

Do blood parasites infect Magellanic penguins (*Spheniscus magellanicus*) in the wild? Prospective investigation and climatogeographic considerations

RALPH ERIC THIJL VANSTREELS^{1*}, MARCELA UHART^{2,3}, VIRGINIA RAGO^{3,4}, RENATA HURTADO⁵, SABRINA EPIPHANIO⁶ and JOSÉ LUIZ CATÃO-DIAS¹

¹ Laboratório de Patologia Comparada de Animais Selvagens, Departamento de Patologia, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, Avenida Prof. Dr. Orlando Marques de Paiva 87, São Paulo, SP 05508-270, Brazil

² Karen C. Drayer Wildlife Health Center, School of Veterinary Medicine, University of California Davis. One Shields Avenue, Davis, CA 95616, USA

³ Wildlife Conservation Society (WCS), Amenabar 1595, Ciudad de Buenos Aires C1426AKC, Argentina

⁴ Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET). Av. Rivadavia 1917, Ciudad de Buenos Aires C1033AAJ, Argentina

⁵ Institute of Research and Rehabilitation of Marine Animals (IPRAM). Rodovia BR-262 Km 0, Cariacica, ES 29140-130, Brazil

⁶ Laboratório de Imunopatologia Celular e Molecular da Malária, Departamento de Análises Clínicas e Toxicológicas, Faculdade de Ciências Farmacêuticas, Universidade de São Paulo. Avenida Prof. Lineu Prestes 580, Cidade Universitária, São Paulo, SP 05508-000, Brazil

(Received 17 September 2016; revised 24 October 2016; accepted 3 November 2016; first published online 11 January 2017)

SUMMARY

Magellanic penguins (*Spheniscus magellanicus*) are native to Argentina, Chile and the Falkland Islands. Magellanic penguins are highly susceptible to blood parasites such as the mosquito-borne *Plasmodium* spp., which have been documented causing high morbidity and mortality in zoos and rehabilitation centres. However, to date no blood parasites have been detected in wild Magellanic penguins, and it is not clear whether this is reflective of their true absence or is instead related to an insufficiency in sampling effort or a failure of the diagnostic methods. We examined blood smears of 284 Magellanic penguins from the Argentinean coast and tested their blood samples with nested polymerase chain reaction tests targeting *Haemoproteus*, *Plasmodium*, *Leucocytozoon* and *Babesia*. No blood parasites were detected. Analysing the sampling effort of previous studies and the climatogeography of the region, we found there is strong basis to conclude that haemosporidians do not infect wild Magellanic penguins on the Argentinean coast. However, at present it is not possible to determine whether such parasites occur on the Chilean coast and at the Falkland Islands. Furthermore, it is troubling that the northward distribution expansion of Magellanic penguins and the poleward distribution shift of vectors may lead to novel opportunities for the transmission of blood parasites.

Key words: avian malaria, climate change, disease, Haemosporida, health, vector-borne pathogen, Piroplasmida, seabird.

INTRODUCTION

Magellanic penguins (*Spheniscus magellanicus*) are native to Argentina, Chile and the Falkland (Malvinas) Islands. The species' population size is estimated between 1.2 and 1.6 million breeding pairs distributed in a minimum of 138 colonies (Boersma *et al.* 2014). The Argentinean Patagonian coast is the most critical habitat for the species, concentrating approximately 75% of its population in 63 breeding colonies (Birdlife International, 2012).

Blood parasites are relevant pathogens to penguins, with a potential to cause substantial morbidity and mortality (Vanstreels *et al.* 2016a). Because of their obligatory heteroxenous life cycle, the spatiotemporal distribution of protozoan blood

parasites is inherently related to that of their invertebrate vectors and, as a result, is strongly influenced by climatic factors (Rogers and Randolph, 2000; Harvell *et al.* 2002; Garamszegi, 2011). In particular, the mosquito-borne *Plasmodium* spp. (avian malaria) are considered significant pathogens to Magellanic penguins due to their well-documented ability to cause rapid outbreaks with high mortality in individuals held in captivity (Vanstreels *et al.* 2016a).

Plasmodium spp. have been extensively reported infecting Magellanic penguins in captivity worldwide (Fix *et al.* 1988; Tollini *et al.* 2000; Ko *et al.* 2008; Bueno *et al.* 2010; Vanstreels *et al.* 2016b) and in wild specimens undergoing rehabilitation in Chile (Carvajal and Alvarado, 2009) and Brazil (Silveira *et al.* 2013; Cabana *et al.* 2014; Campos *et al.* 2014; Vanstreels *et al.* 2014, 2015). However, *Plasmodium* has not been detected in wild Magellanic penguins, nor have any of the other blood

* Corresponding author. Universidade de São Paulo, Av. Prof. Dr. Orlando Marques de Paiva 87, São Paulo, SP 05508-270, Brazil. E-mail: ralph_vanstreels@yahoo.com.br

parasites that infect other species of penguins, which comprise *Babesia*, *Borrelia*, *Haemoproteus*, *Leucocytozoon*, *Trypanosoma* and nematode microfilariae (Vanstreels *et al.* 2016a). It is not clear whether the absence of records of blood parasites in wild Magellanic penguins is reflective of their true absence or is instead related to an insufficiency in sampling effort and/or a failure of the diagnostic methods employed to detect them.

In this study, we investigate the occurrence of blood parasites in Magellanic penguins on the northern Argentinean Patagonian coast, combining traditional (blood smear examination) and molecular methods [nested polymerase chain reactions (PCR) targeting *Babesia*, *Haemoproteus*, *Plasmodium* and *Leucocytozoon*]. Furthermore, we evaluate the climatogeography of the natural distribution of Magellanic penguins in relation to studies on the occurrence of blood parasites, aiming to identify climatic patterns that can be used to predict the occurrence of such parasites.

