outbreaks (Graczyk, Shaw, Cranfield & Beali, 1994c). Graczyk, Cranfield, Skjoldager & Shaw (1994a) stated the death of African penguins (*Spheniscus demersus*) in the Baltimore Zoo as having its peak in August. This phenomenon positively correlated with the density of local vector populations.

Also, climate change is increasing malaria infection in wild birds. This impact has the strongest effects in Europe and Africa and it is more evident in the last years. Three mechanisms may be behind this occurrence: by spreading the vectors populations, by extending the duration of vector's breeding season and by improving the parasite sexual reproduction (as result of increasing temperatures) (Huijben, Schaftenaar, Wijsman, Paaijmans & Taaken, 2007; Garamszegi, 2011).

Figure 11 - View of the Humboldt penguins (*Spheniscus humboldti*) exhibition in “Oceanogràfic” (Valencia, Spain) (Courtesy of Daniel García Párraga, Jose Luis Crespo Picazo, Mónica Valls Torres and Teresa Álvaro Álvarez – “Oceanogràfic” Veterinary Team).



2.2. Immune status

The risk of developing an infection is associated to previous exposure to malaria. Chicks and juvenile birds, as well adults that had never been in an outdoor exhibition or never have contacted with the mosquitoes, show the highest susceptibility for becoming infected (Alves, 2002; Wallace & Walsh, 2005; Weissenböck *et al*., 2011). The most severe presentations of disease in penguins occur after primary exposure. The infection occurs very promptly after being placed in outdoor exhibitions (Graczyk, Cranfield, McCutchan & Bicknese, 1994b). Most of the animals die. The following episodes usually are not fatal (Graczyk, Shaw, Cranfield & Beali, 1994c) and surviving animals will build up viable humoral responses that control endothelium parasite stages, while developing low parasitaemia with no clinical signs (Graczyk, Cranfield, Skjoldager & Shaw, 1994a; Graczyk, Brossy, Plost & Stoskopf, 1995a; Alves, 2002). These animals will act as reservoirs and harbour the parasite while there are not vectors during winter (Alves, 2002). Older penguins that experienced more outdoor seasons have a lower antibody titer than those that experienced one or two seasons, suggesting that they do not become reinfected when bitten by the vector or that they are capable of clearing the reinfection, probably due to an antibody-mediated equilibrium of immunity in naturally immunized penguins presenting endothelial phanerozoites (Graczyk, Cranfield, Skjoldager & Shaw, 1994a).

Vertebrate hosts with mild infections will have a higher probability of surviving and develop an immune response capable of controlling the infection (Zehtindjiev *et al*., 2008).

It is known that anti-*Plasmodium* spp. immunoglobulins are passed due to maternal antibody transfer in the egg yolk and are detectable for up to eight weeks post hatching (Graczyk,

Cranfield, McCutchan & Bicknese, 1994b). This will mean that juvenile penguins will be naïve to *Plasmodium* spp. in the moment of the outdoor exposure (April-May), the moment when parasitemia in wild birds reaches the peak. Adults develop these antibodies by a low-level exoerythrocytic infection (Graczyk, Cranfield, Shaw & Craig, 1994d).

The equilibrium between the immune system and the parasite can be disturbed by chemical or physical stimulus, causing immunosuppression and recrudescence (Graczyk, Cranfield, Skjoldager & Shaw, 1994a). The effects of corticosteroids can be explained by weakening cell-mediated immunity that control phanerozoites in the endothelial tissues (Cranfield *et al*., 1994). Parasite recrudescence after corticosteroid administration was demonstrated in non-symptomatic humans (Ng *et al*., 1997).

Some situations causing stress on penguins, like molt (Figure 12) and poor husbandry, increase the probability of mortality by avian malaria (Cranfield, 2003; Campos, 2011).

Figure 12 – A wild African penguin (*Spheniscus demersus*) molting (Source: http://creativecommons.org/publicdomain/zero/1.0/).

2.3. Penguin species

A higher frequency of infection in species from the genus *Spheniscus* has been reported. This can be explained by the fact that species from colder habitats (the genus *Spheniscus* inhabit temperate habitats) require indoor housing all year (Wallace & Walsh, 2005f). Infections from all avian orders have been reported, with the exception of the Struthioniformes, the Coliiformes and the Trogoniformes. However, about only half of existing avian species have been examined for malaria parasites (Goswami & Swamy, 2013).

2.4. Reservoirs

Wild bird species, especially passerines, are know to be infected with malaria (Huijben, Schaftenaar, Wijsman, Paaijmans & Taaken, 2007). These animals, that share habitats in zoos with the exhibited colony, act as sources of infection to vectors, which might infect

penguins (Weissenböck *et al*., 2011). Without infections in wild birds, malaria in penguins would probably not occur, since their infection overlaps the highest infection rates periods in wild birds (Beier & Stoskopf, 1980).
Since their parasitaemia persist for up to three weeks, they can not be ruled out as possible reservoirs. However, the probability of chronically infected penguins (animals that have recovered from the infection) serve as reservoirs is low, since they rarely exhibit circulating parasites (Beier & Stoskopf, 1980).
Infected birds living in the zoo perimeters, like penguins, can also have important effects on the spread of malaria (Huijben, Schaftenaar, Wijsman, Paaijmans & Taaken, 2007). Another important factor is the existence of suitable breeding sites to the vector near the penguins' exhibition. When this is favorable to the vector, the contact with the host is enhanced (Beier & Trpis, 1981).

2.5. *Plasmodium* spp.

The two species of *Plasmodium* spp. most recorded in penguins in captivity are *P. relictum* and *P. elongatum* (Chitty, 2011). The first is considered more prevalent (Cranfield, 2003) and also more pathogenic (Graczyk, Cranfield, McCutchan & Bicknese, 1994b; Chitty, 2011), since it causes a higher mortality rate (Cranfield, 2003)
P. elongatum has shown a low degree of cross immunity against other malaria parasites (Draper, 1953).

3. Pathogenesis

Malaria has a strong effect on vertebrate hosts, like penguins, since these parasites cause dramatic changes on the efficacy of the metabolism (Marzal, 2012). This disease affects primarily blood and the reticuloendothelial system (Goswami & Swamy, 2013) and it is known that the severity of clinical signs and their progression is proportional to the parasitaemia (Campos, 2011; Goswami & Swamy, 2013).
Unmanaged situations may result in mortalities of about 50 to 60%. In recovered penguins, the probability of mortality decreases to 3 to 4% (Cranfield, 2003).

3.1. Lesions

3.1.1. Macroscopic lesions

Lesions associated with acute infection include hepatomegaly (Figure 13), splenomegaly, discoloration of liver and spleen; and pulmonary edema (Alves, 2002; AAZV, 2013; Goswami & Swamy, 2013). Cardiomegaly and pericardial effusion as also been detected (Fix,

Waterhouse, Greiner & Stoskopf, 1988; Grim *et al*., 2003; Campos, 2011). Renomegaly was detected in a malaria infection in Humboldt penguins (*Spheniscus humboldti*). Fungal granulomas, due to secondary aspergillosis, were also found (Figure 14 and 15) (D. García Párraga, personal communication, August 7, 2014) Fleischman, Laden & Melby (1968) referred extensive lesions in the kidneys of clinically and subclinically malaria infected African penguins (*Spheniscus demersus*).

Figure 13 - Hepatomegaly in a Humboldt penguin (*Spheniscus humboldti*) infected with malaria (Courtesy of Daniel García Párraga, Jose Luis Crespo Picazo, Mónica Valls Torres and Teresa Álvaro Álvarez – "Oceanogràfic" Veterinary Team).

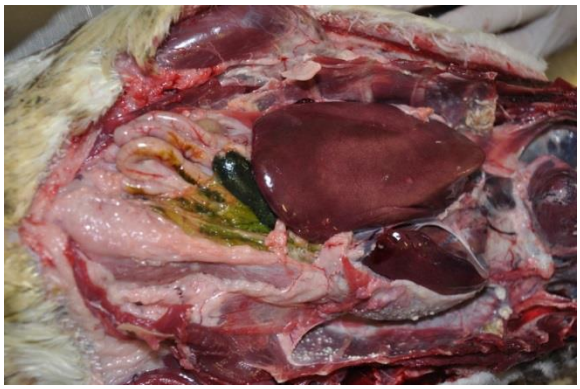
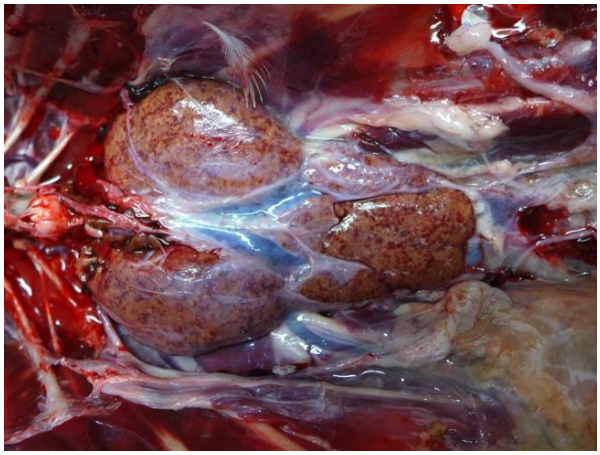


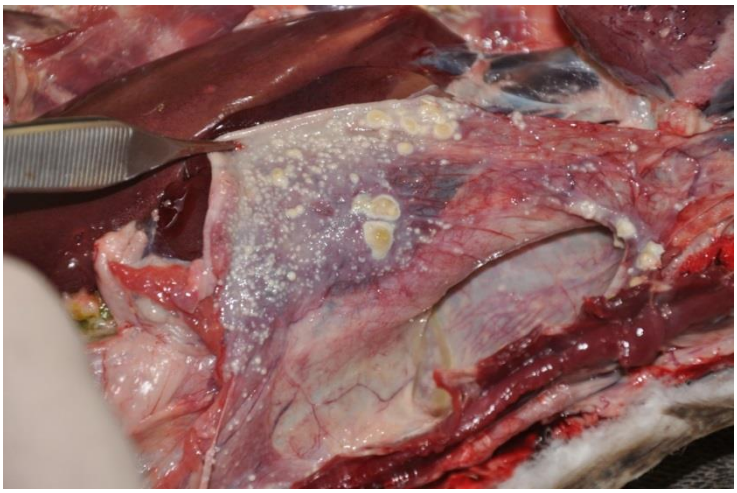
Figure 14 - Renomegaly in a Humboldt penguin (*Spheniscus humboldti*) infected with malaria (Courtesy of Daniel García Párraga, Jose Luis Crespo Picazo, Mónica Valls Torres and Teresa Álvaro Álvarez – "Oceanogràfic" Veterinary Team).



Hemodilution, giving the blood a watery feature, can also be noticed (Alves, 2002; AAZV, 2013; Goswami & Swamy, 2013).

Silveira *et al*. (2013) described gross and histologic findings in Magellanic penguins (*Spheniscus magellanicus*) infected with *P. tejerai* as similar to those found in penguins with *P. relictum* and *P. elongatum*.

Figure 15 - Fungal granulomas in a Humboldt penguin (*Spheniscus humboldti*) concomitantly infected with malaria and Aspergillus spp. (Courtesy of Daniel García Párraga, Jose Luis Crespo Picazo, Mónica Valls Torres and Teresa Álvaro Álvarez – "Oceanogràfic" Veterinary Team).



3.1.2. Microscopic lesions

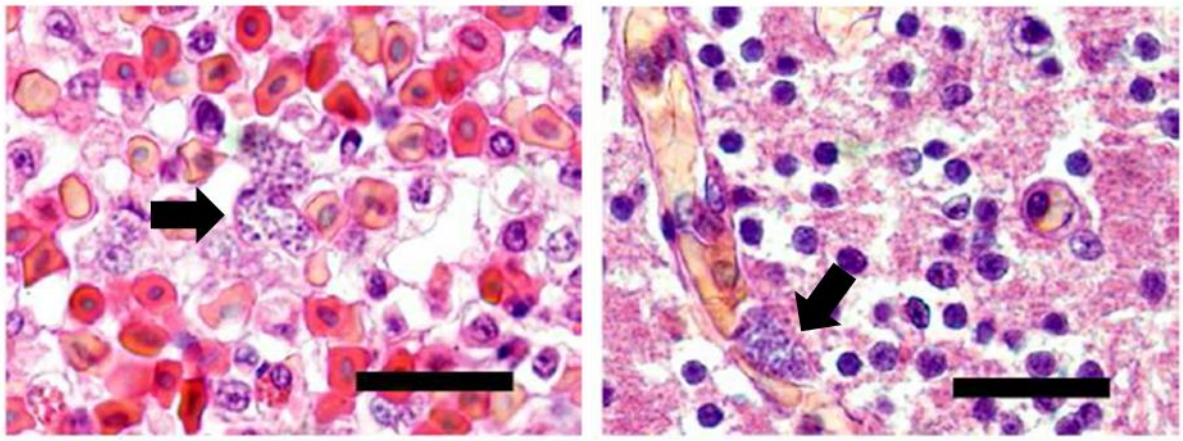
Primary exoerythrocytic meronts are unlikely to cause serious disease since their number is usually low, they have a small size and the inflammatory reaction they cause is not pronounced. Parenchymal organ dysfunction and failure present in malaria infection results from vascular occlusion from the presence of phanerozoites and sometimes metacryptozoites (Valkiūnas, 2005). These characteristics are crucial for the death of the host (Fix, Waterhouse, Greiner & Stoskopf, 1988), resulting in anoxia, apoptosis and necrosis of tissues (Marzal, 2012). Fix, Waterhouse, Greiner & Stoskopf (1988) stated that the consequences of intraendothelial schizogony (Figure 16) were solely capable of causing mortality, with no need of the action of primary exoerythrocytic merogony and erythrocytic merogony.

One of the most severe consequences of secondary exoerythrocytic merogony it's the blockage of brain capillaries (Campos, 2011; Marzal, 2012; AAZV, 2013), resulting in cerebral paralysis (Valkiūnas, 2005).

Another problem is the destruction of erythrocytes by erythrocytic meronts (Valkiūnas, 2005; Campos, 2011; Marzal, 2012). Aggravating this condition, reticuloendothelial cells from spleen, liver and bone marrow remove infected erythrocytes from circulation (Campos, 2011). Also, changes in the chemical composition of the blood plasma are observed. A decrease in the plasma pH and an increase in the proteins concentration occurs when parasitemia starts to increase, inducing a reduction of the hemoglobin's oxygen-binding ability, the decreasing of effective circulation in the capillaries and the intensification of the osmotic fragility of normal erythrocytes. These events result in an acute hemolytic anaemia, since erythropoiesis can not compensate the losses of erythrocytes (Valkiūnas, 2005; Marzal, 2012). Diffuse areas of extramedullary erythropoiesis in liver sinusoids and kidney

interstitium, as well as occasional multifocal mild extramedullary granulopoiesis in hepatic parenchyma, red pulp and subcapsular areas of the spleen and perivascular areas of the kidneys are present (Goswami & Swamy, 2013). Some authors (Cranfield *et al*., 1994; Weissenböck *et al*. 2011) suggest that because penguins have low parasitaemia, the destruction of erythrocytes is not enough to cause clinical anaemia.

Figure 16 - Histological sections of lung (left) and brain (right) of penguins infected with *P. elongatum* with exoerythrocytic meronts (arrow) in the capillary endothelium. In the brain elongated exoerythrocytic meronts (arrow) are present (H&E). Bar - 40 µm. (Courtesy of Dr. Herbert Weissenböck - Institute of Pathology and Forensic Veterinary Medicine, Department of Pathobiology, University of Veterinary Medicine – Vienna).



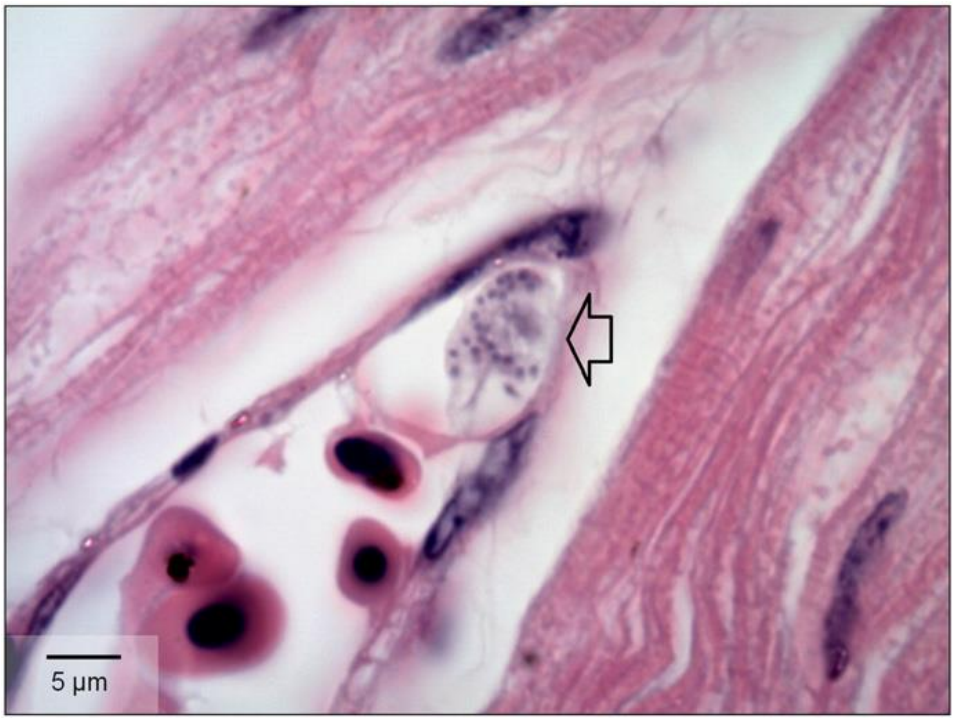
Secondary exoerythrocytic meronts can also block lung capillaries. (AAZV, 2013). Adding to this fact, macrophage infiltrate (Marzal, 2012) and minimal to moderate interstitial pneumonia are obvious (Goswami & Swamy, 2013). These will lead to pneumonia-like symptoms (eg. respiratory failure) (Fix, Waterhouse, Greiner & Stoskopf, 1988; Marzal, 2012).

Macrophage, lymphocytes and plasma infiltrates take place in the liver and the spleen, in a multifocal way (Ko, Kang, Jung, Bae & Kim, 2008; AAZV, 2013). In liver, this is more pronounced in perivascular regions. Hepatic sinusoids are usually dilated and it is possible to find activated Kupffer cells. Red pulp of spleen presents diffuse infiltration of histiocytic cells, while in the white pulp it is possible to find moderate atrophy and mild lympholysis (Ko, Kang, Jung, Bae & Kim, 2008). The typical enlargement of these organs is due to hypercellularity and increased phagocytic activity of macrophages (Goswami & Swamy, 2013). In fact, a hyperplasia of lymphoid macrophage cells in these organs is a characteristic feature of haemosporidiosis. Excessive enlargements due to heavy infections can result in rupture (Valkiūnas, 2005).

Kupffer cells and splenic macrophages contain diffuse haemozoin deposition (AAZV, 2013) as well as vacuolated cytoplasm. This pigment deposition will grant a black shade to the liver and spleen (Valkiūnas, 2005; Goswami & Swamy, 2013).

Overall, histopathological examination will reveal numerous intraendothelial phanerozoites in spleen, lung and liver (Cranfield, 2003; Goswami & Swamy, 2013). Findings in heart, brain, kidney and intestine are less common (Fix, Waterhouse, Greiner & Stoskopf, 1988).
In Magellanic penguins (*Spheniscus magellanicus*) infected with *P. tejerai*, it is described the occurrence of "moderate to severe diffuse interstitial granulocytic pneumonia, moderate pulmonary edema and congestion, severe acute necrotizing splenitis, moderate multifocal to coalescent mixed necrotizing hepatitis and nephritis and mild diffuse granulocytic myocarditis with multifocal to coalescent areas of cardiomyolisis" (Silveira *et al*., 2013, p.166) (Figure 17). Most of the avian malaria pathogenic processes known are based on conclusions resulting from experiments using domestic birds (Campos, 2011).

Figure 17 - Meront (arrow) of *P. tejerai* in an endothelial cell of the myocardium in Magellanic penguin (*Spheniscus magellanicus*) (H&E) (Courtesy of Dr. Érika M. Braga, Departamento de Parasitologia, Instituto de Ciências Biológicas - Universidade Federal de Minas Gerais, Brazil).

