Do terrestrial ectoparasites disperse with penguins?

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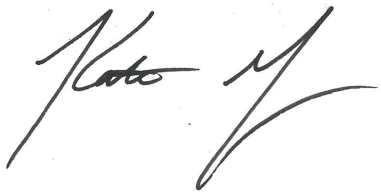
Declaration by author

This thesis contains no material that has been accepted for the award of any other degree or diploma in any university. To the best of the author's knowledge and belief it contains no material previously published or written by another person, except where reference is made in the text or chapter statements of contribution.



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~ For my family ~

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Abstract

Dispersal plays a critical role in evolution. Rare long-distance movements can lead to allopatric speciation, whereas frequent movements can facilitate gene flow among disjunct populations and prevent divergence. Dispersal between populations of a species may be difficult to observe directly, and is often inferred from indirect measures such as species occurrence data. Increasingly, however, high resolution genomic data are being used to clarify dispersal and gene flow, in many cases contradicting past assumptions.

Islands are excellent model regions for investigating dispersal as they offer replicated habitats with clear geographic boundaries. The sub-Antarctic comprises some of the most geographically isolated island ecosystems in the world, representing an ideal model system for assessing the evolutionary consequences of long-distance dispersal. Strong winds, circumpolar oceanic currents, and extreme climatic cycles are thought to have effectively isolated many sub-Antarctic ecosystems, but a growing body of molecular evidence is beginning to question this rhetoric, with numerous species showing connectivity across the region. Connectivity patterns are, however, complex and are not always predictable from an organism's inferred dispersal capacity. With environmental change placing unprecedented pressure on isolated ecosystems, there is a pressing need for improved understanding of dispersal processes and population connectivity via genomic analyses of diverse taxa.

A number of sub-Antarctic species exhibit gene flow across the region despite lacking active long-distance dispersal capabilities. Brooding, sedentary crustaceans have, for example, rafted on buoyant kelp across thousands of kilometres of open ocean in the sub-Antarctic. The close symbiotic or parasitic relationships that such species maintain with the kelp has resulted in whole communities dispersing together. Indeed, active dispersal is often limited in parasites, which can depend almost entirely on mobile hosts for long-distance movement. A parasite that is unable to travel far with its host would, therefore, be expected to show considerable phylogeographic structure. For example, penguins primarily travel underwater but are hosts to terrestrial ectoparasites (most commonly ticks - *Ixodes* spp.) when they come ashore to breed. Aquatic host movements may represent a challenge to the survival of penguin ticks, restricting gene flow across their range.

This thesis first reviews connectivity patterns and challenges throughout the sub-Antarctic, and then uses a multidisciplinary approach (genomic and physiological data) to test whether some terrestrial parasites (ticks: Acari) are able to travel long distances at sea with their aquatically dispersing hosts (penguins). Results indicate that penguin ticks are physiologically resilient, and may be capable of surviving the conditions faced during aquatic penguin movements between colonies. However, these movements appear to be too sporadic to maintain gene flow across the ticks' ranges, resulting in broad-scale geographic structure. In contrast, movement on fine scales (within colonies) is inferred – based on lack of genomic structure – to be common, possibly facilitated by social interactions of hosts. These results emphasise the important role of dispersal in isolated

regions for range expansion and diversification, and highlight the adaptability of parasites to their hosts' environments.

Key words

Sub-Antarctic, phylogeography, dispersal, *Ixodes* ticks, genomics, host-associated movement, physiological tolerances.

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Chapter One

General introduction

King penguins on Macquarie Island. Taken by Laura Phillips, 2016.

1.1 The history of dispersal biogeography

The processes underpinning species distributions have long fascinated researchers. Occurrence data collected during early natural history expeditions hinted at the influence and selective pressure of environmental factors (e.g. habitat area, latitude, and elevation) on assemblages (Darwin, 1859; Lomolino *et al*, 2004; Wallace, 1876). Later, distributions were used to corroborate plate tectonic theory, particularly in the Southern Hemisphere (Raven and Axelrod, 1972). However, long-distance dispersal events were long seen as implausible and / or untestable, and their frequency and biogeographical importance was thus largely ignored (Cowie and Holland, 2006).

Phylogeography is a relatively young field (Fig. 1) that employs molecular methods to investigate inter- and intra-specific relationships over time and space. Phylogeographic studies are providing increasing empirical evidence that dispersal has played a critical role in biogeography (Avise, 2000), dynamically influencing global patterns of biodiversity (Hanski, 2012). The largely oceanic Southern Hemisphere has presented particularly powerful systems for testing hypotheses in dispersal biogeography (McGlone, 2005; Waters and Craw, 2006). Just as we begin to identify baseline dispersal patterns in the region, however, these systems are changing. Biogeography may have begun as a field concerned with observing natural processes, but its role in conservation has grown considerably in recent years (Lomolino, 2004; Richardson and Whittaker, 2010). Increasingly, high-resolution molecular data are helping to predict the influence of changing conditions on biodiversity in systems already under threat.

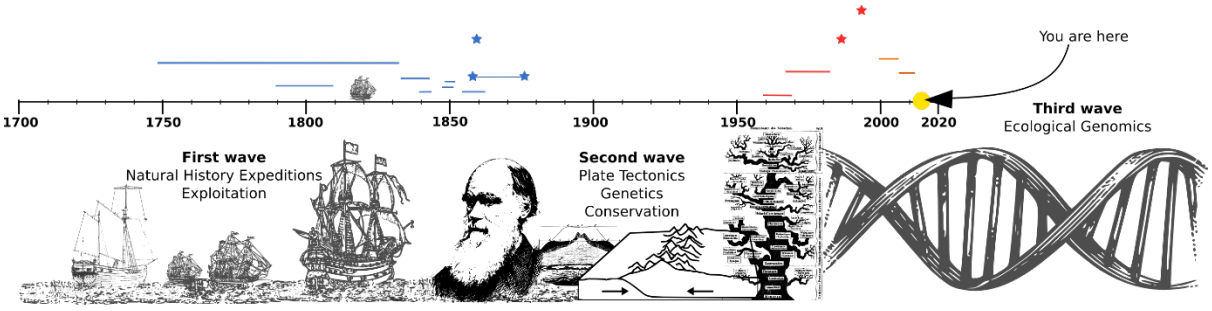

Figure 1. The history of biogeography. All blue lines and stars indicate major natural history expeditions and advancements, and the publication of Darwin's Origin of Species. Red lines and stars indicate major advancements for plate tectonic theory.

In the late 1960s, MacArthur and Wilson published the Theory of Island Biogeography based on comparisons of species richness, island size, and isolation (MacArthur and Wilson, 1967; Warren *et al*, 2015). Despite the name, the theory applies to all spatially explicit regions, where areas of suitable habitat are separated by a matrix of unsuitable habitat (e.g. fragmented systems). At its heart is dispersal, and the factors shifting the equilibrium between immigration and extinction. Wilson subsequently gained support for the theory by experimentally fumigating mangrove islands, and monitoring their recovery (Simberloff and Wilson, 1969). Molecular studies have been able to take the theory one step further, measuring gene flow among discrete populations and examining the processes influencing diversification across a range of scales. In this way, phylogeography has opened up the study of biodiversity and dispersal in fragmented systems.

The dispersal capacity of a wide range of species has now been inferred from phylogeographic studies, but a number of key knowledge gaps persist. Dispersal has often been tested in species that are observed moving (e.g. butterflies: Hanksi, 2012), but is less well understood for species that lack an obvious mechanism of mobility. Movements are often assumed to be limited in such groups, but many are nonetheless distributed over long distances. For example, despite having no swimming capability, some intertidal species have wide distributions (Johannesson, 1988; Nikula *et al*, 2010; Waters, 2008). Likewise, active movement is extremely limited in ticks (Ixodidae) (Falco and Fish, 1991), but the common seabird tick *Ixodes uriae* is present across the polar regions of both hemispheres (Dietrich *et al*, 2014). In both cases, dispersal associated with more mobile organisms has apparently facilitated movement in species with no active mobility.

Parasites often exhibit limited active movement, and so rely entirely host-associated dispersal at all scales (e.g. *I. uriae* among host colonies: McCoy *et al,* 2003a, and within host colonies: McCoy *et al,* 2003b). However, the host and the parasite can have different environmental requirements, which could have implications for the latter's dispersal. For example, several terrestrial ectoparasite groups parasitise pinnipeds, penguins and otters, which spend large amounts of time in aquatic environments, but little is known about the physiological capacity of these ectoparasites to survive in water. The most well-studied system is the Echinophthiriidae family of sucking-lice, comprised of five genera that are specific to pinnipeds and the river otter (Kim, 1971; Kim, 1975; Kim and Emerson, 1974; Leonardi and Lazzari, 2014; Leonardi and Palma,

2013; Murray and Nicholls, 1965; Murray *et al*, 1965) (Fig. 2). Echinophthiriids possess various adaptations not found in other sucking lice (Kim, 1971; Murray and Nicholls, 1965), including modified legs, spines, scales and hairs, and specialised structures (e.g. altered spiracles) that facilitate underwater respiration (Kim, 1971; Kim, 1975) and allow them to survive at sea for several months (Leonardi and Lazzari, 2014). These findings suggest that sucking lice, at least, might be able to disperse with their hosts, but other ectoparasites (e.g. ticks) have not similarly been shown to have adapted to aquatic dispersal. Phylogeographic analysis provides an effective means to test hypotheses about the long-distance dispersal capacity of terrestrial parasites with aquatically-dispersing hosts.

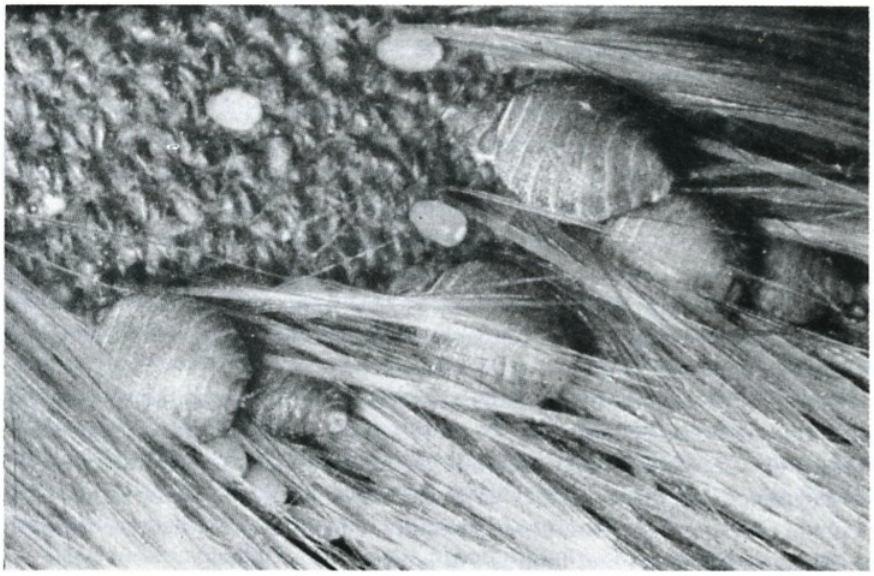

Figure 2. *Antarctophthirus ogmorhini*, the sucking louse of Weddell (*Leptonychotes weddellii*) and leopard (*Hydrurga leptonyx*) seals. Photograph is from the UN Atlas of the Oceans and was taken by I. Roper.

1.2 Model system: dispersal in the sub-Antarctic

The sub-Antarctic region – which consists of physically isolated oceanic islands, and the southern tip of South America – is an ideal model system to test dispersal. Each island is unique in terms of its geological age, its proximity to continental or island land masses, the extent of historic climate fluctuations and human intervention, and the influence of strong winds and oceanic currents (Convey, 2013; Hodgson *et al*, 2014). Importantly, because some of the islands are commonly oceanic in origin, and many are geologically young (e.g. volcanic Marion Island, which appeared < 0.5 Ma, see Table 1 Chown *et al*, 1998), most if not all species present have arrived with dispersal. Patterns of biodiversity in the region are also thought to be dominated by geological, glaciological and geographic isolation, in concert with extreme environmental conditions (Gressitt, 1970; Greve *et al*, 2005; Starý and Block, 1998), but molecular studies are provide strong support for the ongoing importance of dispersal in the sub-Antarctic.

A key finding emerging from the growing number of phylogenetic studies that are being conducted in the sub-Antarctic is that dispersal capacity cannot readily be inferred from life history. Even some species that appear poorly-suited to dispersal show signatures of recent long-distance movements. I therefore began this thesis by synthesizing all available genetic studies conducted across the sub-Antarctic to date. I used the region as a model system to evaluate the influence of dispersal in fragmented systems, for species with and without active dispersal, with an emphasis on understanding the mechanisms involved in dispersal. I found evidence of movement of diverse species across the region, in some cases with entire communities being widely dispersed, whereas some taxa (including apparently dispersive taxa) show genetic regionalisation. The sheer

complexity of signals from a region previously believed to be dominated by isolation highlights the need for improved understanding, ideally via high-resolution genomic studies, to ascertain dispersal capacities (and thus potential to respond to environmental change) for a broad range of taxa.

1.3 Penguin ticks

Penguins are seabirds – found primarily in the sub-Antarctic and greater Antarctic – that come ashore to breed, but that forage and disperse at sea. Like pinnipeds, the ancestors of modern penguins returned to the oceans after evolving for life on land (Davis and Renner, 2003). Sucking lice, ticks and fleas are the most common terrestrial ectoparasites of these aquatically-dispersing vertebrates (Murray, 1967). The waterproof feathers of a penguin trap air during aquatic movements, creating a virtually aerial environment, and so the ecology of penguin sucking lice are not thought to be dissimilar to those on flighted seabirds (Murray, 1967). Ticks are large non-permanent ectoparasites that exploit penguins within their colonies. When attached to the body, and especially when engorged from feeding, their size causes penguin ticks to breach the protective feather layer and likely exposes them to aquatic conditions (Fig. 3). Aquatic dispersal is believed to present a challenge to penguin ticks, which, unlike Echinophthiriidae, exhibit no clear adaptations to aquatic dispersal. Despite these assumed physical limitations (Pugh, 1997), recent phylogenetic studies that included penguin ticks (*I. uriae*) from the greater Antarctic region suggests they may be host-species specific and yet moving between colonies (McCoy *et al*, 2012; McCoy *et al*, 2005). The remainder of this thesis therefore aimed to understand penguin tick dispersal,

including the physiological limitations of penguin ticks, as well as phylogeographic evidence for movement across a colony (terrestrial movement) and between colonies (aquatic movement). I focussed most work on a model system in Australia and New Zealand, but used multispecies colonies in the sub-Antarctic to confirm and extend results.

Figure 3. Photos of penguin ticks attached to their hosts. Taken by Katherine L Moon, Phillip Island.

