Unusual Prevalence of Avian Malaria (*Plasmodium* sp.) in the Adult Population of Humboldt Penguins (*Spheniscus humboldti*) at an Israeli Zoo Between Years 2012 and 2015

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ABSTRACT

Avian Malaria is a vector borne disease caused by protozoan blood parasites of the genus *Plasmodium*, transmitted exclusively by mosquitoes (*Culicidae*) to a variety of birds. However, a few avian groups, including penguins (*Sphenisciformes*), are considered highly susceptible and may develop severe disease when exposed to these parasites. Clinical signs vary from asymptomatic presentation to per-acute mortality, depending on the susceptibility of the host species. Outbreaks in captive populations of penguins have often led to high morbidity and mortality rates. We describe an unusual prevalence of Avian Malaria caused by *Plasmodium* spp. in the adult population of Humboldt penguins (*Spheniscus humboldti*) at an Israeli zoo between years 2012 and 2015. The laboratory diagnostic methods included screening for *Plasmodium* by blood or touch smears stained by Giemsa, nested polymerase chain reaction (PCR) and histopathology. During this period, 14 penguins were found dead while 8 of them (3 females, 5 males) were definitively diagnosed as malaria-positive. Some penguins were found dead without previous detected clinical signs, however, some of them presented with non-specific clinical signs such as weakness and anorexia. Most of the mortalities occurred in the summer months between June and August. The age range of those 8 malaria-positive penguins at death was between 2-12 years, with a mean and standard deviation of the mean of 7.2 ± 3.4 years. As malaria is considered usually a disease of young penguins up to three years of age, the unusual advanced age of infection, uncommon in zoos in Israel previously, is worth noting and should require the continuing monitoring for malaria as a significant cause of penguin mortality in Israeli's zoos.

Keywords: Avian; Malaria; Plasmodium; Humboldt Penguin.

INTRODUCTION

Avian Malaria (AM) is a vector borne disease caused by protozoan blood parasites of the genus *Plasmodium*, transmitted exclusively by mosquitoes (*Culicidae*) to a variety of birds, where more than 60 species are known to infect birds (1). However, a few avian groups are considered highly susceptible and may develop severe disease when exposed to these parasites (2) including penguins (*Sphenisciformes*), therefore AM is a significant infectious diseases for these birds, especially in a captive environment (3, 4). Nearly all cases of AM in penguins have been attributed to *P. relictum* and/or *P. elongatum* (3, 4, 5, 6).

Clinical signs vary from asymptomatic presentation to per-acute mortality, depending on the susceptibility of the

host species. Penguins are considered particularly susceptible, as outbreaks in captive populations have often lead to high morbidity and mortality rates. African penguins in the Israeli zoological centers have also suffered from malaria outbreaks with high mortality rates, since the 1990's, and it remains a significant cause of mortality in this population. So far, the prevalence of malaria infection in African penguins in the Israeli zoological centers has not been regularly surveyed.

The Humboldt penguin *(Spheniscus humboldti)* is a South American penguin that breeds in coastal Chile and Peru. Its nearest relatives are the African penguin, the Magellanic penguin and the Galápagos penguin (7). Humboldt penguins are medium-sized penguins, with a black head that has a white border running from behind the eye, around the black ear-coverts and chin, and joins at the throat. They have blackish-grey upperparts and whitish underparts, with a black breast-band that extends down the flanks to the thigh. They have a fleshy-pink base to the bill. Juveniles have dark heads and no breast-band. They have spines on their tongue which they use to hold their prey. In their natural habitat Humboldt penguins nest on islands and rocky coasts in holes they burrow in the soil. Their diet consists of small fish like sardines or anchovies and they complement it with squid. They feature several hunting techniques and stay together as a coordinated group while hunting. They are sexually matured at 3 years of age, incubation period is 39 to 42 days, and the clutch of eggs is normally 2 eggs with up to 4 clutches per year. The life expectancy of these penguins in the wild is 10-12 years. Their approximated world population size is estimated between 24,000 and 37,000, or even 66,000 according another method of estimation (7). However the tendency of the population is considered decreasing.

