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Molecular Survey of Haemosporidian Parasites in Procellariiformes Sampled in Southern Brazil, 2013–22

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ABSTRACT: The order Procellariiformes includes several species of seabirds that perform long-distance migrations crossing all the oceans. These movements may contribute to the dispersal and exchange of hemoparasites, such as haemosporidians. There is a lack of studies regarding the order Haemosporida in Procellariiformes, and, to date, only the genus *Plasmodium* has been reported. This survey investigated the occurrence of the three genera of haemosporidians, *Plasmodium*, *Haemoproteus*, and *Leucocytozoon*, in samples collected between 2013 and 2022 from 95 individuals of 14 species of Procellariiformes from southern Brazil, including live animals in rehabilitation centers, individuals caught as incidental bycatch, and carcasses found along the coast. A total of 171 samples of blood and fragments of liver and spleen were analyzed, with extracted DNA being subjected to a nested PCR followed by phylogeny analysis. All animals were negative for *Plasmodium* spp. and *Leucocytozoon* spp., but one Black-browed Albatross (*Thalassarche melanophris*) and one Manx Shearwater (*Puffinus puffinus*) specimen were positive for *Haemoproteus* spp. The sequences obtained from positive seabirds did not show 100% similarity with other known lineages available in the MalAvi database and thus were probably novel lineages. However, one sequence clustered together with *Haemoproteus noctuae*, a parasite from Strigiformes, while the other was grouped with *Haemoproteus columbae*, which is classically related to Columbiformes. These results suggest that both positive animals may have become infected when beached or in rehabilitation centers by a spillover of vectors from local birds. This highlights the importance of surveillance of the health of Procellariiformes regarding the possibility of dissemination of new pathogens in different bird populations.

Key words: Albatross, *Haemoproteus*, pathogen, petrel, protozoan, vector-borne.

INTRODUCTION

Procellariiforms are a widespread group of seabirds with more than 100 validated species; 30% of this diversity is found in the Brazilian avifauna (Pacheco et al. 2021; International Union for Conservation of Nature 2022). Procellariiforms are known for their long-distance and long-duration movements during migration (Ballance 2007) and may transport, disperse, and exchange parasites and pathogens (Dietrich et al. 2011; McCoy et al. 2016). Their breeding sites are found in offshore islands, inaccessible islets near the continent, and remote coastal habitats (Warham 1996), which generally limits access to new samples for research. This group is currently seriously threatened by invasive alien species, bycatch in fisheries, climate change, pollution, and diseases (Phillips et al. 2016; Dias et al. 2019). Hence, it becomes essential to develop more studies to elucidate the role of different pathogens on their health status.

Among blood parasites, the order Haemosporida is commonly studied in birds in general, although it has been less studied in seabirds

than in other orders such as Passeriformes. The main genera within the Haemosporida are *Plasmodium*, *Haemoproteus*, and *Leucocytozoon* (Valkiūnas 2005). These parasites are transmitted by blood-sucking insects of the order Diptera (Valkiūnas and Iezhova 2022). *Plasmodium* spp. are transmitted by mosquitoes from the Culicidae family, *Haemoproteus* spp. are transmitted by louse flies (Hippoboscida) and biting midges (Ceratopogonidae), and *Leucocytozoon* spp. are transmitted by blackflies (Simulidae) and biting midges (Ceratopogonidae; Quillfeldt et al. 2010). To date, few studies have specifically addressed these parasites in Procellariiformes (e.g., Campioni et al. 2018; Vanstreels et al. 2020; Ilahiane et al. 2022), and only one genus (*Plasmodium*) has been reported in this order. Our study aimed to investigate the occurrence of Haemosporida in this order of seabirds and identify the lineages potentially associated with these animals.

