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Epidemiology and molecular phylogeny of *Babesia* sp. in Little Penguins *Eudyptula minor* in Australia

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ABSTRACT

Blood parasites are potential threats to the health of penguins and to their conservation and management. Little penguins *Eudyptula minor* are native to Australia and New Zealand, and are susceptible to piroplasmids (*Babesia*), hemosporidians (*Haemoproteus, Leucocytozoon, Plasmodium*) and kinetoplastids (*Trypanosoma*). We studied a total of 263 wild little penguins at 20 sites along the Australian southeastern coast, in addition to 16 captive-bred little penguins. *Babesia* sp. was identified in seven wild little penguins, with positive individuals recorded in New South Wales, Victoria and Tasmania. True prevalence was estimated between 3.4% and 4.5%. Only round forms of the parasite were observed, and gene sequencing confirmed the identity of the parasite and demonstrated it is closely related to *Babesia poelea* from boobies (*Sula* spp.) and *B. uriae* from murres (*Uria aalge*). None of the *Babesia*-positive penguins presented signs of disease, confirming earlier suggestions that chronic infections by these parasites are not substantially problematic to otherwise healthy little penguins. We searched also for kinetoplastids, and despite targeted sampling of little penguins near the location where *Trypanosoma eudyptulae* was originally reported, this parasite was not detected.

1. Introduction

Little Penguins *Eudyptula minor* are the smallest extant penguins and breed from Fremantle in Western Australia across the southern Australian coastline to central New South Wales (NSW), in Tasmania, and in New Zealand (including the Chatham Islands) (Marchant and Higgins, 1990). Although the species has been considered to be of "Least Concern" in recent conservation status assessments (e.g. Birdlife International, 2012), significant decreases have occurred in several breeding colonies in Australia in recent decades (Bool et al., 2007; Stevenson and Woehler, 2007). The reasons for these decreases are numerous, and while the role of disease *per se* has not been investigated or implicated to date, disease could potentially contribute to population decreases now or in the future.

Blood parasites are potential threats to the health of penguins and therefore to their conservation and management (Jones and Shellam, 1999; Levin et al., 2009). Known penguin blood parasites comprise *Babesia peircei* (Earlé et al., 1993), *Borrelia* sp. (Yabsley et al.,

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2011), *Haemoproteus* sp. (Levin et al., 2009), *Leucocytozoon tawaki* (Fallis et al., 1976), *Plasmodium* spp. (Fantham and Porter, 1944), *Trypanosoma eudyptulae* (Jones and Woehler, 1989), and nematode microfilariae (Merkel et al., 2007).

There are relatively few reports of blood parasites in little penguins in the wild or in captivity. *Plasmodium relictum* has been reported to cause mortality in captive little penguins in North America (Griner and Sheridan, 1967) and North Island, New Zealand (NZ) (Varney and Gibson, 2006; Harvey and Alley, 2008), and has also been detected in wild little penguins at South Island, NZ (van Rensburg, 2010). Antibodies against *Plasmodium* sp. or antigenically similar organisms were also demonstrated in wild little penguins at Codfish Island, NZ (Graczyk et al., 1995a), and in captive little penguins at South Island, NZ (Graczyk et al., 1995b). The original and only report of *Trypanosoma eudyptulae* was made by Jones and Woehler (1989), who described this parasite based on blood smears collected from wild little penguins at Marion Bay, Tasmania. *Babesia* sp. has been reported to infect little penguins in NSW (Cunningham et al., 1993), and it has been assumed to be the same species that infects African penguins (*Spheniscus demersus*), *B. peircei* (Peirce, 2000). Cannell et al. (2013) identified DNA from *Haemoproteus* sp. in deceased wild little penguins at Penguin Island, Western Australia; however, because intra-hepatocytic meronts were observed, it is unclear if co-infection with *Leucocytozoon* sp. occurred. Although *L. tawaki* has not yet been detected in wild little penguins, Allison et al. (1978) demonstrated that the infection can develop under experimental conditions of forced exposure to simulid flies in South Island, NZ.

In this study, we conducted a survey for blood parasites in little penguins along the coast of southeastern Australia in NSW, Victoria and Tasmania. Our results provide novel molecular and epidemiological information on *Babesia* sp. in little penguins and contribute with insights into the phylogeny of seabird-infecting *Babesia* spp.

2. Materials and methods

2.1. Sampling procedures

A total of 263 wild little penguins were sampled from October 2012 to March 2013 in 20 study sites in NSW, Victoria and Tasmania (Table 1, Fig. 1). An additional 16 captive-bred little penguin chicks were sampled at Taronga Zoo (Mosman, NSW). Wild penguins were captured in their burrows during the day or were manually caught while in their colonies at night, with the exception of one 2–3 week-old chick found dead at Alum Cliffs, Tasmania (site 16). Further details on sampling effort are provided in Supplementary Data S1.