Malaria of birds is a disease caused by the protozoan parasite *Plasmodium* spp. These are parasites of the tetrapod classes (Phylum *Apicomplexa*, Class *Aconoidasida*, Order *Haemosporida*, Family *Plasmodiidae*) (6, 8, 9). About 65 avian-infecting *Plasmodium* spp. species have been isolated from over 1,000 different species of birds (1, 10). In the avian blood, these parasites can be found in the cytoplasm of erythroblasts and erythrocytes in the form of trophozoites, erythrocytic meronts or gametocytes. In the avian tissues, these parasites will invade endothelial cells and macrophages (9). *Plasmodium* parasites that infect birds have a complex life cycle, involving mosquito females (*Culicidae*) as the final host and the sole vector of the parasite, and a wide range of birds as intermediate hosts.

The life cycle of the parasite takes place in the female mosquito, a few minutes after feeding on the blood of an infected bird (10, 11). The adult gametocytes emerge from the blood cells of the mosquito intestine and then sexual reproduction takes place until the production of oocytes. The oocytes mature and rupture, and sporozoites pass into the mosquito's hemocoel and enter its salivary glands (11) to start a new cycle of life in an avian host following a blood meal of the female mosquito on this host. The cycle in the avian host is divided to an extra-erythrocytic stage and an intra-erythrocytic stage. The cycle first begins with transfer of sporozoites from the mosquito to the bird, their entrance into reticular cells and outset of asexual reproduction in the form of meronts (schizonts) in a variety of organs and tissues (spleen, liver, kidneys, even brain). Merzoites are developed in the meronts and are released from the maturing ruptured meronts to infect new cells. The merozoites can repeat the extra-erythrocytic stage either to infect red blood cells and begin a second intra-erythrocytic stage. At this stage, parasitemia occurs, and various parasitic forms are formed in the red blood cells, some of them are asexual forms such as troposophytes and meronts, while others are sexual forms (gametocytes). Parasitemia usually occurs due to stress or immunosuppression, and may result in severe disease, in which internal organs are damaged due to endothelial injury and blockage of the blood vessels by the meronts. Anemia is caused by parasite invasion to the red blood cells (12, 13, 14). When an infected bird is bitten by a female mosquito the migrating sporozoites enter the mosquito's salivary glands to initiate an additional life cycle.

MATERIAL AND METHODS

The penguin population of the zoo during years 2012-2015.

The penguin population of the zoo in 2012-2015 consisted of 29 penguins (concurrently 25 maximum), females and males in almost equal proportion, all belonging to one species, *Spheniscus humboldti*. All penguins have been imported from Sweden or Austria. The penguins were kept in open display area (about 200 m^2) with a fence separating them from the visitors. They lived in a pond of fresh water (about 30 cubes) with supplemented sodium chloride salt passing through a

filtration system. Four industrial fans were erected in the display area as one of the preventative means to handle disease-vector insects. The nutrition of the penguin population consisted of fish (Herring, Capelin). They received a daily supplement of vitamins in tablet form: 1 single tablet per penguin PO (Mazuri® Vita-Zu® Small Bird TabletMazuri®, Purina Mills®, St. Louis, USA). In addition, on their arrival to the zoo and then once a week, they received 1 single tablet of Chloroquine 25 mg PO (Vetmarket, Shoam, Israel, 6.25 mg/kg (from April to January), as a prophylactic measure against malaria.

Post mortem examination.

Dead penguins were examined by standard systematic necropsy methods, and in those that were *Plasmodium*-suspected, organs typical for *Plasmodium* detection were cut and fixed in 10% buffered formalin for histopathology examination. These organs always included liver, spleen and lungs, but in several of the cases also kidneys and brains (15).

Screening for Plasmodium.