METHODS

Sample collection

Biological samples (blood and/or organs) were obtained from 2013 to 2022 from three different sources: animals admitted to two rehabilitation centers, beached carcasses found along the southern Brazil coast, and specimens incidentally caught by fisheries along the southern Brazilian shelf break. The rehabilitation centers that provided samples for this study were the Unidade de Estabilização de Animais Marinhos, Universidade do Vale de Itajaí (UNIVALI), Penha municipality, Santa Catarina state, and the Centro de Estudos Costeiros, Limnológicos e Marinhos (CECLIMAR), Universidade Federal do Rio Grande do Sul (UFRGS), Imbé municipality, Rio Grande do Sul state, Brazil. Samples were also obtained from the Banco Nacional de Amostras Biológicas de Albatrozes e Petréis (BAAP), a national repository of biological samples from albatrosses and petrels, where the material from bycatch specimens collected by Projeto Albatroz is housed, among others. After collection, all samples were stored at –20 C until laboratory analyses. The blood samples were collected at the time of animal admission at the rehabilitation centers. All procedures regarding animal sampling were authorized by Brazilian environmental authorities (SISBIO license numbers 24381 and 87644).

A total of 171 tissue samples from 95 procellariiform seabirds comprising 14 different species (Table 1) were analyzed by molecular tests at Instituto de Pesquisas Veterinárias Desidério Finamor (IPVDF), Eldorado do Sul, Rio Grande do Sul state, Brazil. The samples included 18 frozen blood samples, 18 blood in ethanol, 93 fragments of frozen liver, and 42 fragments of frozen spleen.

Molecular tests

Following the phenol-chloroform method (Sambrook et al. 1989), DNA extraction was conducted in all frozen tissue samples. For the blood kept in ethanol, an adaptation of the phenol-chloroform method was used (Maia et al. 2017). After extraction, DNA concentration for molecular analysis was standardized between 100 and 200 ng/μL and confirmed by spectrophotometry using a Nanodrop spectrophotometer (ThermoScientific, Waltham, Massachusetts, USA). Molecular testing by nested PCR was then executed, targeting a 617-bp fragment of the cytochrome b gene (*cyt b*) from the genera *Haemoproteus*, *Plasmodium*, and *Leucocytozoon* (Hellgren et al. 2004). The DNA from *Plasmodium gallinaceum* strain A8 (MalAvi lineage GALLUS01) was used as a positive control. Ultrapure water was used as negative control. The first PCR reaction contained a final volume of 25 μL and used the primers HaemNFI (5′-CATATATTAAGAGAAITATGGAG-3′) and HaemNR3 (5′-ATAGAAAGATAAGAAATACCATTC-3′) to amplify the DNA from the three above mentioned genera. For the following two nested-PCR reactions, also with a final volume of 25 μL, the product of the first reaction (1 μL) was used as template. Detection of *Haemoproteus* spp. and *Plasmodium* spp. was conducted by PCR targeting a 479 bp fragment with the primers HaemF (5′-ATGGTGCTTTCGATATATGCATG-3′) and HaemR2 (5′-GCATTATCTGGATGTGATAATGGT-3′). Lastly, HaemFL (5′-ATGGTGTTTTAGATACTTACATT-3′) and HaemR2L (5′-CATT ATCTGGATGAGATAATGGIGC-3′) primers were used for detecting *Leucocytozoon* spp., with a fragment of 479 bp. The conditions and details of all PCR reactions were as described previously (Hellgreen et al. 2004). The first reaction was performed with an incubation at 94 C for 3 min, 20 cycles of 30 s at 94 C, 30 s at 50 C, 45 s at 72 C, and a final extension at 72 C for 10 min. The second

TABLE 1. Summary of all samples from procellariformes from southern Brazil tested by molecular analysis for haemosporidian hemoparasites, separated by species and source locations.