1.4 Little penguins and their ticks

The little penguin inhabits temperate regions across southern Australia and New Zealand, where it has recently been split into two species (Grosser *et al*, 2015); the New Zealand little penguin (*Eudyptula minor*) present around the north of New Zealand and the Australian little penguin (*E. novaehollandiae*) present across southern Australia and the south of New Zealand (Fig. 4). This thesis investigated little penguin-tick assemblages across the range of both host species, and so I use the term 'little penguin' *sensu lato* to refer to both species. Little penguins are burrow-nesting seabirds that disperse and forage aquatically, but return to colonies annually to breed, moult and rest. Conditions at each colony are unique, and differ markedly across the range of the little penguin, including the size and structure of colonies, the timing of breeding season,

fledgling success, clutch size, and the structure of colonies as well as foraging range, effort and average dive depths (Bethge *et al*, 1997; Braidwood, 2009; Chiaradia, 1999; Chiaradia *et al*, 2007; Johannesen *et al*, 2002; Reilly and Cullen, 1981; Reilly and Cullen, 1983; Sutherland and Dann, 2014). Phylogenetic analyses suggest the penguin has crossed the Tasman Sea more than once – including colonisation of Australia from New Zealand, followed by a much more recent back-colonisation of Australian penguins to New Zealand – but that effective isolation between the countries was the cause of speciation (Banks *et al*, 2002; Peucker *et al*, 2009). The extent of penguin dispersal is not well-known, but is thought to be relatively infrequent among colonies (Dann, 1992).

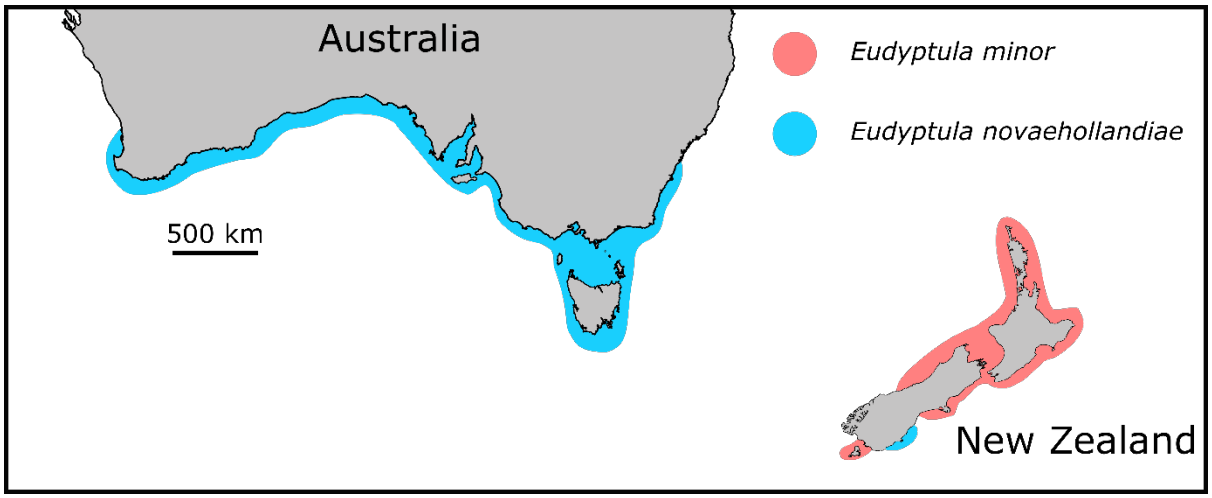

Figure 4. Distribution of the two little penguin species across their range in Australia and New Zealand.

Little penguins are parasitised by two species of ticks: *Ixodes kohlsi* which is only found in Australia, and *I. eudyptidis* which is present in both Australian and New Zealand colonies (Roberts, 1970). The biology of *Ixodes kohlsi* and *I. eudyptidis* (henceforth little penguin ticks) has not been investigated, but is thought to be similar to *I. uriae* (Heath, 2006). Considerable morphological plasticity has meant accepted taxonomic characters

(Roberts, 1970) are unable to split the two species of little penguin tick. The only genetic study previously conducted on little penguin ticks indicated a deep divergence between the two species (Moon *et al*, 2015). The study, which used mitochondrial and nuclear markers, also provided some tantalizing but inconclusive evidence that little penguin ticks may be moving among colonies along the east coast of Australia (Moon *et al*, 2015).

1.5 Ticks and their impacts

Whether tick loads have a direct health impact on little penguins remains largely unknown, but there is no evidence for their influence on body condition (Van Rensburg, 2010). This is also true of some other penguin species (Gauthier–Clerc, 2003), although ticks can have a negative impact penguin breeding success (Mangin *et al*, 2003). Environmental change is already having a variable effect on little penguin populations, with some colonies in decline as a result of changing conditions (Cannell *et al*, 2012) and others benefiting (Cullen *et al*, 2009) depending on their location. Given ticks (Jaenson *et al*, 2012; Korotkov *et al*, 2015) including penguin ticks (Benoit *et al*, 2009) are likely to increase their abundance and/or range as a result of climate change, their influence on their hosts' health remains an important knowledge gap.

Ticks are, however, one of the most important global vectors of disease. Penguins (including the little penguin) harbor several blood-borne pathogens, of which ticks are a confirmed and/or suspected vector (see Vanstreels *et al,* 2016 for a review). For example, little penguin ticks are thought to play a key role in the transmission of

Babesia (Cunningham *et al*, 1993). However, the influence of blood-borne pathogens on little penguins is not well known (but see Cannell *et al*, 2013). More broadly, seabird ticks are one of the most globally important vectors of the bacterial agent of Lyme disease (members of the *Borrelia burgdorferi* sensu lato species complex) (Duneau *et al*, 2008; Gómez-Díaz *et al*, 2010; Gylfe *et al*, 2001; Lobato *et al*, 2011; Olsén *et al*, 1995), and increasing evidence suggests penguins could be competent reservoir hosts of *Borrelia* bacteria (Gauthier-Clerc *et al*, 1999; Schramm *et al*, 2014; Yabsley *et al*, 2012). Given the growing debate over Lyme disease in Australia (Senate Community Affairs Committee Secretariat, 2016), the possibility of little penguins and other wildlife harbouring *Borrelia* is of considerable importance, particularly given the presence of little penguin colonies in the country's two largest cities (Melbourne and Sydney). In the final chapter of this thesis, I test for the presence of *Borrelia* in the largest little penguin colony in Australia.

1.6 Thesis structure

This thesis is a 'thesis by compilation' consisting of six manuscripts intended for publication in peer-review journals, as well as a general introduction (Chapter 1) and a general discussion (Chapter 8). As each chapter was written for a different journal, there are minor formatting changes among chapters.

Chapter 1: General introduction

Chapter 2: Reconsidering connectivity in the sub-Antarctic. In order to assess the complexities of dispersal across a range of organisms, I first reviewed all studies of connectivity in the sub-Antarctic. I identified which species show evidence of isolation and which appear connected, as well as the mechanisms responsible. This chapter has been published in *Biological Reviews* (Moon et al, 2017).

Chapter 3: Can a terrestrial ectoparasite disperse with its marine host? In this chapter I used experimental physiological analyses to assess whether little penguin ticks are capable of surviving at sea with their hosts. The chapter is to be submitted for publication in *Physiological and Biochemical Zoology*.

Chapter 4: Penguin ectoparasite panmixia suggests frequent host movement within a colony. This chapter used genomic techniques to assess the extent of fine-scale movement in little penguin ticks across a large colony in south-eastern Australia. The chapter is intended for submission to *The Auk* but has already benefitted from the constructive feedback of reviewers via previous submission to less-specialised journals (*Molecular Ecology* and *Heredity*).

Chapter 5: Phylogeographic patterns similar in penguins and their ectoparasites. In order to assess the extent of penguin tick movement among colonies across their range, I undertook the first genomic assessment of little penguin ticks from colonies across

Australia and New Zealand. I also compared ticks from sympatric penguins and shearwaters to confirm host-species specificity. This chapter is intended for submission to *Journal of Biogeography*.

Chapter 6: Local, but not long-distance dispersal of ticks between two sub-Antarctic islands. In this chapter I assessed the host-species specificity of seabird ticks from several flighted and swimming hosts in the sub-Antarctic, as well as the extent of movement of penguin ticks between two distant sub-Antarctic islands. The chapter is intended for submission as a 'Brevia' (<1000 words) in *Ecography*.

Chapter 7: Australian penguin ticks screened for novel *Borrelia* species. I used searched for genetic evidence of *Borrelia* bacteria in penguin ticks from south-eastern Australia. This chapter has been accepted for publication as a short communication in *Ticks and Tick-borne Diseases* (Moon et al, in press).

Chapter 8: General conclusion

Except for collections from the TePapa Museum, Marion Island, Montague Island and some from Oamaru, and volunteer assistance, I conducted all of my own fieldwork across Australia, New Zealand and the sub-Antarctic. For the genomic chapters, I did all of my own extractions, amplifications and library preparations, and all of my own bioinformatic analyses. For the physiology chapter, I performed all my own experiments, with the help of professional staff mentioned in the Acknowledgements,

analyzed all of my own data and made all of my own figures. Appendix One is a copy of a paper I co-authored during my thesis, that appeared in *Nature*, that deals primarily with changing perspectives on biodiversity in the Antarctic (Chown *et al*, 2015).

The author at the St Andrews Bay, South Georgia, king penguin colony in March 2017.

1.7 References

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Chapter Two

Reconsidering connectivity in the sub-Antarctic

Macquarie Island Station, taken by Laura Phillips, 2016

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2.1 Abstract

Extreme and remote environments provide useful settings to test ideas about the ecological and evolutionary drivers of biological diversity. In the sub-Antarctic, isolation by geographic, geological and glaciological processes has long been thought to underpin patterns in the region's terrestrial and marine diversity. Molecular studies using increasingly high-resolution data are, however, challenging this perspective, demonstrating that many taxa disperse among distant sub-Antarctic landmasses. Here, we reconsider connectivity in the sub-Antarctic region, identifying which taxa are relatively isolated, which are well connected, and the scales across which this connectivity occurs in both terrestrial and marine systems. Although many organisms show evidence of occasional long-distance, trans-oceanic dispersal, these events are often insufficient to maintain gene flow across the region. Species that do show evidence of connectivity across large distances include both active dispersers and more sedentary species. Overall, connectivity patterns in the sub-Antarctic at intra- and inter-island scales are highly complex, influenced by life-history traits and local dynamics such as relative dispersal capacity and propagule pressure, natal philopatry, feeding associations, the extent of human exploitation, past climate cycles, contemporary climate, and physical barriers to movement. An increasing use of molecular data – particularly genomic data sets that can reveal fine-scale patterns – and more effective international collaboration and communication that facilitates integration of data from across the sub-Antarctic, are providing fresh insights into the processes driving patterns of diversity in the region. These insights offer a platform for assessing the ways in which changing dispersal

mechanisms, such as through increasing human activity and changes to wind and ocean circulation, may alter sub-Antarctic biodiversity patterns in the future.

2.2 Introduction

Dispersal is a fundamental ecological and evolutionary process, providing organisms with access to new environments and fuelling allopatric divergence and adaptation (Clobert *et al.*, 2012; Shaw & Etterson, 2012). The capacity for dispersal varies greatly among taxa (Nathan, 2006; Clobert *et al.*, 2012; Gillespie *et al.*, 2012) and dispersal may occur over a range of spatial and temporal scales, from relatively frequent local exchanges, to rarer long-distance movements between geographically isolated populations or into new habitat (Trakhtenbrot *et al.*, 2005). Dispersal distance influences the scale over which adaptation, diversification and speciation are likely to occur (Kisel & Barraclough, 2010). In consequence, variation in dispersal ability across small and large scales has a considerable impact both on patterns of diversity and on species and community dynamics (Cadotte, 2006; Hendrickx *et al.*, 2009; Vellend, 2010).

Understanding the form, extent and scale of dispersal is therefore a critical component of determining how diversity has evolved. Considerable theoretical and empirical work has characterised dispersal on local, regional and wider biogeographic scales (see Clobert *et al.*, 2012). Much work has focused on islands because they offer discrete, replicated habitats, with well-defined geographic barriers, making them ideal model systems to study the influence of dispersal scale on the ecology and evolution of organisms (MacArthur & Wilson, 1967; Kisel & Barraclough, 2010; Warren *et al.*, 2015). Indeed, much current understanding of the significance of dispersal has emerged from investigations of island species or species occupying island-like situations (Gillespie & Roderick, 2002; Gillespie *et al.*, 2012; Warren *et al*., 2015).

The sub-Antarctic is an isolated region of the Southern Ocean, lying approximately between the Sub-Tropical Convergence (STC) to the north and the Antarctic Polar Front (APF) to the south. Although dominated by the Southern Ocean, the sub-Antarctic includes several isolated islands and the southern tip of South America (see Fig. 1). The islands have diverse geological and glaciological histories (Hodgson *et al*., 2014). Their geological ages span approximately 0.5 to 100 million years, they have origins which range from typical basaltic oceanic islands to sections of raised seafloor, and their glacial histories range from almost complete cover by glaciers during the Last Glacial Maximum (LGM) to no evidence of any glaciation. These sub-Antarctic islands have received considerable attention from a typical island biogeographic perspective (e.g. Gressitt, 1970; Abbott, 1974; Chown, Gremmen & Gaston, 1998). Until recently, however, investigations of the importance of dispersal over various scales in the region have tended to be restricted to inferences drawn by comparing patterns of species occurrence (Gressitt, 1970; Dreux & Voisin, 1987, 1989; Starý & Block, 1998; Øvstedal & Gremmen, 2001; Pugh, 2004; Greve *et al.*, 2005; Primo & Vázquez, 2007; see Downey *et al.*, 2012 for a recent example). Notable exceptions have been attempts to investigate dispersal events directly either by sampling the aerial plankton (Greenslade, Farrow & Smith, 1999; Hawes & Greenslade, 2013), tracking the movements of large mobile species such as seabirds and marine mammals (Bester, 1989; Weimerskirch *et al.*, 1985; Reisinger & Bester, 2010), or by inferring recent colonisation events based on new species occurrence records (e.g. Chown & Language, 1994; Lee *et al.*, 2007; Lee, Terauds & Chown, 2014).