Screening for *Plasmodium* combined one or more of the following diagnostic methods: thin blood or touch smears for Giemsa staining, nested polymerase chain reaction (PCR) and histopathology.

a. **Giemsa blood/touch smear staining**.
Thin blood or touch smears were always taken on a microscope slide from liver, spleen and lungs, and in several cases also from kidneys and brains. The smears were fixed with methanol (4-5 min), dried and stained with Giemsa stain (16), and viewed using a light microscope. On each sample, 10-20 fields were surveyed at X100 and X500 magnifications (11). The slides were examined by an expert veterinarian at the Avian Diseases Laboratory of the Kimron Veterinary Institute (KVI) blinded to the PCR results. Identification of parasitic forms at different stages of the life cycle were carried out according to Valkiunas (10).

b. **PCR analysis.**
Organ segments (liver, spleen, lungs, sometimes also kidneys and brains) were kept at –20°C until their processing for DNA extraction by using a DNA Extraction Kit (QIAamp® DNA Mini Kit No. 51306 (Qiagen, Ca, USA) according to the manufacturer's instructions. A nested polymerase chain reaction was used according Singh *et al.* (17). Briefly, genus-specific primers (rPLU1, rPLU 5) were designed based on the *Plasmodium* 18s small subunit ribosomal RNA (ssrRNA) genes. First PCR produced an expected fragment of over 1,600 base pairs (bp), and the nested product (rPLU3, rPLU 4) produced a conservative 240 bp band that was sequenced in the Biological Services Department of Weizmann Institute of Science, Rehovot, to determine the *Plasmodium* species.

RESULTS

Three-year (2012-2015) clinical follow-up of the penguin population of the zoo.

During the years 2012-2015 fourteen penguins were found dead consisting of 8 females and 6 males, while 8 of them (3 females, 5 males) were conclusively diagnosed as AM-positive as will be detailed later. The rest of the dead penguins, i.e. the other 6, were found AM-negative, some of these were diagnosed with other diseases and for others a diagnosis could not be determined. Some of AM-positive penguins presented with some non-specific clinical signs during 3 days or less, such as weakness and anorexia. According Vanstreels *et al.* (9), most penguins with AM in captivity are in good body condition, do not present clinical signs and suddenly die. When clinical signs are present, they are not specific and may include: anorexia, depression, lethargy, weakness, regurgitation, green faeces, hyperthermia, pale mucosae, and dyspnea.

Most of the penguins of the AM group died in the summer months between June and August. A few of the penguins were treated as follows: subcutaneous fluids (Lactated Ringer's Injection, Teva Medical Marketing, Petah-Tikva, Israel, 200 ml, SC) with dextrose and multivitamins (Duphalyte solution for injection, Zoetis, London, United kingdom, 20 ml, SC), doxycycline (50 mg/ml Suspension, Vetmarket, Shoam, Israel, 10 mg/ kg, PO, SID), and meloxicam (Novacam 0.5 mg/ml, Veterinary, Produlab Pharma B.V, Raamsdonksveer, Netherlands, 0.2 mg/kg, PO, SID). One of the penguins was euthanized as the prognosis was considered very poor.

The age range of those 8 penguins at death was from 2 to 12 years, with a mean and standard deviation of the mean

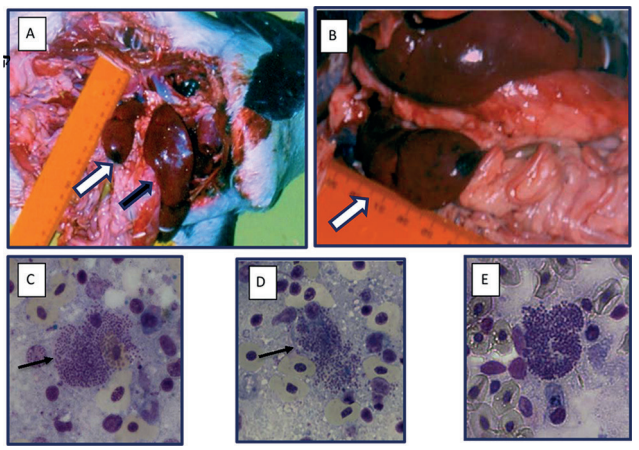

Figure 1: Pathological (A-B) and touch smears (C-E) findings typical to penguin malaria: A-B – Hepatomegaly (black arrow), splenomegaly (white arrows), C-E –meronts (schizonts) (black arrows) in liver (C), spleen (D) and lung (E).

