

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

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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

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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

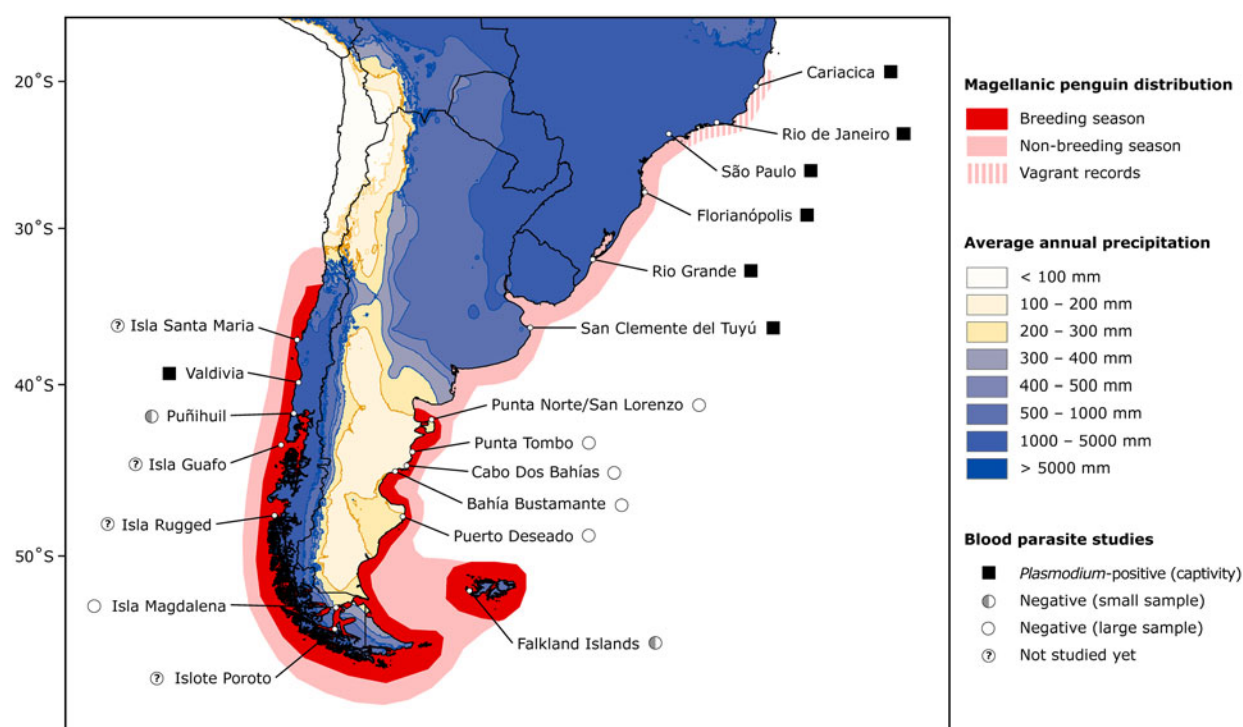


Fig. 1. Geographic distribution of studies investigating the occurrence of blood parasites in Magellanic penguins in relation to the species' natural distribution and historical precipitation data.

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 ($n < 30$):

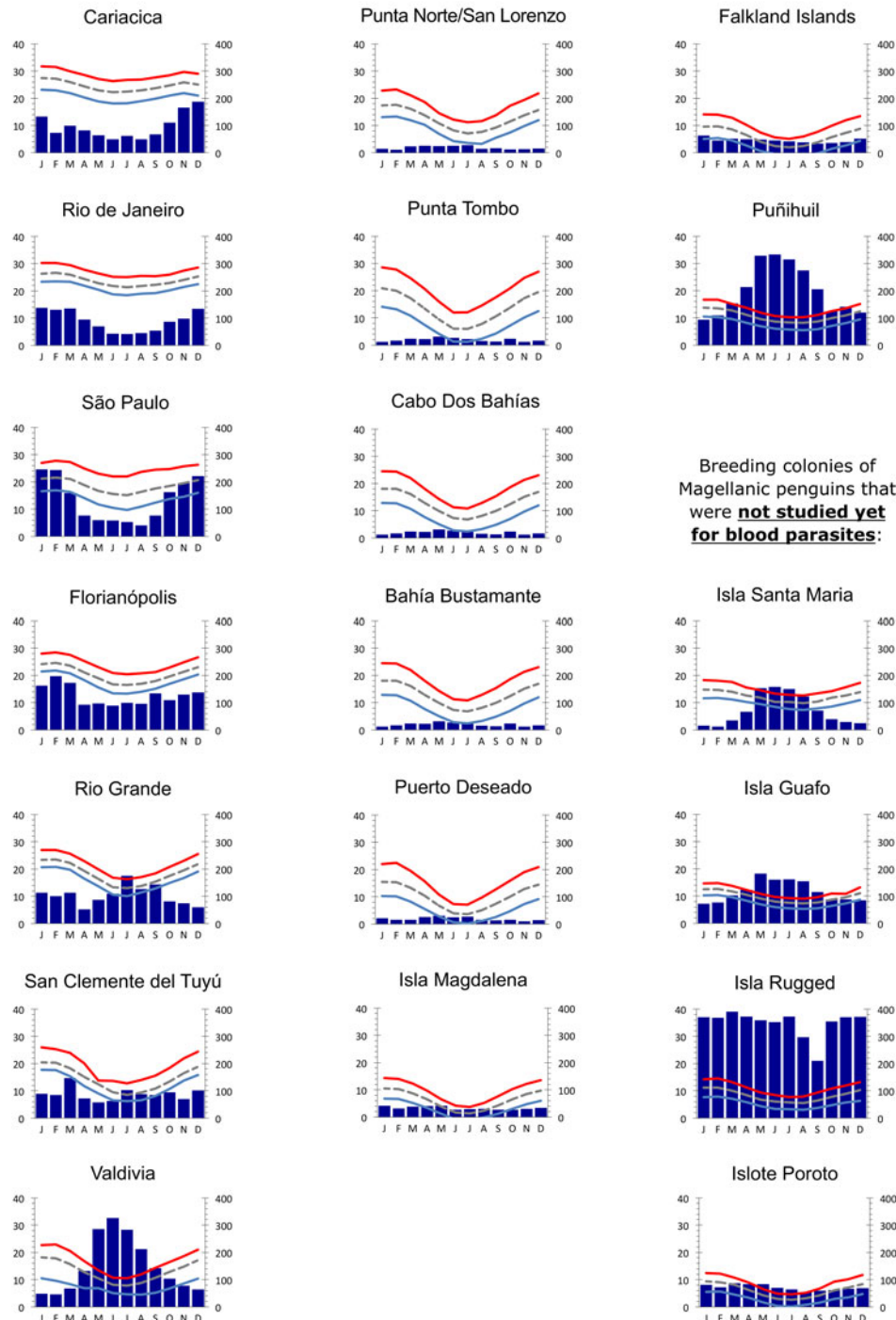


Fig. 2. Comparison of the historical climate normals of different sites in relation to the occurrence of *Plasmodium* in Magellanic penguins. Climatograms present the average maximum (red line), mean daily (dashed grey line) and average minimum (blue line) temperature (in Celsius degrees, left axis)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

						Estimated true prevalence	
Study site	Reference	Year of sampling	Laboratory method	Age group	Sample size	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 sea-birds 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 sea-birds. 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.

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