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

Ralph Eric Thijl Vanstreels [a,*], Eric J. Woehler [b], Valeria Ruoppolo [a,c], Peter Vertigan [d], Nicholas Carlile [e], David Priddel [e], Annett Finger [f], Peter Dann [g], Kimberly Vinette Herrin [h], Paul Thompson [h], Francisco C. Ferreira Junior [i], Érika M. Braga [i], Renata Hurtado [j], Sabrina Epiphanio [k], José Luiz Catão-Dias [a]

[a] *Laboratory of Wildlife Comparative Pathology (LAPCOM), Department of Pathology, School of Veterinary Medicine and Animal Science, University of São Paulo, Brazil*
[b] *Institute for Marine and Antarctic Studies (IMAS), University of Tasmania, Australia*
[c] *International Fund for Animal Welfare, USA*
[d] *Birdlife Tasmania, Australia*
[e] *New South Wales Office of Environment and Heritage, Australia*
[f] *Ecology and Environmental Management Group, College of Engineering and Science, Victoria University, Australia*
[g] *Research Department, Phillip Island Nature Parks, Australia*
[h] *Taronga Conservation Society Australia, Mosman, Australia*
[i] *Department of Parasitology, Institute of Biological Sciences, Federal University of Minas Gerais, Brazil*
[j] *Department of Preventive Veterinary Medicine and Animal Health, School of Veterinary Medicine and Animal Science, University of São Paulo, Brazil*
[k] *Department of Clinical and Toxicological Analyses, Faculty of Pharmaceutical Sciences, University of São Paulo, Brazil*

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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.,

* Corresponding author. Departamento de Patologia, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo. Av. Prof. Orlando Marques de Paiva 87, Cidade Universitária, São Paulo, SP, 05508-000, Brazil. Tel.: +55 11 99917 3082; fax: +55 11 3091 1434.
E-mail address: ralph_vanstreels@yahoo.com.br (R.E.T. Vanstreels).

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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

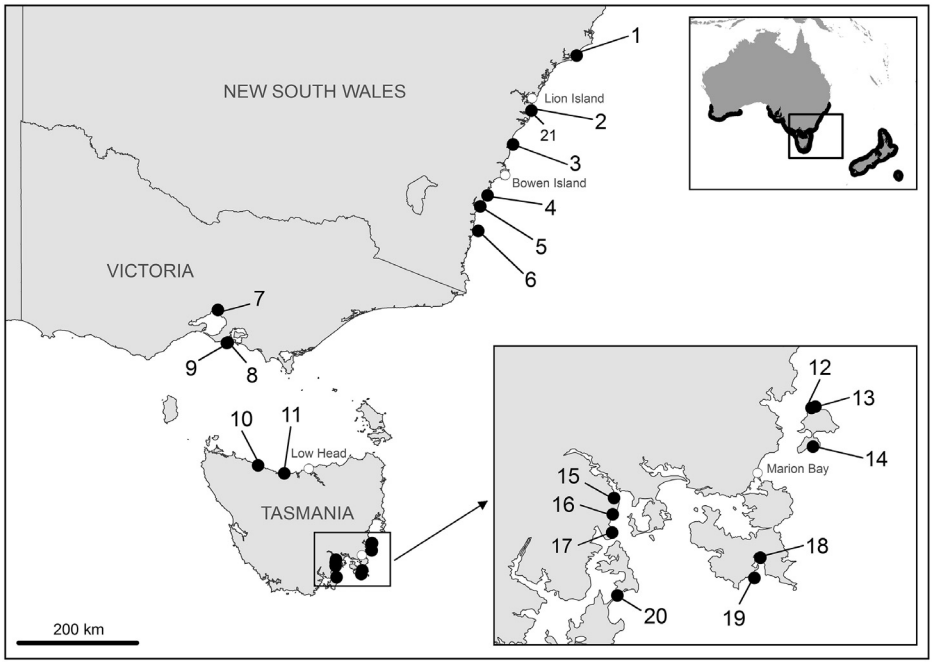

Fig. 1. Geographic distribution of sampling locations, southeast Australia. Site details are given in Table 1. The geographic distribution of little penguins (black area) is shown in the top right map (adapted from Marchant and Higgins, 1990).

quantified through UV spectrophotometry (Nanodrop 2000, Thermo Fisher Scientific – Waltham, USA).