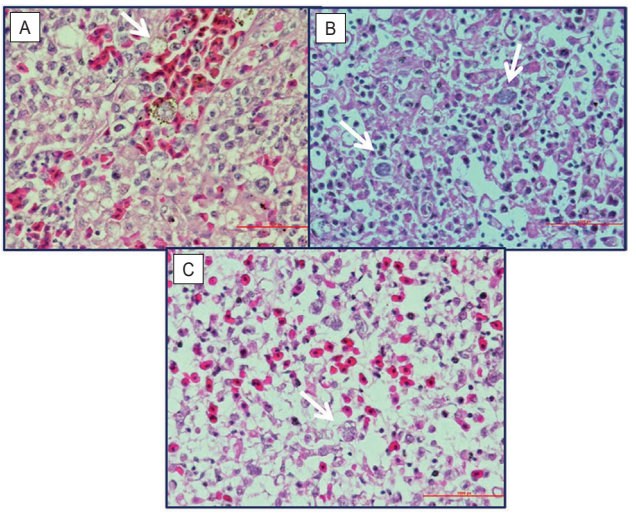

Figure 2: Histopathological findings in penguin malaria: meronts (schizonts) (white arrows) in regions of white cells infiltration, in liver (A), spleen (B) and lung (C).

of 7.2 ± 3.4 years. The time passing from their arrival at the zoo until death was from 3 to 110 months, with a mean of 44.4 months, while the standard deviation of the mean was equal to the mean (44.5 months), indicating a broad range and variability of that parameter.

Pathological & histopathological signs in the AM group.

One of the penguins out of the 8 of the AM group was submitted to post mortem examination at the KVI, the rest were necropsied at the zoo. The first was in good body condition, had hepatomegaly with foci of necrosis, lung congestion with hepatization with fibrinous clots in the abdominal cavity. Post mortem examination of the other seven penguins revealed usually enlarged swollen liver and kidneys, edemetaus lungs, and occasionally serosanguinotic fluid in the pericardium.

Direct organ smears stained with Giemsa revealed many of *Plasmodium* schizonts. Histopathologically, the lesions in the lungs, liver and spleen were characterized by multifocal necrosis with heterophilic and eosinophilic infiltrates with only a slight lymphocytic-plasmacytic infiltrate. Protozoal cysts, 10-20µm diameter (even 30µm in others), probably schizonts, were found in those organs, and contained basophilic structures, 1-2 µm diameter, probably merozoites. Histopathological signs in other penguins revealed necrotic foci with lymphohistiocytic infiltrates in the liver especially in portal regions, and brown gargled pigmented macrophages sometimes including parasitic forms, diffuse fatty hepatocytes, foci of mononuclear cells in liver and kidneys, lung edema with interstitial pneumonia, and multifocal necrosis also in the myocardium. *Plasmodium* parasitic forms could be seen in histopathological sections of all necropsied penguins. Pathological and touch smears findings are presented in Figure 1, histopathological findings are presented in Figure 2.

Giemsa results of the AM group.

Organ smears of 5 of the penguins were stained with Giemsa stain. *Plasmodium* parasitic forms were seen in all 5 penguins including in one penguin that on histopathology had not been diagnosed as *Plasmodium*-positive. The microscopic images of several parasitic forms are presented in Fig. 3.

PCR results of the AM group.

Six of the penguins were molecularly examined to *Plasmodium* in organs or in blood by the nested PCR technique according Singh *et al.* (17). However, one of these gave results that were non-unequivocal, and the other 5 were *Plasmodium* spp.-positive (3 of these are presented in Figure 4). These 5 cases were sequenced in the Biological Services Department of Weizmann Institute of Science, Rehovot. In two of the sequences, segment 4 according rPLU3 primer and segment 1 according rPLU4 primer were 96-98% identical with *P. relictum* type B, with alignment coverage (Query Cover) of 59-95%. In the other positive cases, the PCR bands that