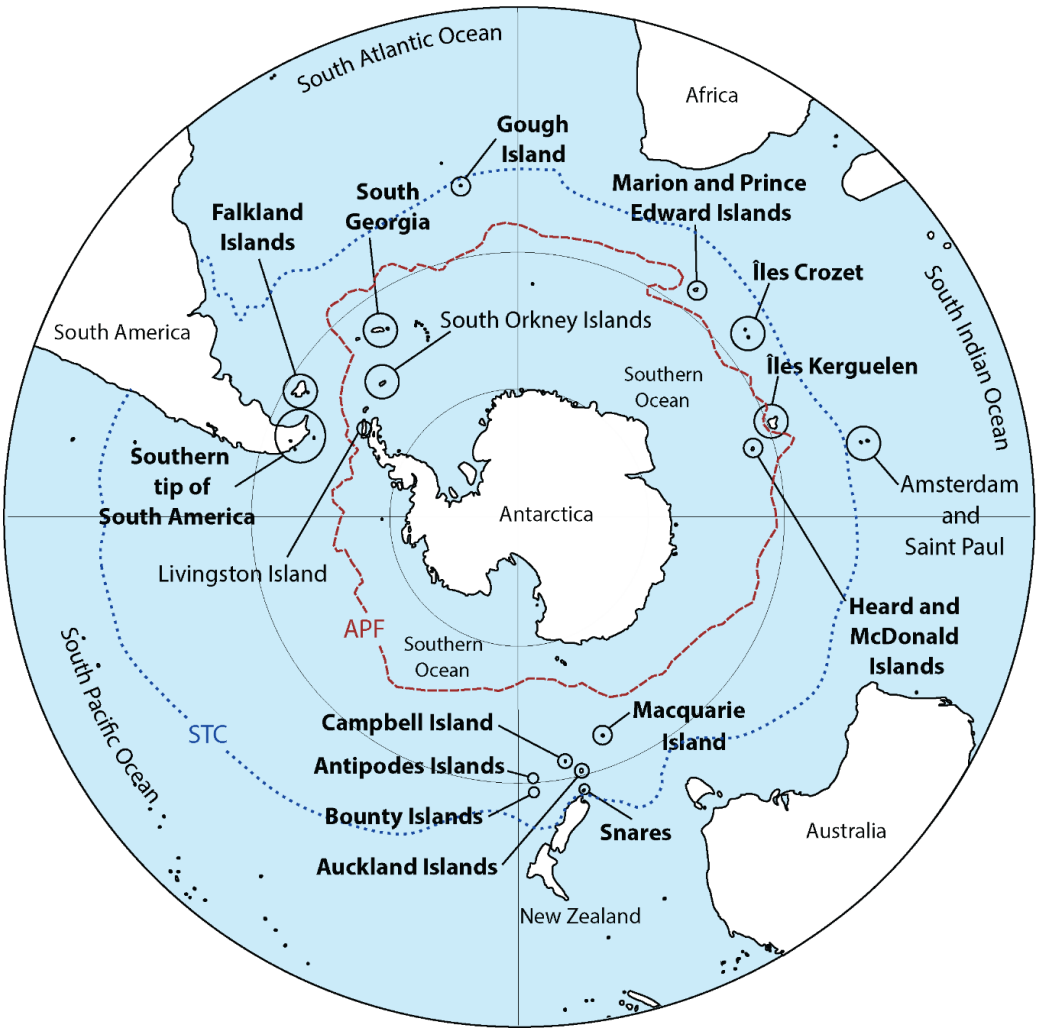

Figure 1. Map of geographic area that we consider the 'sub-Antarctic' region in this review. We here interpret the sub-Antarctic region (**bold** text) to be the area classically delineated by Holdgate (1970) and used widely (Chown & Convey, 2006; Terauds *et al.*, 2012; Convey, 2013), but with a number of additions based on clear biogeographical links (Muñoz *et al.*, 2004; Fraser *et al.*, 2009; Nikula *et al.*, 2010). The classic zonation refers to Prince Edward, Marion, Heard, McDonald, and Macquarie Islands, as well as South Georgia, Îles Kerguelen, Îles Crozet, but we include the southern tip of South America, the New Zealand sub-Antarctic Bounty, Campbell, Auckland, Snares and Antipodes Islands, and the Falkland Islands. The delineation of the sub-Antarctic marine region is inherently more difficult (Terauds *et al.*, 2012), but is traditionally considered to occur between the Antarctic Polar Front (APF) and the Sub-Tropical Convergence (STC) (Fraser *et al.*, 2012). APF and STC locations have been modified from Orsi, Whitworth & Nowlin (1995) and Chapter 13 of Talley *et al.* (2011). New research from Park *et al.* (2014) suggests placing the APF to the south of Îles Kerguelen. Coastline shapefiles downloaded from naturalearthdata.com, version 3.0.0.

With the advent of molecular techniques, the situation has changed profoundly, as it has for systems elsewhere, with a suite of new tools now available to investigate connectivity both within and between islands. Genetic data are providing insights that are changing perceptions of connectivity in the sub-Antarctic, showing that dispersal has clearly taken place extensively among islands, but that ongoing gene flow is relatively rare. Perhaps most significantly, whereas the Antarctic continent was long considered to be effectively biologically isolated, recent work has provided evidence for multiple historic dispersal events by several taxa between the sub-Antarctic islands and the Antarctic continent (e.g. in mites: Mortimer *et al*., 2011). In addition, for some terrestrial species, dispersal has sometimes occurred against the prevailing westerly winds and currents (Grobler *et al*., 2011*b*; Chown & Convey, 2016), in contrast to predominately eastward dispersal of most taxa (Fell, 1962; Sanmartín, Wanntorp & Winkworth, 2007; Waters, 2008).

The molecular genetic data therefore suggest that interpretations of biogeographic processes and patterns in the region, which have until now been based largely on species occurrence records (e.g. Fell, 1962; Gressitt, 1970; Dreux & Voisin, 1987, 1989; McInnes & Pugh, 1998; Greve *et al*., 2005), are in need of revision. For such revision to be an improvement on current knowledge, however, a clear understanding is required of the extent and reliability of the molecular work for marine and terrestrial taxa, the key outcomes from this work at both small and large spatial extents, and the ways in which these outcomes inform previous work. We make a start here by providing a synthetic review of recent molecular studies in the region.

2.3 Mechanisms of dispersal in the sub-Antarctic

Dispersal is a critical process in the sub-Antarctic where thousands of kilometres of open ocean separate small landmasses. Several key dispersal mechanisms operate in the region (Barnes *et al.*, 2006), including wind, ocean currents, transport with mobile animals (zoochory) and humans (anthropogenic transport). These mechanisms are being altered by changing environmental conditions and human activity [winds (Thompson & Solomon, 2002; Gillett & Thompson, 2003), oceanic currents (Klinck & Smith, 1993; Fyfe & Saenko, 2005, 2006), anthropogenic transport (Frenot *et al.*, 2005; Lebouvier *et al.*, 2011; le Roux *et al.*, 2013)], with potentially major ramifications for the connectivity of sub-Antarctic ecosystems.

Movement of biota *via* wind (Gressitt, Leech & O'Brien, 1960; Gressitt *et al*., 1961; Marshall, 1996; Skotnicki, Ninham & Selkirk, 2000; Muñoz *et al.*, 2004; Barnes *et al.*, 2006; Hawes *et al.*, 2007) and oceanic currents [see 2.4 and reviews by Waters (2008) and Fraser *et al.* (2012)] have often been used to explain biogeographic patterns in the sub-Antarctic and the greater Antarctic region. In the sub-Antarctic, aerial transfer has been shown to facilitate the dispersal of plants (Muñoz *et al.*, 2004) and some invertebrates (Hawes & Greenslade, 2013), including moths (Greenslade *et al.*, 1999; Convey, 2005), and over restricted ranges for some Collembola (Hawes *et al.*, 2007). Large animals with active dispersal, such as seabirds, are also influenced by strong winds in the sub-Antarctic (e.g. the foraging ecology of albatross: Weimerskirch *et al.*, 2000, 2012). Microorganisms, which disperse aerially in the Antarctic (Hughes *et al.*, 2004; Pearce *et al.*, 2009, 2010; Bottos *et al.*, 2014), are also probably transported around the sub-

Antarctic with the strong circumpolar winds. These winds help to drive the Antarctic Circumpolar Current (ACC), which travels eastward around the Southern Ocean, and is the strongest ocean current globally (Barker & Thomas, 2004). A wide range of species are inferred to have dispersed around the sub-Antarctic in the path of the ACC, for example the squid *Onykia ingens* (previously known as *Moroteuthis ingens*) (Sands, Jarman & Jackson, 2003), a range of both buoyant and non-buoyant macroalgal species (Fraser *et al.*, 2009, 2013; Macaya & Zuccarello, 2010), and epifaunal organisms associated with algae such as the crustaceans *Limnoria stephenseni* and *Parawaldeckia kidderi* (Nikula *et al.*, 2010). Indeed, the ACC is inferred to have enabled coastal marine organisms to recolonise much of the sub-Antarctic postglacially, as sea ice retreated south (Fraser *et al.*, 2012).

Zoochory and anthropogenic transport have increasingly been identified as important mechanisms of dispersal in both the sub-Antarctic and the greater Antarctic region. Birds are likely to have facilitated movements of many species, including algae, protozoans, and invertebrates such as springtails, flies, mites, lice and spiders which have all been found in association with seabird plumage (Schlichting, Speziale & Zink, 1978; Krivolutsky, Lebedeva & Gavrilo, 2004). In addition, human-mediated transport of organisms is known to facilitate the introduction of alien species to the sub-Antarctic (Frenot *et al.*, 2001). Introductions may be inter- (Lee & Chown, 2009*a*, 2009*b*) and intra-regional (Lee & Chown, 2011), and generally result in founder populations with low diversity (Lee *et al.*, 2007, 2009; Myburgh *et al.*, 2007; van Vuuren & Chown, 2007).

Many of these mechanisms of dispersal in the sub-Antarctic are changing, influencing the rate and efficacy of movements, as well as the likelihood of establishment. For example, wind systems are currently intensifying in the sub-Antarctic (Thompson & Solomon 2002; Gillett & Thompson, 2003), with major implications for biota such as wandering albatross (*Diomedea exulans*) (Weimerskirch *et al.*, 2012). Because wind systems drive the ACC, changes therein are linked to an increase in strength of circulation and a gradual southward migration of the ACC and associated fronts (Klinck & Smith, 1993; Fyfe & Saenko, 2005, 2006). The position and strength of the ACC and associated fronts changed dramatically over long (tens to hundreds of thousands of years) (Gersonde *et al.*, 2005; Kemp *et al.*, 2010) and short (decadal) (Sallée, Speer & Morrow, 2008) timescales, and such changes have the potential to connect or disconnect sub-Antarctic populations. Increasing human activity in the region also poses a risk of increased anthropogenic transport of organisms into and around the sub-Antarctic, which is likely to impact indigenous biota (Frenot *et al.*, 2005; Lebouvier *et al.*, 2011; le Roux *et al.*, 2013). Because increasing temperatures in the greater Antarctic region are also reducing barriers to establishment (Frenot *et al.*, 2005; Aronson *et al.*, 2011; Lebouvier *et al.*, 2011; Chown *et al.*, 2012), changes to dispersal processes will interact with these changing thermal conditions to affect connectivity in the sub-Antarctic.

2.4 Dispersal of sub-Antarctic organisms over small and large scales

Molecular studies investigating connectivity patterns in the sub-Antarctic have been undertaken most commonly at intra-island scales for terrestrial organisms (particularly on Marion Island) (e.g. Mortimer & van Vuuren, 2007; Myburgh *et al*., 2007; Born *et al.*,

2012; Mortimer *et al.*, 2012) and within restricted marine regions (e.g. Shaw, Arkhipkin & Al-Khairulla, 2004; Damerau *et al.*, 2014). Larger-scale, inter-island and even circumpolar studies are, however, becoming increasingly common (e.g. Fraser *et al.*, 2009; Nikula *et al.*, 2010; Nikula, Spencer & Waters, 2012; Mortimer *et al.*, 2011; González-Wevar *et al.*, 2016*a*, *b*).

2.4.1 Intra-island studies

Many terrestrial invertebrate taxa in the sub-Antarctic show little evidence of ongoing genetic connectivity even within islands (Table S1). Although the widespread distributions of many taxa indicate that dispersal events have occurred, gene flow is uncommon judging by the extent of genetic structuring at intra-island scales. For example, genetic sub-structuring at intra-island scales has been observed in mites (Mortimer & van Vuuren, 2007; Mortimer *et al.*, 2012), springtails (Myburgh *et al.*, 2007; McGaughran *et al*., 2010*a*), weevils (Grobler *et al.*, 2006) and in the chironomid midge *Belgica albipes* on Île de la Possession (Allegrucci *et al.*, 2012). Such fine-scale patterns of genetic diversity can provide insights into the physical processes affecting sub-Antarctic organisms. On Marion Island, high haplotype diversity and star-like haplotype network patterns (suggesting demographic expansion) have been found in indigenous mites and springtails, with structure apparently being driven by volcanic and glaciation events, and by current environmental variability (Mortimer & van Vuuren, 2007; Myburgh *et al.*, 2007; McGaughran *et al*., 2010*a*; Mortimer *et al.*, 2012) (Fig. 2). Earlier studies on Marion Island suggested that an east/west divide exists in population processes, and that sites such as Kildalkey Bay (eastern side) contain populations that are similarly distinct in both mite

and springtail species [from a western population in mites (Mortimer & van Vuuren, 2007); from most other populations in two springtail species (Myburgh *et al.*, 2007)]. Furthermore, studies of springtails have found that individuals from the same high-altitude site share a large number of haplotypes with other populations, suggesting the location was a glacial refugium (Katedraalkrans: central refugium in Fig. 2) (Myburgh *et al*., 2007; McGaughran *et al*., 2010*a*). Eastern and western populations may also differ in mean metabolic rates, but a previous study was limited by small sample sizes (McGaughran *et al.*, 2010*a*). A more recent study has clarified patterns, showing populations on the eastern and south-western sides of Marion Island have different patterns of intraspecific genetic diversity, due to differences in long-term survival and demographics driven by volcanic eruptions and glacial periods (Mortimer *et al.*, 2012), and possibly by contemporary climatic differences (McGaughran *et al*., 2010*a*) (Fig. 2; see Hall, Meiklejohn & Bumby, 2011 and Mortimer *et al*., 2012 for details of the geological and glaciological histories of the island).

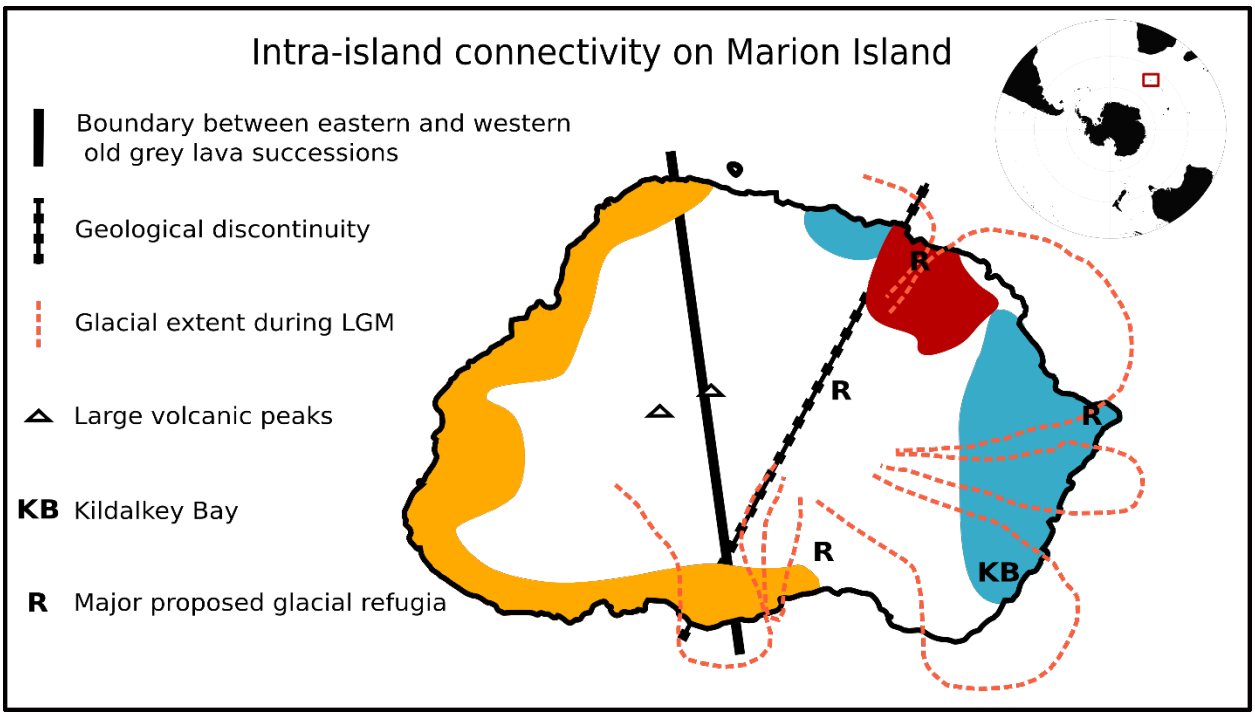

Figure 2. Fine-scale patterns of genetic structure for terrestrial taxa on Marion Island. Yellow regions contain populations that show little genetic divergence from one another and high inferred migration rates (Mortimer *et al.*, 2012), while blue indicates regions where populations show higher genetic divergence from one another and lower inferred migration rates (Mortimer *et al.*, 2012). Red indicates regions of lower genetic divergence (Mortimer *et al.*, 2012), with higher inferred rates of dispersal that are more typical of the south-western side of the island (Mortimer *et al.*, 2008, 2012). Major glaciological and geological features are also shown (see Hall *et al*., 2011 and Mortimer *et al*., 2012 for detailed descriptions). LGM, Last Glacial Maximum.

