Title: Australian penguin ticks screened for novel *Borrelia* species

Authors: Katherine L. Moon, Steven L. Chown, Siew-May Loh, Charlotte L. Oskam, Ceridwen I. Fraser

PII: S1877-959X(17)30387-4
DOI: https://doi.org/10.1016/j.ttbdis.2017.12.007
Reference: TTBDIS 923

To appear in:

Received date: 16-8-2017
Revised date: 7-12-2017
Accepted date: 8-12-2017

Please cite this article as: Moon, Katherine L., Chown, Steven L., Loh, Siew-May, Oskam, Charlotte L., Fraser, Ceridwen I., Australian penguin ticks screened for novel Borrelia species.Ticks and Tick-borne Diseases https://doi.org/10.1016/j.ttbdis.2017.12.007

Australian penguin ticks screened for novel *Borrelia* species

Katherine L. Moon[1, 2], Steven L. Chown[2], Siew-May Loh[3], Charlotte L. Oskam[3], Ceridwen I. Fraser[1]

1. Fenner School of Environment and Society, Australian National University, Acton, ACT 2601, Australia.

2. School of Biological Sciences, Monash University, Clayton, VIC 3800, Australia

3. Vector & Waterborne Pathogens Research Group, School of Veterinary and Life Sciences, Murdoch University, Murdoch, WA 6150, Australia.

Keywords: Tick-borne disease, *Ixodes*, '*Candidatus* Borrelia tachyglossi', Australia.

Word count: 2672

Corresponding author: Katherine L. Moon

Address: Fenner School of Environment and Society, Australian National University, Canberra, ACT 2601, Australia.

Email: katielouisemoon@gmail.com

Abstract

Lyme borreliosis (or Lyme Disease) is an emerging threat to human health in the Northern Hemisphere caused by tick-borne bacteria from the *Borrelia burgdorferi* sensu lato (Bbsl) complex. Seabirds are important reservoir hosts of some members of the Bbsl complex in the Northern Hemisphere, and some evidence suggests this may be true of penguins in the Southern Hemisphere. While the Bbsl complex has not been detected in Australia, a novel *Borrelia* species ('*Candidatus* Borrelia tachyglossi') was recently sequenced from native ticks (*Ixodes holocyclus* and *Bothriocroton concolor*) parasitising echidnas (*Tachyglossus aculeatus*), suggesting unidentified borreliae may be circulating amongst native wildlife and their ticks. In the present study, we investigated whether ticks parasitising little penguins (*Eudyptula novaehollandiae*) harbour native or introduced *Borrelia* bacteria. We chose this penguin species because it is heavily exploited by ticks during the breeding season, lives in close proximity to other potential reservoir hosts (including native wildlife and migratory seabirds), and is known to be infected with other tick-borne pathogens (*Babesia*). We screened over 230 penguin ticks (*Ixodes* spp.) from colonies in south-eastern Australia, and found no evidence of *Borrelia* DNA. The apparent absence or rarity of the bacterium in south-eastern Australia has important implications for identifying potential tick-borne pathogens in an understudied region.

Introduction

Lyme borreliosis (LB) is a multi-organ inflammatory illness of humans that is the most common and widely distributed vector-borne disease in the temperate regions of the Northern Hemisphere (Middleton et al., 2016). LB is caused by spirochaetes of the *Borrelia burgdorferi* sensu lato (Bbsl) complex transmitted by ticks, predominantly in the genus *Ixodes* (Biesiada et al., 2012; Middleton et al., 2016), and leads to disorders of the skin, joints, heart and neurological system (Biesiada et al., 2012; Hercogová, 2015; Halperin, 2016). Late symptoms can include painful radiculitis, arthritis, carditis, meningitis, encephalitis, palsy (Biesiada et al., 2012; Hercogová, 2015; Halperin, 2016), and possibly progressive dementia and chronic fatigue syndrome (Ballantyne, 2008; Minkoff, 2016), although the last remains a matter of contention (Halperin, 2015; 2016).