To test for *Haemoproteus* and *Plasmodium,* we used a nested PCR targeting the cytochrome b mitochondrial gene (Hellgren et al., 2004). The first reaction used 75 ng of sample DNA, 12 µmol of each primer (HaemNFI and HaemNR3), and the following temperature profile: 94 °C for 3 min, 20 cycles (94 °C for 30 sec, 50 °C for 50 sec, 72 °C for 45 sec), 72 °C for 10 min. The second reaction used 1 µL of the first reaction product, 12 µmol of each primer (HaemF and HaemR2), and the following temperature profile: 94 °C for 3 min, 35 cycles (94 °C for 30 sec, 50 °C for 50 sec, 72 °C for 45 sec), 72 °C for 10 min.

To test for *Babesia*, we used two nested PCR targeting the 18S rRNA gene (Medlin et al., 1988; Gubbels et al., 1999; Yabsley et al., 2006). The first reaction used 5 µL of sample DNA, 20 µmol of each primer (Bab5.1 and BabB), and the following temperature profile: 94 °C for 1 min, 30 cycles (94 °C for 1 min, 48 °C for 1 min, 72 °C for 2 min), 72 °C for 5 min. The second reaction used 1 µL of the first reaction product, 20 µmol of each primer (Bab5.1v2 and Bab3.1), and the following temperature profile: 94 °C for 1 min, 30 cycles (94 °C for 1 min, 52 °C for 1 min, 72 °C for 2 min), 72 °C for 5 min. The alternative second reaction used 1 µL of the first reaction product, 20 µmol of each primer (BabRLBF and BabRLBR), and the following temperature profile: 94 °C for 1 min, 30 cycles (94 °C for 1 min, 50 °C for 1 min, 72 °C for 2 min), 72 °C for 5 min.

Table 2 provides the sequences of the primers employed. All reactions were conducted with 12.5 µL GoTaq Green Master Mix (M7122, Promega – Madison, USA) and a total well volume of 25 µL. The following samples were used as controls: blood from a chicken experimentally infected with *Plasmodium gallinaceum,* blood from a tropical screech owl (*Megascops choliba*) naturally infected with *Haemoproteus syrnii*, blood from a brown booby (*Sula leucogaster*) naturally infected with *B. poelea*, and blood from chicken raised in arthropod-free environments. Gel electrophoresis was conducted to visualise amplification products, using 1% agarose gel, SYBR Safe (Invitrogen S33102, Life Technologies – Carlsbad, USA), and a high-resolution imaging system (Gel Doc EZ System 170–8270, Bio-Rad – Hercules, USA).

Table 2
Sequence of the primers employed. "I" stands for inosine, a universal base.

Primer name	Sequence (5′–3′)
HaemNFI	CATATATTAAGAGAAITATGGAG
HaemNR3	ATAGAAAGATAAGAAATACCATTC
HaemF	ATGGTGCTTTCGATATATGCATG
HaemR2	GCATTATCTGGATGTGATAATGGT
Bab5.1	CCTGGTTGATCCTGCCAGTAGT
BabB	CCCGGGATCCAAGCTTGATCCTTCTGCAGGTTCACCTAC
Bab5.1v2	CATATGCTTGTCTTAAA
Bab3.1	CTCCTTCCTTTAAGTGATAAG
BabRLBF	GTAGTGACAAGAAATAACAATA
BabRLBR	TCTTCGATCCCCTAACTTTC

PCR amplification products of positive samples were purified with Polyethylene Glycol 8000. Bi-directional sequencing with dye-terminator fluorescent labelling (Applied Biosystems 4337455, Life Technologies – Carlsbad, USA) was performed using primers Bab5.1v2 and Bab3.1 and an automated sequencer (Applied Biosystems ABI Prism 3100, Life Technologies – Carlsbad, USA). Forward and reversed chromatograms were edited and consensus sequences were deposited in GenBank (KP144322 and KP144323).