Molecular investigations of the cushion plant *Azorella selago* (Apiaceae) at Marion Island have likewise indicated high diversity and population sub-structure, with generally low levels of gene flow (Mortimer *et al.*, 2008; Born *et al.*, 2012). Intra-island gene flow is probably influenced by variable local wind patterns affecting short-distance dispersal (Born *et al.*, 2012). Gene flow in *A. selago* also appears to be influenced by life-history traits (such as reproductive strategy) and landscape features (such as topography and glacial and geological events) that affect dispersal efficiency, and environmental factors

that influence plant establishment (Mortimer *et al.*, 2008; Born *et al.*, 2012). Therefore, as for invertebrates, cushion plant population sub-structure can result from variability in small-scale dispersal capabilities (predominantly dictated by wind dynamics: Born *et al.*, 2012) and is also influenced by vicariance due to past climatic events (Mortimer *et al.*, 2008). These results concur with a growing body of evidence that suggests that flowering plant taxa may have survived long-term on sub-Antarctic islands, including during the LGM, rather than recolonising the region post-glacially (Scott & Hall, 1983; Van der Putten *et al.*, 2010; Bartish *et al*., 2012).

Molecular studies of terrestrial species elsewhere in the world have revealed similar, considerable intra-island sub-structure, consistent with expansion from refugial populations (e.g. the Canary and Hawaiian Islands: Juan *et al.*, 2000 and references within) and fine-scale recolonisation and extinction events (Emerson *et al*., 2006), although haplotype diversities appear to be high on Marion Island (Mortimer & van Vuuren, 2007; Myburgh *et al.*, 2007; Mortimer *et al*., 2012). For example, a phylogeographic study of woodlouse hunter spiders (*Dysdera verneaui*) across one of the Canary Islands has revealed genetic patterns consistent with recolonisation events from refugial populations following volcanic cycles (Macías-Hernández *et al.*, 2013), similar to patterns found for terrestrial biota on volcanic Marion Island (Mortimer & van Vuuren, 2007; Myburgh *et al.*, 2007; Mortimer *et al.*, 2012). On La Palma in the Canary Islands, the population structure of weevils (*Brachyderes rugatus rugatus*) revealed a more complex intra-island phylogeographic history than expected, indicating historic fragmentation, range expansion, and secondary contact events (Emerson *et al.*, 2006).

Though on a much smaller scale, similar complexity was also found within *Azorella selago* mats on Marion Island, which comprise multiple plants in a structure that results from multiple local fragmentation, expansion and extinction events (Mortimer *et al*., 2008; Cerfonteyn *et al.*, 2011).

In the marine realm, molecular studies also indicate that gene flow is influenced by passive dispersal mechanisms (in this case, ocean currents and oceanographic features) and life-history traits (most notably dispersal abilities). For example, a fine-scale study of the brooding sea urchin *Abatus cordatus* around Îles Kerguelen has shown substantial spatial structuring, even over tens of metres, highlighting the effect of naturally low dispersal abilities on local population connectivity (Ledoux *et al.*, 2012). Conversely, most studies have shown little or no fine-scale genetic structure within populations of highly mobile taxa such as fish (*Champsocephalus gunnari* and *Notothenia rossii*: Williams, Smolenski & White, 1994; Duhamel *et al.*, 1995) and within the Falkland Islands population of gentoo penguins (*Pygoscelis papua*) (although the Shallow Harbour colony was found to be differentiated: Levy *et al*., 2016). However, while a recent fine-scale study of king penguins (*Aptenodytes patagonicus*) at Île de la Possession found a high degree of population panmixia, the authors suggested that genetic structure remained heterogeneous across the colony (Cristofari *et al*., 2015). This heterogeneity was thought to be a result of variable habitat quality and rates of local philopatry driving patches of higher and lower gene flow (Cristofari *et al*., 2015). Patagonian toothfish (*Dissostichus eleginoides*) also only appear to show significant spatial genetic structuring on large spatial scales (thousands of kilometres, see 2.4.2) (Smith & McVeagh, 2000; Appleyard,

Ward & Williams, 2002; Shaw *et al.*, 2004; Toomey *et al.*, 2016) and Antarctic octopus *Pareledone turqueti* populations are genetically homogeneous around South Georgia, with wider structure due to deep water (Allcock *et al.*, 1997; Strugnell *et al.*, 2012). However, the extent of variation observed in toothfish populations in different studies and across different scales was strongly influenced by marker type and sample size (see discussion in Toomey *et al*., 2016), emphasising the importance of using high-resolution data and large sample sizes for accurate assessment of population connectivity.

Indeed, a weakness of the majority of the aforementioned intra-island studies (both for the sub-Antarctic and elsewhere) is that they were based typically on either a single gene, or just a few genes (see Table S2), and thus had limited power to resolve fine-scale population structure or even, in some cases, to resolve phylogenetic relationships among closely related taxa. By contrast, genome-wide single nucleotide polymorphism (SNP) data, which are now easily within reach for most groups [e.g. see recent genomic research from elsewhere in the world on invertebrates (Misof *et al*., 2014; Dussex, Chuah & Waters, 2015; Ebel *et al*., 2015; Darwell, Rivers & Althoff, 2016; Kjer *et al*., 2016), plants (Eaton & Ree, 2013; Wang *et al*., 2013; Hipp *et al*., 2014; Zimmer & Wen, 2015) and aquatic insects (Rutschmann *et al*., 2017)] would improve the resolution of molecular studies and their power to detect fine-scale relationships in the sub-Antarctic. Genomic data have already been used to clarify patterns in recently diverged species (Eaton & Ree, 2013), those that went through rapid evolution on large scales (Ebel *et al*., 2015; Darwell *et al*., 2016), and those that exhibit variable structure on fine scales (Dussex *et al*., 2015), highlighting the benefit that high-resolution genomic data can bring to phylogeographic

studies. There are, as yet, only a few examples of genomic studies from the sub-Antarctic, but those that have been published also emphasise the power of such data over traditional approaches. As a case in point, a recent study of introduced brown rats (*Rattus norvegicus*) across South Georgia found vastly different genetic patterns depending on whether mitochondrial DNA (mtDNA) or SNP data were used (Piertney *et al*., 2016). While the mtDNA data only resolved two haplotypes across the entire island, probably reflecting two separate colonisation events, the SNP data revealed fine-scale population sub-structure, inferred to have been driven by contemporary isolation by glacial barriers (Piertney *et al*., 2016). Likewise, mtDNA and chloroplast DNA (cpDNA) analysis of southern bull-kelp (*Durvillaea antarctica*) throughout the high latitudes showed no genetic diversity among and within most sub-Antarctic islands, with a single haplotype dominating (Fraser *et al*., 2009). By contrast, recent SNP data revealed fine-scale population structure for bull-kelp on Chatham Island, and allowed resolution of long-standing taxonomic questions for the genus (Fraser *et al*., 2016*b*).

2.4.2 Inter-island studies

The last decade has seen an increase in the number of studies using genetic methods to investigate the relationships of sub-Antarctic organisms among multiple islands (Tables S1, S2; Fig. 3). Molecular evidence indicates that long-distance dispersal has taken place in several groups. Historic movements between islands (and to the Antarctic Peninsula in some cases) has occurred on multiple occasions for taxa such as springtails, weevils and mites (Stevens *et al.*, 2006; McGaughran, Stevens & Holland, 2010*b*; Grobler *et al*.,

2011*b*; Mortimer *et al.*, 2011), and two separate colonisation events are evident for *Pseudhelops* beetles on the Antipodes Islands (Leschen *et al.*, 2011).

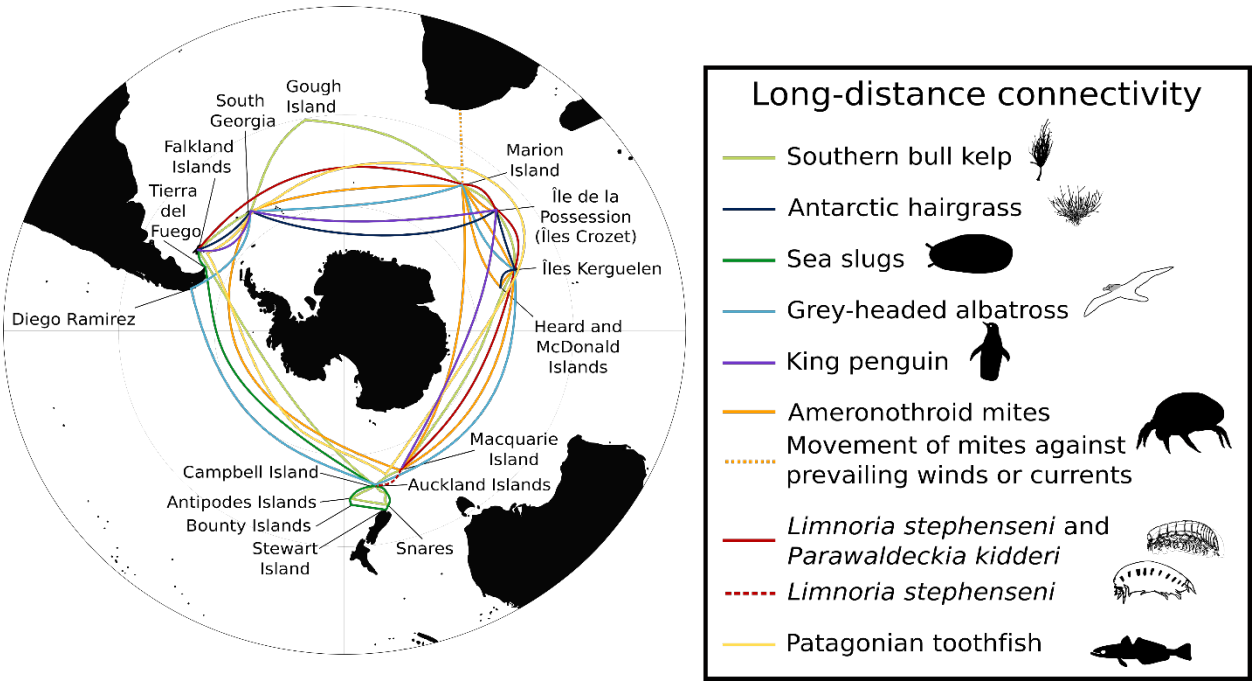

Figure 3. Examples of species that have achieved long-distance dispersal in the sub-Antarctic, with lines showing inferred dispersal routes: southern bull-kelp (*Durvillaea antarctica*) (Fraser *et al.*, 2009), Antarctic hairgrass (*Deschampsia antarctica*) (van de Wouw *et al.*, 2008), sea slugs (*Onchidella* sp.) (Cumming *et al.*, 2014), grey-headed albatross (*Thalassarche chrysostoma*) (Burg & Croxall, 2001), king penguins (*Aptenodytes patagonicus*) (Clucas *et al.*, 2016), Ameronothroid mites (Mortimer *et al.*, 2011), two species of epifaunal crustaceans (*Limnoria stephenseni* and *Parawaldeckia kidderi*) (Nikula *et al.*, 2010), and the Patagonian toothfish (*Dissostichus eleginoides*) (Smith & McVeagh, 2000; Toomey *et al.*, 2016).

The presence of invertebrates on young volcanic islands, and close genetic relationships found between species of different islands, further indicates that trans-oceanic dispersal must have occurred, and molecular dating indicates this usually happened shortly after the emergence of habitat (Grobler *et al.*, 2006; Stevens *et al.*, 2006; van Vuuren *et al.*, 2007;

McGaughran *et al.*, 2010*b*; Mortimer *et al.*, 2011). A recent study from Marion Island, recording two new invertebrates inferred to have arrived recently *via* natural dispersal likewise supports the importance of ongoing long-distance movements of invertebrates (Lee *et al.*, 2014). Ecological factors can, however, have a major influence on a species' ability to achieve long-distance dispersal. For example, differences in habitat use by weevils may explain historic differences in the frequency of their dispersal between Marion and Prince Edward Islands, a distance of only 19 km (Grobler *et al.*, 2006, 2011*a*). *Ectemnorhinus* weevils, which seem to have dispersed between the islands several times, occur on vegetation that is more commonly occupied by birds, whereas *Bothrometopus huntleyi* frequents rock surfaces where birds are rare and therefore dispersal may be less likely (Grobler *et al*., 2011*a*).

These findings align with those from other parts of the world, where colonisation has been inferred or observed to have occurred rapidly after island formation or emergence of habitat, even for relatively remote sites [e.g. plants and animals on Surtsey, Iceland (Fridriksson, 1989), weevils on the Canary Islands (Emerson, Oromí & Hewitt, 2000), rapid recovery of mantis shrimp (*Haptosquilla* sp.) following volcanic eruption on Krakatau (Barber, Moosa & Palumbi, 2002), the progressive colonisation of *Metrosideros* plants across islands in the Hawaiian archipelago as they emerged from the sea (Percy *et al*., 2008)]. Moreover, they are in keeping with molecular work from other islands (reviewed by Gillespie *et al*., 2012) showing that multiple, independent colonisations, over substantial periods, are the norm, supporting a prominent role for dispersal in the sub-Antarctic as is the case elsewhere (Waters, 2008). For example, genetic data from

Calathus beetles (Emerson, Oromí & Hewitt, 1999; Juan *et al.*, 2000) suggests the genus undertook multiple independent colonisations of the Canary Islands. For sub-Antarctic taxa that are flightless, such as the weevils, springtails and mites, exactly how dispersal has occurred remains largely the subject of speculation, but there is nonetheless overwhelming evidence that trans-oceanic dispersal has happened in all of these groups.