An increasing number of people bitten by ticks in Australia are presenting with similar symptoms to those of LB (Chalada et al., 2016). These reports have sparked considerable debate over the causative agent, triggering a Senate Inquiry (Senate Community Affairs Committee Secretariat, 2016) and raising the profile of tick-borne diseases nationwide. Studies to date have failed to detect any members of the Bbsl complex in Australia (Wills and Barry, 1991; Russell et al., 1994) or establish native human-biting ticks, such as *Ixodes holocyclus* (Australian paralysis tick), as competent Bbsl vectors (Piesman and Stone, 1991). The current consensus is that the Bbsl complex is not present in Australia and that Australian Lyme-like illness is probably caused by an unidentified microorganism transmitted by native ticks (Wills and Barry, 1991; Russell et al., 1994; Gofton et al., 2015; Senate Community Affairs Committee Secretariat, 2016).

Natural vertebrate reservoir hosts are integral to maintaining cycles of infection, in that they carry pathogens but are often asymptomatic themselves (Chambert et al., 2012; Voordouw et al., 2015). Hosts that form large, spatially and temporally predictable aggregations (e.g. packs, colonies or herds) and exhibit considerable long-distance movements are of particular epidemiological interest, due to the high potential for pathogen spread. Seabirds are important reservoir hosts for some members of the Bbsl complex, most notably *Borrelia garinii* vectored by the generalist seabird tick *Ixodes uriae* (Olsén et al., 1995; Gylfe et al., 2001; Duneau et al., 2008; Gómez-Díaz et al., 2010; Lobato et al., 2011). Over 60 seabird species are parasitised by this tick (Dietrich et al., 2011), and as most are highly migratory, global transmission of *Borrelia* has occurred, followed by diversification within seabird colonies (Olsén et al., 1995; Gylfe et al., 2000; Gylfe et al., 2001; Gómez-Díaz et al., 2011). *Borrelia* species associated with both LB and relapsing fever (RF) borreliae have now been found in penguins (Gauthier-Clerc et al., 1999; Yabsley et al., 2012; Schramm et al., 2014) suggesting they are competent reservoir hosts of the bacteria in the Southern Hemisphere. Thus far, however, only penguins in the sub-Antarctic and Antarctic regions have been investigated for the presence of *Borrelia* DNA.

In Australia, the roles of native ticks and of wildlife reservoir hosts in the cycling of tick-borne pathogens are well documented. For example, Australian ticks are known to transmit *Coxiella* and *Rickettsia* species that can cause illness in humans (Stenos et al., 2003; Cooper et al., 2013; Graves and Islam, 2016; Oskam et al., 2017). Although research aiming to identify the causative agent(s) of Australian Lyme-like illness remains in its infancy, recent studies have used advanced genetic techniques to screen Australian ticks for tick-borne pathogens (Cooper et al., 2013; Gofton et al., 2015; Graves et al., 2016; Loh et al., 2016; Oskam et al., 2017). To date, four borreliae have been identified in Australia, including two introduced with domestic animals (*Borrelia theileri* and *Borrelia anserina*), and two native species (*Borrelia queenslandica* – though this species remains unconfirmed – and '*Candidatus* Borrelia tachyglossi') (Gofton et al., 2015; Chalada et al., 2016; Loh et al., 2016, 2017). *Borrelia theileri*, *B. anserina* and *B. queenslandica* had been identified by the end of the 1960s and cause borreliosis in animals (in cattle, poultry, and rodents respectively). These species have never been associated with Lyme-like illness in humans, despite an attempt to infect a human volunteer with one of the species (Chalada et al., 2016). '*Candidatus* B. tachyglossi' was only recently sequenced from ticks (*I. holocyclus* and *Bothriocroton concolor*) parasitising echidnas (*Tachyglossus aculeatus*) (Gofton et al., 2015; Loh et al., 2016, 2017). Research has yet to establish whether the echidna is a reservoir host for the bacterium, whether *I. holocyclus* and *B. concolor* are vectors, or whether the bacterium can be transmitted to humans. Although '*Candidatus* B. tachyglossi' is closely related to the RF and reptile-associated (REP) *Borrelia* groups, it forms its own clade within the genus *Borrelia* and has unknown pathogenic consequences (Loh et al., 2017).