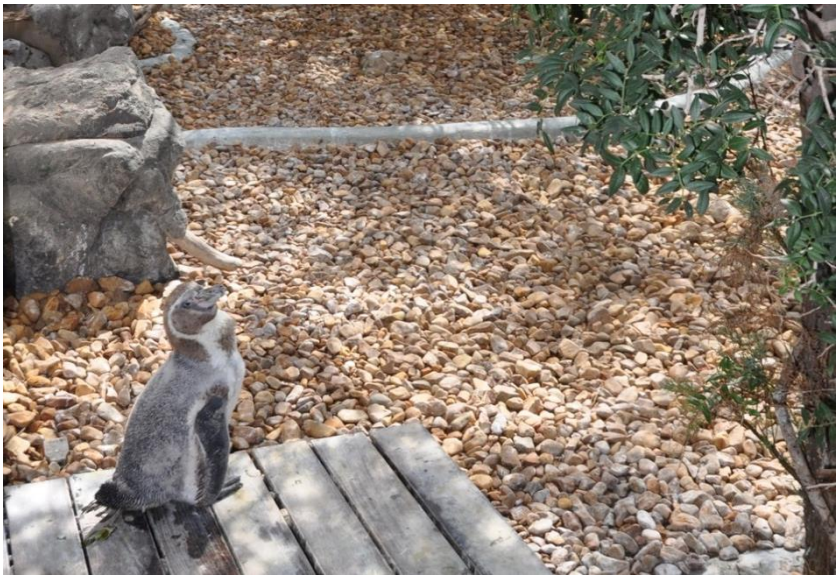


3.2. Clinical Signs

The symptoms of malaria infection may not be evident in the beginning of the infection (Stoskopf & Beier, 1979). It is frequent to find dead animals with no apparent suspicion (Alves, 2002; Wallace & Walsh, 2005).
Typical signs include lost of weight, respiratory distress, lethargy, weakness, pale mucous membranes, separation from the group (Figure 18), vomit, regurgitation when forced feeding and greenish feces (Fleischman, Sladen & Melby, 1968; Fix, Waterhouse, Greiner &

Stoskopf, 1988; Alves, 2002; Grim *et al*., 2003; Valkiūnas, 2005; Wallace & Walsh, 2005; Campos, 2011; AAZV, 2013). Severe forms of the disease have been described to induce motor incoordination, neurological signs (like convulsions) and paralysis (Grim *et al*., 2003; Campos, 2011), usually in a terminal state (Valkiūnas, 2005; AAZV, 2013).

Figure 18 - A Humboldt penguin (*Spheniscus humboldti*) separated from the group at "Oceanogràfic" (Valencia, Spain) (Courtesy of Daniel García Párraga, Jose Luis Crespo Picazo, Mónica Valls Torres and Teresa Álvaro Álvarez – "Oceanogràfic" Veterinary Team).



The presented signs only develop in an advanced phase of the infection. Because of their non-specificity, misinterpretation with other typical penguin diseases (e.g. aspergillosis and bacterial gastroenteritis) is possible (Alves, 2002).

Although Goswami & Swamy (2013) stated that increases in the cloacal temperature have been registered in chickens infected with *P. gallinaceum* (acute phases), like human malaria, penguin malaria parasites are non-pyrogenic agents (Graczyk, Cranfield, Skjoldager & Shaw, 1994a; Alves, 2002; Valkiunas, 2005). The normal internal temperature for an African Penguin (*Spheniscus demersus*) varies from 37.3ºC, when sleeping, to 40.8º, in moments of high activity (Wilson & Grémillet, 1996).

4. Diagnosis

Diagnosis is, perhaps, one of the most important steps when controlling penguin malaria. Usually, when symptoms appear, the effectiveness of treatment decreases greatly (Campos, 2011).

An accurate and early diagnosis and monitoring of malaria in penguins kept in zoological collections are both fundamental tools and challenges to the clinician.

Direct and indirect methods have been developed and improved for diagnosing avian malaria.

Microscopy and PCR-based methods have been used together, improving the hemosporidian's diagnostic in wild birds (Silveira *et al*., 2013). In human medicine, microscopy and rapid diagnostic tests represent the two diagnostics tools with the largest impact on malaria control (Wongsrichanalai, Barcus, Muth, Sutamihardja & Wernsdorfer, 2007).

Combining different methods, the clinician increases the likelihood of an accurate diagnosis (United States Geological Survey [USGS], 2006).

Lack of a good diagnostic method to detect malaria is a significant limiting factor to effective control programs (Alves, 2002).

4.1. Parasitological diagnosis

Giemsa-stained thin blood smear exam using common light microscopy is still considered the "gold standard" in avian malaria diagnosis, being extremely accurate when parasites are detectable (USGS, 2006; Campos & Almosny, 2011). The diagnosis of malaria infection can be fulfilled by detecting schizonts on host's erythrocytes through a blood smear. The light microscopy visualization allows the detection of parasitaemias as low as 0.001% in penguins (Alves, 2002).

Cranfield (2003) refers the use of blood collected with an incision on the jugular of dead animals up to 72 hours. Also, it is a low cost technique, allowing its execution by most zoological parks in the world, providing an estimation of the intensity of parasitaemia (Waldenström, Bensch, Hasselquist & Östman, 2004).

Some disadvantages are associated with this tool. It is a low sensibility method (Campos & Almosny, 2011), missing more than 70% of chronic infections (USGS, 2006). High qualification and expertise of the technician is fundamental, since identification of *Plasmodium* spp. can be difficult. A minimum of 40.000 erythrocytes is required to detect low parasitaemia, making it a time consuming procedure (Beier & Stoskopf, 1980; Zehtindjiev *et al*., 2008). Krone *et al*. (2008) suggested the observation of the blood smear for a minimum of ten minutes, using different objectives. Finding parasites in blood smear establishes malaria infection with certainty (Stoskopf & Beier, 1979). However, during chronic stages, as parasites abandon circulation or stay there in an undetectable stage, detection by microscopy it's difficult and infection can not be ruled out (Alves, 2002). Identification and differentiation of malaria parasites based on morphological features can be difficult due to low parasitaemia (Weissenböck *et al*., 2011). In normal practice, identification of *Plasmodium* species does not influence the treatment options.

4.2. Hematologic and biochemical diagnosis

Some hematological values have been suggested to be used as markers of malaria infection. Penguins infected with malaria can present a total white blood cell counts superior to $20x10^3/\mu l$ and relative lymphocytosis over 60% (Stoskopf & Beier, 1979; Alves, 2002; Wallace & Walsh, 2005; Campos *et al*., 2011). Increases in circulating lymphocytes can be a response to primary exoerythrocytic meronts, before parasitaemia is detectable (Stoskopf & Beier, 1979). Campos *et al*. (2011) describe monocytosis in infected Magellanic penguins (*Spheniscus magellanicus*) (13 penguins from a population of 27 infected animals). However, in a work developed by Graczyk, Shaw, Cranfield & Beali (1994c), the differences between total white blood cell counts and relative lymphocytosis of infected and uninfected penguins were not significant, alerting that these parameters do not have diagnostic value. Presentations of a moderate to severe anaemia have been documented (Alves, 2002; Wallace & Walsh, 2005; Campos *et al*., 2011), with decreases in hematocrits up to 50% or more (AAZV, 2013).

Graczyk, Cranfield & Bicknese (1995c) suggested that eight serum chemistry parameters - gamma-glutamyltranspeptidase (GGTP), alanine aminotransferase (ALT), alkaline phosphatase (AP), creatinine, uric acid, triglyceride, phosphates and very light-density lipoprotein (VLDL) – may be useful for dictating the prognosis of infected African penguins (*Spheniscus demersus*) during preerythrocytic development stages. Only three of those - ALT, GGTP and creatinine – were found to have highly indicative prognostic or diagnostic value. As explained before, malaria infection causes kidney pathology. ALT's highest tissue activity occurs in the kidney (elevated values indicate kidney dysfunction), the same as GGTP (damage to the renal epithelium and the hepatobiliary system) and creatinine (renal failure).

4.3. Molecular diagnosis

The use of molecular methods for the diagnosis of avian malaria is in great expansion (Campos & Almosny, 2011).

Polymerase chain reaction (PCR) based-methods have significantly higher sensitivity than light microscopy (Richard, Sehgal, Jones & Smith, 2002; Krams *et al*., 2012), but may still not identify low level parasitemia or mixed infections (USGS, 2006; AAZV, 2013). Species and subspecies identification is possible using these techniques (Waldenström, Bensch, Hasselquist & Östman, 2004; Okanga, Cumming, Hockey, Grome & Peters, 2013).

Different methods have been developed. Richard, Sehgal, Jones & Smith (2002) compared four PCR assays – two that amplify fragments of the cytochrome b gene of *Plasmodium* and two that target 18S ribosomal subunit gene – concluding that the firsts detected a more substantial number of positives than light microscopy. Christe, Glaizot, Strepparava,

Devevey, & Fumagalli (2012) describe a quantitative PCR assay to quantify malarial parasitaemia in great tits (*Parus major*). Waldenström, Bensch, Hasselquist & Östman (2004) describe a nested PCR that increases the proportion of infection detection to 63.2%, rather than 51.6% with PCR and 7.4% with light microscopy.

Some drawbacks include the high cost, time consumed (although faster than blood smear analysis), possible need of additional sequencing procedures to check the products originated from malaria genome and variations in parasite sequences that inhibit specific alignment of primers (Richard, Sehgal, Jones & Smith, 2002; USGS, 2006).

False negatives may occur due to insufficient concentration of parasite DNA or mistakes during DNA extraction from the sample (Richard, Sehgal, Jones & Smith, 2002).

Because these techniques and blood smear analysis are likely to underestimate avian malaria infection, the two methods should be considered complementary (Valkiūnas *et al*., 2006; Okanga, Cumming, Hockey, Grome & Peters, 2013).

Weissenböck *et al*. (2011) developed a chromogenic in-situ hybridization (ISH) test with a digoxigenin-labelled probe, which targets a fragment of the 18S ribosomal subunit RNA of malaria parasites using paraffin wax-embedded tissues. Samples were used from captive penguins (Humboldt penguins [*Spheniscus humboldti*], Rockhopper penguins [*Eudyptes chrysocome*] and King penguins [*Aptenodytes patagonicus*]) that died from the infection. The method was validated using histology and PCR and cross-reactivity with other protozoa and fungi was ruled out. *Plasmodium* meronts were identified without trouble by a purple to black signal within the capillary endothelium (Figure 19). Using this method, confounding fragmented nuclei within necrotic tissue with meronts does not occur, like in histopathology.

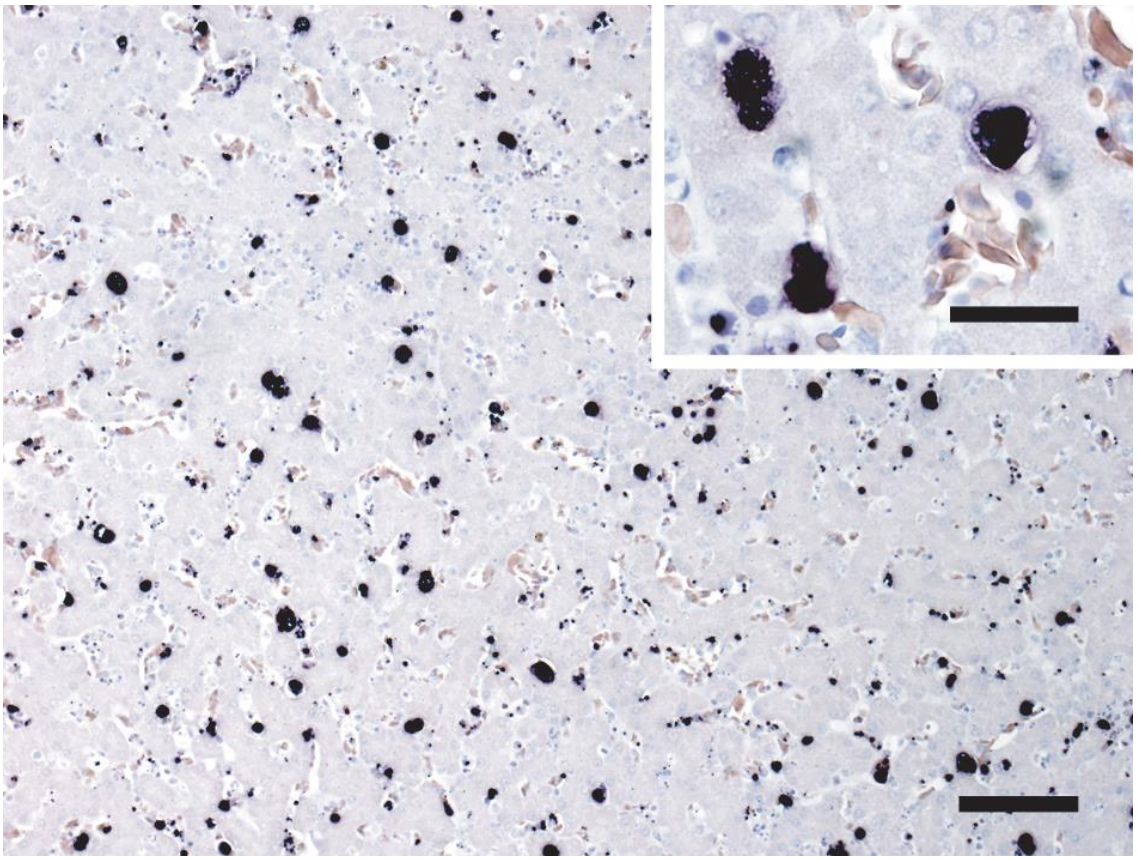
4.2. Serologic diagnosis

Serological methods for detecting malaria infection in penguins are also available (AAZV, 2013).

Graczyk, Cranfield, Skjoldager & Shaw (1994a) developed an Enzyme-linked immunosorbent assay (ELISA) to detect anti-*P. relictum* and anti-*P. elongatum* antibodies from infected African penguins (*Spheniscus demersus*) using *P. falciparum* antigens.

This technique is simple (allowing collection of blood samples on filter paper), sensitive, rapid and relatively inexpensive (Atkinson & Paxton, 2013). Other major advantage is the possibility of testing several antigens at the same time (Graczyk, Cranfield, Skjoldager & Shaw, 1994a). Also, it's possible to access exposure to malaria in adult birds without bleeding them, by performing an ELISA with 50 µl of egg-yolk (Graczyk *et al*., 1995b; Graczyk & Cranfield, 1996).

Figure 19 - ISH of a penguin's liver infected with *P. elongatum* shows numerous meronts (purple to black signal). Bar - 150 mm, inset bar - 40 mm. (Courtesy of Dr. Herbert Weissenböck - Institute of Pathology and Forensic Veterinary Medicine, Department of Pathobiology, University of Veterinary Medicine – Vienna).



Antibody levels measured by ELISA do not correlate with the parasitaemia. This technique is not good to determine the onset of treatment or when monitorization can safely be stoped. On other hand, it's a good tool for detecting exposure and measuring the effects of vaccination in penguins (Cranfield, Graczyk & McCutchan, 2000).

Western Blotting is also available and, however ELISA is a more sensitive assay, this technique is the most specific and can be useful to verify positive results from ELISA and PCR (USGS, 2006).

It is important to refer that serological methods do not provide information about parasite intensity, and are not useful for very early acute infections (USGS, 2006).

In human medicine, another serological method has a great use in general practice. Rapid Diagnostic tests are accurate tools that greatly decrease the time needed to perform a diagnostic. These tests identify *Plasmodium* histidine rich protein 2 (HRP2) and lactate dehydrogenase (pLDH) (Orogade, 2012). Because they present sensitivity limitations, the results must always be associated with the clinical signs. The Veterinary Department of Bristol Zoo Gardens (2008) described the use of rapid diagnostic tests in two bird species, including penguins, for malaria detection. This is a quick (20 minutes), quite inexpensive

diagnostic method, which can be performed in the zoo, with a small sample (one drop of blood), providing an accurate diagnosis.

4.3. Necropsy

Postmortem diagnosis is made by examining the abdomen for hepatomegaly and splenomegaly combined with the detection of organisms in spleen and liver smears (Cranfield, 2003). Other pathological changes are stated in the chapter “3.1 Lesions”.
This broad used technique presents limitations, like the difficulty to differentiate protozoal tissue stages from fragmented nuclei in necrotic tissue (Weissenböck *et al*., 2011).

4.2. Differential diagnosis

Like many animals, especially all wild species, penguins are well adapted for survival in their natural environment. So, signs of illness may be hidden (Wallace & Walsh, 2005). The previous symptoms presented as typical for malaria are, in fact, common for a variety of diseases when working with these species in captivity.
Some diseases may be taken in consideration when dealing with a malaria infection suspicion in penguins.
Aspergillosis is caused by *Aspergillus* spp. fungus. These organisms can exist in low numbers without causing problems, but the disease may occur in stressed or debilitated animals (Wallace & Walsh, 2005). This, along with malaria, is one of the major causes of mortality in penguins kept in zoological collections (Penrith, Huchzermeyer, De Wet & Penrith, 1994; Graczyk & Cranfield, 1996). Symptoms like respiratory distress, lethargy, weight loss and isolation are also common. Non-specific signs of central nervous system may also be present (Wallace & Walsh, 2005).
West Nile Virus infection as been described in penguins from genus *Spheniscus* as causing lethargy, weakness, anorexia and vomiting when force-feeding. Neurological signs are rare and only appear late in the course of the disease, if the animals survive.
Chlamydophila psittaci outbreaks have been also reported. Lethargy and lime green feces and urates, as well as hepatomegaly and splenomegaly, were the most common findings.
Neurological signs due to thiamine deficiency when fish quality is compromised it is an important factor when dealing with these animals (Wallace & Walsh, 2005).
Other important statement is that concurrent diseases were also noticed in penguins with malaria infections (like aspergillosis and bacterial gastroenteritis involving *Clostridium perfringens*) (Penrith, Huchzermeyer, De Wet & Penrith, 1994).

5. Treatment

An important reminder when dealing with this disease is that once clinical signs are present, the success of treatment is highly compromised. This does not mean constant treatment is preferable, since unnecessary or early treatment may interfere with the building of natural immunity. Penguins could be more susceptible in the following outdoor seasons (Cranfield, 2003). When administered at the right time, treatment suppresses malaria parasites to a low-level of exoerythrocytic stages, stimulating immunity (Graczyk, Shaw, Cranfield & Beali, 1994c). Cranfield, Graczyk & McCutchan (2000) stated that a routine of weekly examination of blood smears and treatment with primaquine and chloroquine when penguins were parasitemic reduced the experienced mortality from 50% to 10-15%.

Although isolation of infected animals may be necessary to facilitate the treatment process, as penguins are very sociable animals, mate's company should be arranged. If this is not possible, mates should be within visual or vocal range of the isolated penguin (Wallace & Walsh, 2005).

5.1. Treatment protocols

Treatment protocols described for malaria infections on captive penguins can present variations on dosage, but overall, primaquine and chloroquine associations have been the most popular choices. A summary of protocols described in the literature over a period of 25 years is presented in Table 4.

American Association of Zoo Veterinarians (2013) refers the use of the association of atovaquone-proguanil hydrochloride in penguins. To the author's knowledge, references to a protocol are not available.

In an experiment with *P. gallinaceum* in broilers, Sohsuebngarm, Sasipreeyajan, Nithiuthai & Chansiripornchai (2014) deduced that chloroquine and doxycycline were the most effective drugs in the malaria treatment.

5.2. Side effects

No side effects have been reported in the treatments above described. Some problems related with relapses of parasitemia were detected but not investigated (Valkiūnas, 2005).

Table 4 – Examples of treatment protocols described in the literature specifically designed for penguins.

Reference	Drug(s)	Observations	Target(s)
Stoskopf & Kennedy-Stoskopf, 1986	Primaquine & Chloroquine	Oral administration of 0.3 mg/kg primaquine phosphate SID for 10 days and an initial dose of 10 mg/kg chloroquine phosphate and additional doses of 5 mg/kg at 6, 10 and 24 hours.	Primaquine – tissue schizonticide, gametocytocide Chloroquine – rapidly acting blood schizonticide, gametocytocide, sporontocide
Fix, Waterhouse, Greiner & Stoskopf, 1988	Primaquine & Chloroquine	Administration of 3 mg/bird of primaquine phosphate and 30 mg/bird of chloroquine phosphate SID for 4 months. After that, the dose of primaquine was increased to 7.5 mg/bird SID and chloroquine finished.	
Graczyk, Shaw, Cranfield & Beali, 1994c	Primaquine & Chloroquine	Treatment after detection of parasites with 10 mg/kg of chloroquine phosphate. After 6 hours, administration of 5 mg/kg of chloroquine phosphate and 1 mg/kg primaquine phosphate SID for 10 days.	
Rebêlo *et al*., 2005	Sulfadoxine & Pyrimethamine	Administration of 500 mg/animal of sulfadoxine and 25 mg/animal of Pyrimethamine five times every two days.	Sulfadoxine - slower acting bloog schizonticide Pyrimethamine – slower acting bloog schizonticide
Valkiūnas, 2005	Primaquine & Chloroquine	Oral intubation of a normal (0.85%) saline suspension containing primaquine phosphate and chloroquine phosphate. Primaquine phosphate's dose is 0.003 mg/kg SID for 3 days. Chloroquine phosphate's posology is equal to the one presented by Stoskopf & Kennedy-Stoskopf (1986).	Primaquine – tissue schizonticide, gametocytocide Chloroquine – rapidly acting blood schizonticide, gametocytocide, sporontocide
Wallace & Walsh, 2005	Primaquine & Chloroquine	Initiate, at diagnosis, 1.25 mg/kg of primaquine and 10 mg/kg of chloroquine SID during 10-14 days. Then continue 5 mg/kg of chloroquine BID during 3 days. Some institutions stop at this point and other continue 5 mg/kg of chloroquine SID with primaquine.	
Bueno *et al*., 2010	Primaquine & Chloroquine	Administrate orally 10 mg/kg of chloroquine diphosphate at 0, 6, 12, 18 and 24 h. Then administrate 5 mg/kg of chloroquine diphosphate and 1 mg/kg of primaquine phosphate SID for 3 days.	
Chitty, 2011	Doxycycline	Administrate 20–33 mg/kg of doxycycline subcutaneously along with 60 ml saline solution.	Doxycycline - slower acting bloog schizonticide, tissue schizonticide

5.3. Mechanism of action

5.3.1. Atovaquone

This drug is highly active against asexual blood stages (schizonts and gametocytes). Its action is selective against mitochondrial cytochrome bc1 complex to disable electron transport. Associating proguanil allows enhancing the mitochondrial toxicity of atovaquone (Vinetz, Clain, Bounkeua, Eastman & Fidock, 2011), resulting in a highly efficient protocol for blood stages (AAZV, 2013).