Molecular studies are also providing evidence for long-distance dispersal having facilitated retreat to, or postglacial recolonisation of the sub-Antarctic by some seemingly sedentary organisms, although many do not show evidence of ongoing connectivity. Long-distance retreat of an ancestor to the sub-Antarctic to escape extinction following the glaciation of the Antarctic continent has been proposed for the origin of a monotypic genus of flowering cushion plant endemic to Îles Kerguelen (*Lyallia kerguelensis*), but ongoing movement in the species is restricted (Wagstaff & Hennion, 2007). Likewise, despite being seemingly ill-suited to long-distance dispersal, it is clear that multiple long-distance dispersal events are responsible for the colonisation of sub-Antarctic islands by *Pringlea antiscorbutica* (Bartish *et al*., 2012), Antarctic hairgrass (*Deschampsia antarctica*) (van de Wouw, Dijk & Huiskes, 2008) and across the southern continents and islands for the genera *Azorella, Laretia,* and *Mulinum* (Nicolas & Plunkett, 2012), although contemporary inter-island gene flow appears to be limited in all organisms. For example, a stepping-stone-like pattern of recolonisation was inferred for Antarctic hairgrass, which likely sought refuge on ice-free regions of the Îles Crozet or Îles Kerguelen during the LGM before expanding and recolonising other Indian Ocean islands, such as Heard Island, which was completely glaciated at the LGM (van de Wouw

et al., 2008). Genetic investigation suggests limited contemporary gene flow between Indian Ocean islands for the hairgrass, however, and some populations are already showing signs of genetic isolation and drift (van de Wouw *et al.*, 2008). Likewise, genetic structure of the limpet genus *Nacella* in the region has been heavily influenced by glaciation events, with evidence for historic bottlenecks, postglacial expansions and persistence during glacial events (González-Wevar *et al.*, 2010, 2012*b*, 2013, 2016*a*, *b*, *c*, *d*). Historic long-distance oceanic movements must have occurred for colonisation events, but movements have apparently not been sufficient to maintain current connectivity between provinces, and in some cases have led to speciation (González-Wevar *et al.*, 2010, 2016*a*, *b*, *c*, *d*).

Some sub-Antarctic shallow-marine taxa, such as southern bull-kelp (*Durvillaea antarctica*) and giant kelp (*Macrocystis pyrifera*), are inferred to have recolonised the sub-Antarctic postglacially (Fraser *et al.*, 2009; Macaya & Zuccarello, 2010). For these buoyant marine species, passive oceanic transport of detached, floating plants is the predominant long-distance dispersal mechanism. Furthermore, they act as rafts in the sub-Antarctic, transporting organisms that lack a pelagic stage or have a pelagic stage of short duration, those that rely on kelp habitats [e.g. crustaceans (Nikula *et al.*, 2010, 2012; Fraser, Nikula & Waters, 2011), molluscs (Helmuth, Veit & Holberton, 1994; Donald, Keeney & Spencer, 2011; Nikula *et al.*, 2012; Cumming *et al.*, 2014), and limpets (Reisser *et al.*, 2011)], and non-buoyant macroalgal species, which would otherwise have poor dispersal capacity (Fraser *et al.*, 2013). Molecular evidence (Amplified Fragment Length Polymorphism, AFLP) supports gene flow between populations of some sub-

Antarctic invertebrates *via* rafting (Nikula *et al.*, 2012), whereas other species show phylogeographic structure implying limited recent gene flow (Donald *et al.*, 2011; Reisser *et al.*, 2011). The extent of gene flow between trans-oceanic populations depends on the availability of rafts in the region, the rafting host species, the predisposition and ability of the species to raft, as well as the frequency and consistency of movement (Helmuth *et al.*, 1994; Smith, 2002; Thiel & Gutow, 2005; Thiel & Haye, 2006; Donald *et al.*, 2011; Nikula *et al.*, 2012). Lack of gene flow between populations of dispersive species can also be due to the influence of density-dependent processes, whereby dispersing individuals may have limited or no genetic influence when they arrive at shores already densely populated with conspecifics. If some populations go extinct, however, their habitat becomes available for colonisation by immigrants. Thus, dispersal is critical for colonisation or recolonisation of new territory, but is often ineffective at maintaining gene flow among established populations [see reviews by Waters, Fraser & Hewitt (2013) and Fraser, Banks & Waters (2015)].

Patterns of genetic connectivity in sub-Antarctic benthic taxa suggest far more complex interactions with the ACC than traditionally thought. Increasingly, molecular studies are showing that long-distance dispersal with ocean currents has been vital for benthic colonisation, but that few organisms remain connected *via* this mechanism. For example, a molecular study of the strictly benthic isopod *Septemserolis septemcarinata* has found evidence for recent dispersal between remote islands. This colonisation was likely facilitated by passive dispersal *via* the ACC, but rare movements are too asymmetric and episodic to ensure ongoing gene flow and connectivity (Leese, Agrawal & Held, 2010).

Genetic structuring of sea cucumbers around the sub-Antarctic and greater Antarctic (O'Loughlin *et al.*, 2011) and the genetic isolation of populations of *Cellana strigilis* (limpets) species complex despite its distribution across the New Zealand sub-Antarctic islands (Goldstien, Gemmel & Schiel, 2009; Reisser *et al.*, 2011) also reflects the role of ocean currents in facilitating colonisation of new territory, but with regional diversification resulting from limited ongoing gene flow.

Circumpolarity of species and population panmixia was long thought to be a common pattern of Southern Ocean benthic taxa; a result of the strength of the ACC and the lack of continental barriers to movement (Burton, 1932; Hedgpeth, 1969, 1971; Koltun, 1970; Sarà, *et al*., 1992; Clarke & Johnston, 2003), although some regionalisation had previously been recognised (Hedgpeth, 1969; Arntz, Brey & Gallardo, 1994; Arntz, Gutt & Klages, 1997). Conclusions from molecular studies of benthic organisms in the sub-Antarctic and greater Antarctic regions are challenging these long-held views about the processes affecting Southern Ocean connectivity. These genetic studies are showing that the dispersal capacity of a species (see Thatje, 2012 for an Antarctic review) and environmental conditions (e.g. temperatures, glacial cycles and oceanic features) have driven regional diversification in benthic taxa, including cryptic speciation in some cases (Wilson *et al.*, 2007; Wilson, Schrödl & Halanych, 2009; Hunter & Halanych, 2008; Thornhill *et al.*, 2008; Krabbe *et al.*, 2010; Allcock *et al.*, 2011; Arango, Soler-Membrives & Miller, 2011; Baird, Miller & Stark, 2011; Hemery *et al.*, 2012; Weis *et al.*, 2014; Harder, Halanych & Mahon, 2016).

Evidence from a growing number of species also indicates that occasional dispersal has occurred across the APF (between sub-Antarctic and Antarctic regions) for some taxa on long (evolutionary) timescales, but that little ongoing gene flow occurs across the front, suggesting that the APF might limit latitudinal movement of species enough to act as an effective barrier to connectivity (e.g. Page & Linse, 2002; Hunter & Halanych, 2008; Thornhill *et al.*, 2008; Wilson *et al.*, 2009; Krabbe *et al.*, 2010; O'Loughlin *et al.*, 2011; O'Hara *et al.*, 2013; Poulin *et al*., 2014; Hüne *et al*., 2015; González-Wevar *et al.*, 2012*a*, 2016*b*, *c*). New evidence from observations of floating kelp at sea, however, indicates that the APF can be readily traversed by some marine organisms, suggesting that the absence of many sub-Antarctic taxa in the Antarctic may be largely due to environmental differences on either side of the front (Fraser *et al.*, 2016*a*).

There appears to be limited gene flow among benthic marine populations in southern South America and the sub-Antarctic islands, regardless of dispersal capacity (brooding or broadcasting characteristics). For example, South American and South Georgian populations of the broadcast spawning ribbon worm *Parborlasia corrugatus* were found to be genetically distinct, despite a high dispersal capacity and population panmixia across sub-Antarctic and Antarctic regions (Thornhill *et al.*, 2008). Likewise, a molecular study of the brooding shallow-water isopod *Serolis paradoxa* inferred no gene flow between populations in the Falkland Islands and the southern tip of South America (Leese *et al.*, 2008) and two recent studies of a sea spider genus (*Pallenopsis*) found populations in the Falklands, Antarctica and South America were genetically distinct (Weis *et al.*, 2014; Harder *et al.*, 2016). Despite this, many molecular studies do suggest connections between

South America and the sub-Antarctic, for example sea slugs (*Onchidella*) share a haplotype between New Zealand sub-Antarctic islands and southern Chile (Cumming *et al.*, 2014). However, many of these studies are based on genes with slow mutational rates (e.g. mtDNA), and patterns may reflect recolonisation from ice-free areas following glacial cycles rather than evidence of ongoing gene flow (see Fraser *et al*., 2009).

As might be expected, genetic structure is generally less marked in pelagic than benthic species in the Southern Ocean [see Rogers (2007) and Rogers *et al.* (2012) for reviews, and Van de Putte *et al.* (2012*a*) for an example]. For example, some fish (such as the humped rockcod (*Gobionotothen gibberifrons*), the Antarctic silverfish (*Pleuragramma antarcticum*) and a lanternfish (*Electrona antarctica*)) show little to no significant genetic structuring across broad spatial scales in the Southern Ocean, and recent population expansions have been inferred (Near, Pesavento & Cheng, 2003; Zane *et al.*, 2006; Matschiner, Hanel & Salzburger, 2009; Near *et al*., 2012; Van de Putte *et al*., 2012*b*). Despite this, structure remains evident in some pelagic species, such as krill populations between South Georgia and the Weddell Sea (Zane *et al.*, 1998). Likewise, regionalisation is evident in notothenioid fish species, especially over great distances (Volckaert, Rock & Van de Putte, 2012; Matschiner *et al.*, 2015). For example, though homogeneity has been found on smaller scales [e.g. between Heard and McDonald Islands (Appleyard *et al.,* 2002; Toomey *et al.*, 2016), between South Georgia and Shag Rocks (Appleyard *et al.,* 2002; Shaw *et al.*, 2004), within the West Indian Ocean sector (Appleyard, Williams & Ward, 2004), between Îles Kerguelen and Îles Crozet (Toomey *et al.*, 2016)], Patagonian toothfish are structured between oceanic regions (Indian, Pacific and Atlantic) of the

Southern Ocean (Smith & McVeagh, 2000; Toomey *et al.*, 2016). Genetic structure is evident between the South Georgia/Shag Rocks population and the Patagonian shelf/Falkland Islands population (Shaw *et al.*, 2004; Rogers *et al.*, 2006) and between Heard/McDonald Islands, Macquarie Island and Shag Rocks/South Georgia populations (Appleyard *et al.*, 2002). A more recent genetic study that included only one sub-Antarctic site found that differences in the strength of genetic structure in two fish species were due to the duration of planktonic early life stages, with shorter planktonic phases leading to stronger regional differentiation and longer phases leading to no significant structure (Young *et al*., 2015). Notothenioid fish dominate the diversity, abundance and biomass of the region and their history is punctuated with colonisations and expansions into areas after the opening of ecological niches followed by adaptation to new environments and subsequent diversification (Near *et al.*, 2012; Matschiner *et al.*, 2015; Papetti *et al.*, 2016), suggesting that dispersal capacity is not a limiting factor for gene flow.

Several causes are plausible for geographic structure in sub-Antarctic pelagic species despite planktotrophic larval phases or other dispersive stages. Structure may be driven by geographical isolation (Appleyard *et al.*, 2002; Toomey *et al.*, 2016), oceanographic features such as deep water troughs and the APF (Zane *et al.*, 1998; Smith & McVeagh, 2000; Appleyard *et al.*, 2002; Shaw *et al.*, 2004; Kuhn & Gaffney, 2006; Rogers *et al.*, 2006; Hüne *et al*., 2015; Toomey *et al.*, 2016) or the directionality of the ACC (Matschiner *et al.*, 2009), and life-history traits (Van de Putte *et al.*, 2012*a*; Young *et al*., 2015; Toomey *et al.*, 2016). For example, life-history differences may explain contrasting

patterns of genetic structure in two squid species in the sub-Antarctic; *Martialia hyadesi*, a species that appears to form reproductive stocks that admix during feeding (Brierley *et al.*, 1993) had strong population structure relative to *Onykia ingens*, a species whose egg and paralarval dispersal may facilitate a low level of gene flow across deeper water (Sands *et al.*, 2003). However, low levels of genetic variation were found in both squid species (Brierley *et al.*, 1993; Sands *et al.*, 2003) and both studies were conducted with traditional molecular techniques, which may mean sub-structure would be hard to detect [see Eaton & Ree (2013), Dussex *et al*. (2015), Ebel *et al*. (2015) and Darwell *et al*. (2016) for examples of studies using next-generation sequencing to clarify patterns of shallow phylogenetic relationships]. More broadly for the greater Antarctic region, oceanographic features and life-history traits of the Antarctic toothfish (*Dissostichus mawsoni*) are likely to underpin the genetic differentiation of populations in the continental Antarctic that has been revealed with SNPs (Kuhn & Gaffney, 2008).

Complete panmixia of seabird and marine mammal populations might be expected in such highly dispersive organisms. Indeed this appears true of species such as the grey-headed albatross (*Thalassarche chrysostoma*) and wandering albatross (*Diomedea exulans*) (Burg & Croxall, 2001, 2004; Friesen, Burg & McCoy, 2007; Milot, Weimerskirch & Bernatchez, 2008; but see discussion about extent of contemporary gene flow in Milot *et al*., 2008), leopard seals (*Hydrurga leptonyx*) (Davis *et al.*, 2008) and king penguins (Clucas *et al*., 2016) in the sub-Antarctic, Adélie penguins (*Pygoscelis adeliae*) in the maritime and continental Antarctic (Roeder *et al*., 2001; Clucas *et al*., 2014) and emperor penguins (*Aptenodytes forsteri*) around the continental Antarctic (Cristofari *et al*., 2016).

Among-population structure has, however, been detected at the inter-island scale in gentoo penguins (Clucas *et al*., 2014; Levy *et al*., 2016; Vianna *et al*., 2016), black-browed albatross (*Thalassarche melanophris*) (Burg & Croxall, 2001) and the white-chinned petrel (*Procellaria aequinoctialis*) (Techow, Ryan & O'Ryan, 2009) across the sub-Antarctic, despite high dispersal capacities in these species. Furthermore, high philopatry and differences in foraging distributions following long-distance colonisation are likely the reason for the isolation of distinct evolutionary lineages in the wandering albatross species complex (Burg & Croxall, 2004; Milot *et al*., 2008; Rains, Weimerskirch & Burg, 2011).