Little penguins (*Eudyptula novaehollandiae*) are native to Australia and are heavily parasitised by *Ixodes* ticks (*I. eudyptidis* and *I. kohlsi*) when breeding. The penguins are also known to harbour *Babesia* spp., which is a protozoan parasite that causes piroplasmosis in vertebrates, and is a common co-infection partner of *B. burgdorferi* in North America (Dunn et al., 2014; Diuk-Wasser et al., 2016; Walter et al., 2016). To date, there has only been one human babesiosis fatality due to the tick-borne protozoan, *Babesia microti* (Senanayake et al., 2012), which is genetically distinct from the *Babesia* species described in little penguins. Phillip Island Nature Reserve (Victoria, Australia) represents the

largest colony of little penguins, and is also home to a range of other iconic native Australian animals, including echidnas and koalas (Phillip Island Nature Parks, 2015). At least 10 species of ticks from four genera are known to parasitise echidnas, and five of these tick species also exploit other animals and humans (see Fig. 1). Furthermore, *Bothriocroton* ticks have recently been found in penguin burrows at Phillip Island Nature Park (K.L. Moon pers. obs.), suggesting echidnas and penguins on the island may share parasites and associated pathogens (see Fig. 1). The island is also visited annually by migratory seabirds including short-tailed shearwaters (*Ardenna tenuirostris*), which breed in considerable numbers (Phillip Island Nature Parks, 2014). Despite the potential for the presence of a native *Borrelia* species (due to associations with native Australian wildlife), and the presence of *B. garinii* (due to associations with migratory seabirds), no study has previously investigated whether borreliae are cycling in Australian penguin colonies. We screened over 230 *Ixodes* ticks from penguin hosts at Phillip Island for borreliae, representing the first large-scale assessment of the presence of *Borrelia* spp. DNA in ticks from south-eastern Australia.

Method

Sample collection

A total of 232 *Ixodes* ticks (representing *I. eudyptidis* and *I. kohlsi* species) from 46 little penguin hosts at Phillip Island, Victoria (38.4899° S, 145.2038° E), and two *Ixodes* ticks from two penguins at Montague Island (New South Wales: 36.2510° S, 150.2270° E), were taken directly from the host animal, or from inside their nest burrows, during the course of regular monitoring activities (Moon et al., 2015). Ticks were immediately placed in 96% ethanol for preservation.

DNA extraction and analysis

Ticks were sorted into categories based on host individual and life cycle stage (unfed nymphs, fed nymphs, unfed males, unfed females and fed females). Genomic DNA (gDNA) extractions were then carried out as described by Gofton et al., (2015), using a Qiagen DNeasy Blood and Tissue Kit, with specimens from the same host and life cycle stage extracted as one sample, leaving a total of 72 pooled samples.

Three *Borrelia*-genus specific nested PCR assays were conducted, targeting two genes (*flaB* and *gyrB*) as described by Loh et al. (2016, 2017) (see Table 1 for primer details). '*Candidatus* B. tachyglossi' genotype B described in Loh et al. (2016, 2017) was used as a positive control in all assays. Template-free controls and extraction reagent blank controls were included at every step in the assays to rule out the possibility of contamination. Amplicons of expected sizes were excised, purified and sequenced as described by Loh et al. (2016). Aligned sequences were compared to previously detected sequences using a BLAST nucleotide search in GenBank (https://blast.ncbi.nlm.nih.gov/BLAST/).

Results

Nested PCR assays resulting in amplicons of the correct length were identified in four samples using the *flaB* fragment 1 primers, three samples using the *flaB* fragment 2 primers, three samples using the g*yrB* primers, and in the positive controls. The PCR products of one sample from Phillip Island had amplicons of appropriate sizes for both the *flaB* (fragment 2) and *gyrB* assays. Nested PCR assays amplified the *Borrelia* genes in all (100%) of our positive controls, whereas none of the template-free controls or extraction reagent blank controls produced bands. All amplifications from penguin tick samples resulted in faint bands relative to the positive controls. PCR products from all 10 amplicons were sequenced using BigDye v.3.1 terminator on an ABI 373096 Capillary Sequencer (Life Technologies, USA). Though some amplicons produced clean sequences, these bore no significant similarity to any existing sequences in GenBank, suggesting that they were the result of non-specific primer binding and amplification. *Borrelia* gDNA was therefore not present in any of the ticks sampled from the Phillip Island or Montague Island penguins.

Discussion

Using highly conserved genus-specific housekeeping genes (*flaB* and *gyrB*), we found no genetic evidence for the presence of *Borrelia* in over 230 little penguin ticks from Phillip Island Nature Reserve in Victoria, nor in two ticks from Montague Island in New South Wales. Non-detection does

not conclusively demonstrate absence, but our large-scale sampling of the Phillip Island colony strongly suggests that *Borrelia* is either absent or has an extremely low prevalence in little penguin ticks at this site.