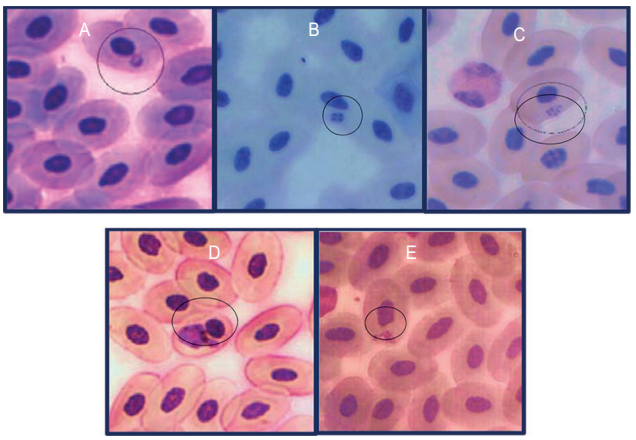

Figure 3. Microscopic images of *Plasmodium* parasitic forms that were found in the penguin blood smears (A-E): A – trophozoite, B, C - intraerythrocytic meronts, D-E – gametocytes.

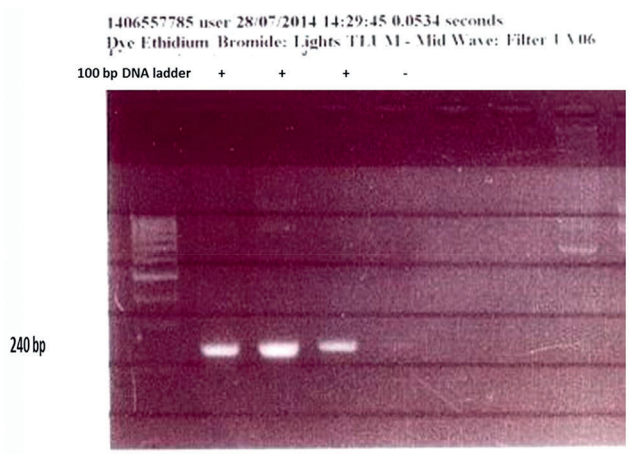

Figure 4. Results of nested polymerase chain reaction (PCR) assay of 3 *Plasmodium*-suspected penguins (lanes 2-4). Genus-specific primers were used for both nest 1 and 2. The PCR products of nest 2 amplifications of the 3 penguins are fit to *Plasmodium* spp. Molecular size markers (100-basepair [bp] ladder) are shown in lane 1. Negative control is shown in lane 5.

characterized *Plasmodium* had not been submitted for sequencing for the definition of the species.

DISCUSSION

Avian malaria is a major cause of mortality in captive penguins both in zoological collections and in rehabilitation systems (15). Thirteen species of penguins have been shown to be susceptible to *Plasmodium* in the wild or in captivity, one of them is the Humboldt penguin (*Spheniscus humboldti*) (18).

This paper reports mortality due to malaria infection in the course of three years in a group of mostly adult Humboldt penguins (up to 12 years of age). Only one of the penguins in this group died at a relatively young age of about 2 years. As AM is considered usually a disease of young penguins not older than 3 years (9, 19), the unusual older age of infection in this group of penguins (up to 12 years, 7.2 years in average), not common in zoos of Israel before (12, 13), is worth noting. According other researchers (20. 21, 22), chicks and juvenile birds but also adults that have not had previous exposure to mosquitoes (such as recently wild-caught birds or those raised in an arthropod-free environment), may show the highest levels of susceptibility.

It is well established that AM outbreaks in zoos result from local mosquitoes infecting penguins with *Plasmodium* spp. acquired from the native birds in the surroundings of the penguin exhibit (23, 24). Because mosquito abundance is markedly seasonal, cases of AM in captive penguins tend to concentrate in spring-summer, particularly late summer (20, 25). In this reported outbreak also, six of the cases occurred between June and August and 5 of those in July or August, in correlation to the high abundance of the mosquito vectors in that period.

Deaths of penguins due to AM are most commonly attributed to *Plasmodium relictum*, with a lower prevalence of *P. elongatum* and *P. juxtanucleare* (26, 27), as we found in two of the penguins that their DNA were sequenced to species definition. Still more sequencing should be carried out.