Species	Popular name	No. specimens	Samples (*n*)	Sample source[a] (*n*[b])
Thalassarche chlororhynchos	Atlantic Yellow-nosed Albatross	8	liver (8), spleen (6), blood (6)	UNIVALI (1), CECLIMAR (5), BAAP (2)
Thalassarche melanophris	Black-browed Albatross	14	liver (13), spleen (2), blood (10)	UNIVALI (1), CECLIMAR (1), BAAP (12)
Diomedea dabbenena	Tristan Albatross	1	liver (1), blood (1)	BAAP (1)
Diomedea epomophora	Southern Royal Albatross	1	liver (1), blood (1)	BAAP (1)
Ardenna grisea	Sooty Shearwater	2	liver (2), spleen (2)	UNIVALI (1), CECLIMAR (1)
Ardenna gravis	Great Shearwater	3	liver (3), spleen (3)	CECLIMAR (3)
Puffinus puffinus	Manx Shearwater	27	liver (27), spleen (10), blood (2)	UNIVALI (17), CECLIMAR (10)
Calonectris borealis	Cory's Shearwater	3	liver (3), spleen (2),	CECLIMAR (3)
Calonectris sp.	Shearwater	4	liver (4), spleen (2)	UNIVALI (4)
Procellaria aequinoctialis	White-chinned Petrel	23	liver (23), spleen (9), blood (11)	UNIVALI (6), CECLIMAR (9), BAAP (8)
Pterodroma sp.	Gadfly Petrel	1	liver (1)	UNIVALI (1)
Daption capense	Cape Petrel	1	liver (1), spleen (1)	CECLIMAR (1)
Macronectes halli	Northern Giant Petrel	1	liver (1), spleen (1), blood (1)	CECLIMAR (1)
Macronectes giganteus	Southern Giant Petrel	6	liver (5), spleen (4), blood (4)	CECLIMAR (4), BAAP (2)
Total	14 species	95 specimens	liver = 93, spleen = 42, blood = 36	UNIVALI = 31, CECLIMAR = 38, BAAP = 26

[a] BAAP=Banco Nacional de Amostras Biológicas de Albatrozes e Petréis; CELIMAR=Centro de Estudos Costeiros, Limnológicos e Marinhos; UNIVALI=Universidade do Vale de Itajaí.
[b] Number of seabirds of each species provided by each source or institution.

reaction was similar, but instead of 20 cycles, 35 cycles were conducted. Samples that yielded positive results from nested PCR were sent for DNA purification and sequencing at ACTGene, Porto Alegre, Rio Grande do Sul, Brazil. Obtained sequences were then compared with published sequences available at MalAvi, an online database of avian haemosporidian parasites (Bensch et al 2009), and at the GenBank nucleotide database (Sayers et al. 2022). All sequences generated in this study were deposited at both MalAvi and GenBank.

Phylogenetic analysis

Phylogenetic analysis was carried out using MEGA11 software (Tamura et al. 2021). For the phylogenetic tree, available sequences from GenBank and MalAvi haemosporidian parasites from aquatic birds and seabirds were used, both those with highest identity similarity to our lineages and those previously identified in aquatic birds. The only sequences of haemosporidians from terrestrial birds used were those with high similarity to our positive samples. As an outgroup, a lineage of *Leucocytozoon* sp. (Genbank accession no. AY393796) was used. First, an alignment was built using the ClustalW plug-in on MEGA11. The phylogeny model chosen was based on the goodness-of-fit of each model available in the software for the data, measured by the Bayesian information criterion and corrected by the Akaike information criterion (Tamura et al. 2011). The final model with the best score was chosen to be used. The phylogenetic tree was built using the maximum likelihood method and TN93 and GI as parameters of nucleotide substitution with 500 bootstraps.

RESULTS

Out of 171 tissue samples tested for haemosporidians, none were positive for *Leucocytozoon*.

However, two of them yielded PCR-positive results for *Haemoproteus-Plasmodium*. One was from a spleen sample of a female Black-browed Albatross (*Thalassarche melanophris*), found in an advanced state of decomposition at the Praia da Armação, Penha, Santa Catarina state, southern Brazil. The other positive sample was from the liver of a male Manx Shearwater (*Puffinus puffinus*), found at the Praia de Imbé, Rio Grande do Sul state, southern Brazil. The shearwater was lethargic although in normal body condition; it received supportive treatment at the rehabilitation center but died the next day.