5.3.2. Proguanil

Proguanil acts as a selective inhibitor of folate biosynthesis, making impossible DNA synthesis and depleting folate cofactors. It has effects on tissue and erythrocytic schizonts. Although it does not inhibit gametocytes, oocysts fail to develop in the mosquito when it's used (Vinetz, Clain, Bounkeua, Eastman & Fidock, 2011).

5.3.3. Pyrimethamine

This is a slow acting drug against bloods schizonts. It has similar action to proguanil but with greater efficacy. However, efficacy on tissue stages is inferior to proguanil. Efficacy on folate biosynthesis can be achieved by sulfonamides or sulfones synergy (Vinetz, Clain, Bounkeua, Eastman & Fidock, 2011).

5.3.4. Chloroquine

Generally, chloroquine is thought of as a drug acting in the circulating stages (Vanstreels *et al*., 2014b). This quinolone derivative is a weak base and it concentrates in the highly acidic digestive vacuoles of blood parasite stages (schizonts and gametocytes). Here, it binds to heme and interrupts its sequestration. Due to the impossibility of inactivating heme or due to increased toxicity of the complex formed by chloroquine and heme it may kill the parasites due to oxidative damage to membranes, digestive proteases or other important parasite molecules. This drug is used for clinical cure (Krettli, Andrade-Neto, Brandão & Ferrari, 2001; Remple, 2004; Vinetz, Clain, Bounkeua, Eastman & Fidock, 2011). Degenerated erythrocytic parasites are detected on blood smears when using this substance, suggesting effective action (Vanstreels *et al*., 2014b).

5.3.5. Primaquine

Primaquine is lethal to exoerythrocytic tissue stages of malaria parasites (schizonts). It is also responsible for avoiding relapses. It possesses some activity against gametocytes but it's inactive against asexual blood stages. Although it's mechanism it's not known, it is believed to act as oxidation-reduction mediator, interfering with mitochondrial electron transport in *Plasmodium* spp. (Remple, 2004; Vinetz, Clain, Bounkeua, Eastman & Fidock, 2011; Vanstreels *et al*., 2014b).

5.3.6. Sulfonamides

These groups of drugs are blood schizonticides of slow action. As stated before, they are used to enhance the inhibition of folate biosynthesis. Sulfadoxine and sulfadiazine are commonly used sulfonamides in combination with pyrimethamine (Vinetz, Clain, Bounkeua, Eastman & Fidock, 2011).

5.3.7. Tetracyclines

These antibiotics act by inhibiting protein translation in *Plasmodium*'s plastid. However, since their action is very slow, it is advised to use them in association with chloroquine, for example. One example of a used tetracycline is doxycycline (Vinetz, Clain, Bounkeua, Eastman & Fidock, 2011).

5.4. Resistance to anti malarial drugs

Stoskopf & Kennedy-Stoskopf (1986) alerted to the development of primaquine-resistant strains of *P. elongatum*. This creates an obligation to monitor penguins under treatment to better understand the therapy's response.

5.5. Other treatments

Regarding the symptoms and concurrent diseases, other treatment actions should be considered.

For severely anemic penguins, because of the blood stages, blood losses or clotting disorders, transfusion may be an option. It is indicated when hematocrit rapidly decreases to less than 20% or it's not stable. When in low hematocrits, transfusion appears to abbreviate the convalescent period until chloroquine and primaquine start to make effect.

When stable, blood transfusion is not needed, as penguins have a good bone marrow response. In this case, supportive care alone is the best option (fluids, iron and complex B vitamins supplementation, and oxygen therapy) (Wallace & Walsh, 2005).

Penguins showing respiratory distress have beneficiated from oxygen therapy as well (Figure 20). Bueno *et al*. (2010) described the use of injectable aminophylline and hydrocortisone to diminish respiratory symptoms in malaria infected Magellanic Penguins (*Spheniscus magellanicus*).

Tollini, Brocksen & Sureda (2000) refer the administration of Fluconazole (100 mg SID) to prevent opportunistic aspergillosis.

In human medicine, plants, fungi, bacteria and marine organisms are extensively researched for their use as anti malaria drugs. Quinine and artemisinin, two substances provided by plants, have been used clinically with proved effects (Kaur, Jain, Kaur & Jain, 2009).

Figure 20 - Hyperbaric Oxygen Therapy procedure to a Humboldt penguin (*Spheniscus humboldti*) with malaria and presenting respiratory distress (Courtesy of Daniel García Párraga, Jose Luis Crespo Picazo, Mónica Valls Torres and Teresa Álvaro Álvarez – "Oceanogràfic" Veterinary Team).

6. Prophylaxis

Prophylaxis is a key aspect to solve malaria mortality in penguins (Valkiūnas, 2005). Manipulation of the effects of this disease can be achieved in three different levels: reducing or eliminating reservoirs of the parasites (e.g. native birds), reducing or eliminating vectors, and adapting the penguin's immune system to better dealing with the disease (Cranfield, Graczyk & McCutchan, 2000).

However, when prophylaxis is used, it consists of a yearly routine, since some individuals may not have had the chance to built natural immunity against *Plasmodium* parasites with some protocols (Cranfield, 2003).

6.1. Drug protocol

As in treatment protocols, prophylactic drug protocols can also present some variations. Some of these protocols are presented in Table 5.

Chitty (2011) hypothesized that prophylactic treatment with doxycycline could be administered in penguin collections, although referring that no attempts have been made. In this scenario, the use in adults while rearing chicks wouldn't be important, since doxycycline appears safe and effective.

Table 5 - Examples of prophylactic drug protocols described in the literature specifically designed for penguins.

Reference	Drug(s)	Observations
Rebêlo *et al*., 2005	Chloroquine & Primaquine	Administration of 250 mg/animal of chloroquine and 15 mg/animal of primaquine once a week.
Wallace & Walsh, 2005	Primaquine	Administrate 1.25 mg/kg of primaquine SID during vector season.[1]
	Sulfadiazine & Pyrimethamine	Administrate one capsule orally for a 3-5 kg penguin containing 125 mg of sulfadiazine and 4 mg of pyrimethamine every two days during vector season.[1]

[1]- Because adult penguins regurgitate food to chicks, usage of these regimens must be considered regarding very small chicks.

6.2. Side effects

An obvious and probably the most important side effect when administrating prophylactic drugs is the extra stress induced in the penguins, possibly creating problems with reproduction (Chitty, 2011).

Pyrimethamine is a folic acid inhibitor causing teratogenicity problems. When using in laying females in reproductive season, caution must be taken. Oral supplementation of folic acid may be given when animals are on prophylactic regimen (Wallace & Walsh, 2005). A recommendation of discontinue Pyrimethamine use ten days before breeding/laying season starts is reported in the literature (Tollini, Brocksen & Sureda, 2000).

Sulfadiazine has been shown to cause diarrhea in penguins (Tollini, Brocksen & Sureda, 2000).

Wünschmann *et al*. (2006) described a neuronal storage disease in five female Humboldt penguins (*Spheniscus humboldti*). The penguins, although coming from different zoos, had

genetically closeness. All the penguins were treated prophylactically with 25 mg/kg of chloroquine (about 100 mg for each penguin) SID for all the mosquito season (June until end of October or death). Clinical signs included lethargy, anorexia, progressive weakness, progressive ataxia, difficulty in standing and inability of accepting food. The birds were not parasitemic, although parasites were found at the necropsy. Histologic neuronal changes were found in all penguins, consistent with a neuronal storage disease. Since all animals simultaneously expressed the disease, an inherited storage it is unlikely. Chloroquine is considered the probable cause upon its ability to cause similar structural changes in neurons of miniature pigs and rats. Caution regarding high and cumulative doses of this drug must be taken.

6.3. Vaccination

An experimental DNA vaccine using DNA sequences of *P. relictum* and *P. elongatum* was produced for use in penguins. Vaccination occurred intradermally above the eyes and intramuscularly in gastrocnemius muscles. Booster injections were given three to four weeks later and until then penguins were housed indoors. Antibody levels increased after vaccination and the reduction of mortality was 75% (Cranfield, 2003; Cranfield, Graczyk & McCutchan, 2000). Grim *et al*. (2004) reported that parasitemia rates in African penguins (*Spheniscus demersus*) decreased from 50% to 17% despite intense mosquito infection rate. Additionally, no mortalities or side effects were recorded in the vaccination year.

However, even though birds are still stimulated by natural infection after immunization, the long-term immunity was low (Cranfield, Graczyk & McCutchan, 2000; Cranfield, 2003). In a study using the vaccine on canaries, McCutchan *et al*. (2004) stated that vaccinated canaries were statically different in terms of protection against malaria of unvaccinated ones. However, two seasons after the vaccination, this difference was no longer existent. Also, the mortality registered in second year belonged to vaccinated birds, while the ones that build natural immunity survived. Hypothesis that the vaccine eliminates or significantly reduces the parasite load and therefore prohibits acquisition of natural immunity seems to be the explanation.

This means boosters must be administrated every season (Cranfield, Graczyk & McCutchan, 2000; Cranfield, 2003).

6.4. Other preventive measures

Zoological gardens use different approaches when dealing with malaria in their penguin colonies. The most obvious way to prevent malaria infection is always keeping penguins in indoor facilities all year long (Graczyk, Shaw, Cranfield & Beali, 1994c; AAZV, 2013). The indoor facilities should have mosquito-free conditions to prevent malaria transmission

(Graczyk, Shaw, Cranfield & Beali, 1994c). Using pesticide strips proves effective to remove mosquitoes (Beier & Stoskopf, 1980). Covering aviaries with fine-mesh bolting silk can prevent mosquito entrance (Valkiūnas, 2005; Goswami & Swamy, 2013).

Options to do it in mosquito season or during its activity hours are also protective measures (Valkiūnas, 2005; Wallace & Walsh, 2005). Beier & Stoskopf (1980) determined that feeding periodicity for the vectors had a peak between midnight and 2 a.m., as diurnal contact is not likely to happen. Also, observations showed that juvenile penguins spend a great amount of time outdoors at night compared with adults. This is probably due to territorial behavior of adults regarding the indoor nesting areas. Bueno *et al*. (2010) referred that no mortalities where reported in São Paulo's Zoo penguin colony while penguins were maintained in an enclosure during the night. Since they started to be freed at night to treat a bumble foot problem (pododermatitis), infections developed.

However, when this is not possible, other measures may help diminish malaria impact.

The usage of fans to circulate air and create wind currents in the outdoor exhibits may help to control vector infestation (Valkiūnas, 2005; Wallace & Walsh, 2005) (Figure 21 and 22).

Figure 21 - Fan (left) and air extractor (right) at Humboldt penguins (*Spheniscus humboldti*) outdoor exhibition at "Oceanogràfic" (Valencia. Spain) (Courtesy of Daniel García Párraga, Jose Luis Crespo Picazo, Mónica Valls Torres and Teresa Álvaro Álvarez – "Oceanogràfic" Veterinary Team).

Figure 22 - Fan situated near nesting area at Humboldt penguins (*Spheniscus humboldti*) outdoor exhibition at “Oceanogràfic” (Valencia. Spain) (Courtesy of Daniel García Párraga, Jose Luis Crespo Picazo, Mónica Valls Torres and Teresa Álvaro Álvarez – “Oceanogràfic” Veterinary Team).

Setting up mosquito traps may also be a good option (Valkiūnas, 2005; London Evening Standard, 2012) (Figure 23).

Other measures to prevent vectors from coming near the penguin exhibition is the use of spray repellent products to the mosquitos, like lavender oil, in the nest boxes and having mosquito repellent plants, like lavender (*Lavandula angustifolia*), near the exhibit (The Independent, 2012; The New York Times, 2013).

Figure 23 - Mosquito trap at Humboldt penguins (*Spheniscus humboldti*) outdoor exhibition at “Oceanogràfic” (Valencia. Spain) (Courtesy of Daniel García Párraga, Jose Luis Crespo Picazo, Mónica Valls Torres and Teresa Álvaro Álvarez – “Oceanogràfic” Veterinary Team).

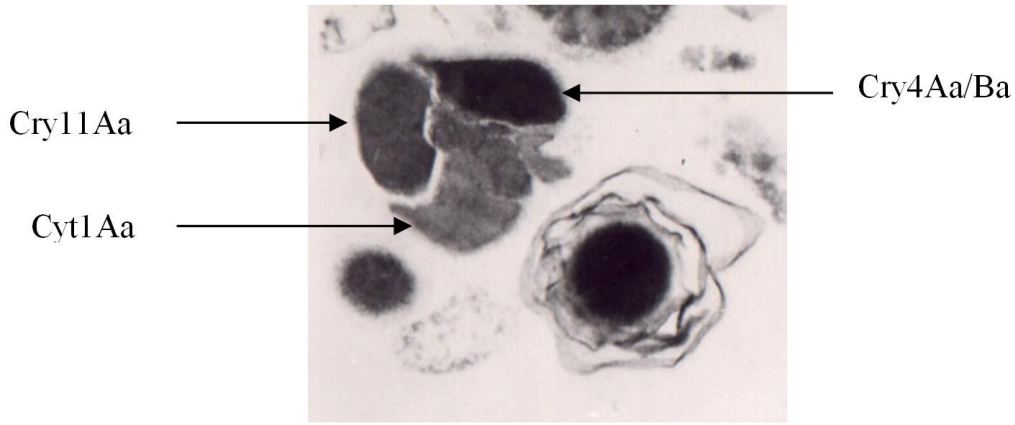
Allowing animals to build up natural immunity may be a good option. However, this requires a very tight monitorization of infection to rapidly initiate treatment (Graczyk, Shaw, Cranfield & Beali, 1994c; WBALTV11, 2013). When immunity is established, testing for malaria is still essential to control possible relapses. This should be made every 1-2 weeks (Valkiūnas, 2005; Wallace & Walsh, 2005).

Immunity and resistance are good tools when trying to control this disease (Atkison & Paxton, 2013). Some bird species with chronic infections may carry resistance genes that can be used to obtain more resistant individuals (USGS, 2006). Graczyk, Cranfield, Shaw & Craig (1994d) proposed that females which produce high titers of anti-*Plasmodium* spp.

antibodies should be used preferentially for reproduction. Also, they propose that serological profiles of each individual should be part of breeding programs of outdoor colonies.

Trying to eliminate the vector population is also an option. Draining and cleaning water bodies in and around the zoo or using pumps to keep water moving helps to reduce larval habitat of the mosquito vectors (Cereghetti *et al*., 2012; Goswami & Swamy, 2013; The New York Times, 2013). The same way, counts of the larvae density in ponds may help to understand the probability of infection, making this a monitorization measure (Bureau of Medicine and Surgery, 2000). The bacteria *Bacillus thuringiensi israelensis* (Figure 24) is highly effective controlling larvae of many mosquito species, including *Aedes*, *Culex* and *Anopheles* larvae (ISSG, 2010). Unlike chemical insecticides, such as organophosphates and carbamates, which harm the environment and have already presented resistances by some insect populations, *Bacillus thuringiensi israelensis* is environmental-friendly and its endotoxins with different action mechanisms prevent resistance from larvae. Some disadvantages are presented, like possibility of the bacteria to sink in the bottom of the pond, adsorption onto organic matter, inactivation by sunlight and ingestion by organisms to which it is not toxic. Alternatives to these disadvantages are being tested using recombinant bacteria (Ben-Dov, 2014). Other possibility is the use of larvae-eating fish, like the Fathead Minnow (*Pimephales promelas*), in the ponds (The New York Times, 2013).

Figure 24 - *Bacillus thuringiensi israelensis* with crystal and endotoxins (Cry11Aa, Cyt1Aa and Cry4Aa/Ba) (left) and spore (right) (Courtesy of Dr. Eitan Ben-Dov, Department of Life Sciences, Achva Academic College; and to the open access journal “Toxins”).



Aims of the study

This work has as main objective the better understanding of penguin malaria prevalence of in zoological collections around the world, as well as, a better insight on the different methods used in zoological gardens towards its diagnosis, treatment and prevention of disease in their outdoor exhibited penguin colonies.

Other goals included: a) understand the routine malaria diagnostic protocol among different zoos, b) evaluate if there are significant differences in the prevalence of infected versus non-infected penguins, in the demonstration of clinical signs and in the mortality rate; c) identify cases of mixed infections of blood parasites in penguins kept in captivity; d) determine the frequency of the typical clinical signs of malaria infection in penguins; e) evaluate the effectiveness of different prophylaxis and treatment protocols, as well as their side effects, implemented in different zoos; and f) associate the clinical signs presented with the prognosis of the animals.

Material and Methods

7. Inclusion criteria

In this research, only zoological gardens that had penguin colonies in total or partial outdoor exhibitions (with access to the exterior) in the period between January 2013 and May 2014 were included.

8. Survey

8.1. Survey Design

In order to achieve the goals proposed, a survey was carried out for veterinarians working in zoological gardens with outdoor exhibited penguins.

This survey was designed according to the current literature on avian malaria (Beier & Stoskopf, 1980; Graczyk, Cranfield, Skjoldager & Shaw, 1994a; Graczyk, Cranfield, McCutchan & Bicknese, 1994b; Graczyk, Shaw, Cranfield & Beali, 1994c; Graczyk, Cranfield & Bicknese, 1995c; Alves, 2002; Richard, Sehgal, Jones & Smith, 2002; Cranfield, 2003; Grim *et al*., 2003; Grim *et al*., 2004; Valkiūnas, 2005; Wallace & Walsh, 2005; Huijben, Schaftenaar, Wijsman, Paaijmans & Takken, 2007; Campos *et al*., 2011; Chitty, 2011; Weissenböck *et al*., 2011; Cereghetti *et al*., 2012; Christe, Glaizot, Strepparava, Devevey & Fumagalli, 2012; Krams *et al*., 2012; AAZV, 2013; Atkinson & Paxton, 2013; Palmer *et al*., 2013; Silveira *et al*., 2013) and survey design guidelines (Dohoo, Martin & Stryhn, 2003;

Nielsen, Agger & Ersbell, 2004). Previous works did not combine data about zoo location, prevalence of *Plasmodium* spp. and other blood parasites, infection in different penguin species, diagnosis methods, treatment and prophylaxis protocols and side effects, preventive measures and prognosis.

One of the considerations for the desing of the survey was that did not require much time of the veterinarian so the number of answers would be the higher possible.The survey has 15 questions, but only 8 are mandatory. Most of the questions are closed. Open questions were left to answers where the variety of possibilities was big (like institution, when there was the possibility of other answers than the stated ones, or when specification of drug active ingredients was requested). If all the possibilities were placed in the survey, this would make it very long and difficult to follow.

The English language is used in order to reach a bigger population. Questions are direct and easy to understand.

8.2. Survey Test and Validation

An initial survey was developed containing 42 questions (Annex 1). The majority were open questions. A panel composed by an epidemiologist, a parasitologist and a zoo veterinarian answered to the survey. The length of the survey and the type of questions were criticized because of the time consumed answering and the difficulty in statistical analysis of open questions. A final survey was elaborated containing 15 questions (Annex 2) and being reviewed by three persons, two with veterinary education and one with no medical education. The survey was classified as simple, easy to understand and quick. No changes were made.

8.3. Application

The survey was performed using the website Survio® (www.survio.com). Databases containing the zoological gardens which held penguin colonies in their collection were given by ISIS (International Species Identification System), with updated content on 31th March 2014. The only exception was Oceania's zoological gardens, which were not contemplated in ISIS list. In this case, individual searchs using Google® (www.google.pt) were made in order to establish which zoological gardens had penguin colonies. Email's containing a direct link to the online survey were sent to every zoological gardens electronic address (Veterinarian Department's email address when available or into general address of the zoo).

Help in divulgation of the survey was requested to WAZA (World Association of Zoos and Aquariums), EAZA (European Association of Zoos and Aquariums), BIAZA (British Association of Zoos and Aquariums), AIZA (Associación Ibérica de Zoos y Acuarios), AFDPZ (Association Française des Parcs Zoologiques), ALPZA (Asociación Latinoamericana de

Parques Zoológicos y Acuarios), AAWV (American Association of Wildlife Veterinarians), EAZWV (European Association of Zoo and Wildlife Veterinarians), EWDA (European Wildlife Disease Association), WDA (Wildlife Disease Association), ECZM (European College of Zoological Medicine), ACZM (American College of Zoological Medicine) and AAZV (American Association of Zoo Veterinarians).

9. Data Analysis

The results were recorded in a Microsoft Excel 2010® file and statistically analyzed with the R program, version 3.1.1, using the extension R Commander (The R Foundation for Statistical Computing, 2014).