2.4. Phylogenetic analysis

Phylogenetic relationships of the *Babesia* lineages identified in this study were inferred using published sequences for which species was identified based on morphological evidence (Criado et al., 2006; Lack et al., 2012), in addition to avian-infecting *Babesia* lineages from published studies (Criado et al., 2006; Jefferies et al., 2008; Yabsley et al., 2009; Quillfeldt et al., 2013; Martínez et al., 2014). Sequences were aligned using ClustalW (Thompson et al., 1997) as implemented in MEGA 5.2.2 (Tamura et al., 2011). A maximum likelihood phylogenetic tree for the parasite sequences was produced using MEGA 5.2.2 with the GTR + Gamma model of nucleotide evolution, with 1000 bootstrap replications.

2.5. True prevalence estimate

True prevalence of blood parasites was estimated from blood smear examination using an adapted Rosan and Gladen procedure (Reiczigel et al., 2010). Test sensitivity (Se) of blood smear examination for *Plasmodium* sp. is estimated between 68% and 81% (Richard et al., 2002; Valkiūnas et al., 2008), and we therefore used values of 60% (worst-case) and 80% (best-case) to estimate true prevalence. Test specificity was fixed at 100% to produce the most conservative estimates, and confidence level was fixed at 95%.

3. Results

Round intracytoplasmatic parasites were observed in the erythrocytes of seven wild little penguin blood smears (Fig. 2). These parasites were most compatible with round forms of piroplasmids (*Babesia* sp.), but early stages of haemosporidians (*Haemoproteus* sp., *Plasmodium* sp., *Leucocytozoon* sp.) could not be discarded on the basis of morphology. No other parasite forms were observed in any of the blood smears, and no blood parasites were detected in the blood smears of the captive-bred penguins sampled in this study.

Molecular testing was applied to two blood smear-positive samples and demonstrated that both were positive in the nested PCR targeting the 18S rRNA gene of piroplasmids and were negative to mitochondrial cytochrome b gene of haemosporidians (see Fig. 3). The identity of the parasite was conclusively established through the sequencing of the 18S rRNA gene amplicons, which demonstrated high phylogenetic similarity with published sequences from *Babesia* spp., particularly with seabird-infecting lineages (Fig. 4, Table 3).

Additionally, it should be noted that the *Haemoproteus*-positive control also yielded amplification products for the nested PCR targeting the 18S rRNA gene; these products were only slightly lighter than those for *Babesia*-positive samples (lane "d" in Fig. 3). Sequencing of these amplicons, however, revealed high identity (>98%) with published 18S rRNA sequences from Strigiformes (owls – data not shown), indicating that this was a false positive result due to unintentional amplification of host DNA.

Apparent prevalence based on blood smears was 2.7% (7/263). True prevalence is estimated to have been between 3.4% (best-case diagnostic performance) and 4.5% (worst-case diagnostic performance). Table 4 provides estimates for each study site and state separately.