The sequences from the two birds were compatible with *Haemoproteus* spp. The sequence from the albatross revealed the highest identity, 98.5% (97% query cover) with the MalAvi lineage CIRCUM01, *Haemoproteus noctuae* clone H01-14CC (GenBank no. KY653757), originally reported in a Northern Long-eared Owl (*Asio otus*). The sequence from the shearwater showed the highest identity, 99.1% (95% query cover) with the MalAvi lineage COQUI05, *Haemoproteus columbae* (GenBank no. KU131585) from a Rock Dove (*Columba livia*) in Brazil. Both sequences acquired in this survey were deposited in the MalAvi database as novel lineages since they did not show 100% identity with other lineages: lineage THAMEL01 for that found in the *T. melanophris* specimen (GenBank no. OQ579011) and lineage PUFPUF01 for the one recovered in the *P. puffinus* specimen (GenBank no. OQ579012).

Phylogenetic analysis (Fig. 1) revealed that lineages THAMEL01 and PUFPUF01 clustered in different groups and clades. *Haemoproteus* sp. THAMEL01 grouped with *Haemoproteus* spp. variants from raptors, in a cluster of *Haemoproteus noctuae* variants. It also clustered in a great clade with species of the subgenus *Haemoproteus* (Fig. 1). The lineage PUPUF01 clustered with *Haemoproteus* spp. from pigeons, within a clade of *Haemoproteus columbae* variants. The PUFPUF01 lineage fell within a different great clade of the subgenus *Parahaemoproteus* (Fig. 1). To allow a better analysis of differences among lineages identified here and other available in the literature, the number of nucleotide substitutions, as well as the identity among nucleotide sequences of *Haemoproteus* sp. isolates genetically related to PUFPUF01 and THAMEL01 lineages, are provided in Supplementary Material Tables S1 and S2. At the amino acid level, PUFPUF01 lineage showed 100% identity with three isolates of *H. columbae* (lineages COQUI05, HAECOL1, and paunal) shown in the phylogenetic tree. Similarly, THAMEL01 lineage showed 100% amino acid identity isolates of *H. noctuae* (lineages AH0031H, AH1650Hc1, and hCIRCUM01) shown in the phylogenetic tree.

DISCUSSION

Haemosporidians are usually considered scarce in seabirds (Quillfeldt et al. 2010, 2011; Vanstreels et al. 2014; Campioni et al. 2018; Kleinschmidt et al. 2022). Among these hosts, they are common only in penguins, frigatebirds, and gulls (Quillfeldt et al. 2010). In Procellariiformes, to date, there have been a few reports of *Plasmodium* spp. (Warner 1968; Quillfeldt et al. 2010; Inumaru et al. 2017; Parsons et al. 2017; Vanstreels et al. 2020), but no reports of *Haemoproteus* and *Leucocytozoon* spp. In our study 2/95 specimens were positive for *Haemoproteus* spp. on molecular analysis.

The low prevalence (2%) that we detected is in agreement with the low prevalence of haemosporidian for seabirds in general (Quillfeldt et al. 2014; Sallaberry-Pincheira et al. 2015; Mariano and Dantas 2021). Some studies have discussed the hypotheses for this pattern (Martínez-Abraín et al. 2004; Quillfeldt et al. 2010, 2011), and it has been suggested that more than one factor could be responsible. Procellariiformes mostly breed in higher latitude sites of colder climates (Onley and Scofield 2007), which impairs the development of the larval vector stage, leading to vector absence or scarcity (Quillfeldt et al. 2010, 2011). This has particular importance since for most seabirds, vector-borne disease transmission commonly occurs during the breeding season and nestling period (Quillfeldt et al. 2011; Campioni et al.