For the categorical variables, absolute and relative frequencies were formulated and the graphics were done using Microsoft Excel 2010®.

Shapiro-Wilk normality test was performed regarding all the quantitative variables.

Since the sample size was small, Fisher's exact test (FET) was used to evaluate the association (contingency) between categorical and binominal variables. The 5% significance level was used.

Two-sample Wilcoxon test was used to evaluate the association between sample means and hypothesized values.

10. General

This survey had the duration of a month (27th April – 27th May, 2014). The survey was completed by 40 zoological gardens. 60% (24/40) of the veterinarians completed this survey in less than 10 minutes.

11. Geographical distribution

The distribution by regions is represented in Graphic 1. The countries included in each category are: Northern Europe – Sweden and United Kingdom; Southern Europe – Italy and Spain; Western Europe – France, Germany, Luxembourg, Switzerland and The Netherlands; Eastern Europe – Poland and Slovakia; North America – Canada and United States of America; and Asia – Israel. The geographical categorization served to facilitate the comprehension of the zones involved and was based on the current political geographic division. A more precise location of each zoological garden can be seen in Figure 25.

Graphic 1 – Spatial distribution of the Zoological Gardens that fulfilled the survey (n=40).

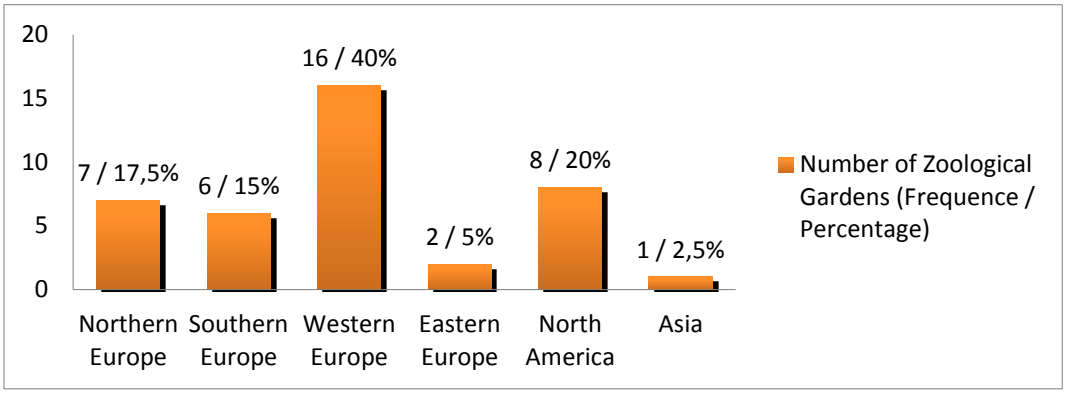
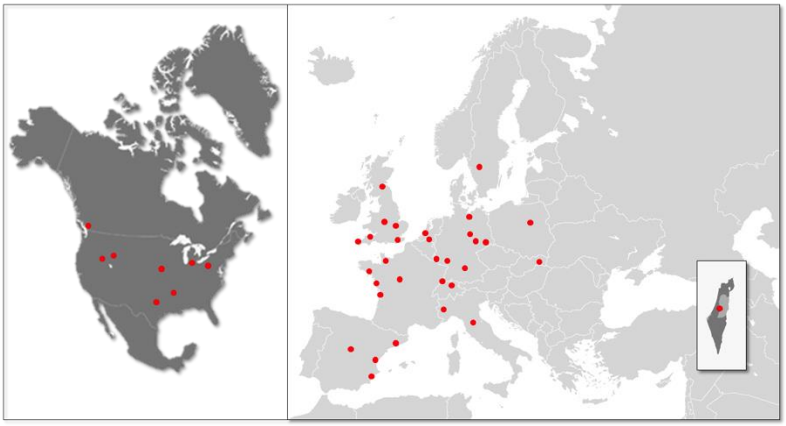


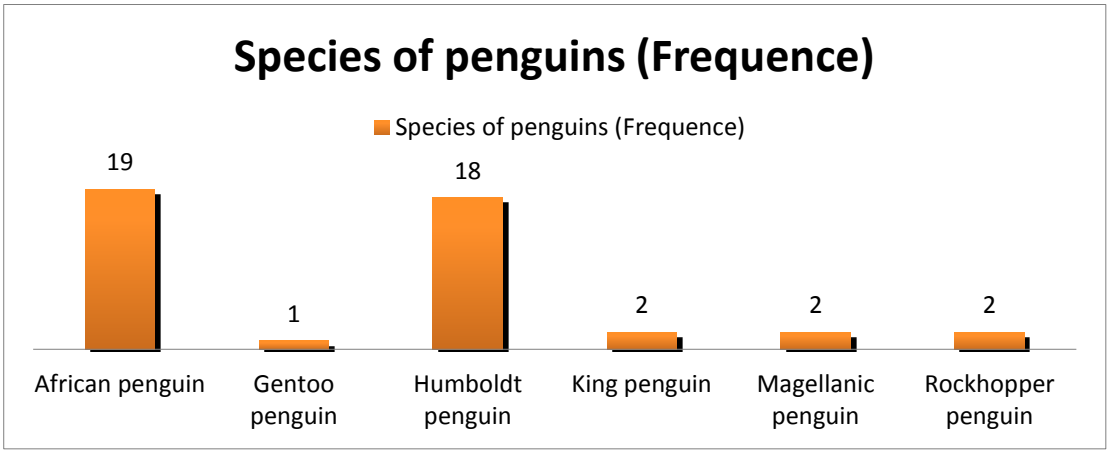
Figure 25 - Geographical distribution of the Zoological Gardens that fulfilled the survey (Left figure – North America; Right bigger figure – Europe; Right smaller figure – Israel) (Map source: Free Vector Maps, 2013).



12. Penguin species

The African penguin (*Spheniscus demersus*) and the Humboldt penguin (*Spheniscus humboldti*) were the most prevalent species exhibited, being present in 19 and 18 zoological gardens, respectively. The absolute frequence of the penguin species exhibited in the Zoological Gardens is stated in Graphic 2.

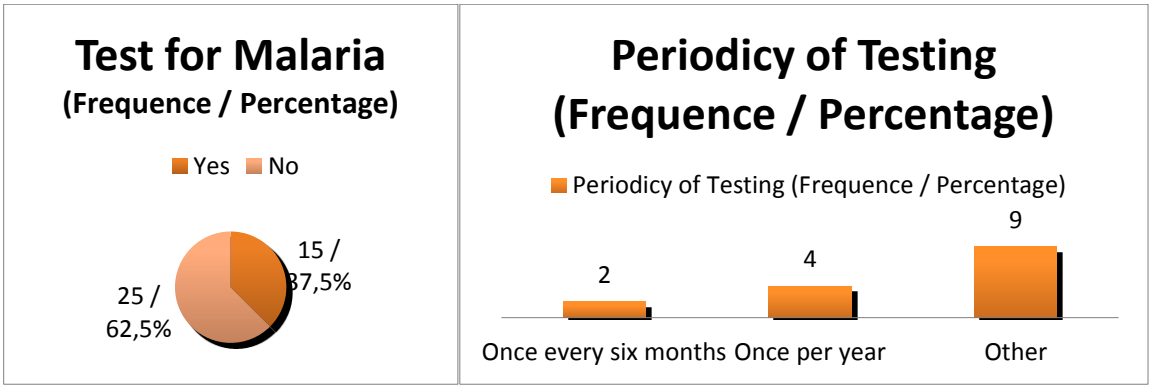
Graphic 2 – Frequence of penguin species exhibited outdoors at the Zoological Gardens that completed the survey.



13. Prevalence of Infection

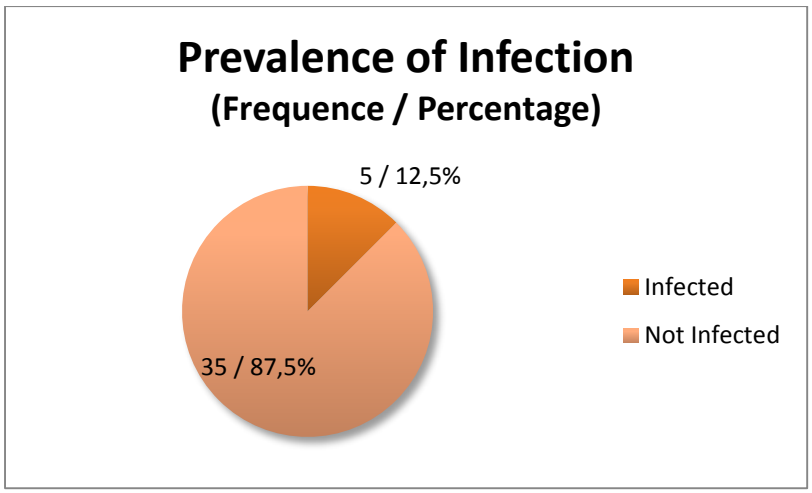
Regarding performing tests for *Plasmodium* spp. infection, 25 (62,5%) Zoological Gardens answered that they do not test for malaria and 15 (37,5%) stated that they test (Graphic 3). Questioned about the periodicity of testing, 2 (13,33%) zoos stated that they test once every six months, 4 (26,67%) stated they test once per year and 9 (60%) stated they use other type of testing frequency plan (Graphic 4).

Graphic 3 (left) – Prevalence of testing for *Plasmodium* spp. in the Zoological Gardens that completed the survey (n=40).
Graphic 4 (right) – Periodicity of testing for *Plasmodium* spp. in the Zoological Gardens that completed the survey (n=40).

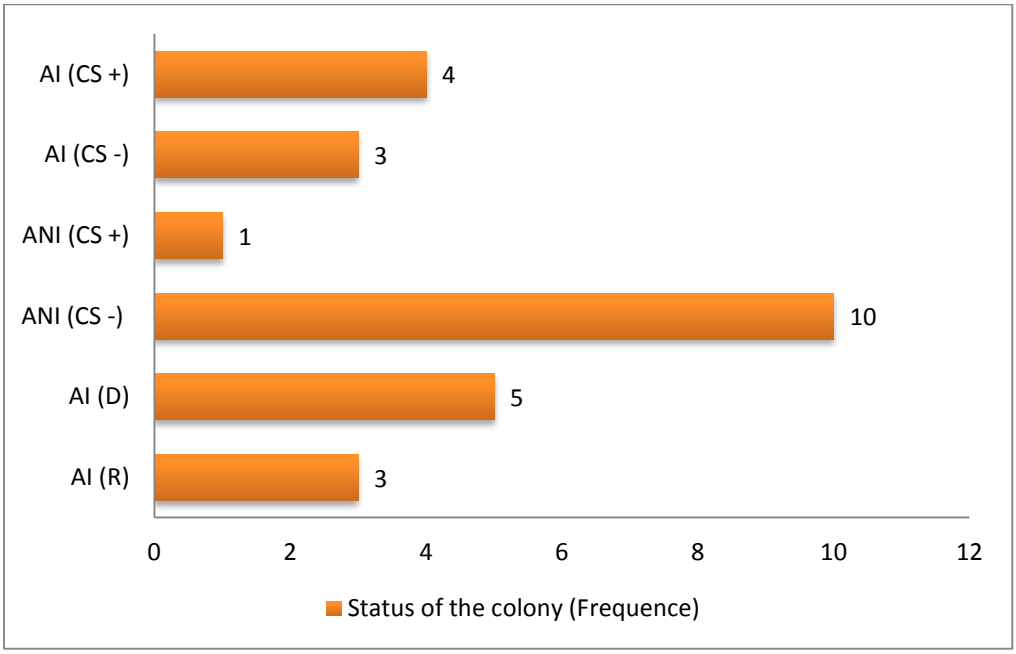


The prevalence of *Plasmodium* spp. infection in the penguin colonies was of 12,5% (5 zoos) (Graphic 5). The distribution by health status of the penguin population in the Zoological Gardens regarding *Plasmodium* spp. infection is presented in Graphic 6.

Graphic 5 – Prevalence of *Plasmodium* spp. infection in the penguin colonies of the Zoological Gardens that completed the survey (n=40).



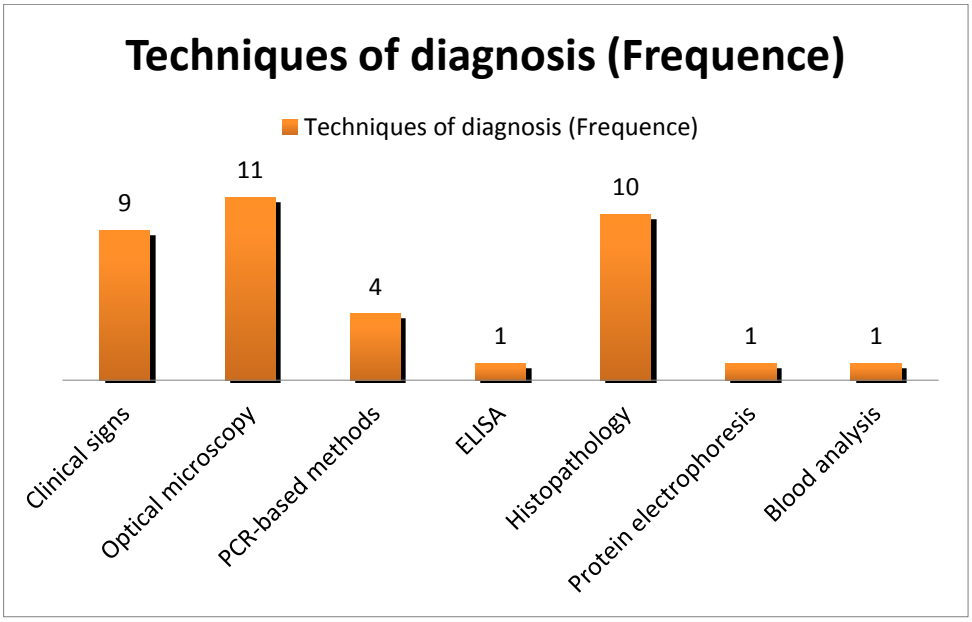
Graphic 6 – Distribution of health status of the penguin colonies of the Zoological Gardens that completed the survey (AI (CS+) - animals infected with *Plasmodium* spp. showing clinical signs; AI (CS-) - animals infected with *Plasmodium* spp. not showing clinical signs; ANI (CS+) - animals not infected with *Plasmodium* spp. showing clinical signs; ANI (CS-) - animals not infected with *Plasmodium* spp. not showing clinical signs; AI (D) - animals infected with *Plasmodium* spp. that died; AI (R) - animals infected with *Plasmodium* spp. that recovered from the infection).



14. Diagnosis

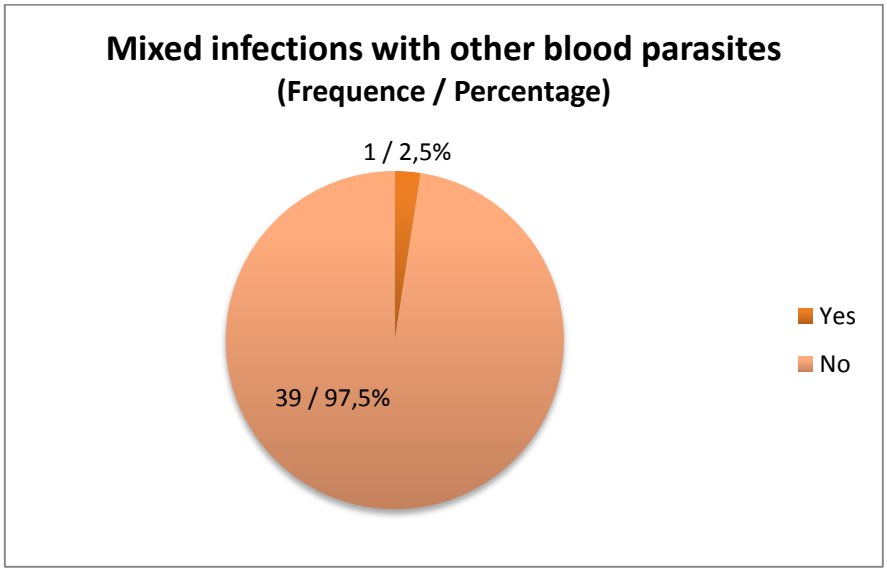
The frequence of techniques used by the Zoological Gardens to detect *Plasmodium* spp. in penguins is shown in Graphic 7.

Graphic 7 – Techniques used to detect *Plasmodium* spp. in the penguin colonies of the Zoological Gardens that completed the survey.



When asked if mixed infections with other blood parasites were detected, one zoological garden answered “Yes” (2,5%) (Graphic 8). The parasites detected were *Haemoproteus* spp. and *Leucocytozoon* spp.

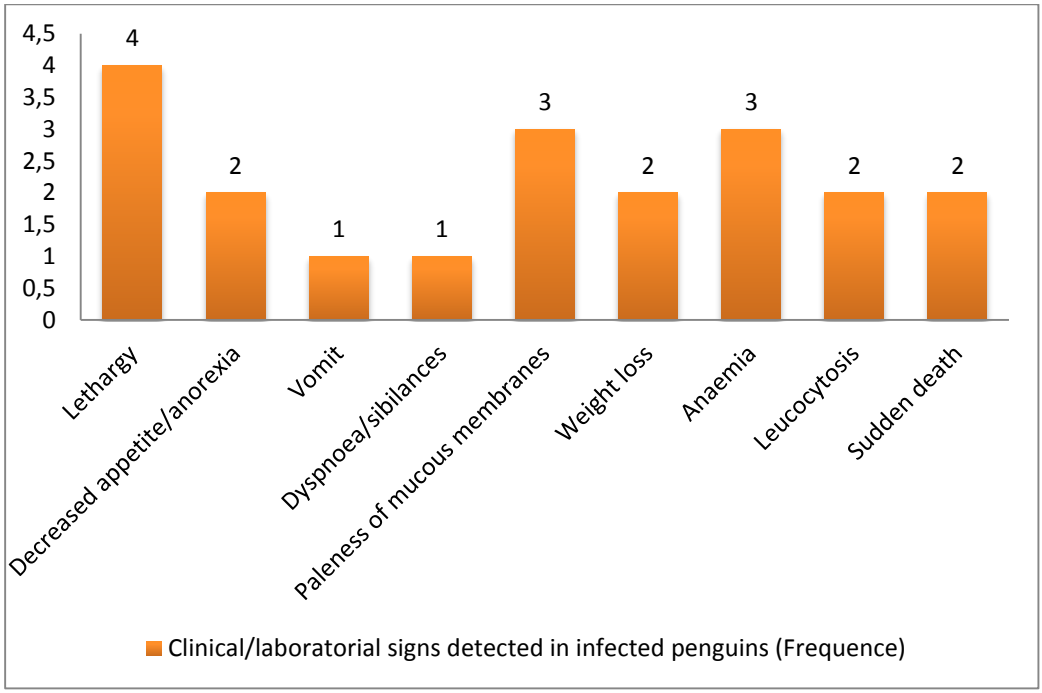
Graphic 8 – Prevalence of mixed infection with other blood parasites in the penguin colonies of the Zoological Gardens that completed the survey (n=40).



15. Clinical and Laboratory Signs

The frequence of clinical signs detected in infected penguins is recorded in Graphic 9.

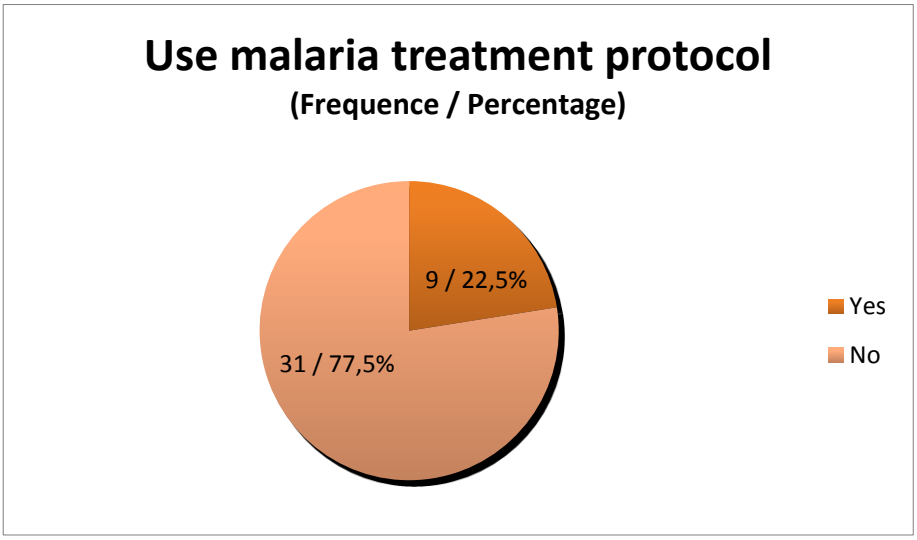
Graphic 9 – Clinical and laboratory signs detected in the penguin colonies of the Zoological Gardens that completed the survey.