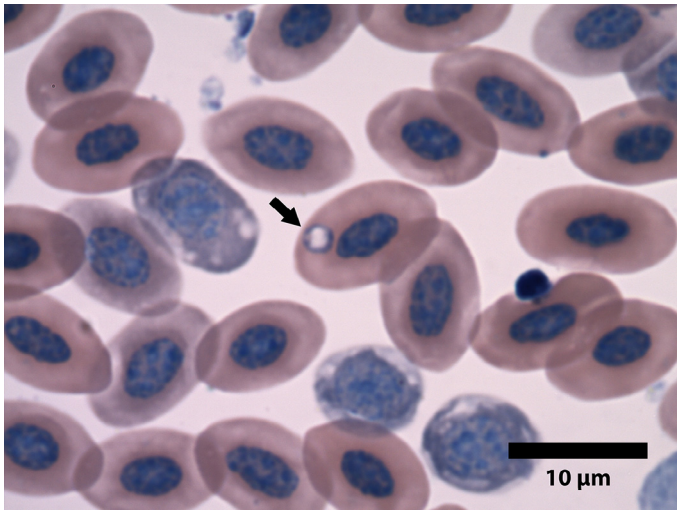

Fig. 2. *Babesia* sp. in the blood smear of a little penguin. Individual details: TAS-124, male, adult, moulting, sampled at "Darlington Foreshore" (Maria Island, Tasmania) in 21/02/2013, Genbank ascension number KP144323, Giemsa stain.

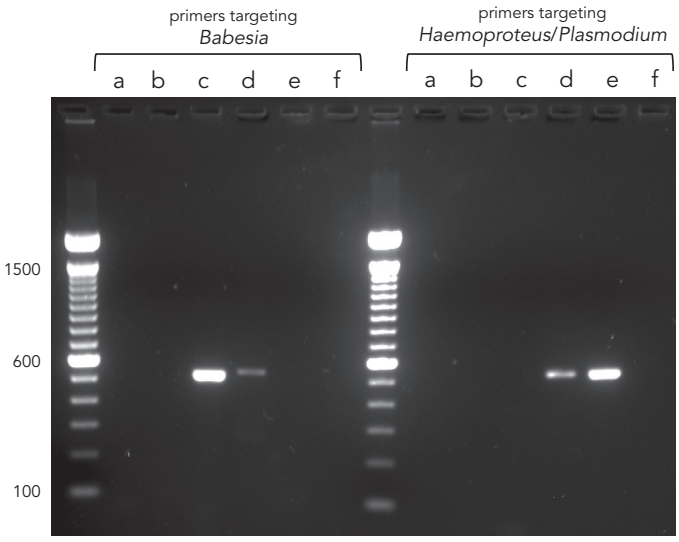

Fig. 3. Agarose gel electrophoresis of amplification products obtained through nested PCR tests targeting the 18S rRNA gene of *Babesia* (primers Bab5.1/BabB followed by RLBF/RLBR) or the mitochondrial cytochrome b gene of *Haemoproteus*/*Plasmodium* (primers HaemNFI/HaemNR3 followed by HaemF/HaemR2). The following samples are represented: (a) captive-born little penguin chick, negative blood smear; (b) adult wild little penguin, negative blood smear; (c) *Babesia*-infected adult wild little penguin, as confirmed through blood smear; (d) *Haemoproteus*-infected adult tropical screech owl, as confirmed through blood smear; (e) *Plasmodium*-inoculated chicken, raised in arthropod-free environment; (f) blood parasite-free chicken, raised in arthropod-free environment.

Table 3
Estimates of evolutionary distance (% expected base substitutions per site) of 18S rRNA gene sequences of avian-infecting *Babesia* spp.

	Morphospecies (Genbank number) Host	2	3	4	5	6	7	8
1	*Babesia kiwiensis* (EF551335) *Apteryx mantelli*	1.71	9.13	9.13	9.15	8.28	8.89	8.89
2	*Babesia* sp. (JX984667) *Turdus falklandii*		8.33	8.33	8.35	8.03	8.22	8.22
3	*Babesia poelea* (DQ200887) *Sula leucogaster*			0.00	0.42	8.54	0.42	0.42
4	*Babesia* sp. (KC754965) *Sula leucogaster*				0.42	8.54	0.42	0.42
5	*Babesia uriae* (FJ717705) *Uria aalge*					8.76	0.84	0.84
6	*Babesia bennetti* (DQ402155) *Larus cachinnans*						8.42	8.42
7	*Babesia* sp. (KP144322) *Eudyptula minor*							0.00
8	*Babesia* sp. (KP144323) *Eudyptula minor*							

We did not maintain records on the presence of ectoparasites or haematophagous insects in NSW or Victoria; however, in Tasmania we observed soft ticks (Argasidae), hard ticks (Ixodidae), fleas (Siphonaptera), lice (*Austrogoniodes* sp.), mosquitoes (Culicidae) and black flies (Simuliidae) (R.E.T. Vanstreels, pers. obs.).