METHODS

Sample collection

All procedures were authorized by local authorities (99/2011-DFyFS-SRRN, N° 083 SsCyAP/12) and approved by the Animal Ethics Committee of the University of São Paulo (CEUA-USP 601415). Sample collection occurred during two expeditions (24–27 January 2012 and 21–25 January 2014; i.e. late chick-rearing) to four breeding colonies in the Argentinean province of Chubut (Table 1). Two hundred and eighty-four non-moulting adult Magellanic penguins were caught at the colonies and manually restrained; sampled individuals presented no external signs of illness or lesions. Blood samples (<0.5% of body mass) were collected through venipuncture of the jugular vein with a heparinized syringe, then birds were marked with a temporary stain (to prevent recapture) and released back to where they had been caught. Blood was then transferred to heparin tubes and kept in a cool container.

Laboratory procedures

Within 3–6 h after collection, blood samples were homogenized then used to prepare two thin blood smears and 1 mL of heparinized blood was frozen in liquid nitrogen (−196 °C) and later transferred to a −80 °C freezer. Blood smears were air-dried, then fixed with methanol; within 1–4 days, one slide was stained with 8% Giemsa and another with Wright-Rosenfeld (Rosenfeld, 1947). One slide from each individual was examined for intracellular and extracellular blood parasites in 150 fields under 1000× magnification (approx. 20–25 min per slide;

Table 1. Number of samples analysed in this study

Breeding colony	2012	2014	Total
Punta Norte/San Lorenzo (42°04'31"S, 63°47'19"W)	36	33	69
Punta Tombo (44°03'08"S, 65°13'20"W)	37	36	73
Cabo Dos Bahías (44°53'59"S, 65°34'43"W)	35	36	71
Bahía Bustamante (45°09'36"S, 66°34'35"W)	36	35	71
Total	144	140	284

field of view area = 0.126 mm²) by an experienced observer (R.E.T. Vanstreels).

Samples were pooled into 96 triplets, and DNA extraction was conducted using the DNEasy Blood and Tissue Kit (69506, Qiagen – Valencia, USA) and was verified and quantified through UV spectrophotometry (Nanodrop 1000, Thermo Fisher Scientific – Waltham, USA). Using the protocols detailed by Vanstreels *et al.* (2015) with no modifications, we conducted nested PCR targeting: (a) the mitochondrial *cyt-b* gene of *Haemoproteus* and *Plasmodium* (using primers HaemNFI/HaemNR3 and HaemF/HaemR2 originally described by Hellgren *et al.* 2004 and Waldenström *et al.* 2004), (b) the mitochondrial *cyt-b* gene of *Leucocytozoon* (using primers HaemNFI/HaemNR3 and HaemFL/HaemR2L originally described by Hellgren *et al.* 2004), and (c) the 18S rRNA gene of *Babesia* (using primers Bab5.1/BabB and RLBF/RLBR originally described by Medlin *et al.* 1988 and Gubbels *et al.* 1999). Each reaction was conducted with positive controls for *Plasmodium*, *Leucocytozoon* and *Babesia*, as well as a negative control (chicken raised in an arthropod-free environment). Gel electrophoresis was conducted to visualize amplification products, using 2% agarose gel and SYBR Safe (Invitrogen S33102, Life Technologies – Carlsbad, USA).

Estimated true prevalence

Because sampling and diagnostic tests are imperfect, we estimated the highest bound of the true prevalence (i.e. the highest prevalence at which parasites could have occurred but gone undetected) for our sampling effort as well as previous studies on wild Magellanic penguins. For this purpose, the Blaker's exact confidence interval was calculated (Reiczigel *et al.* 2010; Sergeant, 2016) based on the sample size of each study and the diagnostic method employed. Because test sensitivity has been estimated between 72 and 81% for thin blood smears and between 64 and 89% for PCR (Richard *et al.* 2002; Valkiūnas *et al.* 2008), we used the following values of test sensitivity as worst-case and best-case scenarios, respectively: 70 and 80% for