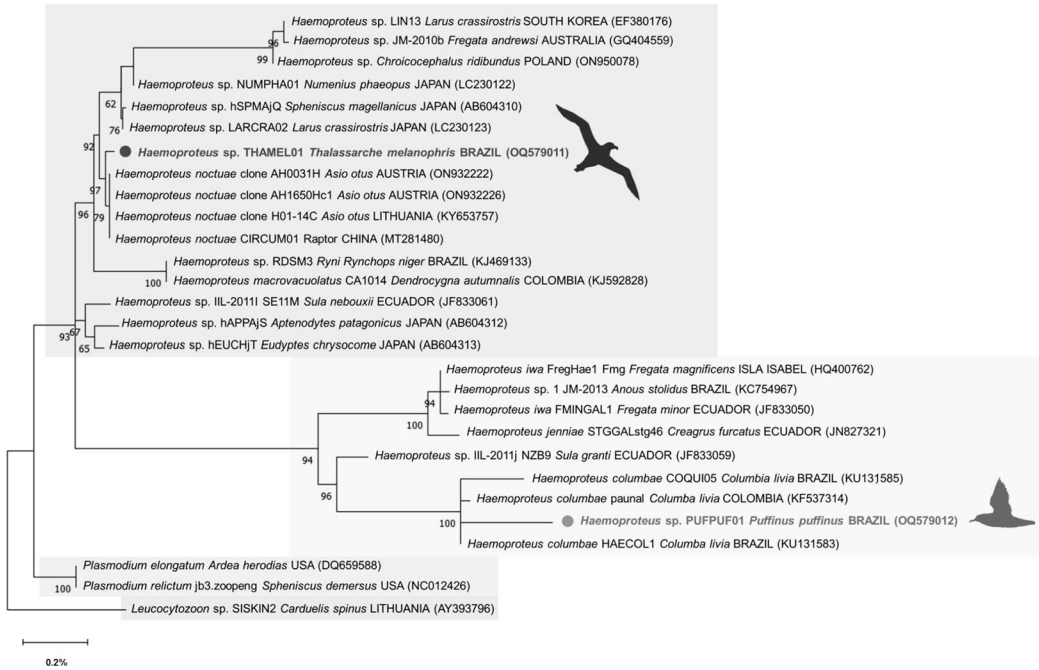

FIGURE 1. Phylogenetic analysis of the cytochrome b gene (*cyt b*) of haemosporidian parasites. Analysis was performed using MEGA 11 software (Tamura et al. 2021); the phylogeny model chosen was based on the goodness-of-fit of each model available in the software for the data, measured by the Bayesian information criterion and corrected by the Akaike information criterion; thus, the phylogenetic tree was built using the maximum likelihood method and TN93 and GI as parameters of nucleotide substitution with 500 bootstraps; the percentage of trees in which the associated taxa clustered together is shown next to the branches (bootstrap value). The tree is drawn to scale, with branch lengths indicating the number of substitutions per each 100 sites. The scale bar indicates percent nucleotide substitutions (0.2%). This analysis involved 28 nucleotide sequences, and there was a total of 472 bp per sequence in the final dataset; the sequences described in this study (*Haemoproteus* sp. lineage THAMEL01 and *Haemoproteus* sp. lineage PUFPUF01) are shown in bold and indicated by a filled circle; in all sequences, the GenBank accession numbers are indicated in parentheses; the shaded boxes indicate the classical clades that divide *Haemoproteus* into two subgenera, the upper box containing *Haemoproteus* subgenus *Haemoproteus*, and the lower box for *Haemoproteus* subgenus *Parahaemoproteus*.

2018). Additionally, this order of seabirds is known for their pelagic habits and for the extensive time of migration in the open sea (Beal et al. 2021), also decreasing the chance of vector exposure. Alternatively, it has been suggested that the immune system of Procellariiforms might be highly effective against infections by hemoparasites, in general, perhaps because of their long embryonic development, which is associated with long-lived characteristics (Esparza et al. 2004; Martínez-Abraín et al. 2004).

Considering the low exposure of Procellariiformes to haemosporidian parasites and their vectors according to the literature (Martínez-Abraín et al. 2004; Quillfeldt et al. 2010, 2011), potential vector-pathogen exposure after the seabirds were beached should be considered. It is important to consider the (unknown) elapsed time on land until the bird was found, during which time these seabirds may have been exposed to a wide variety of vectors, such as biting midges (Ceratopogonidae) and hippoboscid flies (Hippoboscidae) from local birds. Although at this time we cannot rule out that parasite acquisition occurred during the breeding season, it seems plausible to assume that it occurred after they beached. Beached seabirds are usually in already poor health conditions and predisposed to acquire novel pathogens (Parsons et al. 2017). For those that are

rescued, the period of captivity may be a further stressor leading to reduced immunocompetence, in addition to the chance of infection by a spillover of parasites from other animals kept in the facility (Vanstreels et al. 2015; Parsons et al. 2017).