4. Discussion

Five species of *Babesia* are known to infect seabirds: *B. bennetti* (host: Caspian gull *Larus cachinnans*) (Criado et al., 2006), *B. peircei*

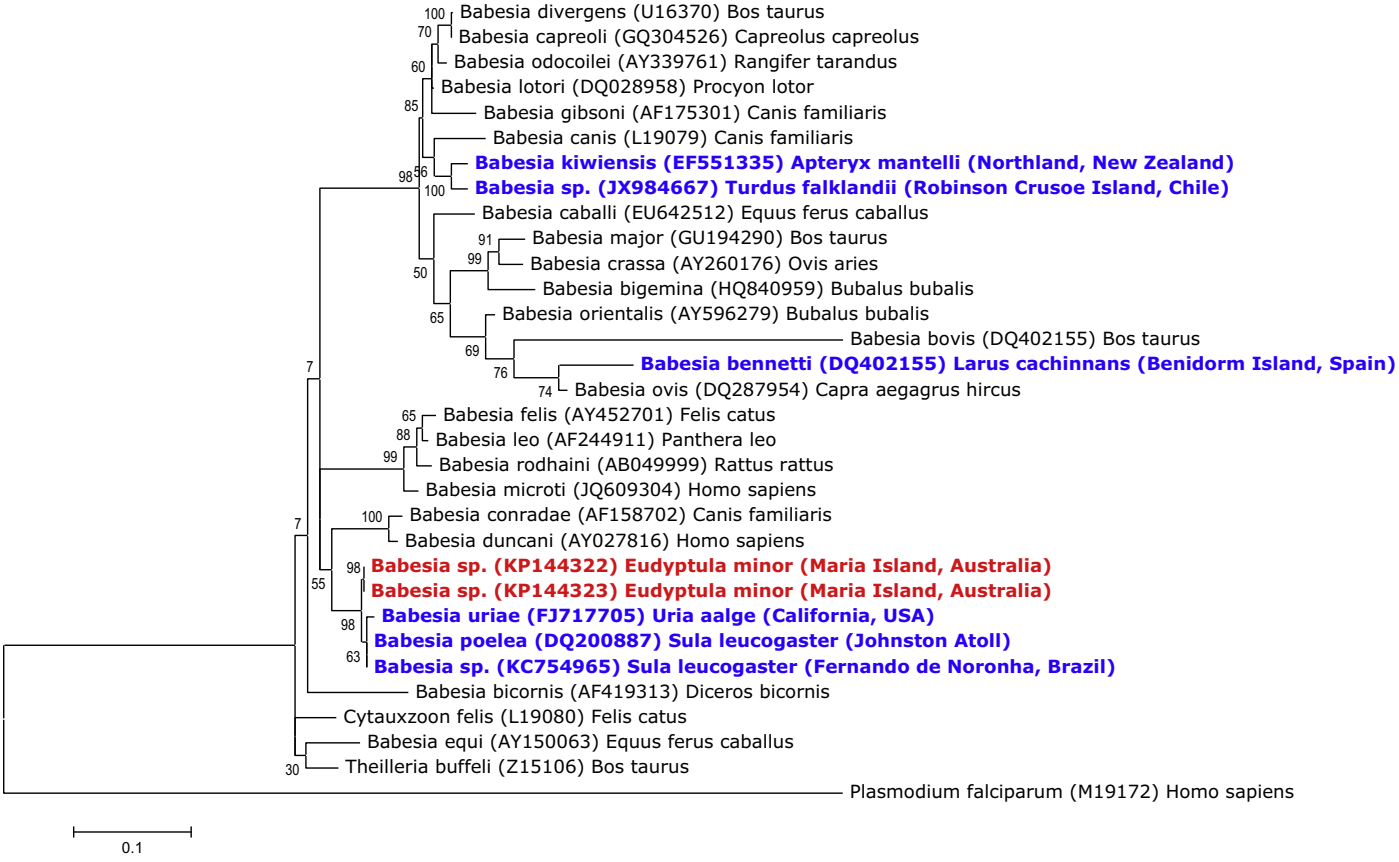

Fig. 4. Maximum likelihood phylogenetic tree of the 18S rRNA gene of the studied *Babesia* lineages. Lineages identified in this study are emphasized in red, and other avian-infecting lineages are emphasized in blue. For each lineage, the following information is provided: morphospecies (Genbank ascension number) host species. For avian-infecting lineages, the geographic location is also provided. Branch lengths are drawn proportionally to evolutionary distance (scale bar is shown). For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.

(African penguin) (Earlé et al., 1993), *B. poelea* (boobies *Sula* spp.) (Work and Rameyer, 1997), *B. uriae* (common murre *Uria aalge*) (Yabsley et al., 2009), and *B. ugwidiensis* (cormorants *Phalacrocorax* spp.) (Peirce and Parsons, 2012). *B. peircei* and *B. poelea* share remarkable morphological similarity, both with the distinctive feature of concentrating the chromatin in the distal half of the merozoites, which led Peirce (2000) to suggest that these taxa may in fact be synonymous. The later-described *B. uriae* (Yabsley et al., 2009) also shares this morphological characteristic.

In this study, the lack of schizonts in blood smears has precluded morphospecies identification, as the round forms we observed are common to all avian-infecting *Babesia* spp. (Peirce, 2000). This has been a recurring characteristic of *Babesia* infections in little penguins (Cunningham et al., 1993; van Rensburg, 2010), and has made it impossible to determine whether little penguins are infected by *B. peircei* or by a distinct and presumably novel species. Unfortunately, there are no 18S rRNA gene sequences from *B. peircei* to which ours could be compared. Future studies will hopefully allow for this comparison, but it is our opinion that the parasite found in little penguins in this study is most likely *B. peircei*, as proposed by Peirce (2000).

Table 4