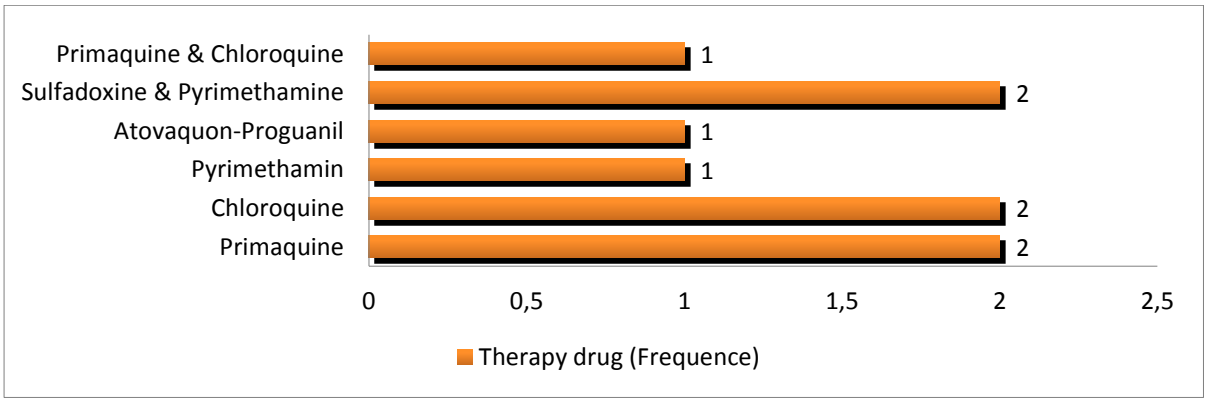
16. Treatment

The use of malaria treatment was applied in penguins living in 9 (22,5%) zoological gardens (Graphic 10). Regarding the drugs used, the frequence is stated in Graphic 11.

Graphic 10 – Use of malaria treatment in the penguin colonies of the Zoological Gardens that fulfilled the survey (n=40).

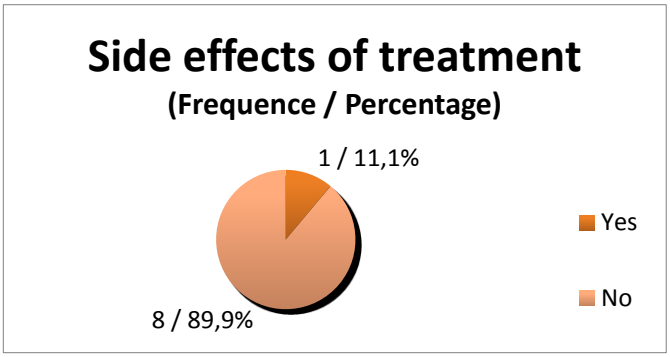


Graphic 11 – Frequency of malaria treatment drugs in the penguin colonies of the Zoological Gardens that completed the survey.



Regarding side effects of treatment, one zoological garden (11,1%) stated that the penguins showed side effects after malaria treatment (Graphic 12). The side effects were anorexia and vomit.

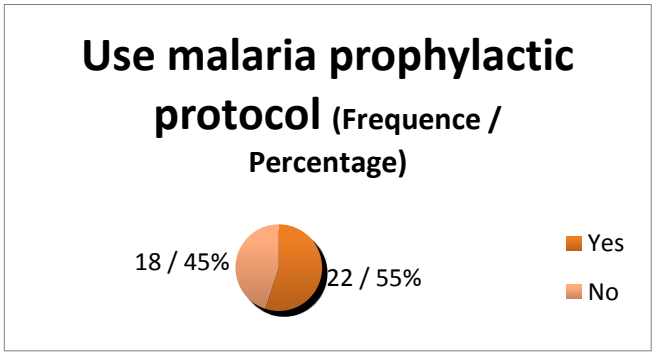
Graphic 12 – Prevalence of side effects of malaria treatment drugs in the penguin colonies of the Zoological Gardens that completed the survey (n=9).



17. Prophylaxis

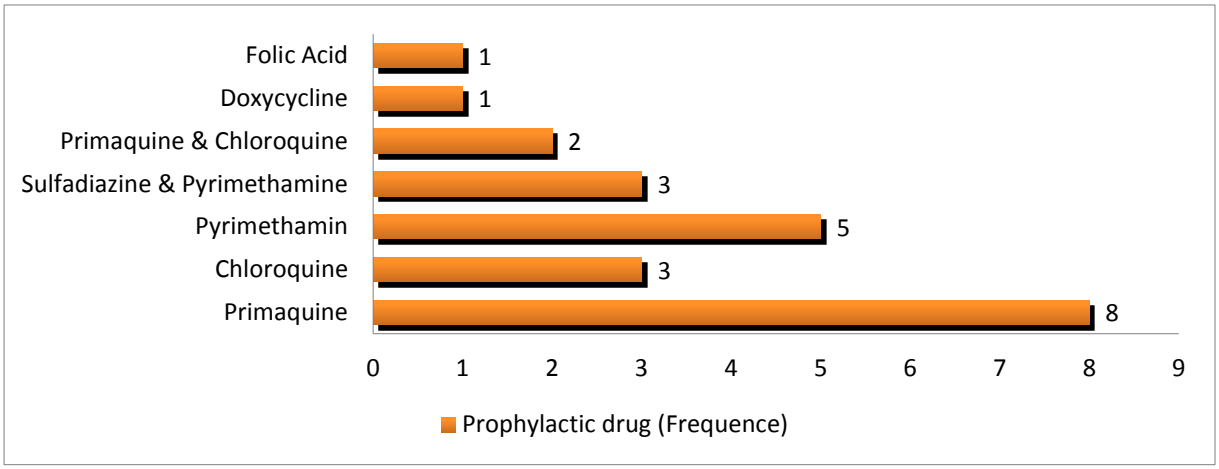
The use of malaria prophylactic protocol was applied in 22 zoological gardens (55%) (Graphic 13).

Graphic 13 – Use of malaria prophylactic protocol in the penguin colonies of the Zoological Gardens that completed the survey (n=40).



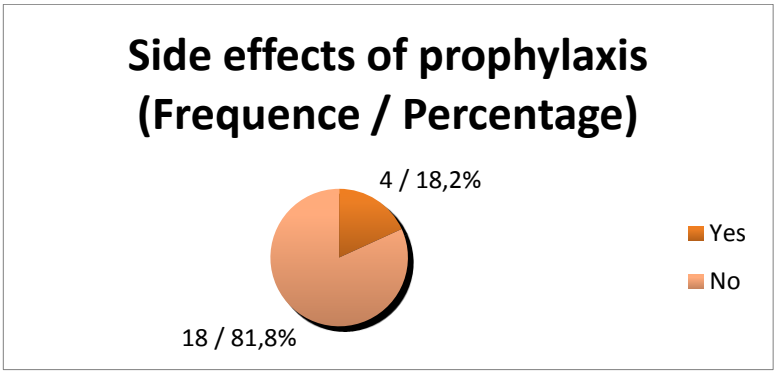
Regarding the drugs used, the frequence is stated in Graphic 14.

Graphic 14 – Frequency of malaria prophylactic drugs in the penguin colonies of the Zoological Gardens that completed the survey.

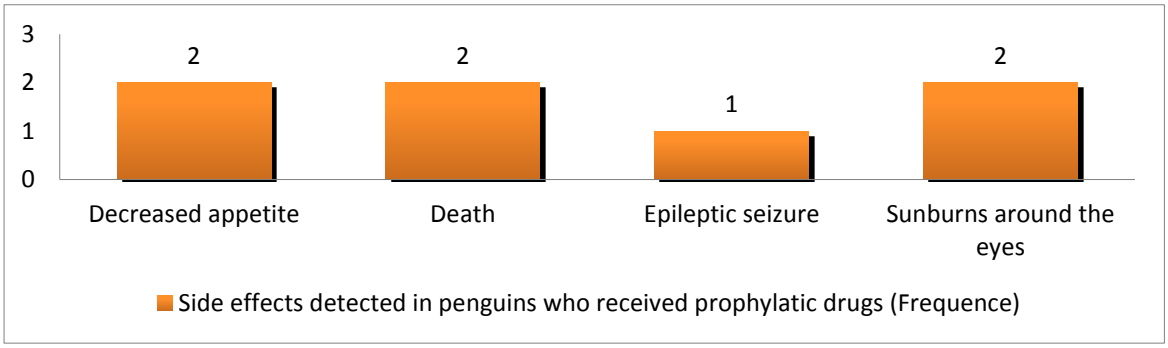


Regarding side effects of prophylactic protocol, four zoological gardens (18,2%) stated that the penguins showed side effects after prophylactic protocol (Graphic 15). The side effects are stated in Graphic 16.

Graphic 15 – Prevalence of side effects of malaria prophylactic drugs in the penguin colonies of the Zoological Gardens that completed the survey (n=22).

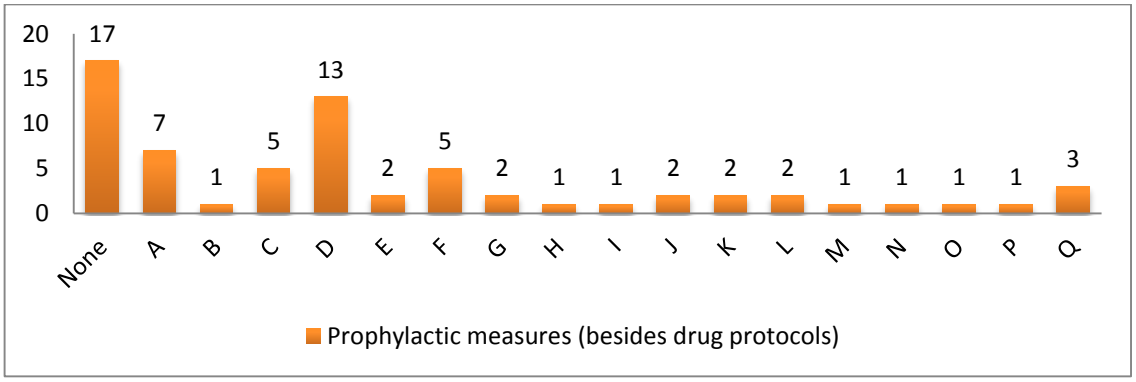


Graphic 16 – Side effects detected in infected penguins in the penguin colonies of the Zoological Gardens that completed the survey.



The frequence of prophylactic measures, besides drug protocols, used in the zoological gardens that completed the survey was recorded in Graphic 17.

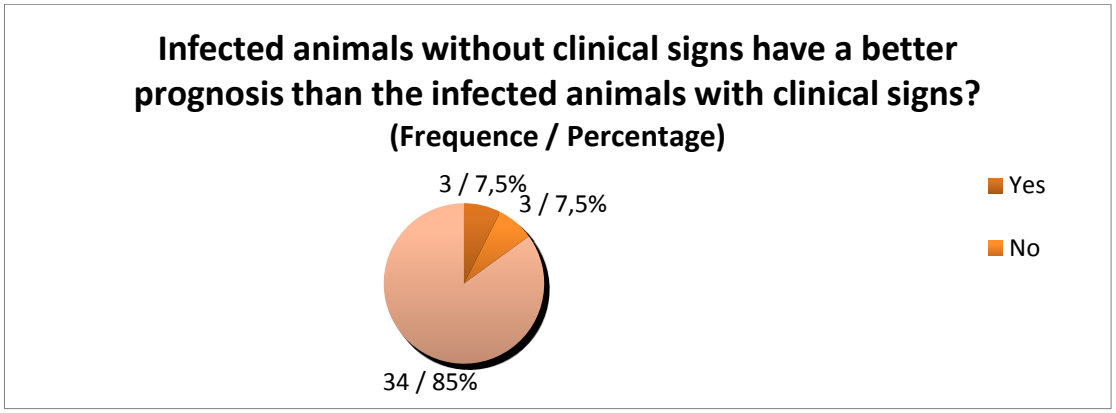
Graphic 17 – Frequence of prophylactic measures used by the Zoological Gardens that completed the survey (A - Set up mosquito traps at the exhibit site; B - Have mosquito repellent plants near the exhibit; C - Allow birds to be exposed to the vector to develop natural immunity; D - Reduce the number of potential water catchment containers in order to reduce the mosquito breeding sites available; E - Keep animals in inside enclosures during the mosquito season; F - Bring the animals to inside enclosures in the evening; G - Use of fans to circulate the air near the exhibit site; H - Spray repellent products to the mosquitos in the nest boxes; I - Keep larvae-eating fish in ponds near the penguins exhibit; J - High levels of hygiene and disinfection programs of the exhibits; K - Daily empting and filling of the penguins water pond; L - Use of sprinklers around the exhibit; M - Full post-mortem examination; N - Checking the amount of mosquito larvae in the water ponds; O - Use of pumps to move water in all ponds; P - Prohibition of bushes around exhibit to keep air moving; and Q - Biological anti larvae treatment using *Bacillus thurigiensis* var *israelensis*)



18. Prognosis

When asked if there was a association between the absence of clinical signs in infected animals and a better prognosis, 34 (85%) zoological gardens answered “Do not know”, 3 (7,5%) answered “Yes” and the same percentage answered “No” (Graphic 18).

Graphic 18 – Results of the question “In general did the infected animals without clinical signs have a better prognosis than the infected animals with clinical signs?” (n=40).



19. Association between categorical variables

Associations using F.E.T. were made to the following variables:

- Animals infected with malaria and:
    - Location of the zoo;
    - Species of penguins species;
    - Diagnostic method;
    - Periodicity of testing;
    - Preventive measures;
    - Prophylactic drug protocol;
- Animals not infected with malaria and:
    - Location of the zoo;
    - Periodicity of testing;
    - Preventive measures;
    - Prophylactic drug protocol;
- Animals that recovered from the infection and the treatment protocol;
- Animals that died from malaria and the treatment protocol.

Associations using two-sample Wilcoxon test were made to the following variables:

- Cumulative number of clinical signs and:
    - Animals that recovered from the infection;
    - Animals that died from malaria;
- Cumulative number of prophylactic measures and:
    - Animals infected with malaria;
    - Animals not infected with malaria.

The variables that presented a significant difference (*p*-value <0,05) are stated in Table 6.

Table 6 – Variables that presented significant difference using FET and two-sample Wilcoxon test.

Variables	*p*-value
Zoological Gardens in Southern Europe x Animals not infected with malaria	0.003611
Use of Biological anti larvae treatment using *Bacillus thurigiensis* var. *israelensis* x Animals infected with malaria	0.03644
Cumulative number of clinical and laboratorial signs x Animals that died from malaria	0.01964

Discussion

This study allowed a perception of the current methodologies to control malaria affecting penguin colonies in 40 zoological collections located in different regions of the globe. Initial goals to identify cases of mixed infections of blood parasites in penguins kept in zoological collections and determine the frequency of the typical clinical signs of malaria infection in penguins were achieved. Additionally, the survey dissemination created awareness of the impact of this disease, noted by the feedback of some veterinarians' replies.

However, the sample size makes it impossible to generate statistically significant associations. The goals to evaluate significant differences in the prevalence of infected versus non-infected penguins, in the demonstration of clinical signs and in the mortality rate, in the determination of the effectiveness of different prophylaxis and treatment protocols, comparing it with the death or recovery of animals, and association of the clinical signs presented with the prognosis of the animals, were not achieved.

Huijben, Schaftenaar, Wijsman, Paaijmans & Taaken (2007) stated that all the eight zoos in their survey experienced mortality. In this study, mortality rate is of 12,5% (5 of the 40 zoos) in the period between January 2013 untill May 2014. On the other hand, recovery rate was of 7,5% (3 of 40 zoos). Although in their study they state that the cumulative number of fatal cases was increasing, our results may be due to a larger sample (40 zoological gardens) or to a more effective control of the disease (association can not be calculated, since similar data were not presented in Huijben, Schaftenaar, Wijsman, Paaijmans & Taaken (2007) study). The fact that recovery rate is lower than the mortality rate may be due to a well known susceptibility of these animals to the disease (Bueno *et al*., 2010) or that the control programs are not totally effective (ISSG, 2010).

20. General

A sample of 40 zoological collections compared with the 248 institutions that have penguin colonies worldwide, according to ISIS databases, may seem small. However, some aspects must be analyzed. From all of the zoological gardens that have penguin colonies, a part has the animals in indoor exhibitions without access to the exterior. Quantification of how many zoological gardens have outdoor and indoor exhibitions is difficult and not practical, since the data is not compiled.

Only some of the Zoo and Aquarium's related associations helped in the dissemination of this survey. Although all the zoological gardens were contacted individually, some of the institutions refused to participate in scientific studies that are not made or approved by these associations.

Another factor contributing to the number of answers could be the means of contact. Email was used in this case and the possibility that it did not reach the veterinary staff is significant.

However, since the survey had 264 views (according to Survio® final report), this may not be the main cause.
Other possibilities for some of the visitors who did not complete the survey (19 started to answer but did not complete) were the length of the survey or disagreement with its composition.

21. Geographical distribution

The majority of answers came from European zoological gardens. According to ISIS databases, Europe has in fact a bigger number of zoological gardens containing penguin colonies than other parts of the world. However, North America has a relatively similar number of zoological gardens with penguin colonies. Differences in the amount of answers of each region may be due to the dissemination made by associations, since the two associations who advertised the survey are European.
Within Europe, most zoological gardens with penguin colonies are located in Western Europe (42,3%, 58 zoological gardens) and Northern Europe (37,2%, 51 zoological gardens). Southern and Eastern Europe present a significantly inferior number (10,2%, 14 zoological gardens). Differences between the number of answers coming from Western (16) and Northern Europe (7) and between Southern (6) and Eastern Europe (2) may be due to better vector development features of Western and Southern Europe. Although vectors are present in all Europe (Vinogradova, 2000), their optimal development conditions involve hot wet climates (Hudson County Mosquito Control, 2013). It is known that river water levels in the center of Europe favor the development of the mosquito eggs (Becker *et al*., 2010) and Southern Europe was a historically zone of high endemicity even for human malaria (Alten, Kampen & Fontenille, 2007). Adequate conditions for vector development, as well as possible longer transmission seasons, have the potential to enhance the possibility to develop infections in these regions. This would make veterinarians from these countries more conscious about the disease, participating in the survey.

22. Penguin species

According to ISIS databases, Humboldt (*Spheniscus humboldti*) and African penguins (*Spheniscus demersus*) are the penguin species more prevalent in zoological gardens, representing 44,4% (3038 individuals) and 35,4% (2423 individuals) of the world's penguin population in zoological collections, respectively. So, the results of the penguin species distribution from the zoos that answered the survey were in accordance to the expected.

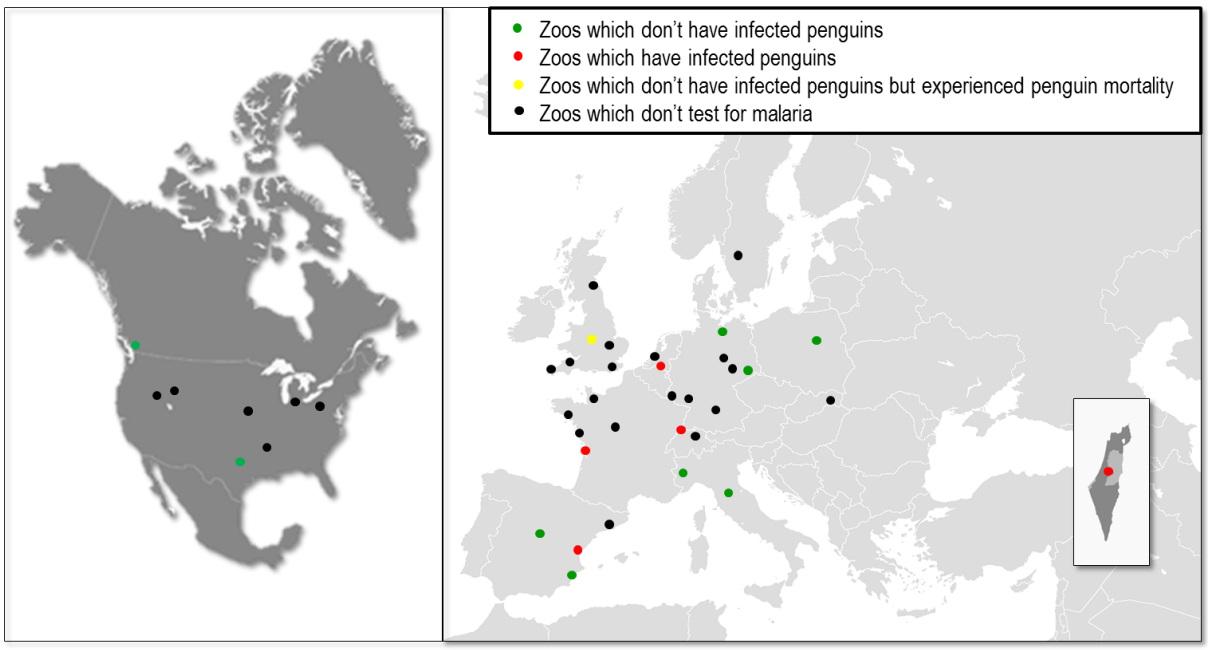
23. Prevalence of Infection

A limitation for this survey was the lack of a question asking the reasons why in some zoological gardens testing for malaria was not part of the routine. Location may be the most obvious cause. However, transmission of avian malaria can occur in the area of the Arctic Circle in Northern Europe (Krams *et al*, 2012). Also, at least one zoo from each region represented, tested for malaria. Awareness of the disease and risk of infection may be other explanations for not testing. The same way, this fact can explain why 5 of the 6 zoological gardens from Southern Europe test their penguin colonies.