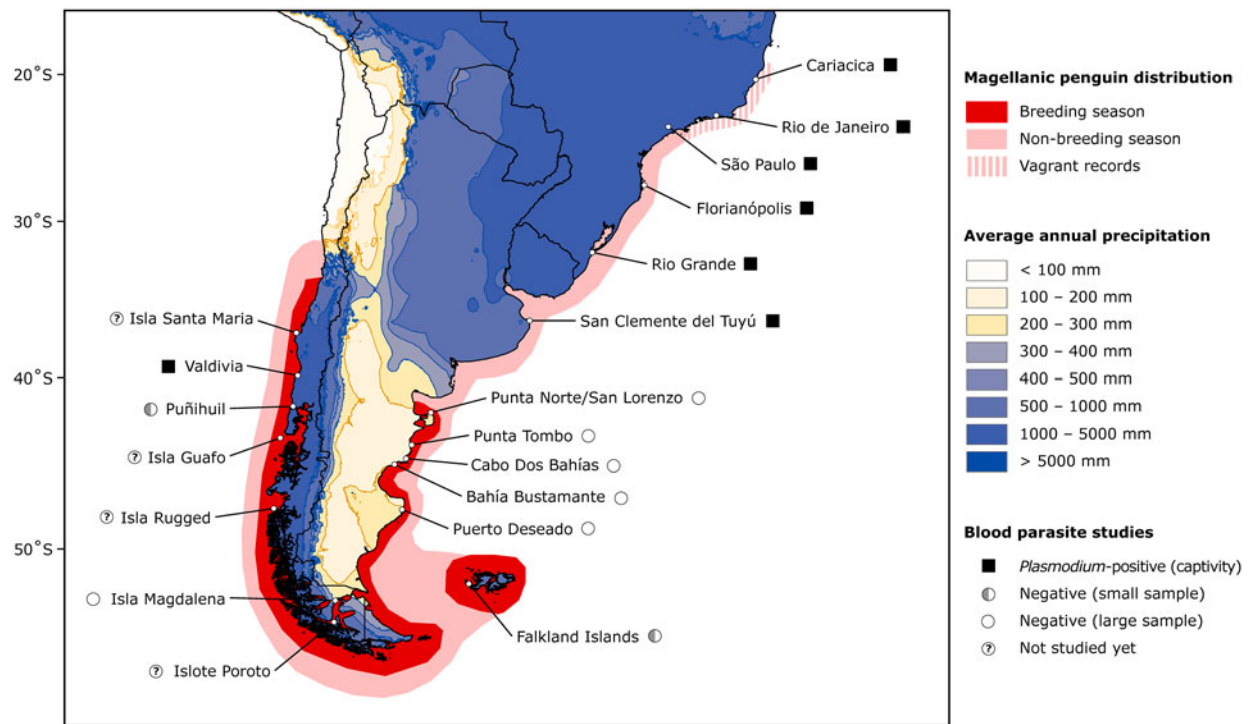


Fig. 1. Geographic distribution of studies investigating the occurrence of blood parasites in Magellanic penguins in relation to the species' natural distribution and historical precipitation data.

studies employing only blood smears, 65 and 90% for studies employing only PCR, and 80 and 90% for studies combining both methods. Test specificity was fixed at 100% to produce the most conservative estimates, and confidence level was fixed at 95%.

Geospatial and climate analyses

Maps and climatograms of the historical climate normals of South America (data from 1950 to 2000, depending on the region) were prepared using published data (Hajek and Di Castri, 1975; Fontannaz, 2001; Hijmans *et al.* 2005; Ramos *et al.* 2009; SMN, 2016). The natural distribution of Magellanic penguins was based on Birdlife International (2012), but was extended to Rio de Janeiro and Espírito Santo states as 'vagrant' considering the several hundred individuals that have regularly stranded in that region in recent years (L.F.S. P. Mayorga, personal communication).

We compared the historical climate parameters between locations where *Plasmodium* was recorded in captive Magellanic penguins and locations where wild Magellanic penguins were studied for blood parasites. For this purpose, two-tailed Mann–Whitney tests were used to compare average annual highest temperature, mean daily temperature during hot semester (October–March), mean daily temperature during cold semester (April–September), average annual lowest temperature, and mean annual precipitation, as obtained from the historical climate normals datasets.

RESULTS

No blood parasites were seen in blood smears, and all samples were negative in the nested PCR tests targeting *Haemoproteus/Plasmodium*, *Leucocytozoon* or *Babesia*. Figures 1 and 2 compare the geographic distribution and the climate of locations where investigations on the occurrence of blood parasites in Magellanic penguins have been conducted. Table 2 summarizes the sampling and laboratory details of this and previous studies on wild specimens, providing the estimated true prevalence for each study.

Locations where *Plasmodium* was recorded in captive Magellanic penguins differed from locations where studies failed to identify blood parasites with regards to: mean daily temperature during hot semester (respectively 21.4 *vs* 13.8 °C; $P=0.007$), mean daily temperature during cold semester (16.6 *vs* 7.1 °C; $P<0.001$), average annual lowest temperature (11.5 *vs* 1.0 °C; $P<0.001$) and mean annual precipitation (1342.2 *vs* 300.2 mm; $P<0.001$). On the other hand, no difference was identified in relation to average annual highest temperature (27.7 *vs* 21.7 °C; $P>0.9$).