A review of the causes of phylogeographic structure in flighted seabirds (Friesen *et al.*, 2007) has predominantly implicated non-breeding (failed or pre-breeding) movements and foraging behaviour in the population genetic structure of seabirds. Differences in foraging grounds of albatross species (Burg & Croxall, 2001), and in winter foraging behaviours of three species of penguin (Clucas *et al.*, 2014) probably underpin population structure differences. While gentoo penguins remain close to their colonies during winter periods and exhibit significant genetic structuring, Adélie and (to a lesser extent) chinstrap (*Pygoscelis antarctica*) penguins forage hundreds to thousands of kilometres away and show limited or no population structure (Clucas *et al.*, 2014), as do sub-Antarctic king penguin populations separated by thousands of kilometres (based on SNP data: Clucas *et al*., 2016). Studies have found that gentoo penguins have historically colonised the greater Antarctic region (likely *via* the ACC in a clockwise manner: Vianna *et al*., 2016), traversing the APF and its associated temperature and salinity gradients in

the process. However the front, in conjunction with the species' restricted, coastal foraging habits, high philopatry and the great distances between islands, has restricted ongoing gene flow both between the Antarctic continent and the sub-Antarctic islands, and between the sub-Antarctic islands themselves (de Dinechin *et al*., 2012; Clucas *et al.*, 2014; Levy *et al.*, 2016; Vianna *et al*., 2016). Oceanographic features such as temperature and salinity differences across oceanic fronts are also probably responsible for speciation in the rockhopper penguin (*Eudyptes chrysocome sensu lato*) (Banks *et al*., 2006; Jouventin, Cuthbert & Ottvall, 2006; de Dinechin *et al*., 2009). Generally, population structure in penguins has been inferred to have been influenced by responses to past climatic fluctuations (Friesen *et al.*, 2007; Clucas *et al*., 2014), responses to different environmental conditions on either side of oceanic fronts (de Dinechin *et al*., 2009, 2012; Clucas *et al*., 2014, 2016), life-history traits (particularly foraging ecology) differing between species and across their range (de Dinechin *et al*., 2009, 2012; Levy *et al*., 2016) and density-dependent processes (Clucas *et al*., 2016). Variable responses to local habitat conditions such as habitat quality (Cristofari *et al*., 2015) and parasites might also influence seabird population structure, but these interactions have not yet been examined in detail (McCoy *et al.*, 2002; McCoy, Boulinier & Tirard, 2005*a*; Friesen *et al.*, 2007).

Evidence for life history (such as high philopatry and breeding biology) acting as a barrier to gene flow is also found in vagile marine animals in the sub-Antarctic (for a review of species movements over time, see Younger, Emerson & Miller, 2016). For example, life-history traits appear to dictate contemporary gene flow in marine mammals such as the southern elephant seal (*Mirounga leonina*) (Slade *et al.*, 1998; Hoelzel, Campagna &

Arnbom, 2001; Fabiani *et al*., 2003). In this species, long-distance foraging trips and changes in sea ice facilitated historic colonisation events (de Bruyn *et al.*, 2009), but contemporary gene flow appears constrained (Slade *et al.*, 1998; Hoelzel *et al*., 2001) by breeding-site fidelity, which is sex dependent (Fabiani *et al*., 2003) as in other seal species (e.g. Hoffman, Trathan & Amos, 2006). Conversely, no significant genetic differentiation has been found in leopard seals among Macquarie and Heard Islands, South Georgia, the Ross Sea, the Antarctic Peninsula and the South Orkney Islands, despite evidence for geographical variation in vocalisations and fidelity to feeding areas (Davis *et al.*, 2008). This lack of structure may be due to the tendency of leopard seals not to aggregate in large numbers (Davis *et al.*, 2008).

Almost all native mammals present in the sub-Antarctic region are marine (Convey, 2007, 2013) and many have extensive histories of heavy exploitation by humans (Bonner, 1984; Trathan & Reid, 2009). For example, fur seals (Otariidae) were extensively hunted during the late 18th and 19th centuries in the Southern Ocean. Uncontrolled exploitation severely reduced numbers, leading to population bottlenecks (Hoffman *et al.*, 2011). The cessation of such activities has led to an increase in seal numbers across the sub-Antarctic (Wynen *et al.*, 2000; Hoffman *et al.*, 2011; Bonin *et al.*, 2013). Recolonisation of such highly vagile, long-lived animals would be expected to create a pattern of very low genetic diversity. Generally, however, there has been consistently high genetic diversity discovered in rebounding fur seal populations in the sub-Antarctic, which can be attributed to the short duration of the bottleneck and survival of diverse refugial populations during sealing (Wynen *et al.*, 2000; Hoffman *et al.*, 2011; Bonin *et al.*, 2013).

Likewise, panmictic populations may be expected in recolonised parts of the distributional ranges of long-lived exploited animals [see Dickerson *et al.* (2010) and Lancaster, Arnould & Kirkwood (2010) for examples of genetic patterns of other recovering fur seal species], but population structure is evident in the sub-Antarctic fur seal (*Arctocephalus tropicalis*) and weak but significant population structure is evident in the Antarctic fur seal (*Arctocephalus gazella*). Genetic patterns are likely due to different exploitation and recolonisation histories (e.g. size of pre-sealing colonies, severity of bottleneck, location of source population and speed of recovery), the presence of pre-sealing genetic structure, the importance of relict populations, the large distances between colonies and sex-dependent migration rates (Wynen *et al.*, 2000; Hoffman *et al.*, 2011; Bonin *et al.*, 2013). Despite structuring, there is evidence for some contemporary gene flow occurring between geographically separated fur seal populations, for example between South Georgia and Livingston Island in the Antarctic fur seal (Bonin *et al.*, 2013). Some contemporary movement is explained by emigration from colonies that have reached carrying capacity (such as South Georgia: Bonin *et al.*, 2013).

Worldwide, the availability of whale genetic data is growing. Evidence available for the sub-Antarctic remains limited, but studies of killer whales (*Orcinus orca*) (LeDuc, Robertson & Pitman, 2008; Morin *et al.*, 2010) that included some samples from the region suggest that differences in foraging behaviour and prey choice, habitat preferences, vocalisations and social mechanisms have resulted in genetically differentiated populations both globally and within the greater Antarctic (see de Bruyn, Tosh & Terauds, 2013 for a review). Worldwide, these ‘types’ were considered sufficiently

distinct to constitute sub-species and species (Morin *et al.*, 2010). A more recent study using a phylogenomic approach to clarify and resolve shallow phylogenetic killer whale relationships across the world (although with very few samples from the sub-Antarctic) found some differences in phylogenies based on old *versus* new genetic techniques, but nonetheless concluded that phylogenetic and population structure was likely the result of social and behavioural isolation resulting from resource specialisation (Moura *et al*., 2015). Elevation of regional populations to subspecies status has also occurred for humpback whales (*Megaptera novaeangliae*), where a recent global study has found evidence for reproductive isolation of oceanic groups (Jackson *et al.*, 2014). In addition, a separate study has provided evidence for restricted gene flow between blue whales (*Balaenoptera musculus*) from Antarctic populations and those from Chilean and eastern tropical Pacific populations, and that these isolated breeding units may represent subspecies (Torres-Florez *et al.*, 2014). As with killer whales, restricted gene flow in humpback and blue whales has also been attributed to social structure (such as sex-dependent fidelity to breeding site), behavioural differences, differences in migratory and foraging behaviours, exploitation histories or vacariant (e.g. glacial) events (Jackson *et al.*, 2014; Torres-Florez *et al.*, 2014).

Studies of inter-island connectivity in the sub-Antarctic have often been limited by sample locations and sizes, and the investigation of only one or a few genes (see Table S2). As studies employing genomic data sets begin to emerge (Moura *et al*., 2015; Clucas *et al*., 2016; Fraser *et al*., 2016*b*; Toomey *et al*., 2016) comparisons with single-gene studies are possible, and are revealing patterns that the lower-resolution markers could not. For

example, a recent worldwide (including a few sub-Antarctic samples) comparative study of killer whale phylogenies created from mtDNA and SNPs (Moura *et al*., 2015) found that a genomic approach produced a more complex, robust phylogeny, and was able to clarify the processes influencing evolution in this taxon. The application of phylogenomics is particularly warranted in a region such as the sub-Antarctic, where extensive, recent glacial and volcanic activity has driven population changes as well as dispersal and divergence events that would be unresolvable using traditional techniques.

2.5 Anthropogenic dispersal

Much evidence exists for human-mediated biological introductions to the sub-Antarctic (Frenot *et al.*, 2005; Greenslade & Convey, 2012; Hughes, Convey & Huiskes, 2014). Several studies have quantified the extent and numbers of propagules transported annually into the sub-Antarctic (Whinam, Chilcott & Bergstrom, 2005) and Antarctic (Lee & Chown, 2009*a*; Chown *et al.*, 2012), as well as those associated with the construction activities at research stations (Lee & Chown, 2009*b*; Hughes *et al.*, 2010). Whinam *et al.* (2005) found 981 propagules on 64 members of expeditions (including their equipment and clothing) visiting Macquarie Island with the Australian Antarctic Program. Of these propagules, germination trials showed that 163 were viable, from 24 species. Live and dead invertebrates as well as fungus infection were also identified in fresh food transported during the expedition. Surveys of passenger luggage and cargo associated with the South African National Antarctic Programme estimated that some 1400 seeds from 99 taxa were transferred into the greater Antarctic region during each field season (Lee & Chown, 2009*a*). Of these, 30–50% were considered likely to enter the

environment. Further investigation of the seeds found a large proportion were from families and genera of known cosmopolitan and invasive species including those that have invaded the Antarctic region. The seeds were also representative of a large proportion of the vegetation locally found at the site of cargo loading. Finally, Chown *et al.* (2012) quantified the number of vascular plant propagules per person for all visitors to the Antarctic continent (scientists, tourists and support personnel associated with all Antarctic programs) during a field season, and assessed the spatially explicit likelihood of establishment in current and future Antarctic climates. Visitors carrying seeds averaged 9.5 seeds per person. In addition, particular areas of the Antarctic continent were demonstrated to be at higher risk of establishment, such as the western Antarctic Peninsula, where expeditions are more frequent and environmental conditions are comparatively moderate. Furthermore, surveys from all three studies indicated that many members of expeditions had travelled to cold-climate areas such as alpine, cold-temperate or polar environments before visiting the Antarctic (Whinam *et al.*, 2005; Lee & Chown, 2009*a*; Chown *et al.*, 2012), which may increase the likelihood of transfer and establishment of species that are capable of surviving Antarctic conditions (Chown *et al.*, 2012). Invertebrate transfer is likewise substantial with shipping. For example, between 2000 and 2013, 1,376 individuals from 98 families were collected in transit to the Antarctic region, with flies, beetles and moths being most common (Houghton *et al*., 2016). Intra-regional transfer of native Antarctic or sub-Antarctic species is also probably occurring (Lee & Chown, 2009*a*, 2011), suggesting that anthropogenic transport supports intra-regional homogenisation. Anthropogenic introductions are likely to be exacerbated

by increasing traffic and better establishment success with climate amelioration (Bergstrom & Chown, 1999; Whinam *et al.*, 2005).

Anthropogenic transport of marine species into and around the sub-Antarctic is also facilitated by hull fouling of ships associated with scientific and tourist expeditions (Lewis *et al.*, 2003 Lewis, Riddle & Smith, 2005; Lewis, Bergstrom & Whinam, 2006; Lee & Chown, 2009*c*; Hughes & Ashton, 2016). Fouling on the hulls of vessels embarking for sub-Antarctic islands is likely to facilitate the movement of substantial numbers (and in some cases entire assemblages) of marine species across large distances and natural barriers (Lewis *et al.*, 2005; Whinam *et al.*, 2005; Lee & Chown, 2009*c*). Fouling species may be recruited in temperate ports (such as Hobart) during winter (Lewis *et al.*, 2003, 2005) and have been shown to be capable of surviving the journey to the sub-Antarctic region (Lewis, Riddle & Hewitt, 2004; Lee & Chown, 2007), where establishment is more likely than the Antarctic due to a lack of sea-ice and warmer conditions (Lee & Chown, 2009*c*). While transportation in ballast water has been identified as a global mechanism for marine species dispersal, this process is probably less common in the Southern Ocean where smaller vessels unidirectionally transport ballast water from higher to lower latitudes (Lewis *et al.*, 2003, 2005). Moreover, the Antarctic Treaty System has adopted provisions to limit ballast water exchange (e.g. ballast water exchanges must take place in deep water far from the nearest land before arrival in, and prior to leaving, Antarctic waters: Secretariat of the Antarctic Treaty, 2006).

2.6 Conclusions

(1) Recent research has indicated that movement of taxa among sub-Antarctic islands is more common than previously was thought. Most significantly, new evidence from a range of studies indicates that although dispersal among islands is frequent for many taxa, and facilitates colonisation following disturbances, ongoing gene flow is relatively rare. As a result, many sub-Antarctic organisms show much genetic structure and diversity. Further empirical research will be required to determine which dispersal mechanisms are the most important in the sub-Antarctic, and how these have varied over time.

(2) Some evidence exists that changes in the effectiveness of dispersal mechanisms due to climatic changes, for example the migration of the ACC and associated fronts (Allan *et al.*, 2013) and changes in the velocity of the westerly winds, may already be impacting on species inhabiting these regions (Weimerskirch *et al.*, 2012). Diminishing isolation due to increasing human activities is also occurring in the region (Chown *et al.*, 2015), threatening current assemblages *via* the homogenisation and simplification of marine and terrestrial biotas (Gaston *et al.*, 2003; Shaw *et al.*, 2010).

(3) The majority of phylogeographic studies in the sub-Antarctic have used single genes, or have been limited to a few genes. Studies based on next-generation genetic data are, however, beginning to emerge for the sub-Antarctic, particularly in the last few years, and are revealing more complex patterns of contemporary connectivity on a range of spatial scales (Fraser *et al*., 2016*b*; Piertney *et al*., 2016). Their application is particularly important to clarify genetic sub-structure in cases where recent divergence (Fraser *et al*.,

2016*b*) or colonisation (Piertney *et al*., 2016) events cannot be resolved using traditional approaches.

(4) Here, as is the case for island systems worldwide, anthropogenic transport is facilitating movement of both indigenous and non-indigenous species within and among islands, and is likely to obscure molecular signals of natural dispersal and affect indigenous assemblages, respectively.

(5) Spatially explicit model systems with limited anthropogenic activity, such as the sub-Antarctic, are increasingly rare. In consequence, further work on the role of dispersal in the generation of biodiversity patterns and evolutionary responses to climate change in this region could provide many opportunities that more-disturbed systems cannot. Moreover, understanding the contributions of anthropogenic dispersal and natural background dispersal to evolutionary dynamics is also more straightforward in the region than elsewhere given good records of patterns of human activity (e.g. Chown, Hull & Gaston, 2005). Such understanding is of global interest.

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2.9 Supplementary material

2.9.1 Tables

Table S1. Evidence for short- and long-distance dispersal within terrestrial and marine systems in the sub-Antarctic and whether there is evidence for population structure on intra- and inter-island scales.