The lineages with which those we identified clustered, *H. noctuae* and *H. columbae*, have been traditionally associated with Strigiformes and Columbiformes, respectively, comprising terrestrial, cosmopolitan and even synanthropic birds. Thus, it is reasonable to hypothesize that a spillover via vectors from terrestrial, local birds (such as owls and doves) may have occurred. Despite infections with different lineages of *Haemoproteus* spp. being suggested as host-specific, it is reasonable to ponder that captivity or unnatural situations, such as beached seabirds, may provide conditions for the eventual spillover of parasite lineages.

Thus, it is important to consider that seabirds, particularly in rehabilitation, might be exposed both to Haemosporida associated with them and to species traditionally found in other birds. Nevertheless, rehabilitation centers provide a useful opportunity for animal recovery and for studying different species of seabirds, such as Procellariiformes, which normally are difficult to access because of their peculiar habits. Moreover, these rescued animals might serve as sentinels for pathogen surveillance in the rest of the population (Parsons et al. 2017).

Infection with *Haemoproteus* spp. is usually considered asymptomatic (Quillfeldt et al. 2014; Sallaberry-Pincheira et al. 2015; Parsons et al. 2017). However, the presence of other concomitant diseases, stress, or both may affect hemoparasite infections, resulting in increased parasitemia and associated clinical signs (Quillfeldt et al. 2010). Indeed, a recent report informed the death of a *P. puffinus* specimen, due to acute and lethal avian malaria, which was probably related to the stress of a long period in rehabilitation (Vanstreels et al. 2020). Likewise, there are reports of deaths in captive birds associated with *Haemoproteus* spp. infection (Cannell et al. 2013; Yoshimoto et al. 2021). Therefore, a haemosporidian infection, despite the usual absence of clinical signs, cannot be ruled out as a potential cause of reduced animal fitness, whether in captivity or in the wild.

The Black-browed Albatross positive for *Haemoproteus* sp. lineage THAMEL01 highlights the importance of how much valuable information may be generated from the carcasses of rare seabirds, as in the case of many procellariiforms, found on the beaches. Often only fresh carcasses are sampled for laboratory tests. It is necessary to reinforce to the monitoring teams the relevance of acquiring carcasses of rare seabirds found along the coast, even if not fresh, and performing at least a basic necropsy and organ collection.

The negative result for *Plasmodium* spp. and *Leucocytozoon* spp. raises some interesting speculations. Despite the recent expansion of mosquitos on breeding islands of procellariiforms, possibly because of climate changes (Uhart et al. 2018; Dias et al. 2019), or the introduction of it by human actions (Bataille et al. 2009), the occurrence of these haemoparasites still seems rare in these seabirds. Two other studies investigating *Plasmodium* spp. parasites in breeding islands of three procellariiform species (Scopoli's Shearwater, *Calonectris diomedea;* Cory's Shearwater, *Calonectris borealis*; and European Storm-petrel, *Hydrobates pelagicus*) likewise obtained negative results (Campioni et al. 2018; Ilahiane et al. 2022). There appear to have been only five published records of *Plasmodium* spp. infection in this order of seabirds before 2020 (Warner 1968; Quillfeldt et al. 2010; Inumaru et al. 2017; Parsons et al. 2017; Vanstreels et al. 2020). The negative results for *Leucocytozoon* spp. are consistent with the lack of occurrence on Procellariiformes. Other studies have pointed out that this genus may be presented at low prevalence in the area under the influence of the South Atlantic Ocean (Lotta et al. 2019; Morel et al. 2021), which is an important part of the migratory route of various species of Procellariiformes.

Considering all the literature above and our results, the haemosporidian infection in procellariiform seabirds seems to be an issue more associated with exposure outside their

natural habitat (i.e., after they beached) than *in situ* exposure in their breeding sites. This highlights the need for continuous surveillance of those animals to verify any potential new parasite-host relationships, in order to enable early identification of pathogen spillover, which may permit implementation of mitigation measures to reduce pathogen spread among different bird populations and the occurrence of novel epidemics.

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SUPPLEMENTARY MATERIAL

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