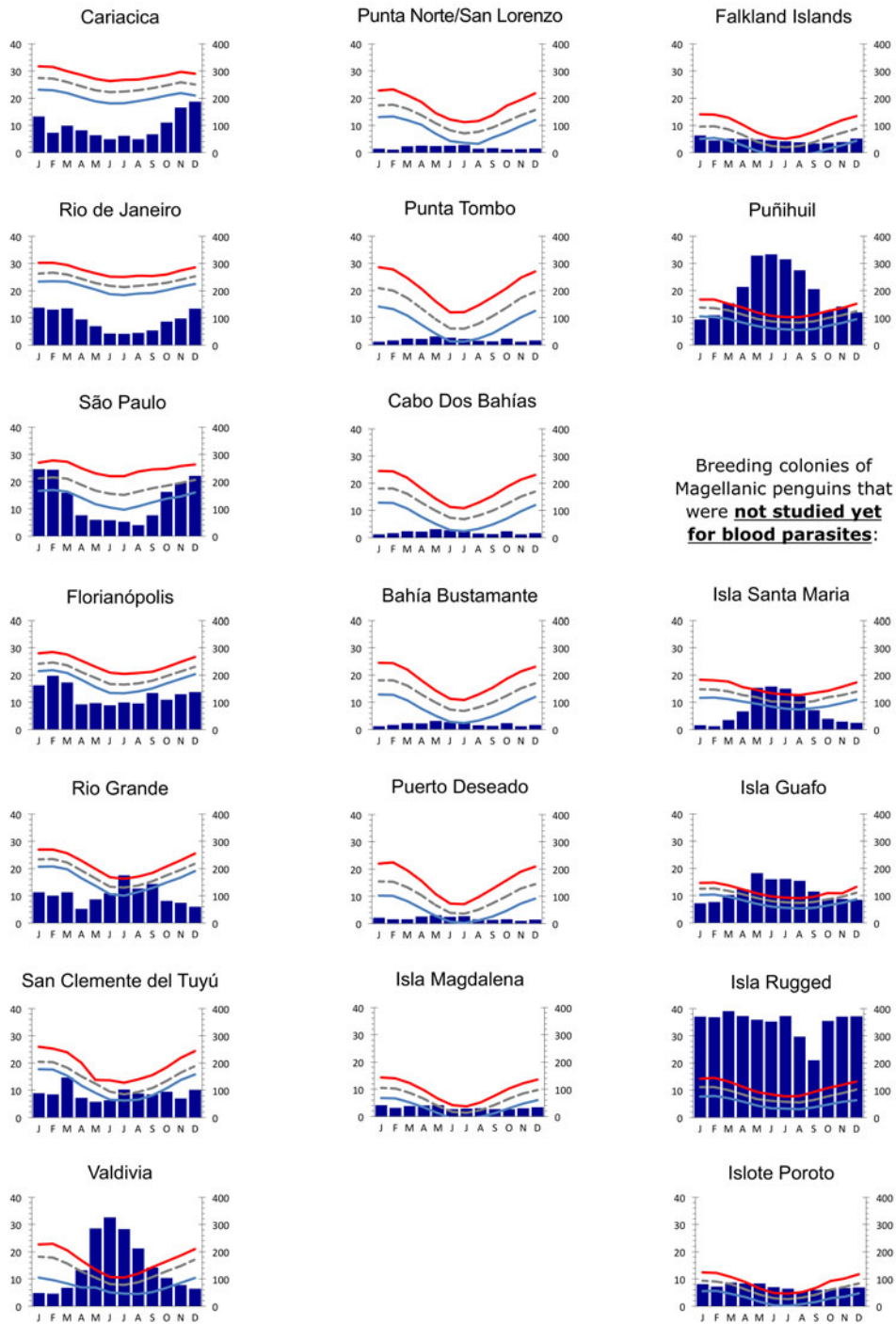
DISCUSSION

To date, the southernmost records of blood parasites in captive penguins in South America are cases of *Plasmodium* spp. infections in Valdivia, Chile (39°49'S) (Carvajal and Alvarado, 2009), and San Clemente del Tuyú, Argentina (36°20'S)

Sites where *Plasmodium* was detected in captive Magellanic penguins:

Breeding colonies of Magellanic penguins that were studied with large sample sizes ($n > 30$):

Breeding colonies of Magellanic penguins that were studied with small sample sizes ($n < 30$):



Breeding colonies of Magellanic penguins that were not studied yet for blood parasites:

Fig. 2. Comparison of the historical climate normals of different sites in relation to the occurrence of *Plasmodium* in Magellanic penguins. Climatograms present the average maximum (red line), mean daily (dashed grey line) and average minimum (blue line) temperature (in Celsius degrees, left axis) and the average monthly precipitation (blue bars; in millimetres, right axis).

(Vanstreels *et al.* 2016b), whereas all studies have failed to detect blood parasites in wild Magellanic penguins sampled south of 40°S (Fig. 1). One could therefore be led to suspect that a latitudinal temperature gradient might be the main constraint to the

occurrence of blood parasites in wild Magellanic penguins. However, because haemosporidians (*Haemoproteus*, *Plasmodium* and *Leucocytozoon*) have been reported in South American forest birds as far south as Navarino Island (54°56'S) (Merino

Table 2. Summary of studies attempting to detect blood parasites in wild Magellanic penguins

Study site	Reference	Year of sampling	Laboratory method	Age group	Sample size	Estimated true prevalence	
						Worst-case (%)	Best-case (%)
Argentinean coast and Magellan strait							
Multiple locations, Chubut, Argentina	3, 4	1998–2001	TBS	adults	36	≤12·6	≤11·0
Multiple locations, Chubut, Argentina	3, 4	1998–2001	TBS	chicks	300	≤1·7	≤1·5
Isla Vernaci Norte, Chubut, Argentina	5	1999–2000	TBS	adults	103	≤4·9	≤4·3
Puerto Deseado, Santa Cruz, Argentina	7	2010–2013	PCR _{HP} , PCR _L	adults	48	≤10·2	≤7·4
Isla Magdalena, Magallanes, Chile	7	2010–2013	PCR _{HP} , PCR _L	adults	287	≤1·9	≤1·4
Multiple locations, Chubut, Argentina	8	2012–2014	TBS, PCR _{HP} , PCR _L , PCR _B	adults	284	≤1·6	≤1·4
Subtotal					1058	≤0·5	≤0·4
Pacific Patagonian coast							
Puñihuil, Los Lagos, Chile	7	2010–2013	PCR _{HP} , PCR _L	adults	25	≤19·6	≤14·2
Falkland (Malvinas) Islands							
Multiple locations	1, 2	1986–1987	TBS	adults	12	≤33·6	≤29·4
New Island	6	2008–2009	TBS, PCR _{HP} , PCR _L	adults	18	≤22·3	≤19·8
Subtotal					30	≤16·3	≤11·8

TBS, thin blood smear; PCR_{HP}, PCR targeting *Haemoproteus* and *Plasmodium*; PCR_L, PCR targeting *Leucocytozoon*; PCR_B, PCR targeting *Babesia*. References: (1) Hawkey *et al.* 1989; (2) Keymer *et al.* 2001; (3) Jovani *et al.* 2001; (4) Tella *et al.* 2001; (5) Moreno *et al.* 2002; (6) Quillfeldt *et al.* 2010; (7) Sallaberry-Pincheira *et al.* 2015; (8) This study.

et al. 2008), where daily mean temperatures range between 1·8 and 9·8 °C throughout the year (Hajek and Di Castri, 1975), it is clear that temperature is not *per se* the constraint to the occurrence of these parasites. Instead, the combination of strong winds and the scarcity of freshwater on the Atlantic Patagonian coast might be the key factor driving the lack of haemosporidian in Magellanic penguins at that region.