True prevalence estimates under different scenarios of test sensitivity.

	Apparent prevalence	Estimated true prevalence	
		Best-case (SE = 80%)	Worst-case (SE = 60%)
New South Wales	3.0% (2 / 67)	3.8%	5.0%
Victoria	1.8% (1 / 55)	2.3%	3.0%
Tasmania	2.8% (4 / 141)	3.5%	4.7%
Total	2.7% (7 / 263)	3.4%	4.5%

Our phylogenetic analysis indicates that *B. poelea*, *B. uriae* and *Babesia* sp. from little penguins form a neat phylogenetic cluster, with high bootstrap value (98) and low evolutionary distance (<0.84% expected base substitutions per site). It is unclear, however, whether (a) *B. poelea*, *B. peircei* and *B. uriae* are distinct species and their morphological and genetic similarities do not reflect the true reproductive isolation that exists, or (b) the three parasites correspond to a single species that can be transmitted among different taxonomic orders of seabirds.

The hypothesis that *B. poelea*, *B. peircei* and *B. uriae* are distinct species is strengthened by the fact that most *Babesia* spp. are host-specific at the family or subfamily level (Peirce, 2000). Additionally, Yabsley et al. (2009) argue that the relatively small genetic difference between *B. uriae* and *B. poelea* is sufficient to indicate a reproductive isolation between these parasites. Alternatively, the hypothesis that *B. poelea* and *B. uriae* are in fact synonymous to *B. peircei* is made plausible by the fact that opportunities for cross-infection of *Babesia* among different taxonomic orders of seabirds are perhaps more common than initially assumed. There are numerous locations in the world where sympatric species nest in close proximity, and can be parasitised by the same species of Ixodidae and Argasidae ticks (Dietrich et al., 2011). Thus, it is reasonable that these ticks could be vectors for the transmission of *Babesia* among different species of seabirds, as has been shown to occur for spirochaetes (Olsén et al., 1995). In this case, the subtle morphological differences observed among *B. poelea*, *B. peircei* and *B. uriae*

could reflect host-specific variations, as has been demonstrated in other avian blood parasites (e.g. Laird and Van Riper, 1981).