The question regarding periodicity of testing is not correct since instead of the choice "Other" it should have been done "Less than once every six months" and "More than once per year". Conclusions about these results can not be made since most of the veterinarians answered "Other" giving no information on the normal testing schedule.

Overall, the prevalence of malaria infection is relevant (12,5%). Real prevalence may be higher since not all the zoological gardens test for malaria. This finding agrees with the opinion of Jones & Shellam (1999) that penguins' infections are more widespread than shown on the records. Location of zoos that reported infected penguins was variable (Southern Europe, Western Europe and Asia) (Figure 26) agreeing with the widespread distribution of the vectors and the avian malaria parasites (Ziegyte *et al*., 2014).

Figure 26 - Categorization of the Zoological Gardens according to their penguin colonies health status regarding malaria (Left figure – North America; Right bigger figure – Europe; Right smaller figure – Israel) (Map source: Free Vector Maps, 2013).



Regarding the health status of the individuals, the most prevalent animals are the ones which are not infected and do not show clinical signs. This finding agrees with the prevalence of infection presented.

The presence of infected animals with or without clinical signs is similar. The last category can be explained by the presence of chronic infected penguins that may have occasional blood stages of the parasite (Alves, 2002), animals that present an initial stage of the disease (Beier & Stoskopf, 1980), zoos that have penguins that recovered from the infection and so veterinarians know they are infected even if no parasites are detected, zoos that use molecular and serological methods to the detection of the parasites - molecular methods can detect low parasitaemia that may not cause clinical signs (Christe, Glaizot, Strepparava, Devevey & Fumagalli, 2012) and serological methods detect antibodies which only reveal that parasite was present (Graczyk, Cranfield, Skjoldager & Shaw, 1994a); and when zoos allow their penguins to gain natural immunity and treat immediately, before clinical signs appear (Graczyk, Cranfield, Skjoldager & Shaw, 1994a). From the zoological gardens that stated that penguins infected with malaria not showing clinical signs were present in their colonies, animals that recovered from the infection are present and PCR-based methods are used for the diagnosis of the disease.

The presence of penguins not infected with malaria showing clinical signs has two possible explanations: either a bad diagnosis has been made or the symptoms of another disease are being mistaken for the symptoms of malaria (Beier & Stoskopf, 1980). Since optical microscopy and PCR based-methods are used as diagnostic tools in the zoo that reported this case, the most likely is that another disease is causing the clinical signs, like aspergillosis.

Regarding the existence of cases of death or recovery of the malaria, significant differences do not exist.

24. Diagnosis

Light microscopy, allied to clinical signs showed by the penguin, is still the most used diagnostic technique. This is much due to the fact that it's a cheap method, only requires that the zoo to has a microscope and it can provide a relatively quick diagnosis (Alves, 2002).

Histopathology is also a broadly used technique. Although sudden death has only been reported in two zoos, it is common practice in zoological medicine to perform necropsy to all dead animals and to send samples to analysis if any suspicion or alteration is present.

Molecular and serological methods are reported to be used. Costs related to these techniques and the fact that positive results may not be associated with active infection may be the reasons why these techniques do not seem as popular as microscopy, although their greater sensitivity (Campos, 2011).

Protein electrophoresis and blood analysis are likely to be used as indicators of the penguins health status and not as diagnostic tools. The use of hematological parameters has been studied and correlation with diagnosis couldn't have been done (Graczyk, Shaw, Cranfield & Beali, 1994c), while protein electrophoresis is likely used to evaluate increases in beta, beta/gamma and gamma globulins associated with infection/inflammation, being referenced as strongly supporting aspergillosis diagnoses (University of Miami, 2014).

Regarding mixed infections with other blood parasites, only one zoological garden reported detection of *Haemoproteus* spp. and *Leucocytozoon* spp. infections with these parasites have already been documented in penguins (Fallis, Bisset & Allison, 1976; Vanstreels *et al*., 2014b). A possible explanation for the low prevalence of mixed infections is that *Haemoproteus* spp. and *Leucocytozoon* spp. have a narrower range of vertebrate hosts than *Plasmodium* spp. Also, the prevalence of *Haemoproteus* spp. and *Leucocytozoon* spp. vectors (hippoboscid flies and simuliid flies, respectively) on the zoo environment is lower that the prevalence of mosquitoes (Valkiunas, 2005).

25. Clinical and Laboratorial Signs

No significant deviations are observed in the clinical and laboratorial signs. Lethargy, paleness of mucous membranes and anaemia are the most frequent observed signals, probably due to the fact that they are easy to observe. All the signs referred are according to the literature (Graczyk, Cranfield, McCutchan & Bicknese, 1994b; Alves, 2002; Wallace & Walsh, 2005).

26. Treatment

Nine of the forty zoological gardens stated that they use or have used treatment protocols, being in accordance to the prevalence of infection plus the past cases of malaria reported.

Treatment drugs used do not present significant differences. All of the drugs are stated in the literature as treatment protocols (Rebêlo *et al*., 2005; Valkiūnas, 2005; AAZV, 2013). No efficacy studies comparing the different treatment protocols have been published in the literature untill present date to the author's knowledge. The diversity of drugs protocols reveals the lack of a strong effective treatment protocol.

Side effects of treatment protocols have only been reported in one zoological garden. This zoo used a Sulfadiazine & Pyrimethamine combination and reported anorexia and vomit. Sulfadiazine causes diarrhea in penguins (Tollini, Brocksen & Sureda, 2000). Although for the pyrimethamine teratogenic effects were reported in penguins (Wallace & Walsh, 2005), in humans it has been shown that it causes anorexia and vomit (World Health Organization [WHO], 1995). To the author knowledge, this is the first report of anorexia and vomit in penguins under Sulfadiazine & Pyrimethamine therapy.

27. Prophylaxis

Although only 15 zoos test their colonies for malaria, 22 apply prophylactic drug protocols. So this gap may represent zoological gardens where veterinarians are aware of the disease, but conditions related to the colony make difficult the testing process. Many zoos do not like to perform such activities with their penguins because of the induced stress (Wallace & Walsh, 2005). Many problems related with stress from human handling include decrease in reproduction rates and even death in sick animals (Ellenberg, Mattern, Seddon & Jorquera, 2006). So, probably, in these zoos prophylaxis is the field where more effort is applied to control the disease.

Regarding the drug protocols, Primaquine and Pyrimethamine are more commonly used than the other options. However, significant differences are not observed. One interesting finding is the use of Doxycycline as a prophylactic drug. To the author's knowledge, no protocols are established for penguins, although Chitty (2011) pointed its possible use as a prophylactic drug.

Side effects have been reported in four of the zoological gardens, varying from decreased appetite, sunburns around the eyes, epileptic seizure to death. One zoo reported decreased appetite using a Primaquine & Chloroquine combination. Both these drugs are known to cause abdominal distress and nausea in humans (Vinetz, Clain, Bounkeua, Eastman & Fidock, 2011). This will be a possible explanation to the decreased appetite showed. Three clinical signs were reported using Pyrimethamine: in one colony only death and in the other reports of death, epileptic seizure and sunburns around the eyes. In human medicine, pyrimethamine has been reported to cause skin rashes, exfoliative dermatitis and urticaria. The same way, it has been shown to make the skin more sensitive to sunlight (Vinetz, Clain, Bounkeua, Eastman & Fidock, 2011). Pyrimethamine can also induce epileptic seizures (Villena *et al*., 1998). This may explain the sunburns and epileptic seizures presented. To author knowledge, no reports of death using pyrimethamine regarding humans and penguins exist. In excessive doses, pyrimethamine causes megaloblastic anaemia in humans (Vinetz, Clain, Bounkeua, Eastman & Fidock, 2011). If this mechanism would happen in penguins, death could be due to anaemia. However, other clinical signs would have been noticed and reported. A zoo using doxycycline reported decreased appetite and sunburns around the eyes in their penguins. Doxycycline is known to cause photosensitivity (Vinetz, Clain, Bounkeua, Eastman & Fidock, 2011), decreased appetite and nausea in humans (Berger, 2013). In penguins, it has been shown to cause gastrointestinal ulceration, regurgitation and photosensitization of the skin (Jencek *et al.*, 2012).

In the author knowledge, there are no previous reports of some of the discussed side effects in penguins receiving primaquine and chloroquine and pyrimethamine therapies.

When asked about other preventive measures implemented on the zoo, 17 veterinarians stated that they do not use preventive measures. Nine of these zoos are from Western Europe, four are from Northern Europe, two are from Southern Europe, one from Asia and one from Eastern Europe. Again, favorable climate conditions would make a higher awareness or increase the necessity of using preventive measures. Many of the zoos that completed the survey do not use prophylactic drugs or test for malaria as well (9 zoos). However, eight zoological gardens either test for malaria, have prophylactic drug protocols or both. Infection (including death and recovery) was reported in only one zoo using light microscopy, PCR-based methods and histopathology as diagnostic techniques and chloroquine as prophylactic drug. Treatment in this zoo is not used. This fact may reflect the need for multiple methods to prevent and control the infection.

A significant number of zoos limit the number of water catchment containers in order to reduce the mosquito breeding sites available. This has proven to be an important measure since reduction of vector population can be achieved (Tollini, Brocksen & Sureda, 2000). However, range of the mosquito population may be larger than the zoo perimeter and this may be an insufficient measure. With the same logic, zoos also use mosquito traps, which are cheap and effective to control the vector. Another popular measure is allowing animals to be exposed to the vector, monitoring the infection and rapidly advance with treatment (WBALTV11, 2013). This method has proven to be effective in order to create individuals resistant to the disease since, unlike vaccination (that none of the zoos reported to use), it makes a long-term immunity, protecting the penguins for other mosquito seasons during their life. A possible explanation for the fact that not all the zoos use this measure is because it requires great monitorization (that might stress the animals) and it risks not detecting parasitaemia soon enough or even that the treatment is not successful in stabilizing the infection (Graczyk, Cranfield, McCutchan & Bicknese, 1994b).

An important observation is that keeping colonies indoor all year round or during mosquito season is not a very used method. Practical disadvantages like the lack of such facilities in certain zoos or the stress induced even for a little transport are evident.

Some measures referred by the veterinarians were not seen by the author in the literature. Restraint barriers like bushes or trees can, like the fans, create wind currents that difficult the flight of mosquitos and thus prevent their prevalence near the exhibition. Water sprinklers will work with the same logic of impeding the flight by soaking the vectors.

28. Prognosis

A question was made regarding the association between the presence of clinical signs and the prognosis of the disease. This was a subjective topic since it was based on the veterinarian's opinions and not on scientific data.

Results are obvious that veterinarians do not find a direct association between these variables. It would be logical to think that an animal without clinical signs would have been suffering in less extent to the infection or that parasite load was lower, allowing the immune system to control the infection and recovery from the infection. However, situations like sudden death, often reported in the literature, would alter this perception.

29. Association between categorical variables

The only understandable association present is the one stating that zoos where more clinical and laboratorial signs were reported, presented penguin mortality. This is logical, since greater degrees of infection will result in bigger expression of symptoms and would be more difficult for the penguins to control the infection, eventually succumbing to the disease.
However, given the small size of the sample, the possibility of occurring an error exists. In other words, it may exist significant difference in some of the variables analysed. However, regarding the sample size, it is not possible to show these differences unless they are very pronounced.

30. Monitorization and eradication program

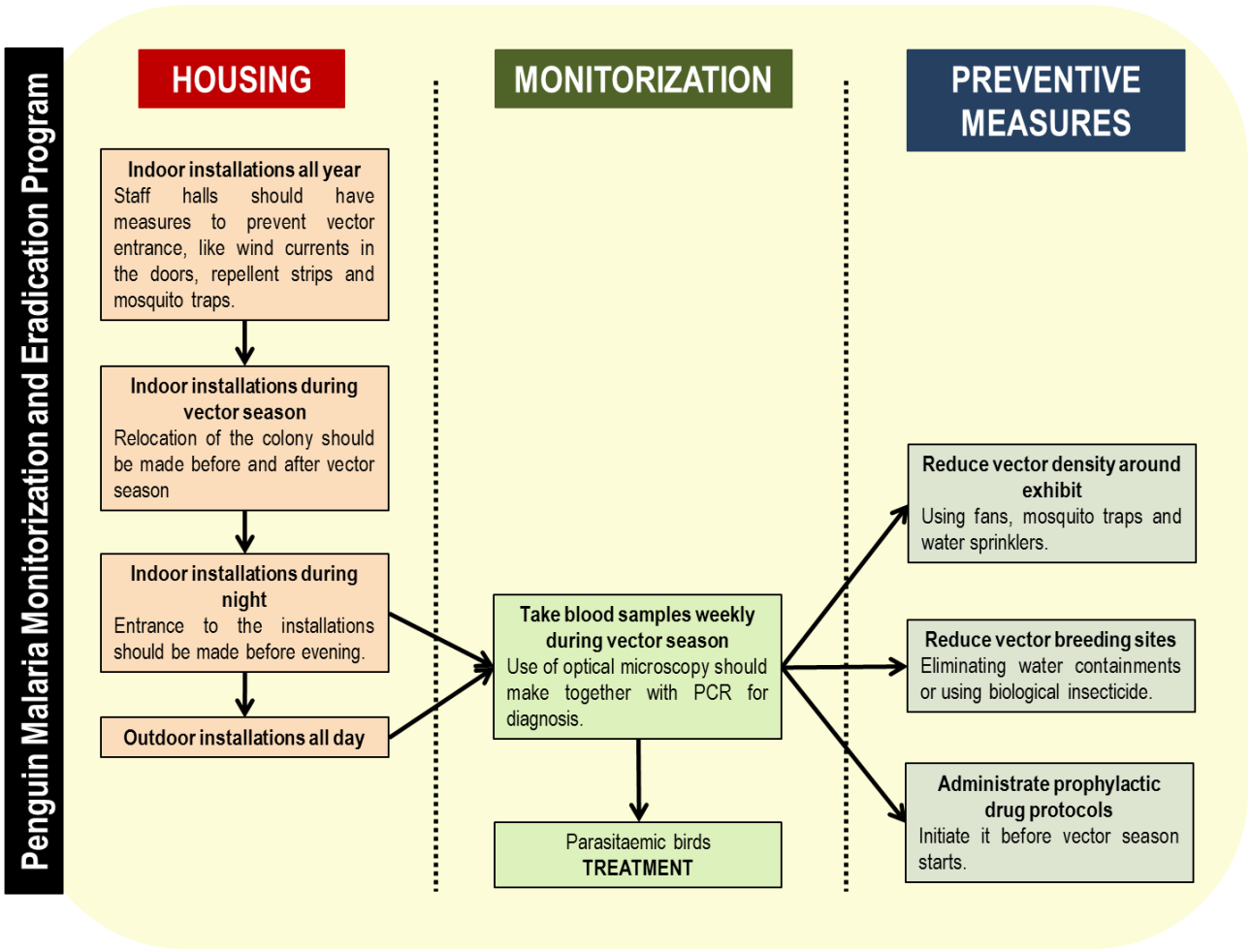
A proposal for a program of monitorization and eradication of malaria in penguin colonies in zoos is presented (Figure 27). Major aspects include housing, monitorization and preventive measures. The present compilation is thought to be used by zoological gardens around the world with outdoor penguin colonies. Other important aspects reported by Tollini, Brocksen & Sureda (2000) include using the same keepers to recognize more easily behavioral changes in the animals, training of hand-feeding the penguins to control food and medication administration and written track of these two parameters.

30.1. Housing

Ideal housing conditions include vector-free indoor installations where penguins are housed all year. Staff halls should be equipped with measures to reduce possible vector entrance, like wind currents in the doors, repellent strips and mosquito traps.
If such conditions are not possible all year long, this could be made only in vector season. Vector prevalence and activity should be searched for the localization of the zoo. Relocation of the colony should be made before and after vector season. Another option is to make it only by night, since that’s when vectors have maximal activity. When this choice is made, preventive measures and prophylactic drug protocols should be used to ensure infection does not happen, since there is vector activity in the day.

Using a silky net, an enclosure can be made for housing the penguins by night. Areas and housing conditions can be consulted in Wallace & Walsh (2005).

Figure 27 - Program of monitorization and eradication of malaria in penguin colonies in zoological gardens (Original).



30.2. Monitorization

This action is indicated to animals that do not go to inside enclosures. It should be started before vector season and made weekly during it. Bleeding the animals permits a diagnosis and initiates treatment. Maryland Zoo only tests 1- and 2-year-old penguins (WBALTV11, 2013), since older penguins have theoretically developed immunity. The author suggests this scheme, but also testing newly introduced individuals, whether if they come from indoor exhibitions or from zoos where no or other *Plasmodium* spp. was present. This allows birds to develop natural immunity and stay protected for the next seasons.

Diagnosis should be made using more than one technique to enhance sensitivity. Light microscopy and PCR-based methods are suggested to be used together in order to eliminate false negatives.

30.3. Preventive measures

Environment should be changed in order to reduce vector abundance near the exhibit. Water containers should be avoided or compositions using *Bacillus thurigiensis israelensis* should be added to it. Wind currents should be created with fans, mosquito traps should be placed in different sites and water sprinklers could be used. Extreme importance in protecting nesting area is necessary, since this will be the place where penguins will be when vector is most active.

Prophylactic drug protocols should be initiated before vector season starts.

Conclusion

Avian malaria has shown to cause profound impacts in many of the penguin colonies kept in zoological collections around the globe. Measures can be made to reduce or eliminate the consequences of this disease in highly susceptible animals, like penguins.

Over all acceptance of this study by the zoo community was good, revealing interest in this problem.

Fifteen zoos (37,5%) test their colonies for malaria, and a global prevalence of 12,5% (5 zoos) of institutions with infected penguins was found. Diagnostic techniques most currently used are optical microscopy (11) and histopathology (10). Twelve zoos use combinated techniques diagnosis protocols. Mixed infections with other blood parasites were reported by one zoo (*Haemoproteus* spp. and *Leucocytozoon* spp.). No significant differences were found in clinical and laboratorial signs presented, being lethargy (4) the most prevalent. Nine zoos (22,5%) use treatment protocols on detected cases, but no significant differences were observed in the protocols. Twenty two zoos (55%) use prophylaxis protocols, being primaquine the most common drug in eight zoos. Seventeen zoos (42,5%) reported that no preventive measures besides preventive therapeutic protocols are used, while reducing the number of potential water catchment containers in order to eliminate the mosquito breeding sites available, was the measure most commonly adopted in 13 zoos (32,5%). For the first time, there are reports on penguins of anorexia and vomit when using sulfadiazine and pyrimethamine; anorexia when using primaquine and chloroquine and epileptic seizures, sunburns around the eyes and death when using pyrimethamine.

The main goals established for this study were accomplished. Prevalence of infection and techniques for treatment and prevention of malaria were discussed. Also, diagnostic techniques' preferences, detection of mixed infections and clinical and laboratorial signs frequencies were also discussed. The study failed to demonstrate significant differences in the prevalence of infected and non-infected penguins, in the mortality and recovery rates; as well as in determining the effectiveness of treatment and prophylaxis protocols associating it with mortality and recovery rates, and associating the prognosis with the clinical signs presented. New observations regarding the rate of testing and application of preventive measures were made.

Results and conclusions from this study allow a better understanding of the penguin malaria control reality present in the zoological collections. These observations allow compiling data that can be used by other zoological collections in their penguin colonies.

In the future, a better knowledge of the variables studied along with housing conditions, demographics of the penguin population, health status of the colony regarding other common diseases, treatment times and supportive care treatment and clinical signs associated with a good or bad development of the disease should be achieved.

Future studies in this area are needed. Alternative treatments used in human medicine with natural substances from plants should be tested in penguin colonies to reduce the use of treatment with synthetic drugs and their side effects. Apart from that, pharmacokinetic studies are fundamental to know the optimal concentration of the malaria treatment options for penguins. Also, prevalence studies should be made in zoo's birds and native birds to understand which species can function as reservoirs of the disease. Development and testing of new cheap and quick methods of diagnosis, like rapid diagnostic tests, will increase the possibility of an accurate diagnosis that permits treatment in time.

After almost 90 years of the first report of avian malaria in a penguin, this is still the number one cause of mortality in captive penguins. Also, with climate changes and human action, wild and endangered species are being affected by this problem. Zoological gardens containing penguin colonies should implement measures that could serve in prevention of malaria dissemination in their collections, but also be committed with formal research towards the better knowledge of the disease and especially on how to intervene to control it when necessary in wild populations on risk.

References

Alten, B., Kampen, H. & Fontenille, D. (2007). Malaria in Southern Europe: resurgence from the past? In W. Takken & B.G.J. Knols (Eds.), *Emerging pests and vector-borne diseases in Europe*. (pp. 59-74). Wageningen: Wageningen Academic Publishers.

Alves, A.C.R. (2002). *Estudos de Malária Aviária em pinguins da espécie Spheniscus demersus no Jardim Zoológico de Lisboa*. Dissertação de Mestrado em Parasitologia Médica. Lisboa: Instituto de Higiene e Medicina Tropical – Universidade Nova de Lisboa.