The combined study effort conducted along the Atlantic Patagonian coast, especially with the recent studies employing high-sensitivity nested PCR tests, provides strong basis to conclude that haemosporidians are absent or near-absent in wild Magellanic penguins in that region. However, it is worth noting that sampling efforts to date have been predominantly focused on the Argentinean Patagonia and the Magellan strait (Fig. 1), a remarkably arid region, with average rainfall generally lower than 300 mm per year, strong winds and scarce freshwater. The emphasis in sampling Magellanic penguins in this region is not surprising considering it is where most of the species' population is concentrated (Birdlife International, 2012). However, the very limited sampling effort on the Pacific Patagonian coast and the Falkland Islands precludes a categorical conclusion that

haemosporidian parasites do not infect Magellanic penguins in the wild.

The Chilean Patagonian coast has a remarkably diverse climate, with generally higher rainfall than its Argentinean counterpart (>1000 mm/year, see Figs 1 and 2), and ecological modelling indicates it provides a more favourable environment for dipteran vectors (WRBU, 2016). Albeit relatively small for Magellanic penguin standards, the breeding colonies in Chile congregate several hundreds to thousands of pairs (Boersma *et al.* 2014), representing a significant fraction of the species' population. Similarly, the Falkland Islands are not as dry as the Argentinean Patagonian coast, with an average rainfall of 680 mm/year. Although the archipelago is reportedly mosquito-free (Medlock *et al.* 2010), a *Plasmodium* sp. infection was recently identified in a thin-billed prion (*Pachyptila belcheri*) breeding on New Island (Quillfeldt *et al.* 2010). Additional studies with extensive sampling of wild Magellanic penguins on the Chilean Patagonian coast and the Falkland Islands are therefore warranted.

With regards to *Babesia*, we did not find evidence of this parasite in wild Magellanic penguins. However, it is still early to conclude whether this parasite is present or absent on the Argentinean Patagonian coast, since this was the first study to

employ molecular methods for its diagnosis. *Ixodes* spp. ticks are thought to be responsible for the transmission of *Babesia* to penguins (Cunningham *et al.* 1993; Earlé *et al.* 1993; Montero *et al.* 2016), and *Ixodes uriae* has been sporadically recorded on seabirds in Argentina, Chile and the Falkland Islands (Muñoz-Leal and González-Acuña, 2015). While *I. uriae* does not seem to be a common parasite of wild Magellanic penguins, it can clearly thrive in penguin colonies at harsh environmental conditions such as those of the southern tip of South America (Barbosa *et al.* 2011; Muñoz-Leal and González-Acuña, 2015) and therefore the occurrence of tick-borne blood parasites should be further investigated throughout the breeding distribution of Magellanic penguins.

It is worth noting that besides these broader trends in climatic and geographic distribution, other factors may also affect the detection of blood parasites in seabirds. Because most studies on wild Magellanic penguins have targeted the sampling of non-moulting apparently healthy adults, it is possible that blood parasites were not detected because they are confined to other life stages. Previous studies have shown that blood parasite infections tend to be more frequent and more acute in penguin chicks as they approach fledging and in adult penguins during moult (Fallis *et al.* 1976; Allison *et al.* 1978; Hill *et al.* 2010; Argilla *et al.* 2013), and future studies on Magellanic penguins might therefore benefit from sampling these life stages.

Perspectives for climate change and distribution shifts

Even if future studies conclude that blood parasites are absent in wild Magellanic penguins throughout the species' distribution, there is evidence to indicate this could change in the future. A recent northward expansion of the population of Magellanic penguins has been noted in Argentinean Patagonia, with new colonies being established and rapidly increasing (Gómez-Laich *et al.* 2015; Pozzi *et al.* 2015). For example, the Punta Norte/San Lorenzo colony (42° 04'S) was founded in 1977 and rapidly escalated to become one of the species' largest colonies, with more than 134 000 breeding pairs by 2008. Similarly, small northern colonies established in the last decade such as Complejo Isote Lobos (41°26'S, founded in 2002) and El Pedral (42°56'S, founded in 2009) have speedily grown, contrasting with the stable or declining trends of some of the larger breeding colonies in central Patagonia (Wilson *et al.* 2005; Pozzi *et al.* 2015). This northward distributional shift of the Magellanic penguin, which is possibly linked to a decreased prey availability in the central Argentinean coast (Gómez-Laich *et al.* 2015; Pozzi *et al.* 2015), could lead the species to breed in areas that are suitable for mosquitoes and other vectors.

On the other hand, recent studies indicate a poleward extension of the distribution of dipteran insects, and there is evidence of a distribution shift of mosquito-borne pathogens in response to climate change (Rogers and Randolph, 2000; Harvell *et al.* 2002; Garamszegi, 2011). In this scenario, the clash between the northward expansion of Magellanic penguins and the southward expansion of mosquitoes could provide novel opportunities for the transmission of blood parasites on the northern Argentinean Patagonian coast, with potentially grave consequences for this species' conservation.