To summarize, we were faced in the adult population of Humboldt penguins (*Spheniscus humboldti*) of an Israeli zoo between years 2012 and 2015 with unusual prevalence of Avian Malaria caused by *Plasmodium* spp., defined as *P. relictum* at least for the sequenced cases. At this point of time we cannot assess what are the reasons for the extension of *Plasmodium* infectivity age sensitivity from young to older penguins, and whether it is associated only to the *Spheniscus humboldti* species. Monitoring of malaria as a cause of penguin mortality in Israeli's zoos should be proceed further.

REFERENCES

1. Martinsen, E.S. and Perkins, S.L.: The diversity of *Plasmodium* and other *Haemosporidians*: The interesection of taxonomy, phylogenetics and genomics. In: Carlton, J.M., Perkins, S.L. and Deitsch, K.W. (Eds.): Malaria parasites: comparative genomics, evolution and molecular biology. Caister Academic Press, Norfolk, pp. 1-15, 2013.
2. Levin, I.I. and Parker, P.G.: Hemosporidian parasites: impacts on avian hosts. In: Miller E., Fowler M., eds. Fowler's zoo and wild animals medicine. Missouri: Elsevier Saunders, pp. 356-363, 2011

3. Clarke, J.R. and Kerry, K.R.: Diseases and parasites of penguins, Kor. J. Polar Res. 4:79-96, 1993.
4. Jones, H.I. and Shellam, G.R.: Blood parasites in penguins, and their potential impact on conservation. Mar. Ornithol. 27:181-184, 1999.
5. Jones, H.I. and Shellam, G.R.: The occurrence of blood-inhabiting protozoa in captive and free-living penguins. Polar Biol. 21:5-10, 1999.
6. Quillfeldt, P., Martínez, J., Hennicke, J., Ludynia, K., Gladbach, A., Masello, J.F., Riou, S. and Merino, S.: Hemosporidian blood parasites in seabirds - a comparative genetic study from Antartic to tropical habitats. Naturwissenschaften 97:809-817, 2010.
7. Vianna, .A., Cortes, M., Ramos, B., Sallaberry-Pincheira, N., González-Acuńa, D., Dantas, G, P.,S., Morgante, J., Simeone, A. and Luna-Jorquera, G.: Canges in abundance and distribution of Humboldt penguin *Spheniscus humboldti*. Marine Ornithol. 42: 153-159, 2014.
8. Marzal, A.: Recent advances in studies on Avian Malaria parasites. In: Okwa, O. (Ed.): Malaria Parasites. In Tech, Chap. 8, pp 135-158, 2012. www.intechopen.com/books/malaria-parasites/recent-advances-in-studies-on- avian-malaria-parasites.
9. Vanstreels, R.E.T., Braga, É.M. and Catao-Dias, J.L.: Blood parasites of penguins: a critical review. Parasitol. 143: 931-956, 2016.
10. Valkiūnas, G.: Avian Malaria Parasites and Other Haemosporidia, Boca Raton: CRC Press, 1ST ed., 2004. Valkiunas, G. (2004). Avian Malaria Parasites and other Haemosporidia. Boca Raton: CRC Press. Valkiunas, G. (2004). Avian Malaria Parasites and other Haemosporidia. Boca Raton: CRC Press.
11. Palinauskas, V., Valkiūnas, G. and Bensch, S.: Experimental investigation of avian malaria Parasites (*Plasmodium, haemosporida*): linkage of traditional and molecular data. Inst. Ecol. Biomed. Sci. Zoology. Doctoral Dissertation, p. 138, 2009.
12. Lublin, A., Mechani, S., Perl, S., Horowitz, H.I., Levison, M. and Weisman, Y.: Diagnosis of malaria (*Plasmodium* spp.) in penguins in Israel during 1992-1997. Isr. J. Vet. Med. 53:43, 1998.