American Association of Zoo Veterinarians (2013). *Infectious Disease Committee Manual 2013 Plasmodium.* Acedido em Mai. 25, 2014, disponível em http://c.ymcdn.com/sites/www.aazv.org/resource/resmgr/IDM/IDM_Plasmodium_2013.pdf

Atkinson, C.T. & Paxton, E.H. (2013). Immunological markers for tolerance to avian malaria in Hawai`i `Amakihi: new tools for restoring native Hawaiian forest birds? *Technical Report HCSU-042.* Acedido em Mar. 17, 2014, disponível em http://hilo.hawaii.edu/hcsu/documents/TR042_Atkinson_Immunologicalmarkers.pdf.

Becker, N., Petric, D., Zgomba, M., Boase, C., Madon, M., Dahl, C. & Kaiser, A. (2010). Biology of Mosquitoes. *Mosquitoes and Their Control*. Acedido em Ago. 20, 2014, disponível em file:///C:/Users/Miguel%20Grilo/Downloads/9783540928737-c1%20(1).pdf.

Beier, J.C. & Stoskopf, M.K. (1980). The epidemiology of Avian Malaria in Black-footed penguins (*Spheniscus demersus*). *Journal of Zoo Animal Medicine*, 11, 99-105.

Beier, J.C. & Trpis, M. (1981). Incrimination of natural culicine vectors which transmit *Plasmodium elongatum* to penguins at the Baltimore Zoo. *Canadian Journal of Zoology*, 59(3), 470-475.

Ben-Dov, E. (2014). *Bacillus thuringiensis* subsp. *israelensis* and its dipteran-specific toxins. *Toxins,* 6(4), 1222-1243.

Bennett, G.F., Bishop, M.A. & Peirce, M.A. (1993). Checklist of the avian species of *Plasmodium* Marchiafava & Celli, 1885 (Apicomplexa) and their distribution by avian family and Wallacean life zones. *Systematic Parasitology*, 26, 171-179.

Berger, R.S. (2013). A Double-Blind, Multiple-Dose, Placebo-Controlled, Cross-Over Study to Compare the Incidence of Gastrointestinal Complaints in Healthy Subjects Given Doryx R and Vibramycin R. *The Journal of Clinical Pharmacology*, 28(4), 367-370.

Bristol Zoo Gardens (2008). *Bristol Zoo Gardens scoops top award for zoo and wildlife medicine at British Zoo "Oscars"*. Acedido em Jul. 31 2014, disponível em http://www.bristolzoo.org.uk/bristol-zoo-gardens-scoops-top-award-for-zoo-and-wildlife-medicine-at-british-zoo-oscars.

Bueno, M.G., Lopez, R.P.G., Menezes, R.M.T., Costa-Nascimento, M.J., Lima, G.F.M.C., Araújo, R.A.S., Guida, F.J.V. & Kirchgatter, K. (2010). Identification of *Plasmodium relictum* causing mortality in penguins (*Spheniscus magellanicus*) from São Paulo Zoo, Brazil. *Veterinary Parasitology,* 173, 123-127.

Bureau of Medicine and Surgery (2000). Unit Protective Measures. *Navy Medical Department Pocket Guide to Malaria Prevention and Control.* Acedido em Ago. 19 2014, disponível em http://fas.org/irp/doddir/milmed/malaria.pdf.

Campos, S.D.E. & Almosny, N.R.P. (2011). A malária aviária causada por agentes do gênero *Plasmodium* pode ser um desafio durante a reabilitação. *Boletim Nº02 Pinguins no Brasil*. Acedido em Jan. 15 2014, disponível em http://pt.scribd.com/doc/77710678/Boletim-Pinguins-no-Brasil-N-2.

Campos, S.D.E. (2011). *Estudo clínico e laboratorial da infecção por Plasmodium spp. e parasitos gastrointestinais em pinguins-de-Magalhães (Spheniscus magellanicus Foster 1781) resgatados por três instituições no litoral sudeste do Brasil.* Dissertação de Pós-Graduação em Medicina Veterinária (Clínica e Reprodução Animal). Niterói: Universidade Federal Fulminense.

Campos, S.D.E., Silva, L.G., Pereira, B.B.N., Magalhães, B.S.N., Pires, J.R., Brener, B. & Almosny, N.R.P. (2011). Aspectos hematológicos da infecção por *Plasmodium* spp. em Pinguins-de-Magalhães (*Spheniscus magellanicus*) capturados no litoral do Rio de Janeiro. *CD-ROM de Resumos do 38º Congresso Brasileiro de Medicina Veterinária.* Acedido em Jul. 13, 2014, disponível em http://www.sovergs.com.br/site/38conbravet/resumos/348.pdf.

Cannell, B.L., Krasnec, K.V., Campbell, K., Jones, H.I., Miller, R.D. & Stephens, N. (2013). The pathology and pathogenicity of a novel *Haemoproteus* spp. infection in wild Little Penguins (*Eudyptula minor*). *Veterinary Parasitology*, 197 (1-2), 74-84.

Cereghetti, N., Wenker, C., Hoby, S., Müller, P., Marti, H. & Lengeler, C. (2012). Avian malaria and 1st prevention strategies in the Zoo Basel. In Chappuis, F., Lengeler, C. & Müller, N. (Eds.) *Posters SSTMP Joint Annual Meeting 2012, Olma Messen St. Gallen, Switzerland, 21-22 June*, p. 2.

Chitty, J. (2011). Use of Doxycycline in Treatment of Malaria in Penguins. In Bergman, E. (Ed.) *Proceedings of the Association of Avian Veterinarians 32nd Annual Conference & Expo with the Association of Exotic Mammal Veterinarians, Seattle, Washington, USA, 6-12 August*, pp. 63-65.

Christe, P., Glaizot, O., Strepparava, N., Devevey, G & Fumagalli, L. (2012). Twofold cost of reproduction: an increase in parental effort leads to higher malarial parasitaemia and to a decrease in resistance to oxidative stress. *Proceedings of the Royal Society – Biological Sciences*, 279, 1142-1149.

Cranfield, M.R. (2003). Sphenisciformes (Penguins). In M.E. Fowler & R.E. Miller (Eds.), *Zoo and Wildlife Medicine*. (5th ed.). (pp. 107-108). Saint Louis: Saunders.

Cranfield, M.R., Graczyk, T.K. & McCutchan, T.F. (2000). ELISA Antibody Test, PCR and a DNA vaccine for use with Avian Malaria in African Penguins. *Proceedings of the American Association of Zoo Veterinarians / International Association for Aquatic Animal Medicine Joint Conference*. Acedido em Mai. 28, 2014, disponível em https://getinfo.de/app/ELISA-ANTIBODY-TEST-PCR-AND-A-DNA-VACCINE-FOR-USE/id/BLCP%3ACN037713958.

Cranfield, M.R., Graczyk, T.K., Beall, F.B., Ialeggio, D.M., Shaw, M.L. & Skjoldager, M.L. (1994). Subclinical avian malaria infections in African black-footed penguins (*Spheniscus demersus*) and induction of parasite recrudescence. *Journal of Wildlife Diseases*, 30(3), 372-376.

Dohoo, I., Martin, W. & Stryhn, H. (2003). Questionnaire Design. In I. Dohoo, W. Martin & H. Stryhn (Eds.), *Veterinary Epidemiologic Research.* (pp. 53-64). Charllotetown: AVC Inc.

Draper, C.C. (1953). Observations on the reciprocal immunity between some avian *Plasmodia*. *Parasitology,* 43, 139-142.

Earle, R.A., Huchzermeyer, F.W., Bennett, G.F. & Brassy, J.J. (1993). *Babesia peircei* sp. Nov. from the jackass penguin. *South African Journal of Zoology,* 28(2), 88-90.

Ellenberg, U., Mattern, T., Seddon, P.J. & Jorquera, G.L. (2006). Physiological and reproductive consequences of human disturbance in Humboldt penguins: The need for species-specific visitor management. *Biological Conservation*, 133, 95-106.

Fallis, A.M., Bisset, S.A. & Allison F.R. (1976). *Leucocytozoon tawaki* n.sp. (Eucoccida: Leucocytozoidae) from the penguin *Eudyptes pachyrhynchus*, and preliminary observations on its development in *Austrosimulium* spp. (Diptera: Simuliidae). *New Zealand Journal of Zoology*, 3(1), 11-16.

Fantham, H.B. & Porter, A. (1944). On a *Plasmodium* (*Plasmodium relictum* var. *spheniscidæ*, n. var.), observed in four species of Penguins. *Proceedings of the Zoological Society of London,* 114(3), 279-292.

Fix, S.A., Waterhouse, C., Greiner, E.C. & Stoskopf, M.K. (1988). *Plasmodium relictum* as a cause of avian malaria in wild-caught Magellanic penguins (*Spheniscus magellanicus*). *Journal of Wildlife Diseases*, 24(4), 610-619.

Fleischman, R.W., Sladen W.J.L. & Melby, E.C. (1968). Malaria (*Plasmodlum elongatum*) in captive African penguins (Spheniscus demersus). *Journal of the American Veterinary Medical Association*, 153, 928-935.

Fordyce, R.E. & Jones, C.M. (1990). Penguin history and new fossil material from New Zealand. In L.S. Davis & J.T. Darby (Eds), *Penguin biology*. (pp. 417-446). London: Academic Press.

Gailey-Phipps, J. (1978). A world survey of penguins in captivity. *International Zoo Yearbook*, 18(1), 7-13.

Garamszegi, L.Z. (2011). Climate change increases the risk of malaria in birds. *Global Change Biology,* 17, 1751–1759.

Goswami, P. & Swamy, M. (2013). Avian Malaria: Diagnosis and management. *JNKVV Research Journal*, 47(1), 19-24.

Graczyk, T.K. & Cranfield, M.R. (1996). A Model for the Prediction of Relative Titres of Avian Malaria and *Aspergillus* spp. IgG in Jackass Penguin (*Spheniscus demersus*) Females Based on Maternal IgG in Egg-yolk. *International Journal for Parasitology*, 26(7), 749-754.

Graczyk, T.K., Brossy, J.J., Plost, A. & Stoskopft, M.K. (1995a). Avian malaria seroprevalence in Jackass penguins (*Spheniscus demersus*) in South Africa. *Journal of Parasitology,* 81(5), 703-707.

Graczyk, T.K., Cranfield, M.R. & Bicknese, E.J. (1995c). Evaluation of serum chemistry values associated with avian malaria infections in African black-footed penguins (*Spheniscus demersus*). *Parasitology Research*, 81, 316-319.

Graczyk, T.K., Cranfield, M.R., Brossy, J.B., Cockrem, J.F., Jouventin, P. & Seddon, P.J. (1995b). Detection of Avian Malaria Infections in Wild and Captive Penguins. *Journal of the Helminthological Society of Washington*, 62(2), 135-141.

Graczyk, T.K., Cranfield, M.R., McCutchan, T.F. & Bicknese, E.J. (1994b). Characteristics of naturally acquired avian malaria infections in naive juvenile African black-footed penguins (*Spheniscus demersus*). *Parasitology Research*, 80, 634-637.

Graczyk, T.K., Cranfield, M.R., Shaw, M.L. & Craig, L.E. (1994d). Maternal antibodies against *Plasmodium* spp. in African Black-footed Penguin (*Spheniscus demersus*) chicks. *Journal of Wildlife Diseases*, 30(3), 365-371.

Graczyk, T.K., Cranfield, M.R., Skjoldager, M.L. & Shaw, M.L. (1994a). An ELISA for detecting anti-*Plasmodium* spp. antibodies in African black-footed penguins (*Spheniscus demersus*). *Journal of Parasitology*, 80(1), 60-66.

Graczyk, T.K., Shaw, M.L., Cranfield, M.R. & Beali, F.B. (1994c). Hematologic characteristics of avian malaria cases in African Black-footed penguins (*Spheniscus demersus*) during the first outdoor exposure season. *Journal of Parasitology*, 80(2), 302-308.

Grim, K.C., McCutchan, T., Li, J., Sullivan, M., Graczyk, T.K., McConkey, G. & Cranfield, M. (2004). Preliminary results of an anticircumsporozoite DNA vaccine trial for protection against avian malaria in captive African black-footed penguins (*Spheniscus demersus*). *Journal of Zoological Wildlife Medicine,* 35(2), 154-161.

Grim, K.C., Van der Merwe, E., Sullivan, M., Parsons, N., McCutchan, T.F. & Cranfield, M. (2003). *Plasmodium juxtanucleare* associated with mortality in black-footed penguins (*Spheniscus demersus*) admitted to a rehabilitation center. *Journal of Zoo and Wildlife Medicine,* 34(3). 250-255.

Hudson County Mosquito Control (2013). General Information. *Mosquito Biology*. Acedido em Ago. 20, 2014, disponível em http://www.hudsonregional.org/mosquito/mosquitobio.htm.

Huff, C.G. & Shiroish, T. (1962). Natural infection of Humboldt's penguin with Plasmodium elongatum. Journal of Parasitology, 48, 495.

Huff, C.G. (1965). Susceptibility of mosquitoes to avian malaria. *Experimental Parasitology*, 16, 107-132.

Huijben, S., Schaftenaar, W., Wijsman, A., Paaijmans, K. & Takken, W. (2007). Avian malaria in Europe: an emerging infectious disease? In W. Takken & B.G.J. Knols (Eds.), *Emerging pests and vector-borne diseases in Europe*. (pp. 59-74). Wageningen: Wageningen Academic Publishers.

Invasive Species Specialist Group (2005). *Plasmodium relictum (micro-organism)*. Acedido em Abr. 20, 2014, disponível em http://www.issg.org/database/species/ecology.asp?si=39&fr=1&sts=&%20ang=EN&ver=print&prtflag=false.

Invasive Species Specialist Group (2010). *Invasive Species Management and Control: Avian malaria (Plasmodium relictum)*. Acedido em Abr. 20, 2014, disponível em http://www.issg.org/database/species/reference_files/plarel/plarel_man.pdf.

Jencek, J.E., Beaufrère, H., Tully, T.N.J., Garner, M.M., Dunker, F.H. & Baszler, T.V. (2012). An outbreak of *Chlamydophila psittaci* in an outdoor colony of Magellanic penguins (*Spheniscus magellanicus*). *Journal of Avian Medicine and Surgery*, 26(4), 225-231.

Jones, H.I. & Shellam, G.R. (1999). Blood parasites in penguins, and their potential impact on conservation. *Marine Ornithology*, 27, 181–184.

Jones, H.I. & Woehler, E.J. (1989). A New Species of Blood Trypanosome from Little Penguins (*Eudyptula minor*) in Tasmania. *The Journal of Protozoology*, 36(4), 389–390.

Kaur, K., Jain, M., Kaur, T. & Jain, R. (2009). Antimalarials from nature. *Bioorganic & Medicinal Chemistry*, 17, 3229–3256.

Ko, K.N., Kang, S.C., Jung, J.Y., Bae, J.H. & Kim, J.H. (2008) Avian malaria associated with *Plasmodium* species infection in a penguin in Jeju Island. *Korean Journal of Veterinary Research*, 48(2), 197-201.

Krams, I., Suraka, V., Cirule, D., Hukkanen, M., Tummeleht, L., Mierauskas, P., Rytkönen, S., Rantala, M.J., Vrublevska, J., Orell, M. & Krama, T. (2012). A comparasion of microscopy and PCR diagnostics for low intensity infections of haemosporidian parasites in the Siberian tit *Poecile cinctus*. *Annales Zoologici Fennici*, 49, 331-340.

Krettli, A.U., Andrade-Neto, V.F., Brandão, M.G.L. & Ferrari, W.M.S. (2001). The Search for New Antimalarial Drugs from Plants Used to Treat Fever and Malaria or Plants Randomly Selected: a Review. *Memórias do Instituto Oswaldo Cruz*, 96(8), 1033-1042.

Krone, O., Waldenström, J., Valkiūnas, G., Lessow, O., Müller, K., Iezhova, T.A., Fickel, J. & Bensch, S. (2008). Haemosporidian blood parasites in European birds of prey and owls. *Journal of Parasitology*, 94(3), 709-715.

Laird, M. (1950). Some parasites of New Zealand birds. *Victoria University College Zoological Publishings*, 5, 1–20.

Levin, I.I., Outlaw, D.C., Hernán-Vargas, F.G. & Parker, P.G. (2009). Plasmodium blood parasite found in endangered Galapagos penguins (*Spheniscus mendiculus*). *Biological Conservation,* 142(12), 3191-3195.

London Evening Standard (2012). *London Zoo's penguins hit by outbreak of killer malaria.* Acedido em Ago. 19 2014, disponível em http://www.standard.co.uk/news/london/london-zoos-penguins-hit-by-outbreak-of-killer-malaria-8204752.html.

Loupal, G. & Kutzer, E. (1996). Infektionen mit *Plasmodium* spec. bei Papageitauchern (*Fratercula arctica*). *Kleintierpraxis*, 41(12), 901-906.

Marzal, A. (2012). Recent Advances in Studies on Avian Malaria Parasites. In O. Okwa (Ed.), *Malaria Parasites.* (pp. 135-158). Rijeka: InTech.

McCutchan, T.F., Grim, K.C., Li, J., Weiss, W., Rathore, D., Sullivan, M., Graczyk, T.K., Kumar, S. & Cranfield, M.R. (2004). Measuring the Effects of an Ever-Changing Environment on Malaria Control. *Infection and Immunity*, 72(4), 2248–2253.

Miller, G.D., Hofkin, B.V., Snell, H., Hahn, A. & Miller, R.D. (2001). Avian Malaria and Marek's disease: potential threats to Galapagos penguins (Spheniscus mendiculus). *Marine Ornithology*, 29, 43–46.

Ng, C.K., Mak, A.Y., Au, T.S., Au, T.C., Lai, S.T. & Lai, J.Y. (1997). *Plasmodium* infection unmasked by corticosteroid therapy. *Hong Kong Medical Journal*, 3(3), 328-330.

Nielsen, A.C., Agger, J.F. & Ersbell, A.K. (2004). Questionnaires. In H. Houe, A.K. Ersbell & N. Toft (Eds.), *Introduction to Veterinary Epidemiology.* (pp. 187-204). Gylling: Biofolia.

O'Brien, J. (1999). UK penguins struck by avian malaria. *BBC News – Sci/Tech*. Acedido em Mai. 24, 2014, disponível em: http://news.bbc.co.uk/2/hi/science/nature/467989.stm.

Okanga, S., Cumming, G.S., Hockey, P.A.R., Grome, M. & Peters, J.L. (2013). A comparison of techniques employed in detection of avian malaria infection, South Africa. *African Zoology*, 48(2), 309-317.

Orogade, A. (2012). Current Issues in Clinical and Laboratory Diagnosis in Malaria. In O. Okwa (Ed.) *Malaria Parasites*. (pp. 161-172). Rijeka: InTech.

Palmer, J.L., McCutchan, T.F., Hernan-Vargas, F., Deem, S.L., Cruz, M., Hartman, D.A. & Parker, P.G. (2013). Seroprevalence of malarial antibodies in Galapagos penguins (*Spheniscus mendiculus*). *Journal of Parasitology*, 99(5), 770–776.

Penrith, M., Huchzermeyer, F.W., De Wet, S.C. & Penrith, M.J. (1994). Concurrent infection with *Clostridium* and *Plasmodium* in a captive king penguin *Aptenodytes patagonicus*. *Avian Pathology*, 23(2), 373-380.

Pérez-Tris, J., Hasselquist, D., Hellgren, O., Krizanauskiene, A., Waldenström, J. & Bensch, S. (2005). What are malaria parasites? *Trends in Parasitology*, 21(5), 209-211.

Rebêlo, E., Baptista, R., Afonso, A., Monteiro, M., Lapão, N., Sogorb, A., Carvalho, P. & Mendonça, P. (2005). Casos de malária em pinguins *Spheniscus demersus* do Jardim Zoológico de Lisboa [abstract]. In Ferreira, L.A., Leitão, A.C., Vaz, Y., Monteiro, A.S., Godinho, C., Ribeiro, J.R. & Horta, A. (Eds.) C*ongresso Ciências Veterinárias 2005: Livro de Resumos, Estação Zootécnica Nacional, Portugal, 13-15 Outubro,* p. 222.

Remple, J.D. (2004). Intracellular Hematozoa of Raptors: A Review and Update. *Journal of Avian Medicine and Surgery*, 18(2), 75-88.

Richard, F.A., Sehgal, R.N.M., Jones, H.I. & Smith, T.B. (2002). A Comparative Analysis of PCR-Based Detection Methods for Avian Malaria. *Journal of Parasitology*, 88(4), 819-822.

Sano, Y., Aoki, M., Takahashi, H., Miura, M., Komatsu, M., Abe, Y., Kakino, J. & Itagaki, T. (2005). The First Record of *Dirofilaria immitis* Infection in a Humboldt Penguin, *Spheniscus humboldti*. *Journal of Parasitology*, 91(5), 1235-1237.

Scott, H.H. (1927). Report on the deaths occurring in the Society's gardens during the year 1926. *Proceedings of the Zoological Society of London*, 173–198.

Silveira, P., Belo, N.O., Lacorte,G.A., Kolesnikovas, C.K.M., Vanstreels, R.E.T., Steindel, M., Catão-Dias, J.L., Valkiūnas, G. & Braga, E.M. (2013). Parasitological and new molecular-phylogenetic characterization of the malaria parasite *Plasmodium tejerai* in South American penguins. *Parasitology International*, 62, 165-171.