Additionally, even in regions with arid and mosquito-adverse climate it is possible that human presence near penguin colonies – in the form of towns, settlements, farms, ranches, tourist visitation centers, resorts, park ranger stations, etc. – may provide micro-environments of wind protection and freshwater availability that favour the thriving of dipteran vectors. A troubling example of this is the recent increase in mosquito and fly populations in the coastal Argentinean city of Puerto Madryn (42°46'S), which prompted a fumigation program to prevent outbreaks of vector-borne diseases that may pose a risk to public health (MPM, 2009, 2016). It is therefore urgent to conduct studies examining the presence of invertebrates and synanthropic birds in areas where Magellanic penguins breed within close proximity to human settlements or facilities, to evaluate the potential for blood parasite transmission.

ACKNOWLEDGEMENTS

We are grateful to Felix Capellino, Gastón Delgado, Sergio Heredia, Julio Loureiro, David Verón, Cecilia Decker, Mónica Jacobsen, Leonhard Schnittger, Marcos Amaku, Susan Kutz, Claudia Niemeyer, Luciana Gallo, Matias Di Martino, Lucas Beltramino, Jorge Oyakawa, Daniela Debone, Luana Ortolan, Michelle Secundes, Luis Felipe S. P. Mayorga, Nola Parsons, Lisa Nupen for their valuable contributions. This study was supported by Laboratório de Patologia Comparada de Animais Selvagens da Universidade de São Paulo, Fundação Mundo Marino, Instituto de Patobiología del Instituto Nacional de Tecnología Agropecuária, Wildlife Conservation Society and the One Health Institute at University of California, Davis.

FINANCIAL SUPPORT

Financial support was provided by Fundação de Amparo à Pesquisa do Estado de São Paulo (grant numbers FAPESP 2009/53956-9 and 2010/51801-5) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

REFERENCES

- Allison, F. R., Desser, S. S. and Whitten, L. K. (1978). Further observations on the life cycle and vectors of the haemosporidian *Leucocytozoon tawaki* and its transmission to the Fiordland crested penguin. *New Zealand Journal of Zoology* 5, 371–374.
- Argilla, L. S., Howe, L., Gartrell, D. and Alley, M. R. (2013). High prevalence of *Leucocytozoon* spp. in the endangered yellow-eyed penguin