Blood samples (between 0.05 and 3 mL) were collected through venepuncture (25 × 0.7 mm needle, 3 mL syringe) of the dorsal metatarsal vein or right jugular vein. For one deceased penguin chick, blood was collected directly from the heart. For the 11 penguins at Haunted Bay (site 14), we also collected additional blood samples by pinching the anterior flipper muscle (*M. extensor metacarpi radialis*) with a 25 × 0.7 mm needle, then collecting a blood drop with a heparinised capillary tube. Sampling procedures were approved by the relevant Animal Research Ethics Committees (New South Wales 021028/02, Phillip Island Nature Park 32011, University of Tasmania A12394, University of São Paulo 2790/12) and authorities (New South Wales SL100668, Victoria 10005200, 10006148, Tasmania FA12284).

2.2. Morphological analysis of blood parasites

Two thin blood smears were freshly prepared from each sample, air-dried and then fixed with absolute methanol within 6 hours. One slide was stained with Giemsa and another with Wright-Rosenfeld (Rosenfeld, 1947). One slide (preferably Giemsa-stained) from each individual was examined for intracellular and extracellular blood parasites in 200 fields under 1000× magnification (approx. 30 minutes per slide; field of view area = 0.126 mm^2) by an experienced observer (R.E.T. Vanstreels). Based on a sample of 100 randomly selected microscope fields (obtained from 10 different individuals, 10 fields each), we found that each field contained an average 208 ± 44 erythrocytes; we therefore examined approximately 40,000 erythrocytes per individual. Additionally, blood smears from penguins sampled at Haunted Bay (site 14) were further examined under 500× magnification for 20–30 min to increase the probability of detecting *Trypanosoma* sp.

Table 1
Details of the study sites and sample sizes. Superscript numbers within brackets correspond to the number of individuals with *Babesia*-positive blood smears.

Study sites	Geographic coordinates	N
New South Wales		
1 - Cabbage Tree Island (Shoal Bay)	32°41′17.37″ S 152°13′30.67″ E	10[2]
2 - Manly Point (Sydney)	33°48′32.88″ S 151°16′57.76″ E	7
3 - Big Island, Five Islands (Port Kembla)	34°29′24.81″ S 150°55′38.04″ E	10
4 - Brush Island (Bawley Point)	35°31′39.66″ S 150°24′54.80″ E	10
5 - "Northern Islet", Tollgate Islands (Batemans Bay)	35°44′53.54″ S 150°15′37.93″ E	10
6 - Montague Island (Narooma)	36°15′02.20″ S 150°13′35.60″ E	20
Victoria		
7 - St. Kilda (Melbourne)	37°52′01.82″ S 144°58′23.39″ E	16
8 - "Summerland Estate" (Phillip Island)	38°30′38.70″ S 145°08′31.74″ E	12[1]
9 - "Summerland Southwest" (Phillip Island)	38°30′58.62″ S 145°07′44.04″ E	27
Tasmania		
10 - "Doctor's Rocks West" (Wynyard)	40°59′50.76″ S 145°46′05.58″ E	18
11 - Lillico Beach (Devonport)	41°09′36.00″ S 146°18′02.28″ E	22
12 - "Darlington Foreshore" (Maria Island)	42°34′41.46″ S 148°03′56.16″ E	7[2]
13 - Fossil Cliffs (Maria Island)	42°34′21.60″ S 148°04′45.48″ E	22
14 - Haunted Bay (Maria Island)	42°43′07.14″ S 148°04′08.40″ E	11
15 - Red Chapel Beach (Hobart)	42°54′29.58″ S 147°20′44.70″ E	4
16 - Alum Cliffs (Taroona)	42°57′35.04″ S 147°20′31.14″ E	5
17 - Lucas Point (Tinderbox)	43°02′09.90″ S 147°20′18.24″ E	2
18 - Stinking Bay (Tasman Peninsula)	43°07′30.66″ S 147°52′43.74″ E	10
19 - Maignon Bay (Tasman Peninsula)	43°11′57.25″ S 147°51′23.34″ E	13
20 - The Neck (Bruny Island)	43°16′12.66″ S 147°20′54.30″ E	27[2]
***Ex-situ* (New South Wales)**		
21 - Taronga Zoo (Mosman)	33°50′34.88″ S 151°14′30.89″ E	16

2.3. PCR testing and gene sequencing

After blood smears were freshly prepared, the remaining volume of the blood samples collected in Tasmania and Taronga Zoo was transferred to cryotubes and frozen (−20 °C). Frozen blood samples from a few selected individuals were used for PCR testing and gene sequencing. DNA extraction was conducted using the DNEasy Blood and Tissue Kit (69506, Qiagen – Valencia, USA) and was verified and