Species	Evidence of short-distance dispersal	Evidence for intra-island population structure	Evidence of long-distance dispersal	Evidence for inter-island population structure
		Terrestrial		
Invertebrates				
Weevils	Grobler *et al.* (2006)	Yes	Grobler *et al*. (2011*a*, *b*)	Yes
Mites	Mortimer & van Vuuren (2007); Mortimer *et al.* (2012)	Yes Yes	Mortimer *et al.* (2011)	Yes
Springtails	Myburgh *et al.* (2007); McGaughran *et al.* (2010*a*)	Yes	Stevens *et al.* (2006); McGaughran *et al.* (2010*b*)	Yes

Pseudhelops beetles			Leschen *et al.* (2011)	Yes
Ticks (*Ixodes uriae*)	McCoy *et al.* (2005*b*)	Yes	Dietrich *et al.* (2014)	Yes
Chironomid midge (*Belgica albipes*)	Allegrucci *et al.* (2012)	Yes		
Plants				
Antarctic hairgrass (*Deschampsia antarctica*)			van de Wouw *et al.* (2008)	Yes
Flowering cushion plants (*Azorella*)	Mortimer *et al.* (2008); Cerfonteyn *et al*. (2011); Born *et al.* (2012)	Yes		
Azorella, Laretia and *Mulinum* genera			Nicolas & Plunkett (2012)	Yes
Moss (*Bryum argenteum*)			Pisa *et al.* (2014*)	No

Moss (*Ceratodon purpureus*)	Skotnicki *et al*. (2004)	Yes	Skotnicki *et al*. (2004)	Yes
Lyallia kerguelensis			Wagstaff & Hennion (2007)	Yes
Pringlea antiscorbutica			Bartish *et al*. (2012)	Yes
		Marine		
Plants				
Kelp			Fraser *et al.* (2009, 2016*b*); Macaya & Zuccarello (2010)	No (but low genetic diversity)
Invertebrates				
Epifaunal crustacean species (*Limnoria stephenseni* and *Parawaldeckia kidderi*)			Nikula *et al.* (2010)	No

Diloma nigerrima	Donald *et al*. (2011)	Yes
Sea slugs (*Onchidella*)	Cumming *et al.* (2014)	Yes
Crustacean and mollusc species (*Cantharidus roseus*, *Onithochiton neglectus* and *Parawaldeckia karaka*)	Nikula *et al.* (2012)	No
Intertidal limpet (*Cellana strigilis* species complex)	Goldstien *et al.* (2009); Reisser *et al.* (2011)	Yes Yes
Multiple genera of benthic invertebrates	González-Wevar *et al.* (2012*a**); Poulin *et al*. (2014)	Yes

Nacella	González-Wevar *et al.* (2010, 2012*b*, 2013*, 2016*a*, *b*, *c*, *d*)	Yes
Smooth-shelled mussels (*Mytilus* sp.)	Gérard *et al*. (2008)	Yes
Benthic isopod (*Septemserolis septemcarinata*)	Leese *et al.* (2010)	Yes
Shallow water isopod (*Serolis paradoxa*)	Leese *et al.* (2008)	Yes
Benthic shrimp (*Nematocarcinus lanceopes*)	Raupach *et al.* (2010*)	No
Bivalve (*Limatula* species)	Page & Linse (2002)	Yes
Sea urchin *(Abatus cordatus*)	Ledoux *et al.* (2012)	Yes

Sea urchin genus (*Sterechinus*)			Díaz *et al.* (2011*)	Yes
Pycnogonid (*Colossendeis megalonyx*)			Krabbe *et al.* (2010*)	Yes
Brooding brittle star (*Astrotoma agassizii*)	Hunter & Halanych (2008*)	No (within allopatric South American clades)	Hunter & Halanych (2008*)	Yes (between South America and Antarctica)
Sea cucumbers			O'Loughlin *et al.* (2011)	Yes
Chrinoid (*Promachocrinus kerguelensis*)			Wilson *et al.* (2007*); Hemery *et al.* (2012)	Yes Yes
Antarctic sea slug (*Doris kerguelenensis*)			Wilson *et al.* (2009)	Yes
Ribbon worm (*Parborlasia corrugatus*)			Thornhill *et al.* (2008)	No (but evidence for lack of connectivity

				between South America and sub-Antarctic sites)
Antarctic krill (*Euphausia superba* Dana)			Zane *et al.* (1998*)	Yes
Squid (*Onykia ingens* previously known as *Moroteuthis ingens*)			Sands *et al.* (2003)	Yes (but low)
Squid (*Martialia hyadesi*)	Brierley *et al.* (1993)	Yes		
Antarctic octopus (*Pareledone turqueti*)	Allcock *et al.* (1997); Strugnell *et al.* (2012)	No	Allcock *et al.* (1997); Strugnell *et al.* (2012)	Yes (between Shag Rocks and South Georgia)
Sea star genus (*Odontaster*)			Janosik, Mahon &	No (but evidence for

			Halanych (2011)	lack of connectivity between South American and sub-Antarctic sites)
Sea spider (*Pallenopsis* sp.) – note the taxonomy remains ambiguous			Weis *et al.* (2014); Harder *et al.* (2016)	Yes Yes
Bathyal ophiuroid fauna (from eight families)			O'Hara *et al.* (2013*)	Yes (between Macquarie Ridge and Antarctica)
Vertebrates				
Patagonian toothfish (*Dissostichus eleginoides*)	Appleyard *et al.* (2002, 2004); Shaw *et al.* (2004)	No	Smith & McVeagh (2000); Appleyard *et*	Yes (but homogeneity is observed

			al. (2002, 2004); Shaw *et al.* (2004); Rogers *et al.* (2006); Toomey *et al.* (2016)	within some regions)
Trematomus hansoni			Van de Putte *et al.* (2012*a**)	Yes
Electrona antarctica			Van de Putte *et al.* (2012*b**)	No
Harpagifer species			Hüne *et al*. (2015*)	Yes
Crocodile icefish			Damerau *et al.* (2014*)	Yes
Antarctic icefishes (Channichthyidae lineage)			Near *et al*. (2003)	Yes
Icefish (*Champsocephalus gunnari*)	Williams *et al.* (1994);	No No		Yes

	Duhamel *et al.* (1995)		Kuhn & Gaffney (2006); Young *et al*. (2015*)	
Marbeled rockcod (*Notothenia rossii*)	Duhamel *et al.* (1995)	No	Young *et al*. (2015*)	No
Lepidonotothen nudifrons	Dornburg *et al*. (2016)	No		
Antarctic fur seal (*Arctocephalus gazella*)	Wynen *et al.* (2000)	No	Wynen *et al.* (2000); Bonin *et al.* (2013)	Yes No (between Livingston Island and South Georgia)
Sub-Antarctic fur seal (*Arctocephalus tropicalis*)	Wynen *et al.* (2000)	No	Wynen *et al.* (2000)	Yes
Southern elephant seal (*Mirounga leonina*)			Slade *et al.* (1998); Hoelzel *et al.* (2001);	Yes Yes

			Fabiani *et al*. (2003); de Bruyn *et al*. (2009*)	No (male movement clear) NA (historic study)
Leopard seal (*Hydrurga leptony*x)			Davis *et al.* (2008)	No
King penguin (*Aptenodytes patagonicus*)	Trucchi *et al*. (2014); Cristofari *et al*. (2015)	No (demographically focused study) No (but local heterogeneity was proposed)	Clucas *et al.* (2016)	No
Rockhopper penguin (*Eudyptes chrysocome sensu lato*)			Banks *et al*. (2006); Jouventin *et al*. (2006); de Dinechin *et al*. (2009)	Yes (multiple species proposed)

Gentoo penguin (*Pygoscelis papua)*	Levy *et al.* (2016)	No	de Dinechin *et al*. (2012); Clucas *et al.* (2014); Levy *et al.* (2016); Vianna *et al*. (2016)	Yes
Macaroni penguin (*Eudyptes chrysolophus*)	Jouventin *et al*. (2006*)	No		
Sheldgeese (*Chloephaga rubidiceps* and *Chloephaga picta*)			Kopuchian *et al*. (2016)	Yes
Kelp gull (*Larus dominicanus*)			de Almeida Santos *et al*. (2016*)	Yes (among greater regions)
Black-browed albatross (*Thalassarche melanophris*)			Burg & Croxall (2001)	Yes

Wandering albatross (*Diomedea exulans*)			Burg & Croxall (2004); Milot *et al*. (2008)	No
Wandering albatross (*Diomedea antipodensis* taken to be *D. antipodensis* and *D. gibsoni* reclassified as one species)			Burg & Croxall (2004)	Yes
Grey-headed albatross (*Thalassarche chrysostoma*)			Burg & Croxall (2001)	No
White-chinned petrel (*Procellaria aequinoctialis*)			Techow *et al*. (2009)	Yes
Brown rat (*Rattus norvegicus*)	Piertney *et al*. (2016)	Yes		

Killer whale (*Orcinus orca*)	LeDuc *et al.* (2008); Morin *et al.* (2010*); Moura *et al*. (2015)	Yes

Table S2. Molecular techniques and number of sub-Antarctic samples used for phylogenetic studies in the sub-Antarctic region.

Species	Reference	Genetic technique used	Total sub-Antarctic sample size
		Terrestrial	
Invertebrates			
Weevils	Grobler *et al.* (2006)	mtDNA (partial COI[1])	52
	Grobler *et al*. (2011*a*)	mtDNA (partial COI)	86
	Grobler *et al*. (2011*b*)	mtDNA (partial COI)	73
Mites	Mortimer & van Vuuren (2007)	mtDNA (partial COI)	57
	Mortimer *et al.* (2011)	mtDNA (partial COI), nuclear	86 (SM[3])

[1] COI: cytochrome c oxidase subunit I
[3] SM: supplementary material

		marker (partial H3[2])	
	Mortimer *et al.* (2012)	mtDNA (partial COI)	291
Springtails	Stevens *et al.* (2006)	mtDNA (partial COI)	23
	Myburgh *et al.* (2007)	mtDNA (partial COI)	75
	McGaughran *et al.* (2010*a*)	mtDNA (partial COI)	113
	McGaughran *et al.* (2010*b*)	mtDNA (partial COI), nuclear DNA (partial 18S[4] and partial 28S)	41
Pseudhelops beetles	Leschen *et al.* (2011)	mtDNA (partial COI)	Unknown
Ticks (*Ixodes uriae*)	McCoy *et al.* (2005*b*)	Nuclear DNA (8 microsatellite loci)	331
	Dietrich *et al.* (2014)	mtDNA (partial COIII),	mtDNA (80), microsatellites (403)

[2] H3: Histone 3
[4] 18S: 18S ribosomal RNA

		nuclearDNA (8 microsatellite loci)	
Chironomid midge (*Belgica albipes*)	Allegrucci *et al.* (2012)	mtDNA (partial COI), nuclear DNA (28S)	14
Plants			
Antarctic hairgrass (*Deschampsia antarctica*)	van de Wouw *et al.* (2008)	AFLP[5] (74 markers between 53 and 552 bp[6] in length were scored)	161
Flowering cushion plants (*Azorella*)	Mortimer *et al.* (2008)	Chloroplast DNA (*trn*H-psbA[7]- which had insufficient power for fine-scale analysis), AFLP (over 100 polymorphic bands made up of	42 (includes 15 samples from one mat)

[5] AFLP: Amplified Fragment Length Polymorphism
[6] bp: base pair
[7] *trn*H-psbA: chloroplast intergenic spacer region

		75–500 bp fragments)	
	Cerfonteyn *et al*. (2011)	Nuclear DNA (7 microsatellite markers)	5–8 samples from a total of 42 cushions
	Born *et al.* (2012)	Nuclear DNA (7 microsatellite markers)	1304
Azorella, Laretia and *Mulinum*	Nicolas & Plunkett (2012)	Plastid DNA (rpl16[8] intron and trnD-trnY-trnE-trnT[9] regions were targeted)	Unknown
Moss (*Bryum argenteum*)	Pisa *et al.* (2014*)	nuclear DNA (partial ITS[10])	10 (SM)
Moss (*Ceratodon purpureus*)	Skotnicki *et al*. (2004)	RAPD[11], nuclear DNA (partial ITS)	Unknown
Lyallia kerguelensis	Wagstaff & Hennion (2007)	Chloroplast DNA (partial rbcL[12] and IGS[13])	Unknown

[8] rpl16: plastid intron region
[9] trnD-trnY-trnE-trnT: plastid region containing three intergenic spacers
[10] ITS: nuclear internal transcribed spacer region
[11] ***RAPD:*** ***Random Amplified Polymorphic DNA***

[12] rbcL: Ribulose-1,5-bisphosphate carboxylase/oxygenase large gene
[13] IGS: intergenic spacer region

Pringlea antiscorbutica	Bartish *et al*. (2012)	Nuclear DNA (partial nrITS[14]), chloroplast DNA (partial IGS)	36
		Marine	
Plants			
Kelp	Fraser *et al.* (2009)	mtDNA (partial COI), nuclear DNA (partial chloroplast rbcL region)	170
	Macaya & Zuccarello (2010)	mtDNA (partial intergenic spacer region – region between genes)	48
	Fraser *et al*. (2016*a*)	SNPs[15] (40,912 parsimony-informative SNPs across entire genome)	10
Invertebrates			

[14] nrITS: nuclear ribosomal internal transcribed spacer region
[15] SNP: single nucleotide polymorphism

Epifaunal crustacean species (*Limnoria stephenseni* and *Parawaldeckia kidderi*)	Nikula *et al.* (2010)	mtDNA (partial COI)	*Limnoria stephenseni* (89), *Parawaldeckia kidder*i (62)
Diloma nigerrima	Donald *et al*. (2011)	Nuclear DNA (3 microsattelites)	44
Sea slugs (*Onchidella*)	Cumming *et al.* (2014)	mtDNA (partial COI), nuclear DNA (partial H3 – only a portion of specimens were analysed), AFLP (215 polymorphic loci between 50 and 600 bp in length – only a portion of specimens were analysed)	64 (SM)

Crustacean and mollusc species (*Cantharidus roseus, Onithochiton neglectus* and *Parawaldeckia karaka*)	Nikula *et al.* (2012)	mtDNA (partial COI), AFLP (94–164 polymorphic loci per species)	*Cantharidus roseus* (27), *Onithochiton neglectus* (21), *Parawaldeckia karaka* (48) (SM)
Intertidal limpet (*Cellana strigilis* species complex)	Goldstien *et al.* (2009)	mtDNA (partial Cyt b[16], partial 12S and partial16S)	40
	Reisser *et al.* (2011)	mtDNA (partial COI and partial 16S), nuclear DNA (ATPase β), RAPDs (4 markers were used and a total of 58 loci were scored)	mtDNA (105), RAPD (143)

[16] Cyt b: Cytochrome b

Multiple genera of benthic invertebrates	González-Wevar *et al.* (2012*a**)	mtDNA (partial COI)	121 (from Magellanic Province)
	Poulin *et al*. (2014)	mtDNA (partial COI)	Unknown
Nacella	González-Wevar *et al.* (2010)	mtDNA (partial COI and partial Cyt b)	≈27
	González-Wevar *et al.* (2012*b*)	mtDNA (partial COI)	139
	González-Wevar *et al.* (2013*)	mtDNA (partial COI)	30
	González-Wevar *et al.* (2016*a*)	mtDNA (partial COI) nuclear DNA (partial 28S)	109 (45 from SG for one species, 64 from two sites on Marion for the other species)
	González-Wevar *et al.* (2016*b*)	mtDNA (partial COI and partial Cyt b), nuclear DNA (partial 28S)	Unknown
	González-Wevar *et al.* (2016*d*)	mtDNA (partial COI)	300

	González-Wevar *et al.* (2016*c*)	mtDNA (partial COI)	149 (101 new samples and 48 from González-Wevar *et al.* 2012*b*)
Smooth-shelled mussel (*Mytilus* sp.)	Gérard *et al*. (2008)	mtDNA (partial COI and partial 16S)	≈159
Benthic isopod (*Septemserolis septemcarinata*)	Leese *et al.* (2010)	mtDNA (partial COI), nuclear DNA (8 microsatellites)	mtDNA (95), nuclear DNA (59)
Shallow water isopod (*Serolis paradoxa*)	Leese *et al.* (2008)	mtDNA (partial 16S), nuclear DNA (7 microsatellites)	mtDNA (71), nuclear DNA (91)
Benthic shrimp (*Nematocarcinus lanceopes*)	Raupach *et al.* (2010*)	mtDNA (partial COI and partial 16S), nuclear DNA (partial 28S)	5 (no intraspecific variation was found for 16S or 28S – therefore not informative)
Bivalve (*Limatula* species)	Page & Linse (2002)	mtDNA (partial 16S), nuclear DNA	8