Unlike the generalist tick *I. uriae*, which is responsible for the transmission of Bbsl complex bacteria among seabirds, the *Ixodes* ticks investigated in this study are normally specialists on little penguins (Roberts 1970). Such host specificity would restrict pathogen exposure and spread even if the ticks are competent vectors of *Borrelia* species. Nonetheless, the possible occasional exploitation of penguins by echidna ticks, as indicated by the presence of these ticks in penguin burrows, may expose the penguins to pathogens present in echidnas (such as '*Candidatus* B. tachyglossi'). Experimental work has shown that a *Borrelia*-infected generalist tick may transmit the bacteria to other tick species via a reservoir host that is exploited by both ticks (Heylen et al., 2017). The fact that several tick species parasitising echidnas are generalists (e.g. *I. holocyclus, I. tasmani* and *Haemaphysalis humerosa*: Roberts 1970) therefore broadens the potential host range for pathogens such as '*Candidatus* B. tachyglossi' considerably (see Fig. 1) (McCoy et al., 2013). Collectively these generalist ticks are known to harbour the causative agents of Queensland tick typhus (*Rickettsia australis*), Flinders Island Spotted Fever (*Rickettsia honei*) and Q fever (*Coxiella burnetii*), and probably play a significant role in the maintenance of infection cycles in native Australian animals (Smith and Derrick, 1940; Campbell and Domrow, 1974; Sexton et al., 1991; Graves and Stenos, 2009). Importantly, however, not all ticks parasitising echidnas are likely to be equally capable of acquiring and transmitting pathogens. Studies in the Northern Hemisphere suggest there are differences among related tick species in their competence to act as vectors for Bbsl-complex bacteria (Heylen et al., 2014), and evidence suggests that *I. holocyclus* is not a competent vector for these bacteria (Piesman and Stone, 1991).

Borrelia bacteria may not yet have infected many tick hosts in south-eastern Australia. Indeed, all reports of (non-Bbsl) *Borrelia* species in native Australian animals or their ticks have thus far been restricted to Queensland (Chalada et al., 2016; Loh et al., 2016) or western New South Wales (Gofton

et al., 2015; Loh et al., 2016, 2017). Previously, only a small number of echidna ticks from Victoria (n = 4) have been screened for *Borrelia* spp., with no positive results (Loh et al., 2016). Furthermore, *I. holocyclus* ticks from north-eastern New South Wales were also negative for the bacteria (Graves et al., 2016) and our sample sizes were too small to confirm its presence in penguin ticks from south-eastern New South Wales. Novel Australian *Borrelia* species (including '*Candidatus* B. tachyglossi') may therefore be geographically limited to Queensland and western New South Wales (Gofton et al., 2015; Loh et al., 2016; Loh et al., 2017). While the broad distribution of Lyme-like illness (Senate Community Affairs Committee Secretariat 2016) suggests that the causative agent would need to be broadly distributed, most incidences published in the scientific literature remain restricted to New South Wales, Queensland and Western Australia (Gofton et al., 2015; Chalada et al., 2016).

The identification of '*Candidatus* B. tachyglossi' in adult *B. concolor* ticks removed from echidnas is not conclusive evidence that echidnas are effective reservoir hosts for this bacterium. No larval ticks were tested, and so the presence of '*Candidatus* B. tachyglossi' in the adult echidna ticks may have been the result of feeding on another host during a previous life cycle stage. Five tick species known to parasitise echidnas are not host-specific (see Fig. 1). If the true reservoir host(s) is absent from Phillip Island, '*Candidatus* B. tachyglossi' infection would not be maintained in the local native animals. Our results could therefore indicate that the little penguins at Phillip Island have not been exposed to the bacteria, due to the lack of a competent vector or reservoir host. There is also considerable variation in host-to-tick transmission efficiency in vertebrate species (Tälleklint and Jaenson, 1994; LoGiudice et al., 2003), and little penguins may not be competent reservoir hosts themselves despite evidence for competency in other penguin species (Gauthier-Clerc et al., 1999; Yabsley et al., 2012; Schramm et al., 2014).