- (*Megadyptes antipodes*) in the sub-Antarctic regions of New Zealand. *Parasitology* **140**, 672–682.
- Barbosa, A., Benzal, J., Vidal, V., D'Amico, V., Coria, N., Diaz, J., Motas, M., Palacios, M. J., Cuervo, J. J., Ortiz, J. and Chitimia, L.** (2011). Seabird ticks (*Ixodes uriae*) distribution along the Antarctic Peninsula. *Polar Biology* **34**, 1621–1624.
- BirdLife International** (2012). *Spheniscus magellanicus*. The IUCN Red List of Threatened Species 2012: e.T22697822A37820611. <http://dx.doi.org/10.2305/IUCN.UK.2012-1.RLTS.T22697822A37820611.en>
- Boersma, P. D., Frere, E., Kane, O., Pozzi, L. M., Pütz, K., Raya-Rey, A., Rebstock, G. A., Simeone, A., Smith, J., Van Buren, A., Yorio, P. and Garcia-Borboroglu, P.** (2014). Magellanic penguin (*Spheniscus magellanicus*). In *Penguins: Natural History and Conservation* (ed. Garcia-Borboroglu, P. and Boersma, P. D.), pp. 145–152. Washington University Press, Seattle, USA.
- 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. and Kirchgatter, K.** (2010). Identification of *Plasmodium relictum* causing malaria in penguins (*Spheniscus magellanicus*) from São Paulo Zoo, Brazil. *Veterinary Parasitology* **173**, 123–127.
- Cabana, A. L., Vanstreels, R. E. T., Xavier, M. O., Osório, L. G., Adornes, A. C., Leite, A. M., Soares, M. P., Silva-Filho, R. P., Catão-Dias, J. L. and Meireles, M. C. A.** (2014). Lethal concurrent avian malaria and aspergillosis in Magellanic penguin (*Spheniscus magellanicus*). *Boletín Chileno de Ornitología* **20**, 28–32.
- Campos, S. D. E., Pires, J. R., Nascimento, C. L., Dutra, G., Torres-Filho, R. A., Toma, H. K., Brener, B. and Almosny, N. R. P.** (2014). Analysis of hematologic and serum chemistry values of *Spheniscus magellanicus* with molecular detection of avian malarial parasites (*Plasmodium* spp.). *Pesquisa Veterinária Brasileira* **34**, 1236–1242.
- Carvajal, E. R. and Alvarado, P. M.** (2009). Pesquisa de *Plasmodium* spp. en pinguinos de Magallanes (*Spheniscus magellanicus*) de la Región de los Ríos: malaria aviar como nueva patología de interés en la avifauna local. *Boletín Veterinario Oficial* **10**, 1–4.
- Cunningham, M., Gibbs, P., Rogers, T., Spielman, D. and Walraven, E.** (1993). *Ecology and Health of the Little Penguin Eudyptes Minor near Sydney: A Report Prepared for the Water Board*. Taronga Zoo, Sydney, Australia.
- Earlé, R. A., Huchzermeyer, F. W., Bennett, G. F. and Brossy, J. J.** (1993). *Babesia peircei* sp. nov. from the jackass penguin. *African Zoology* **28**, 88–90.
- Fallis, A. M., Bisset, S. A. and Allison, F. R.** (1976). *Leucocytozoon tawaki* n. sp. (Eucoccidia: Leucocytozoidae) from the penguin *Eudyptes pachyrhynchus*, and preliminary observations on its development in *Austrosimulium* spp. (Diptera: Simuliidae). *New Zealand Journal of Zoology* **3**, 11–16.
- Fix, A. S., Waterhouse, C., Greiner, E. C. and Stoskopf, M. K.** (1988). *Plasmodium relictum* as a cause of avian malaria in wild-caught Magellanic penguins (*Spheniscus magellanicus*). *Journal of Wildlife Diseases* **24**, 610–619.
- Fontannaz, C. C.** (2001). *Estadística Climatológica*. Dirección Meteorológica de Chile, Climatología y Meteorología Aplicada, Santiago, Chile.
- Garamszegi, L. Z.** (2011). Climate change increases the risk of malaria in birds. *Global Change Biology* **17**, 1751–1759.
- Gómez-Laich, A., Wilson, R. P., Sala, J. E., Luzenti, A. and Quintana, F.** (2015). Moving northward: comparison of the foraging effort of Magellanic penguins from three colonies of northern Patagonia. *Marine Biology* **162**, 1451–1461.
- Gubbels, J. M., de Vos, A. P., van der Weide, M., Viseras, J. and Schouls, L. M.** (1999). Simultaneous detection of bovine Theileria and Babesia species by reverse line blot hybridization. *Journal of Clinical Microbiology* **37**, 1782–1789.
- Hajek, E. R. and Di Castri, F.** (1975). *Bioclimatografía de Chile*. Universidad Católica de Chile, Santiago, Chile.
- Harvell, C. D., Mitchell, C. E., Ward, J. R., Altizer, S., Dobson, A. P., Ostfeld, R. S. and Samuel, M. D.** (2002). Climate warming and disease risks for terrestrial and marine biota. *Science* **296**, 2158–2162.
- Hawkey, C. M., Horsley, D. T. and Keymer, I. F.** (1989). Haematology of wild penguins (Sphenisciformes) in the Falkland islands. *Avian Pathology* **18**, 495–502.
- Hellgren, O., Waldenström, J. and Bensch, S.** (2004). A new PCR assay for simultaneous studies of *Leucocytozoon*, *Plasmodium*, and *Haemoproteus* from avian blood. *Journal of Parasitology* **90**, 797–802.
- Hijmans, R. J., Cameron, S. E., Parra, J. L., Jones, P. G. and Jarvis, A.** (2005). Very high resolution interpolated climate surfaces for global land areas. *International Journal of Climatology* **25**, 1965–1978.
- Hill, A. G., Howe, L., Gartrell, B. D. and Alley, M. R.** (2010). Prevalence of *Leucocytozoon* spp. in the endangered yellow-eyed penguin *Megadyptes antipodes*. *Parasitology* **137**, 1477–1485.
- Jovani, R., Tella, J. L., Forero, M. G., Bertellotti, M., Blanco, G., Ceballos, O. and Donazar, J. Á.** (2001). Apparent absence of blood parasites in the Patagonian seabird community: is it related to the marine environment? *Waterbirds* **24**, 430–433.
- Keymer, I. F., Malcolm, H. M., Hunt, A. and Horsley, D. T.** (2001). Health evaluation of penguins (Sphenisciformes) following mortality in the Falklands (South Atlantic). *Diseases of Aquatic Organisms* **45**, 159–166.
- Ko, K.-N., Kang, S.-C., Jung, J.-Y., Bae, J.-H. and Kim, J.-H.** (2008). Avian malaria associated with *Plasmodium* spp. infection in a penguin in Jeju Island. *Korean Journal of Veterinary Research* **48**, 197–201.
- Medlin, L., Elwood, H. J., Stickle, S. and Sogin, M. L.** (1988). The characterization of enzymatically amplified eukaryotic 16-like rRNA-coding regions. *Gene* **71**, 491–499.
- Medlock, J. M., Schaffner, F. and Fontenille, D.** (2010). Invasive mosquitoes in the European associate continental and overseas territories. European Centre for Disease Prevention and Control, Solna, Sweden. http://www.ecdc.europa.eu/en/activities/sciadvicelayouts/forms/view_dispform.aspx?ID=212&List=a3216f4c-f040-4f51-9f77-a96046dbfd72
- Merino, S., Moreno, J., Vázquez, R. A., Martínez, J., Sánchez-Monsalve, I., Estades, C. F., Ippi, S., Sabat, P., Rozzi, R. and McGehee, S.** (2008). Haematocrit in forest birds from southern Chile: latitudinal gradients in prevalence and parasite lineage richness. *Austral Ecology* **33**, 329–340.
- Montero, E., González, L. M., Chaparro, A., Benzal, J., Bertellotti, M., Masero, J. A., Colominas-Ciuró, R., Vidal, V. and Barbosa, A.** (2016). First record of *Babesia* sp. in Antarctic penguins. *Ticks and Tick Borne Diseases* **7**, 498–501.
- Moreno, J., Yorio, P., Garcia-Borboroglu, P., Potti, J. and Villar, S.** (2002). Health state and reproductive output in Magellanic penguins (*Spheniscus magellanicus*). *Ethology, Ecology and Evolution* **14**, 19–28.
- Municipalidad de Puerto Madryn (MPM)** (2009). *Dengue*. <http://www.madrin.gov.ar/dengue/>
- Municipalidad de Puerto Madryn (MPM)** (2016). *Comienzan las fumigaciones por moscas y mosquitos*. <http://lu17.com/destacado/comienzan-las-fumigaciones-por-moscas-y-mosquitos>
- Muñoz-Leal, S. and González-Acuña, D.** (2015). The tick *Ixodes uriae* (Acari: Ixodidae): hosts, geographical distribution, and vector roles. *Ticks and Tick Borne Diseases* **6**, 843–868.
- Pozzi, L. M., García-Borboroglu, P., Boersma, P. D. and Pascual, M. A.** (2015). Population regulation in Magellanic penguins: what determines change in colony size. *PLoS ONE* **10**, e0119002.
- Quillfeldt, P., Martínez, J., Hennessee, J., Ludynia, K., Gladbach, A., Masello, J. F., Riou, S. and Merino, S.** (2010). Hemsporidian blood parasites in seabirds: a comparative genetic study of species from Antarctic to tropical habitats. *Naturwissenschaften* **97**, 809–817.
- Ramos, A. M., Santos, L. A. R. and Fortes, L. T. G.** (2009). *Normais Climatológicas do Brasil 1961–1990*. Instituto Nacional de Meteorologia, Brasília.
- Reiczig, J., Földi, J. and Ózsvári, L.** (2010). Exact confidence limits for prevalence of a disease with an imperfect diagnostic test. *Epidemiology & Infection* **138**, 1674–1678.
- Richard, F. A., Sehgal, R. N. M., Jones, H. I. and Smith, T. B.** (2002). A comparative analysis of PCR-based detection methods for avian malaria. *Journal of Parasitology* **88**, 819–822.
- Rogers, D. J. and Randolph, S. E.** (2000). The global spread of malaria in a future, warmer world. *Science* **289**, 1763–1766.
- Rosenfeld, G.** (1947). Corante pancrômico para hematologia e citologia clínica: nova combinação dos componentes do May-Grunwald e do Giemsa num só corante de emprego rápido. *Memórias do Instituto Butantan* **20**, 329–335.
- Sallaberry-Pincheira, N., González-Acuña, D., Herrera-Tello, Y., Dantas, G. P. M., Luna-Jorquera, G., Frere, E., Valdés-Velasquez, A., Simeone, A. and Vianna, J. A.** (2015). Molecular epidemiology of avian malaria in wild breeding colonies of Humboldt and Magellanic penguins in South America. *EcoHealth* **12**, 267–277.
- Sergeant, E. S. G.** (2016). *EpiTools epidemiological calculators*. AusVet Animal Health Services and Australian Biosecurity Cooperative Research Centre for Emerging Infectious Disease. <http://epitools.ausvet.com.au/>
- Silveira, P., Belo, N. O., Lacorte, G. A., Kolesnikovas, C. K. M., Vanstreels, R. E. T., Steindel, M., Catão-Dias, J. L., Valkiūnas, G. and Braga, É. M.** (2013). Parasitological and new molecular-phylogenetic characterization of the malaria parasite *Plasmodium tejeraei* in South American penguins. *Parasitology International* **62**, 165–171.
- Servicio Meteorológico Nacional (SMN)** (2016). *Atlas Climático*. <http://www.smn.gov.ar/serviciosclimaticos/?mod=elclima&id=1>
- Tella, J. L., Forero, M. G., Bertellotti, M., Donazar, J. A., Blanco, G. and Ceballos, O.** (2001). Offspring body condition and immunocompetence