Regardless, it is clear that these parasites share a close phylogenetic relationship and numerous phenotypic characteristics. In contrast, the phylogenetic relationship between *B. ugwidiensis* and other avian-infecting *Babesia* remains unknown. The hosts of *B. ugwidiensis* and *B. peircei* are sympatric in South Africa and share ectoparasites (Dietrich et al., 2011); however, the morphology of these parasites is clearly distinct (Peirce and Parsons, 2012). On the other hand, *B. bennetti* belongs to a phylogenetic group that is clearly distinct from that of *B. poelea*, *B. uriae* and *Babesia* sp. from little penguins, being more closely related to the *Babesia* spp. identified in domestic mammals (see Fig. 4).

Cunningham et al. (1993) were first to document the occurrence of *Babesia*-like parasites in little penguins at Bowen (apparent prevalence = 2/126 = 1.6%) and Lion islands (8/168 = 4.8%) (NSW, Australia). The protozoan nature of these parasites was demonstrated through electron microscopy, but because only round forms were observed, the identity of the parasites could not be conclusively demonstrated. Likewise, van Rensburg (2010) observed round intracytoplasmatic inclusions compatible with *Babesia* sp. in the blood smears of little penguins at Tiritiri Matangi Island (9/79 = 11.4%) (Hauraki Gulf, NZ), but also could not conclusively identify the parasite. Other studies examining blood smears or histopathology of wild little penguins did not find evidence of *Babesia* sp. infection in Western Australia (Jones and Shellam, 1999; Cannell et al., 2013), Victoria (Obendorf and McColl, 1980; Mortimer and Lill, 2007), Tasmania (Jones and Woehler, 1989; Jones and Shellam, 1999), NSW (Mykytowycz and Hesterman, 1957) or New Zealand (Laird, 1950; Crockett and Kearns, 1975; Allison et al., 1978).

In this study, we confirm the occurrence of *Babesia* sp. in wild little penguins in NSW, and extend the known distribution of these parasites to Victoria and Tasmania. The parasite was identified in 2.7% of the blood smears examined from wild little penguins; however it must be considered that blood smear examination is, as any other diagnostic test, imperfect (Garamszegi, 2011). Considering the information available on the performance of blood smear examination for the detection of other avian blood parasites (Richard et al., 2002; Valkiūnas et al., 2008), we estimate that true prevalence was between 3.4% and 4.5%. We suggest that if each Australian state is examined separately for *Babesia* in little penguins, the true prevalence is estimated between 2.3% and 5.0% (Table 4), based on current evidence.

Blood smear examination is considered to be the standard method to detect blood parasites due to its high specificity, its value for morphological characterisation and its ability to detect mixed infections while requiring no *a priori* decisions on the parasitic species for which searches are to be conducted. Alternatively, PCR has been shown to provide a higher sensitivity, particularly for chronic infections (Richard et al., 2002; Valkiūnas et al., 2008). Our results demonstrate that the nested PCR targeting the 18S rRNA gene is adequate for the detection of *Babesia* sp. in little penguins, and may therefore become a viable tool for future studies on the epidemiology of penguin-infecting *Babesia*. However, because we observed that the avian host's DNA may occasionally be co-amplified and lead to false positive results, it is imperative that positive results obtained by this method are confirmed through sequencing of the amplicons.