The inferred absence of *Borrelia* spp. from little penguin ticks at Phillip Island has broader implications for tick-borne pathogen cycling in native Australian animals. Little penguins are found across the entire south coast of Australia, and often co-occur with other native wildlife and migratory birds. A recent study has found no population structure in penguin ticks taken from Victoria and New

South Wales, suggesting that long-distance tick movement may be facilitated by hosts among the east coast penguin colonies (Moon et al., 2015). Penguin colonies that share tick vectors may share tick-borne pathogens, as is the case for *I. uriae* facilitating the circulation of some members of the Bbsl complex, in particular *B. garinii*, among seabird colonies outside of Australia (Olsén et al., 1993; Olsén et al., 1995; Gylfe et al., 2000; Lobato et al., 2011). Novel *Borrelia* bacteria such as '*Candidatus* B. tachyglossi', as well as agents with known pathogenic consequences (e.g., the agents for Queensland tick typhus, Flinders Island Spotted Fever and Q fever), therefore have the potential to cycle between native Australian host species facilitated by generalist ticks, and among east coast penguin colonies facilitated by penguin ticks. This study is the first to concentrate on ticks from heavily populated south-eastern Australia, and indicates that *Borrelia* spp. do not appear to cycle among penguin colonies in the region.

Acknowledgements

The authors would like to thank the reviewers of an earlier draft for their constructive comments. We thank Allen Heath for his help with tick taxonomy, the Vector & Waterborne Pathogens Research Group at Murdoch University in Western Australia for their advice on experimental design, and for access to their laboratory, the Phillip Island Nature Reserve staff in Victoria, Gemma Carroll at Macquarie University in New South Wales, and Lauren Moon and Grant Duffy for help with sample collections. The authors would also like to thank Frances Brigg and the Western Australia State Agricultural Biotechnology Centre for Sanger sequencing.

Funding

This study was part funded by the Murdoch University School of Veterinary and Life Sciences small grant scheme.

References

Ballantyne, C., 2008. The chronic debate over Lyme disease. Nat. Med. 14, 1135-1140.

Barker, S., A.R. Walker, 2014. Ticks of Australia: The species that infest domestic animals and humans. Zootaxa. 3816, 1-144.

Biesiada, G., J. Czepiel, M.R. Leśniak, A. Garlicki, T. Mach, 2012. Lyme disease: review. Arch Med Sci. 8, 978-982.

Campbell, R., R. Domrow, 1974. Rickettsioses in Australia: isolation of *Rickettsia tsutsugamushi* and *R. australis* from naturally infected arthropods. Trans. R. Soc. Trop. Med. Hyg. 68, 397-402.

Chalada, M.J., J. Stenos, R.S. Bradbury, 2016. Is there a Lyme-like disease in Australia? Summary of the findings to date. One Health. 2, 42-54.

Chambert, T., V. Staszewski, E. Lobato, R. Choquet, C. Carrie, K.D. McCoy, T. Tveraa, T. Boulinier, 2012. Exposure of black□legged kittiwakes to Lyme disease spirochetes: dynamics of the immune status of adult hosts and effects on their survival. J. Anim. Ecol. 81, 986-995.

Cooper, A., J. Stephens, N. Ketheesan, B. Govan, 2013. Detection of *Coxiella burnetii* DNA in wildlife and ticks in northern Queensland, Australia. Vector Borne Zoonotic Dis. 13, 12-16.

Dietrich, M., E. Gómez-Díaz, K.D. McCoy, 2011. Worldwide distribution and diversity of seabird ticks: implications for the ecology and epidemiology of tick-borne pathogens. Vector-Borne and Zoonotic Diseases. 11, 453-470.

Diuk-Wasser, M.A., E. Vannier, P.J. Krause, 2016. Coinfection by *Ixodes* tick-borne pathogens: ecological, epidemiological, and clinical consequences. Trends Parasitol. 32, 30-42.

Duneau, D., T. Boulinier, E. Gómez-Díaz, A. Petersen, T. Tveraa, R.T. Barrett, K.D. McCoy, 2008. Prevalence and diversity of Lyme borreliosis bacteria in marine birds. Infect Genet Evol. 8, 352-359.

Dunn, J.M., P.J. Krause, S. Davis, E.G. Vannier, M.C. Fitzpatrick, L. Rollend, A.A. Belperron, A. Stacey, L.K. Bockenstedt, D. Fish, 2014. *Borrelia burgdorferi* promotes the establishment of *Babesia microti* in the northeastern United States. PLoS One. 9, e115494.