are negatively affected by high breeding densities. *Proceedings of the Royal Society of London B* **268**, 1455–1461.

Tollini, J., Brocksen, A. and Sureda, N. (2000). Prevention and treatment of avian malaria in a captive penguin colony. *Penguin Conservation* **13**, 28–31.

Valkiūnas, G., Zehntindjiev, P., Dimitrov, D., Križanauskienė, A., Iezhova, T. A. and Bensch, S. (2008). Polymerase chain reaction-based identification of *Plasmodium* (*Huffia*) *elongatum*, with remarks on species identity of haemosporidian lineages deposited in GenBank. *Parasitological Research* **102**, 1185–1193.

Vanstreels, R. E. T., Kolesnikovas, C. K. M., Sandri, S., Silveira, P., Belo, N. O., Ferreira-Junior, F. C., Epiphanyo, S., Steindel, M., Braga, É. M. and Catão-Dias, J. L. (2014). Outbreak of avian malaria associated to multiple species of *Plasmodium* in Magellanic penguins undergoing rehabilitation in Southern Brazil. *PLoS ONE* **9**, e94994.

Vanstreels, R. E. T., Silva-Filho, R. P., Kolesnikovas, C. K. M., Bhering, R. C. C., Ruoppolo, V., Epiphanyo, S., Amaku, M., Ferreira-Junior, F. C., Braga, É. M. and Catão-Dias, J. L. (2015). Epidemiology and pathology of avian malaria in penguins undergoing rehabilitation in Brazil. *Veterinary Research* **46**, 30.

Vanstreels, R. E. T., Braga, É. M. and Catão-Dias, J. L. (2016a). Blood parasites of penguins: a critical review. *Parasitology* **143**, 931–956.

Vanstreels, R. E. T., Capellino, F., Silveira, P., Braga, É. M., Rodríguez-Heredia, S. A., Loureiro, J. and Catão-Dias, J. L. (2016b). Avian malaria (*Plasmodium* spp.) in Magellanic penguins (*Spheniscus magellanicus*) captive in northern Argentina. *Journal of Wildlife Diseases* **52**, 734–737.

Waldenström, J., Bensch, S., Hasslequist, D. and 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**, 191–194.

Walter Reed Biosystematics Unit (WRBU) (2016). *VectorMap: know the vector, know the threat*. <http://www.vectormap.org/>

Wilson, R. P., Scolaro, J. A., Grémillet, D., Kierspel, M. A. M., Laurenti, S., Upton, J., Gallelli, H., Quintana, F., Frere, E., Müller, G., Straten, M. T., and Zimmer, I. (2005). How do Magellanic penguins cope with variability in their access to prey? *Ecological Monographs* **75**, 379–401.