The clinical relevance of *Babesia* in penguins is not clear. Brossy et al. (1999) considered that *B. peircei*, "does not cause overt clinical symptoms except under stress or in association with other debilitating diseases" in African penguins. Cunningham et al. (1993) did not find evident signs of illness in *Babesia*-positive little penguins, except for mild regenerative anaemia. Similarly, co-infection by *B. peircei* was observed in 50% of the African penguins infected with Relapsing Fever *Borrelia* at a rehabilitation centre in South Africa, and yet these co-infections are not accompanied by significant signs of disease (Yabsley et al., 2011). None of the *Babesia*-positive penguins in this study presented any obvious sign of disease, supporting earlier proposals that this is not a substantially pathogenic parasite to otherwise healthy little penguins. It is worth considering, however, that the fact that we only observed round forms with no schizonts in the blood smears (as did Cunningham et al., 1993) suggests these were chronic infections, when asexual multiplication is low and most circulating life stages are latent progametocytes; it is possible that the acute stage of the infection could result in more prominent health implications for the host.

The little penguin colony at Marion Bay from which Jones and Woehler (1989) described *T. eudyptulae* was destroyed during a fire in 1994, and the site has not been recolonised since (Stevenson and Woehler, 2007; E.J. Woehler, unpubl. data). We intentionally directed considerable sampling effort on the extant breeding colonies close to Marion Bay (see Fig. 1), but did not detect *T. eudyptulae* in these colonies in this study. This is surprising considering the relatively high prevalence (17.3%) with which the parasite was found in that study, and could indicate that: (a) the parasite eluded detection in this study, (b) the parasite occurred in lower prevalence at the locations and time at which we conducted our sampling compared with the 1989 study, and/or (c) the parasite disappeared along with its host population at Marion Bay (which we consider highly unlikely).

Jones and Woehler (1989) obtained blood samples by superficially scraping the skin near the brachial vein on the flipper with razorblades (E.J. Woehler, pers. obs.), therefore obtaining capillary blood. In this study, we used venipuncture of large vessels (jugular or metatarsal), obtaining peripheral blood. This difference in sampling may be relevant as it has been shown that mammal-infecting trypanosomes tend to concentrate in capillaries rather than larger blood vessels (Hornby and Bailey, 1931; Banks, 1978). It is presently unclear whether avian-infecting trypanosomes behave similarly (Holmstad et al., 2003). We produced smears from capillary blood samples obtained by pinching the flipper muscle from little penguins at Haunted Bay. We found no parasites in those smears; however, the sample size may have been too small (n = 11); we therefore encourage future studies to employ blood sampling methods that yield capillary blood. Furthermore, because the *Trypanosoma* parasitemia is often very low, the development of PCR tests that allow for the detection of *T. eudyptulae* could be of great benefit to future studies on the epidemiology of this parasite.

It is possible that little penguin colonies permanently or intermittently fail to provide adequate environmental conditions suitable for the proliferation of arthropod vectors and/or the presence of other avian species that could act as reservoirs of infection. Factors such as interannual prevalence fluctuations, age (Merino et al., 1996), time of the day (Cornford et al., 1976) and seasonal latency (Valkiūnas et al., 2004) may affect both the occurrence of *Trypanosoma* spp. or the probability of their detection. Arthropod vectors of *T. eudyptulae* remain unknown but could include mites (Acari), mosquitoes (Culicidae) and blood-sucking flies (Hippoboscidae, Simuliidae) (Molyneux, 1977).

In conclusion, it is unclear whether *Babesia* sp. poses a significant concern to the conservation of little penguins. Future studies investigating the health effects of this parasite and its epidemiological dynamics would help in understanding this parasite. The question still remains on whether this parasite corresponds to *Babesia peircei* from African penguins or if it is a distinct species, and future studies in the African species may assist in clarifying this. The failure to detect *T. eudyptulae* in southeastern Tasmania is puzzling, and for the moment this continues to be the most enigmatic blood parasite of penguins.

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Conflict of interest

The authors declared that there is no conflict of interest.

Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.ijppaw.2015.03.002.

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