Gauthier-Clerc, M., B. Jaulhac, Y. Frenot, C. Bachelard, H. Monteil, Y. Le Maho, Y. Handrich, 1999. Prevalence of *Borrelia burgdorferi* (the Lyme disease agent) antibodies in king penguin *Aptenodytes patagonicus* in Crozet Archipelago. Polar Biol. 22, 141-143.

Gofton, A.W., S. Doggett, A. Ratchford, C.L. Oskam, A. Paparini, U. Ryan, P. Irwin, 2015. Bacterial profiling reveals novel "*Ca.* Neoehrlichia", *Ehrlichia*, and *Anaplasma* species in Australian human-biting ticks. PLoS One. 10, e0145449.

Gofton, A.W., C.L. Oskam, N. Lo, T. Beninati, H. Wei, V. McCarl, D.C. Murray, A. Paparini, T.L. Greay, A.J. Holmes, 2015. Inhibition of the endosymbiont "*Candidatus* Midichloria mitochondrii" during 16S rRNA gene profiling reveals potential pathogens in *Ixodes* ticks from Australia. Parasit. Vectors. 8, 345.

Gómez-Díaz, E., T. Boulinier, N. Sertour, M. Cornet, E. Ferquel, K.D. McCoy, 2011. Genetic structure of marine *Borrelia garinii* and population admixture with the terrestrial cycle of Lyme borreliosis. Environ. Microbiol. 13, 2453-2467.

Gómez-Díaz, E., P.F. Doherty Jr, D. Duneau, K.D. McCoy, 2010. Cryptic vector divergence masks vector□specific patterns of infection: an example from the marine cycle of Lyme borreliosis. Evol Appl. 3, 391-401.

Graves, S., J. Stenos, 2009. Rickettsioses in Australia. Ann. N. Y. Acad. Sci. 1166, 151-155.

Graves, S.R., A. Islam, 2016. Endemic Q fever in New South Wales, Australia: a case series (2005–2013). Am. J. Trop. Med. Hyg. 95, 55-59.

Graves, S.R., C. Jackson, H. Hussain-Yusuf, G. Vincent, C. Nguyen, J. Stenos, M. Webster, 2016. *Ixodes holocyclus* tick-transmitted human pathogens in north-eastern New South Wales, Australia. Trop Med and Inf Dis. 1, 4.

Gylfe, Å., S. Bergström, J. Lundström, B. Olsen, 2000. Epidemiology: reactivation of *Borrelia* infection in birds. Nature. 403, 724-725.

Gylfe, Å., M. Yabuki, M. Drotz, S. Bergström, M. Fukunaga, B. Olsen, 2001. Phylogeographic relationships of *Ixodes uriae* (Acari: Ixodidae) and their significance to transequatorial dispersal of *Borrelia garinii*. Hereditas. 134, 195-199.

Halperin, J.J., 2015. Chronic Lyme disease: misconceptions and challenges for patient management. Infect Drug Resist. 8, 119-128.

Halperin, J.J., 2016. Lyme Borreliosis. Human emerging and re-emerging infections: viral and parasitic infections. S. K. Singh. Hoboken, New Jersey, USA, John Wiley & Sons Inc. 1, 887-895.

Hercogová, J., 2015. Lyme borreliosis. European Handbook of Dermatological Treatments. A. D. Katsambas, T. M. Lotti, C. Dessinioti, A. M. D'Erme. Berlin, Germany, Springer-Verlag Berlin Heidelberg, 561-566.

Heylen, D., A. Krawczyk, I. Lopes de Carvalho, M.S. Núncio, H. Sprong, A.C. Norte, 2017. Bridging of cryptic Borrelia cycles in European songbirds. Environ. Microbiol. 19, 1857-1867.

Heylen, D., H. Sprong, K. Oers, M. Fonville, H. Leirs, E. Matthysen, 2014. Are the specialized bird ticks, *Ixodes arboricola* and *I. frontalis*, competent vectors for *Borrelia burgdorferi* sensu lato? Environ. Microbiol. 16, 1081-1089.

Lobato, E., J. Pearce-Duvet, V. Staszewski, E. Gómez-Díaz, J. González-Solís, A. Kitaysky, K.D. McCoy, T. Boulinier, 2011. Seabirds and the circulation of Lyme borreliosis bacteria in the North Pacific. Vector Borne Zoonotic Dis. 11, 1521-1527.