Smith, K.F., Sax, D.F. & Lafferty, K.D. (2006). Evidence for the Role of Infectious Disease in Species Extinction and Endangerment. *Conservation Biology*, 20(5), 1349-1357.

Sohsuebngarm, D., Sasipreeyajan, J., Nithiuthai, S. & Chansiripornchai, N. (2014). The efficacy of artesunate, chloroquine, doxycycline, primaquine and a combination of artesunate and primaquine against avian malaria in broilers. *The Journal of Veterinary Medical Science*, 76(6), 813-817.

Stoskopf, M.K. & Beier, J. (1979). Avian Malaria in African Black-Footed Penguins. *Journal of the American Veterinary Medical Association,* 175(9), 944-947.

Stoskopf, M.K. & Kennedy-Stoskopf, S. (1986). Aquatic birds (Sphenisciformes, Gaviiformes, Podicipediformes, Procellariiformes, Pelecaniformes, and Charadriiformes). In M.E. Fowler (Ed.) *Zoo and Wild Animal Medicine* (2nd Ed.). (p. 306). Philadelphia: Saunders.

The Independent (2012). *Six penguins die after malaria outbreak at London Zoo*. Acedido em Ago. 19 2014, disponível em http://www.independent.co.uk/news/uk/home-news/six-penguins-die-after-malaria-outbreak-at-london-zoo-8205634.html.

The New York Times (2013). *Zoos Try to Ward Off a Penguin Killer*. Acedido em Ago. 19 2014, disponível em http://www.nytimes.com/2013/10/08/science/earth/zoos-aim-to-ward-off-a-penguin-killer.html?_r=1&.

Tollini, J., Brecksen, A. & Sureda, A. (2000). Prevention and treatment of avian malaria in a captive penguin colony. *Penguin Conservation*, 13(1), 26-27.

United States Geological Survey (2006). *Ecology and Diagnosis of Introduced Avian Malaria in Hawaiian Forest Birds*. Acedido em Jan. 17, 2014, disponível em http://pubs.usgs.gov/fs/2005/3151/report.pdf.

University of Miami (2014). Serodiagnostics for Avian Aspergillosis. *Avian and Wildlife Laboratory.* Acedido em Ago. 20, 2014, disponível em http://www.cpl.med.miami.edu/avian-and-wildlife/avian-aspergillosis.

Valkiūnas, G. (2005). *Avian malaria parasites and other Haemosporidia.* Boca Raton: CRC Press.

Valkiūnas, G., Anwar, A.M., Atkinson, C.T., Greiner, E.C., Paperna, I. & Peirce, M.A. (2005). What distinguishes malaria parasites from other pigmented haemosporidians? *Trends in Parasitology,* 21(8), 357-358.

Valkiūnas, G., Bensch, S., Iezhova, T.A., Križanauskienė, A., Hellgren, O. & Bolshakov, C.V. (2006). Nested Cytochrome B Polymerase Chain Reaction Diagnostics Underestimate Mixed Infections of Avian Blood Haemosporidian Parasites: Microscopy is Still Essential. *Journal of Parasitology*, 92(2), 418-422.

Vanstreels, R.E.T., Kolesnikovas, C.K.M., Sandri, S., Silveira, P., Belo, N.O., Junior, F.C.F., Epiphanio, S., Steindel, M., Braga, E.M. & Catão-Dias, J.L. (2014b). Outbreak of Avian Malaria Associated to Multiple Species of *Plasmodium* in Magellanic Penguins Undergoing Rehabilitation in Southern Brazil. *PLoS ONE*, 9(4), e94994.

Villena, I., Aubert, D., Leroux, B., Dupouy, D., Talmud, M., Chemla, C., Trenque, T., Schmit, G., Quereux, C., Guenounou, M., Pluot, M., Bonhomme, A. & Pinon, J.M. (1998). Pyrimethamine-sulfadoxine Treatment of Congenital Toxoplasmosis: Follow-up of 78 Cases Between 1980 and 1997. *Scandinavian Journal of Infectious Diseases*, 30(3), 295-300.

Vinetz, J.H., Clain, J., Bounkeua, V., Eastman, R.T. & Fidock, D. (2011). Chemotherapy of Malaria. In L.L. Brunton (Ed.), *Goodman and Gilman's The Pharmacological Basis of Therapeutics*. (12th ed.). (pp. 1383-1418). New York: McGraw-Hill Professional.

Vinogradova, E.B. (2000). The *Culex pipiens* complex. In S.I. Golovatch (Ed.), *Culex pipien pipiens Mosquitoes: Taxonomy, Distribution, Ecology, Physiology, Genetics, Applied Importance and Control.* (p. 36). Sofia: PENSOFT Publishers.

Waldenström, J., Bensch, S., Hasselquist, D. & Östman, Ö. (2004). A New Nested Polymerase Chain Reaction Method Very Efficient in Detecting *Plasmodium* and *Haemoproteus* Infections From Avian Blood. *Journal of Parasitology*, 90(1), 191-194.

Wallace, R. & Walsh, M. (2005). *American Zoo and Aquarium Association Penguin Husbandry Manual Third edition*. Acedido em Abr. 23 2014, disponível em http://marineanimalwelfare.com/images/Penguin_HB.pdf.

WBALTV11 (2013). *Zoo vets closely monitor penguins for malaria*. Acedido em Ago. 19 2014, disponível em http://www.wbaltv.com/news/maryland/baltimore-city/zoo-vets-closely-monitor-penguins-for-malaria/22488710#!bFZqHE.

Weissenböck, H., Dinhopl, N., Mostegl, M.M., Richter, B., Nedorost, N., Maderner, A. & Fragner, K. (2011). Application of in-situ hybridization for the detection and identification of avian malaria parasites in paraffin wax-embedded tissues from captive penguins. *Avian Pathology*, 40(3), 315-320.

Wilson, R.P. & Grémillet, D. (1996). Body temperatures of free-living African Penguins (*Spheniscus demersus*) and Bank Cormorants (*Phalacrocorax neglectus*). *The Journal of Experimental Biology*, 199, 2215–2223.

Wongsrichanalai, C., Barcus, M.J., Muth, S., Sutamihardja, A. & Wernsdorfer, W.H. (2007). A Review of Malaria Diagnostic Tools: Microscopy and Rapid Diagnostic Test (RDT). *The American Journal of Tropical Medicine and Hygiene*, 77(6), 119-127.

World Health Organization (1995). Drugs used in Parasitic Diseases. *WHO Model Prescribing Information*. Acedido em Ago. 20, 2014, disponível em http://apps.who.int/medicinedocs/pdf/h2922e/h2922e.pdf.

Wunschman, A., Armien, A, Wallace, R, Wictor, M & Oglesbee, M. (2006). Neuronal storage disease in a group of captive Humboldt penguins (*Spheniscus humboldti*). *Veterinary Pathology,* 43(6), 1029-1033.

Zehtindjiev, P., Ilieva, M., Westerdahl, H., Hansson, B., Valkiūnas, G. & Bensch, S. (2008). Dynamics of parasitemia of malaria parasites in a naturally and experimentally infected migratory songbird, the great reed warbler *Acrocephalus arundinaceus*. *Experimental Parasitology*, 119, 99-110.

Ziegyte, R., Bernotienė, R., Bukauskaitė, D., Palinauskas, V., Iezhova, T. & Valkiūnas, G. (2014). Complete sporogony of Plasmodium relictum (lineages pSGS1 and pGRW11) in mosquito Culex pipiens pipiens form molestus, with implications to avian malaria epidemiology. *The Journal of Parasitology*. Advance online publication. doi: 10.1645/13-469.1.

Annex 1 – Survey "Characterization of Malaria Infection on Penguins Kept in Captivity" – Test version

Faculty of Veterinary Medicine of the University of Lisbon
Survey
Characterization of malaria infection on penguins kept in captivity

Goals

a) Know the frequency of the typical clinical signs of malaria infection in penguins;
b) Associate the clinical signs presented with the prognosis of the animals;
c) Check if all species of penguins in zoos exhibit similar levels of infection, associating the results with the location and vectors;
d) Evaluate the effectiveness of different prophylaxis and treatment protocols, as well as their side effects, implemented in different zoos.

NOTE: IT'S NO INTENTION OF THIS STUDY TO EVALUATE THE WORK OF ANY OF THE RESPONDENTS.

Questions

1. **Institution**: _____
   _____
2. **Location (City, Region, Country):**_____
   _____
3. **Contact person (Name, Email, phone):**_____
   _____

PENGUIN SPECIES

4. **Which penguin species do you keep outdoors?** (Please write the scientific name and the quantity of males and females.)

Penguin specie (scientific name)	*Males*	*Females*	*Chicks (< 1-2 years old)*
_____	_____	_____	_____
_____	_____	_____	_____
_____	_____	_____	_____
_____	_____	_____	_____
_____	_____	_____	_____

5. **If previously indoors, how long have the penguins been in the outside enclosure?**
   _____

HOUSING CONDITIONS

6. **Please fill each parameter related to the penguins housing conditions on outdoor.**
   1. Air temperature range along the year:_____
   2. Water temperature range along the year (please specify the measuring system):_____
   3. Photoperiod
      i. Natural ___
      ii. Artificial ___
      iii. Both ___
   4. Ventilation conditions
      i. Natural ___
      ii. Forced with blowers ___
      iii. Other. Which one? _____

5. Sun area on land:_____
6. Shadow area on land:_____
7. Feeding
    i. Fish species fed to the penguins:_____
    _____
    ii. Average intake:_____
    iii. Average percentage of each fish species:_____
    _____
8. Feeding protocol
    i. On water ___
    ii. On land ___
    iii. Number of sessions a day ____
9. Vitamin supplementation
    i. Yes ___ Which one? _____
    ii. No ___
10. Which water disinfection protocol do you use?
    i. Chlorine ___
    ii. Ozone ___
    iii. UV ___
    iv. Other ___ Which one? _____
    v. None ___
11. Population density (number of animals per square meter of land and water surface):_____
12. Mixed enclosures (shared with other species)
    i. Yes ___ Which one?_____
    ii. No ___
13. Existence of fresh water sources close to facility that allow mosquito breeding
    i. Yes ___
    ii. No ___
14. Public shorter distance to the penguins:_____
15. Public activities with animals
    i. Yes ___ Which one? _____
    ii. No ___
16. Breeding rate of the colony:_____
17. Routine management of the individuals. Which periodicity do you do each of the following activities?
    i. Weight _____
    ii. Deworming_____
    iii. Supplementation_____
    iv. Other. Which one and periodicity?_____
    _____
18. Other common clinical issues present in the colony (answer yes or no):
    i. Bumble foot Syndrome ___
    ii. Aspergillosis ___
    iii. Moulting disorders ___
    iv. Other. Which one?_____

PREVALENCE

7. Do you check the health status of your penguins routinely?

1. No ___
2. Yes ___ Please specify which tests and periodicity:_____
_____

8. **Do you test your penguins for malaria?**
    1. No ___
    2. Yes ___ Please specify which tests and periodicity:_____
    _____

(ANSWER FROM QUESTION 9 TO 18 ONLY IF YOU ANSWER "YES" IN QUESTION 8)

9. **Which penguins do you routinely test?**
    1. All penguins ___
    2. Symptomatic penguins ___

10. **How many animals with clinical signs compatible with malaria tested negative?**
    _____

11. **Regarding your penguin population, please fill the number of individuals in each category.**

Age	*Infected with clinical signs*	*Infected without clinical signs*	*Not Infected with clinical signs*	*Not Infected without clinical signs*
Species				
Juveniles				
Adults				
Seniors				

12. **Is there a seasonal pattern of malaria cases in your penguins?**
    1. No ___
    2. Yes ___ Please specify:
        i. The period of the highest risk of infection:_____
        _____
        ii. The period of the lowest risk of infection:_____
        _____

13. **How many animals tested positive for malaria died?**
    _____

14. **How many animals tested positive for malaria recovered from the infection?**
    _____

DIAGNOSIS

15. **Which techniques do you use in malaria diagnosis in penguins?** (It's possible to choose more than one)
    - Clinical signs _____
    - Optical microscopy _____
    - PCR-based methods _____
    - ELISA _____
    - Histopathology _____
    - Other: which one? _____

16. **Of these techniques, sort from 1 to 5 depending on the performance diagnosis of malaria in penguins.** (1 is attributed to the technique with the best performance and 5 to the worst. Techniques with equal performance can be evaluated with the same number)
    - Clinical signs _____
    - Optical microscopy _____
    - PCR-based methods _____
    - ELISA _____
    - Histopathology _____

17. **Did you detect mixed infections in positive malaria tested penguins? If yes, which parasites did you found?**

    _____
    _____

18. **Do you normally detect parasites inside erythrocytes in positive/affected individuals?**
    1. Yes ___
    2. No ___

19. **Do you keep frozen serum or whole blood samples in a bank?**
    1. Yes ___
    2. No ___

CLINICAL SIGNS

20. **Which clinical/laboratorial signs did you detect in infected penguins?** (It's possible to choose more than one)
    - Lethargy _____
    - Decreased appetite/anorexia _____
    - Vomit _____
    - Dyspnoea/sibilances _____
    - Paleness of mucous membranes _____
    - Weight loss _____
    - Anaemia _____
    - Hyperproteinemia _____
    - Leucocytosis _____
    - Other: which one? _____

21. **Did you detect any atypical clinical sign? Which one?**

    _____
    _____

TREATMENT

22. **Did you use any protocol treatment to treat malaria infections in penguins?**
    1. No ___
    2. Yes ___ Which one? (Please specify the active ingredient and the dose used)_____
       _____

(ANSWER FROM QUESTION 27 TO 29 ONLY IF YOU ANSWER "YES" IN QUESTION 26)

23. **When do you start the treatment?**
    1. When the diagnosis is confirmed ____
    2. When there are clinical signs ____

24. **Do you provide any other supportive treatment besides the antimalarial drugs to diseased/infected animals?**
    1. No ___
    2. Yes ___ Which one? (Please specify the active ingredient and the dose used)_____
       _____

25. **Did the treated animals develop any side effects?**
    1. No ___
    2. Yes ___ Which one and under what drug?_____
       _____

PROPHYLAXIS

26. **Do you apply any prophylactic drug protocol to malaria in your penguin collection?**
    1. No ___
    2. Yes ___ Which one? (Please specify the active ingredient and the dose used)_____
27. **Did the treated animals develop any side effects?**
    1. No ___
    2. Yes ___ Which one and under what drug?_____
       _____
28. **What prophylactic measures, besides drug protocols, do you apply in your zoo to control penguin malaria?**

    _____
    _____

PROGNOSIS

29. **In general, did the infected animals without clinical signs have better prognosis than the infected animals with clinical signs?**
    1. Yes ___
    2. No ___
30. **Which clinical signs and diagnostic values do you associate with a good prognosis?**

    _____
    _____
    _____
31. **Which clinical signs and diagnostic values do you associate with a bad prognosis?**

    _____
    _____
    _____

Annex 2 – Survey "Characterization of Malaria Infection on Penguins Kept in Zoological Collections" – Final version

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Characterization of Malaria Infection on Penguins Kept in Zoological Collections

This survey its part of my Integrated Master thesis project at Lisbon University - Faculty of Veterinary Medicine and it is supervised by Dr. Daniel Garcia Párraga (Oceanográfic of Valencia). The major goal of this survey it to access the current status of malaria infection in penguins kept in Zoo's around the world.

It's no intention of this study to evaluate the work performance of any of the respondents.

Goals

a) Understand the routine malaria diagnostic protocol among different zoos;

b) Evaluate if there are significant differences in the prevalence of infected versus non-infected penguins, in the demonstration of clinical signs and in the mortality rate;

c) Identify cases of mixed infections of blood parasites in penguins kept in captivity;

d) Determine the frequency of the typical clinical signs of malaria infection in penguins;

e) Evaluate the effectiveness of different prophylaxis and treatment protocols, as well as their side effects, implemented in different zoos;

f) Associate the clinical signs presented with the prognosis of the animals.

The questionnaire consists of 15 questions, mostly multiple-choice questions, and requires about ten minutes for their completion.

Thank you for your participation!

Miguel Grilo

1. **Institution**: _____
   _____

2. **Which penguin species do you keep outdoors?** (It's possible to choose more than one answer.)

   - ☐ Adelie penguin *(Pygoscelis adeliae)*
   - ☐ Chinstrap penguin *(Pygoscelis antarctica)*
   - ☐ Gentoo penguin *(Pygoscelis papua)*
   - ☐ Emperor penguin *(Aptenodytes fosteri)*
   - ☐ King penguin *(Aptenodytes patagonicus)*
   - ☐ Rockhopper penguin *(Eudyptes crestatus)*
   - ☐ Macaroni penguin *(Eudyptes chrysolophius)*
   - ☐ Fordland crested penguin *(Eudyptes pachyrhynchus)*
   - ☐ Royal penguin *(Eudyptes schllegeli)*
   - ☐ Snares Island penguin *(Eudyptes robustusi)*
   - ☐ Erect crested penguin *(Eudyptes sclateri)*

- Yellow-eyed penguin *(Megadyptes antipodes)*
- African penguin *(Spheniscus demersus)*
- Humboldt penguin *(Spheniscus humboldti)*
- Magellanic penguin *(Spheniscus magellanicus)*
- Galapagos penguin *(Spheniscus mendiculus)*
- Fairy penguin *(Eudyptula minor)*

3. Do you test your penguins for malaria?

- Yes
- No

ANSWER QUESTIONS 4 TO 9 ONLY IF YOU ANSWERED "YES" ON QUESTION 3.

4. With which periodicity do you test your penguins for malaria?

- Once per month
- Once every six months
- Once per year
- Other

5. Regarding your penguin population and the malaria tests made since last year until present (2013-2014), in your colony there are... (It's possible to choose more than one answer.)

- Animals infected with malaria showing clinical signs
- Animals Infected with malaria not showing clinical signs
- Animals not infected with malaria showing clinical signs
- Animals not infected with malaria not showing clinical signs
- Animals infected with malaria that died
- Animals infected with malaria that recovered from the infection

6. Which techniques do you use in malaria diagnosis in penguins? (It's possible to choose more than one answer.)

- Clinical signs
- Optical microscopy
- PCR-based methods
- ELISA
- Histopathology
- Other(s). Which one(s)? _____

7. Did you detect mixed infections with other blood parasites in positive malaria tested penguins?

- Yes
- No

ANSWER QUESTION 8 ONLY IF YOU ANSWERED "YES" ON QUESTION 7.

8. Which blood parasites did you find?

- Other Plasmodium spp. than P. relictum, P. elongatum, P. tejerai and P. juxtanucleare
- Haemoproteus spp.
- Leucocytozoon spp.
- Babesia spp.
- Trypanosoma spp.
- Other(s). Which one(s)?

_____

9. **Which clinical/laboratorial signs did you detect in infected penguins?** (It's possible to choose more than one answer.)
   - ☐ Lethargy
   - ☐ Decreased appetite/anorexia
   - ☐ Vomit
   - ☐ Dyspnoea/sibilances
   - ☐ Paleness of mucous membranes
   - ☐ Weight loss
   - ☐ Anaemia
   - ☐ Hyperproteinemia
   - ☐ Leucocytosis
   - ☐ Other: which one? _____

10. **Did you use any protocol treatment to treat malaria infections in penguins?**
   - ☐ No
   - ☐ Yes. Which one? (Please specify the active ingredient(s)) _____

ANSWER QUESTION 11 ONLY IF YOU ANSWERED "YES" ON QUESTION 10.

11. **Did the treated animals develop any side effect(s)?**
   - ☐ No
   - ☐ Yes. Which one(s)? _____

12. **Do you apply any prophylactic drug protocol to malaria in your penguin colony?**
   - ☐ No
   - ☐ Yes. Which one? (Please specify the active ingredient(s)) _____

ANSWER QUESTION 13 ONLY IF YOU ANSWERED "YES" ON QUESTION 12.

13. **Did the animals receiving the prophylactic drug(s) develop any side effect(s)?**
   - ☐ No
   - ☐ Yes. Which one(s)? _____

14. **What prophylactic measures, besides drug protocols, do you apply in your zoo to control penguin malaria?** (It's possible to choose more than one answer.)

   - ☐ None
   - ☐ Allow birds to be exposed to the vector to develop natural immunity
   - ☐ Reduce the number of potential water catchment containers in order to reduce the mosquito breeding sites available
   - ☐ Vaccination against malaria parasites
   - ☐ Keep animals in inside enclosures during the mosquito season
   - ☐ Bring the animals to inside enclosures in the evening
   - ☐ Use of fans to circulate the air near the exhibit site
   - ☐ Set up mosquito traps at the exhibit site
   - ☐ Spray repellent products to the mosquitos (eg. lavender oil) in the nest boxes
   - ☐ Have mosquito repellent plants near the exhibit
   - ☐ Keep larvae-eating fish in ponds near the penguin's exhibit
   - ☐ Other(s). Which one(s)?

15. **In general, did the infected animals without clinical signs have better prognosis than the infected animals with clinical signs?**
   - ☐ Yes
   - ☐ No
   - ☐ Do not know