13. Lublin, A., Mechani, S., Perl, S., Horowitz, H.I., Levison, M. and Weisman, Y.: Penguin diseases in zoos of Israel with emphasis on Malaria (*Plasmodium* spp.). 6th Eur AAV – DVG Conf. Munich, Germany, pp. 259-261, 2001.
14. Graczyk, T.K., Cranfield, M.R., McCutchan, T.F. and Bicknese, E.J.: Characteristics of naturally acquired avian malaria infections in naive juvenile African black-footed penguins (*Spheniscus demersus*). Parasitol. Res. 80:634-637, 1994.
15. Robinson, I.: Seabirds : Avian Malaria. In: Tully, T.N., Jr., Dorrestein, G.M. and Jones, A.K. (Eds.): Handbook of Avian Medicine, Elsevier, 2nd Ed. Chap. 16, pp. 377-403, 2009.
16. World Health Organization, Giemsa staining of Malaria blood films, version 1, 2016.
17. Singh, B., Bobogare, A., Cox-Singh, J., Snounou, G., Abdullah, M.S. and Rahman, H.A.: A genus- and species-specific nested polymerase chain reaction malaria detection assay for epidemiologic studies. Am. J. Trop. Med. Hyg. 60: 687-692, 1999.
18. Rodhain, J.: L'infection a Plasmodium relicturn chez les pingouins. Annal. Parasitol. Humaine Comp. 17: 139-157, 1939.
19. Knowles, S.C.L., Wood, M.J., Alves, R., Wilkin, T.A., Staffan, B. and Sheldon, B.C.: Molecular epidemiology of malaria prevalence and parasitaemia in a wild bird population, Mol. Ecol. 20: 1062-1076, 2011.
20. Dinhopl, N., Mostegl, M.M., Richter, B., Nedorost, N.,Maderner, A., Fragner, K and Weissenböck, H.: Application of in-situ hybridization for the detection and identification of avian malaria parasites in paraffin wax-embedded tissues from captive penguins. Avian Pathol. 40: 315-320, 2011.
21. Wallace, R.S.: Sphenisciformes (penguins). In M.E. Fowler & R.E. Miller (Eds.), Fowler's Zoo and Wildlife Medicine (8th ed., pp. 82-88). Saint Louis, MO: Saunders, 2014.
22. Grilo, M.L. Vanstreels, R.E.T., Wallace, R, García-Párraga, D., Braga, É.M., Chitty, J., Catão-Dias, J.L. and Madeira de Carvalho, L.M.: Malaria in penguins – current perceptions. Avian Pathol. 45:393-407, 2016.
23. Leclerc, A., Chavatte, J.-M., Landau, I., Snounou, G. and Petit, T.: Morphologic and molecular study of hemoparasites in wild Corvids and evidence of sequence identity with Plasmodium DNA detected in captive black-footed penguins (*Spheniscus demersus*). J. Zoo Wildl. Med. 45:577-588, 2014.
24. Beier, J.C. and Stoskopf, M.K.: The Epidemiology of Avian Malaria in Black-Footed Penguins (*Spheniscus demersus*). J. Zoo Anim. Med. 11: 99-105, 1980.
25. Vanstreels, R.E.T., Filho de Silva, R.P., Kolesnikovas, C.K.M., Bhering, R.C. C., Ruoppolo, V, Epiphanio, S., Amaku, M., Ferreira, F.C. Jr., Braga, E.M., Martins, E. and Catão-Dias, J.L.: Epidemiology and pathology of avian malaria in penguins undergoing rehabilitation in Brazil. Vet. Res. 46:30, 2015.
26. Fix, A.S., Waterhouse, C., Greiner, E.C. and Stoskopf, M.K.: *Plasmodium relictum* as a cause of Avian Malaria in wild-caught Magellanic Penguins (*Spheniscus magellanicus*), J. Wildl. Dis. 24: 610-619, 1988.
27. Grim, K.C., van der Merwe, E., Sullivan, M., Parsons, N., Mc Cutchan, T.F. and Cranfield, M.: *Plasmodium juxtanucleare* associated with mortality in black-footed penguin (*Spheniscus demersus*) admitted to a rehabilitation center. J. Zoo Wildl. Med. 34: 250-255, 2003.