LoGiudice, K., R.S. Ostfeld, K.A. Schmidt, F. Keesing, 2003. The ecology of infectious disease: effects of host diversity and community composition on Lyme disease risk. Proc. Natl. Acad. Sci. USA. 100, 567-571.

Loh, S.-M., A. Gillett, U. Ryan, P. Irwin, C. Oskam, 2017. Molecular characterization of '*Candidatus* Borrelia tachyglossi' family *Spirochaetaceae*) in echidna ticks, *Bothriocroton concolor*. Int. J. Syst. Evol. Microbiol. 67, 1075-1080.

Loh, S.-M., A.W. Gofton, N. Lo, A. Gillett, U.M. Ryan, P.J. Irwin, C.L. Oskam, 2016. Novel *Borrelia* species detected in echidna ticks, *Bothriocroton concolor*, in Australia. Parasit. Vectors. 9, 339.

McCoy, K.D., E. Léger, M. Dietrich, 2013. Host specialization in ticks and transmission of tick-borne diseases: a review. Front Cell Infect Microbiol. 3.

Middleton, J., I. Cooper, A.S. Rott, 2016. Can interventions that aim to decrease Lyme disease hazard at non-domestic sites be effective without negatively affecting ecosystem health? A systematic review protocol. Environ. Evid. 5, 23.

Minkoff, D.I., 2016. Lyme disease: prevalence, diagnosis, and comprehensive treatment. Townsend Letter, 76-78.

Moon, K.L., S.C. Banks, C.I. Fraser, 2015. Phylogeographic Structure in Penguin Ticks across an Ocean Basin Indicates Allopatric Divergence and Rare Trans-Oceanic Dispersal. PloS One. 10, e0128514.

Olsén, B., D.C. Duffy, T.G. Jaenson, Å. Gylfe, J. Bonnedahl, S. Bergström, 1995. Transhemispheric exchange of Lyme disease spirochetes by seabirds. J. Clin. Microbiol. 33, 3270-3274.

Olsén, B., T.G. Jaenson, L. Noppa, J. Bunikis, S. Bergström, 1993. A Lyme borreliosis cycle in seabirds and *Ixodes uriae* ticks. Nature. 362, 340-342.

Oskam, C.L., A.W. Gofton, T.L. Greay, R. Yang, S. Doggett, U.M. Ryan, P.J. Irwin, 2017. Molecular investigation into the presence of a *Coxiella* sp. in *Rhipicephalus sanguineus* ticks in Australia. Vet. Microbiol. 201, 141-145.

Phillip Island Nature Parks, 2014. Phillip Island's Short-Tailed Shearwaters. Nature Notes. Phillip Island, Victoria, State of Victoria.

Phillip Island Nature Parks, 2015. Phillip Island Nature Parks Annual Report 2014-15. Phillip Island, Victoria, State of Victoria.

Piesman, J., B.F. Stone, 1991. Vector competence of the Australian paralysis tick, *Ixodes holocyclus*, for the Lyme disease spirochete *Borrelia burgdorferi*. Int. J. Parasitol. 21, 109-111.

Roberts, F.H.S., 1970. Australian ticks. Melbourne, Australia, Commonwealth Scientific and Industrial Research Organisation (CSIRO).

Russell, R., S. Doggett, R. Munro, J. Ellis, D. Avery, C. Hunt, D. Dickeson, 1994. Lyme disease: a search for a causative agent in ticks in south–eastern Australia. Epidemiol. Infect. 112, 375-384.

Schramm, F., M. Gauthier-Clerc, J.-C. Fournier, K.D. McCoy, C. Barthel, D. Postic, Y. Handrich, Y. Le Maho, B. Jaulhac, 2014. First detection of *Borrelia burgdorferi* sensu lato DNA in king penguins (*Aptenodytes patagonicus halli*). Ticks Tick Borne Dis. 5, 939-942.

Senanayake, S.N., A. Paparini, M. Latimer, K. Andriolo, A.J. Dasilva, H. Wilson, M.V. Xayavong, P.J. Collignon, P. Jeans, P.J. Irwin, 2012. First report of human babesiosis in Australia. Med. J. Aust. 196, 350-352.

Senate Community Affairs Committee Secretariat, 2016. Growing evidence of an emerging tick-borne disease that causes a Lyme-like illness for many Australian patients: Final report. Parliament House, Canberra.

Sexton, D.J., B. Dwyer, R. Kemp, S. Graves, 1991. Spotted fever group rickettsial infections in Australia. Rev Infect Dis. 13, 876-886.

Smith, D., E. Derrick, 1940. Studies in the Epidemiology of Q Fever. I. The Isolation of Six Strains of *Rickettsia burneti* from the Tick *Haemaphysalis humerosa*. Aust J Exp Biol Med Sci. 18, 1-8.

Stenos, J., S. Graves, V.L. Popov, D.H. Walker, 2003. *Aponomma hydrosauri*, the reptile-associated tick reservoir of *Rickettsia honei* on Flinders Island, Australia. Am. J. Trop. Med. Hyg. 69, 314-317.

Tälleklint, L., T.G.T. Jaenson, 1994. Transmission of *Borrelia burgdorferi* sl from mammal reservoirs to the primary vector of Lyme borreliosis, *Ixodes ricinus* (Acari: Ixodidae), in Sweden. J. Med. Entomol. 31, 880-886.

Voordouw, M.J., S. Lachish, M.C. Dolan, 2015. The Lyme disease pathogen has no effect on the survival of its rodent reservoir host. PLoS One. 10, e0118265.

Walter, K.S., K.M. Pepin, C.T. Webb, H.D. Gaff, P.J. Krause, V.E. Pitzer, M.A. Diuk-Wasser, 2016. Invasion of two tick-borne diseases across New England: harnessing human surveillance data to capture underlying ecological invasion processes. Proc R Soc Lond [Biol]. 283, 20160834.

Wills, M., R. Barry, 1991. Detecting the cause of Lyme disease in Australia. Med. J. Aust. 155, 275.

Yabsley, M.J., N.J. Parsons, E.C. Horne, B.C. Shock, M. Purdee, 2012. Novel relapsing fever *Borrelia* detected in African penguins (*Spheniscus demersus*) admitted to two rehabilitation centers in South Africa. Parasitol. Res. 110, 1125-1130.

Tables

Table 1: Primers used for *Borrelia*-specific nested PCR assays.

Gene	Primer	Sequence (5' – 3')	Annealing temperature	Expected product size (bp)	Primer reference
flaB (first primer set)	*External*		52	645	Barbour et al. 1996; Clark et al. 2013; Loh et al. 2016
	FlaB280F	GCAGTTCARTCAGGTAACGG			
	FlaRL	GCAATCATAGCCATTGCAGATTGT			
	Internal		55	407	
	flaB_737R	GCATCAACTGTRGTTGTAACATTAACAGG			
	FlaLL	ACATATTCAGATGCAGACAGAGGT			
*flaB*2 (second primer set)	*Primary*		52	545	Barbour et al. 1996; Toledo et al. 2010; Loh et al. 2017
	Forward	CTGAAGAGCTTGGAATGCAAC			
	Reverse	AGGTACTTGATTTGCTTGTGC			
	Secondary		52	526	
	Forward	CTGAAGAGCTTGGAATGCAAC			
	Reverse	GCAATCATAGCCATTGCAGATTGT			
gyrB (fragment 3)	*Primary*		51	764	Loh et al. 2017
	Forward	CTTTGGGAAACTACTATGAAYCCTG			
	Reverse	ACATCCAGATTTACTACATCAAGYG			
	Secondary		51	713	
	Forward	CTTTGGGAAACTACTATGAAYCCTG			
	Reverse	GGTTCAACWTCATCYCCCAT			

Figure legend

Fig. 1: Host-tick connections between echidnas, little penguins, and other Australian wildlife. There are five Ixodid tick species parasitising echidnas that also exploit other hosts. Solid black lines link recorded hosts for each tick species (Roberts, 1970; Barker and Walker, 2014), with each parasitised host group shown as a coloured silhouette. The number of species parasitised in the group is given inside or next to the silhouette. Groups include a flying fox species (bat silhouette) and the dingo (dog silhouette) as well as a number of birds (bird silhouette), reptiles (snake silhouette), rodents (rat silhouette), marsupials (koala silhouette) and domestic animals and humans (grouped together and represented by the human silhouette). Curved grey lines show where the same host species are parasitised by two tick species, with the thickness of the line relating to how many host species are shared. The figure therefore illustrates the potential size of the pathogen system if *Bothriocroton* ticks link penguin and echidna hosts.