		(partial 18S and partial ITS-1)	
Sea urchin (*Abatus cordatus*)	Ledoux *et al.* (2012)	Nuclear DNA (3 microsatellite markers and 2 EPIC[17] markers)	374
Sea urchin (*Sterechinus*)	Díaz *et al.* (2011*)	mtDNA (partial COI)	13
Pycnogonid (*Colossendeis megalonyx*)	Krabbe *et al.* (2010*)	mtDNA (partial COI)	15 (but includes outgroups from other locations and other species)
Brooding brittle star (*Astrotoma agassizii*)	Hunter & Halanych (2008*)	mtDNA (partial COII and partial 16S)	93
Sea cucumbers	O'Loughlin *et al.* (2011)	mtDNA (partial COI)	22 (including 13 species)
Chrinoid (*Promachocrinus kerguelensis*)	Wilson *et al.* (2007*)	mtDNA (partial COI and partial Cyt b)	8 (SM)

[17] ***EPIC: exon-primed intron-crossing markers***

	Hemery *et al.* (2012)	mtDNA (partial COI, partial Cyt b and partial 16S), nuclear DNA (partial 28S and partial ITS)	<200 (all but COI were only sequenced for a portion of the sample)
Antarctic sea slug (*Doris kerguelenensis*)	Wilson *et al.* (2009)	mtDNA (partial COI and partial 16S)	18 (SM)
Ribbon worm (*Parborlasia corrugatus*)	Thornhill *et al.* (2008)	mtDNA (partial COI and partial 16S)	16 (SM)
Antarctic krill (*Euphausia superba* Dana)	Zane *et al.* (1998*)	mtDNA (partial NDI)	70
Squid (*Onykia ingens* previously known as *Moroteuthis ingens*)	Sands *et al.* (2003)	Nuclear DNA (6 RAPD primers with 30 reproducible polymorphic bands in total)	73
Squid (*Martialia hyadesi*)	Brierley *et al.* (1993)	Horizontal starch gel electrophoresis	134 (the provenance of

		(44 enzymes were stained for)	some specimens was ambiguous due to their confiscation from illegal fishing vessel)
Antarctic octopus (*Pareledone turqueti*)	Allcock *et al.* (1997)	Horizontal starch gel electrophoresis (54 enzymes were stained for, only 2 were found to be polymorphic and used in final analysis)	359
	Strugnell *et al.* (2012)	mtDNA (partial COI), nuclear DNA (10 microsatellites)	mtDNA (132), nuclear DNA (307)
Sea star (*Odontaster*)	Janosik *et al.* (2011)	mtDNA (partial COI and partial 16S)	32
Sea spider (*Pallenopsis* sp.) –	Weis *et al.* (2014)	mtDNA (partial COI)	≈28

note the taxonomy remains ambiguous	Harder *et al.* (2016)	mtDNA (partial COI)	20
Bathyal ophiuroid fauna (from eight families)	O'Hara *et al.* (2013*)	mtDNA (partial COI)	40
Vertebrates			
Patagonian toothfish (*Dissostichus eleginoides*)	Smith & McVeagh (2000)	Allozymes (11 allozyme loci), nuclear DNA (8 microsatellites)	Allozymes (≈162), nuclear DNA (≈200)
	Appleyard *et al.* (2002)	mtDNA (RFLP[18] analysis of ND2[19] and BCL[20]), nuclear DNA (7 microsatellites)	946
	Appleyard *et al.* (2004)	mtDNA (RFLP analysis of ND2 and BCL), nuclear DNA (7 microsatellites)	156

[18] RFLP: Restriction Fragment Length Polymorphism
[19] ND2: NADH dehydrogenase subunit 2 gene
[20] BCL: mitochondrial gene region containing control region or D-loop

	Shaw *et al.* (2004)	mtDNA (RFLP analysis of ND2 and CR[21]), nuclear DNA (5 microsatellites)	429
	Rogers *et al.* (2006)	mtDNA (partial 12S – 16S was also attempted but no variation was found), nuclear DNA (7 microsatellites)	≈220
	Toomey *et al.* (2016)	mtDNA (partial Ctr1, partial CR4[22], partial Cyt B and partial COI), nuclear SNP DNA (Sec61a[23], Mb[24],	419

[21] CR: control region
[22] Ctr1, CR4: control regions
[23] Sec61a: endoplasmic reticulum membrane protein translocator Sec61 alpha
[24] Mb: myoglobin

		Dyst6a[25], LDHA[26])	
Trematomus hansoni	Van de Putte *et al.* (2012*a**)	mtDNA (partial Cyt b), nuclear DNA (6 microsatellites)	21
Electrona antarctica	Van de Putte *et al.* (2012*b**)	Nuclear DNA (7 microsatellites)	47
Harpagifer species	Hüne *et al*. (2015*)	mtDNA (partial CR)	63
Crocodile icefish	Damerau *et al.* (2014*)	mtDNA (partial Cyt B and partial D-loop), nuclear DNA (9 microsatellites)	approx. 100
Antarctic icefishes (Channichthyidae lineage)	Near *et al*. (2003)	mtDNA (complete gene sequences of ND2 and 16S)	Unknown
Icefish (*Champsocephalus gunnari*)	Williams *et al.* (1994)	mtDNA (11 informative	53

[25] Dyst6a: dystrophin 6a
[26] LDHA: L-lactate dehydrogenase A intron 5

		restriction enzymes)	
	Duhamel *et al.* (1995)	Isoenzymes (13 enzymes analysed)	223
	Kuhn & Gaffney (2006)	mtDNA (partial Cyt b, partial CR, partial CR-12S and partial ND2), nuclear DNA (CaM[27], LDHA5[28], MLL[29], RPS7[30])	104
	Young *et al*. (2015*)	Nuclear DNA (11 microsatellites)	Unknown
Marbeled rockcod (*Notothenia rossii*)	Duhamel *et al.* (1995)	Isoenzymes (13 enzymes were analysed)	177
	Young *et al*. (2015*)	Nuclear DNA (9 microsatellites)	Unknown

[27] CaM: calmodulin
[28] LDHA5: lactate dehydrogenase A intron 5
[29] MLL: mixed lineage leukemia-like protein
[30] RPS7: ribosomal protein S7

Lepidonotothen nudifrons	Dornburg *et al*. 2016*	mtDNA (partial ND2), nuclear DNA (partial rag1[31] and partial s7 ribosomal protein 1)	Unknown
Antarctic fur seal (*Arctocephalus gazella*)	Wynen *et al.* (2000)	mtDNA (partial mitochondrial $tRNA^{thr}$ control region), RFLP	mtDNA (120), RFLP (144)
	Bonin *et al.* (2013)	mtDNA (partial HVR1[32]), nuclear DNA (17 microsatellites)	246
Sub-Antarctic fur seal (*Arctocephalus tropicalis*)	Wynen *et al.* (2000)	mtDNA (partial $tRNA^{thr}$ CR), RFLP	mtDNA (97), RFLP (89)
Southern elephant seal	Slade *et al.* (1998)	mtDNA (partial CRI, 3 diagnostic restriction sites),	mtDNA (15 and 115), nuclear DNA (unknown)

[31] rag1: nuclear loci
[32] HVR1: mitochondrial hypervariable region 1

(*Mirounga leonina*)		nuclear DNA (15 gene fragments including microsatellites)	
	Hoelzel *et al*. (2001)	mtDNA (partial CR), nuclear DNA (7 microsatellites and partial DQB[33])	mtDNA (28), nuclear DNA (30–40)
	Fabiani *et al*. (2003)	mtDNA (partial HVR1), nuclear DNA (multiple loci including microsatellites)	57
	de Bruyn *et al.* (2009*)	mtDNA (partial HVR1)	189
Leopard seal (*Hydrurga leptonyx*)	Davis *et al.* (2008)	Nuclear DNA (14 microsatellite loci)	72
	Trucchi *et al*. (2014)	mtDNA (partial CR), 31 452 SNPs	mtDNA (140), SNPs (8)

[33] DQB: exon 2 region of the DQB gene

King penguin (*Aptenodytes patagonicus*)	Cristofari *et al*. (2015)	Nuclear DNA (8 microsatellite loci)	175
	Clucas *et al*. (2016)	5154 unlinked SNPs	64
Rockhopper penguin (*Eudyptes chrysocome sensu lato*)	Banks *et al*. (2006)	mtDNA (partial COI, partial 12S and partial Cyt b)	20
	Jouventin *et al*. (2006)	mtDNA (partial CR and partial ND2)	70
	de Dinechin *et al*. (2009)	mtDNA (partial CR, partial ND2, partial COI and partial Cyt b)	Samples taken from Banks *et al*. (2006) and Jouventin *et al*. (2006) as well as 16 extra samples from the Falkland Islands
Gentoo penguin (*Pygoscelis papua*)	de Dinechin *et al*. (2012)	mtDNA (partial CR and partial ND2)	57
	Clucas *et al.* (2014)	mtDNA (partial HVR1)	129 (SM)

	Levy *et al.* (2016)	mtDNA (partial HVR1), nuclear DNA (8 microsatellites)	395 (only subset used for mtDNA analysis)
	Vianna *et al*. (2016)	mtDNA (partial HVR1), nuclear DNA (12 microsatellites)	mtDNA (56), nuclear DNA (55)
Macaroni penguin (*Eudyptes chrysolophus*)	Jouventin *et al*. (2006*)	mtDNA (partial CR and partial ND2)	19
Sheldgeese (*Chloephaga rubidiceps* and *Chloephaga picta*)	Kopuchian *et al*. (2016)	1706 SNPs (*Chloephaga rubidiceps*), 1862 SNPs (*Chloephaga picta*)	*C. rubidiceps* (15) *C. picta* (27)
Kelp gull (*Larus dominicanus*)	de Almeida Santos *et al*. (2016*)	Nuclear DNA (7 microsatellites)	6
Black-browed albatross	Burg & Croxall (2001)	mtDNA (partial HVR1), nuclear	mtDNA (73), nuclear DNA (765)

(*Thalassarche melanophris*)		DNA (7 microsatellites)	
Wandering albatross (*Diomedea exulans*)	Burg & Croxall (2004)	mtDNA (partial HVR1), nuclear DNA (9 microsatellites)	649
	Milot *et al*. (2008)	AFLP (36 polymorphic markers)	344
Wandering albatross (*Diomedea antipodensis* taken to be *D. antipodensis* and *D. gibsoni* reclassified as one species)	Burg & Croxall (2004)	mtDNA (partial HVR1), nuclear DNA (9 microsatellites)	123
Grey-headed albatross (*Thalassarche chrysostoma*)	Burg & Croxall (2001)	mtDNA (partial HVR1), nuclear DNA (7 microsatellites)	mtDNA (50), nuclear DNA (756)

White-chinned petrel (*Procellaria aequinoctialis*)	Techow *et al*. (2009)	mtDNA (partial Cyt b)	89
Brown rat (*Rattus norvegicus*)	Piertney *et al*. (2016)	mtDNA (partial Cyt B), nuclear DNA (299 SNPs)	349
Killer whale (*Orcinus orca*)	LeDuc *et al.* (2008)	mtDNA (partial HVR1)	Unknown (81 in total)
	Morin *et al.* (2010*)	mtDNA (full length genome ~16,390 bp)	Unknown (139 in total)
	Moura *et al*. (2015)	mtDNA (set of 10 primers based on Morin *et al*. 2010), nuclear DNA (SNPs)	Unknown (possibly only one sub-Antarctic site)

Chapter Three

Can a terrestrial ectoparasite disperse with its marine host?

Little penguin swimming in Half Moon Bay, Stewart Island, New Zealand. Taken by Chris Charles December 10, 2012.

This chapter has been formatted for submission to *Physiological and Biochemical Zoology*.

3.1 Abstract

One of the most extreme examples of parasite adaptation comes from terrestrial ectoparasites exploiting marine hosts. Despite the ubiquity of such ectoparasitism and its ecological and evolutionary importance, investigations of the responses of ectoparasites to conditions encountered on their hosts are rare. In the case of penguins and their ticks, current understanding suggests that ticks freely parasitise their hosts on land, but are incapable of surviving extended oceanic journeys. We examined this conjecture by assessing the physiological capacity of little penguin ticks to endure at-sea foraging and dispersal events of their hosts. Survival in penguins ticks was not significantly compromised by exposure to depths commonly associated with host dives (40 m and 60 m), repeated seawater exposure relevant to the most common (30 seconds) and longest (120 seconds) recorded host dives nor to extended (48 hours) exposure to seawater. In addition, average closed phase durations in adult and nymphal ticks exhibiting discontinuous gas exchange (339 ± 237 and 240 ± 295 seconds, respectively) exceeded that of the maximum recorded host dive duration (120 seconds). NAN respirometry also confirmed spiracle closure. Metabolic rates (0.354 ± 0.220 and 4.853 ± 4.930 $\mu l/hr^{-1}$, at 25°C for unfed and fed adult females, respectively) were significantly influenced by temperature, but the optimal and LT50 temperatures for adult ticks and fed nymphal ticks were higher than swimming penguin body temperatures. Our findings suggest that marine host dispersal is unlikely to present an insurmountable barrier to long-distance tick dispersal. Such dispersal has important implications for evolutionary theory, conservation and epidemiology.

3.2 Introduction

Parasitic species are thought to affect almost every organism at some point in their life cycle. The host environment is a key component of parasite survival, and thus is an important arbiter of selection (Thompson, 1994, 2005). Parasites with limited active movement also rely on their hosts to facilitate dispersal. Local adaptation is influenced by relative rates of gene flow (Gandon et al., 1996; Lion & Gandon, 2015) resulting in an important role for dispersal in evolutionary responses. Because hosts influence both the environmental regime which imposes selection on parasites and, in part, their responses to it, empirical exploration of the form and range of host facilitated movement is an essential component of understanding host-parasite evolutionary dynamics (Prugnolle et al., 2005; Louhi et al., 2010).

Ticks are one of the most important, widely distributed ectoparasite groups globally (Jongejan & Uilenberg, 2004; Boulinier et al., 2016), yet rely almost entirely on host movements for dispersal (Falco & Fish, 1991). Some of the most widespread ticks are those associated with seabirds. For example, *Ixodes uriae* owes its presence across both hemispheres to dispersal with seabird hosts (Dietrich, Gómez–Díaz & McCoy, 2011; Muñoz–Leal & González–Acuña, 2015). Penguins are colonial seabirds that forage and disperse entirely at-sea, and are parasitised by ticks when they come ashore to breed and moult. Terrestrial movements at the intra-colony scale are unlikely to present much of a barrier to tick movement, particularly in heavily populated and highly social penguin colonies (Cristofari et al., 2015).

Among colony movements are comparatively much more problematic. Penguins forage, prospect and overwinter at-sea for both short (day trips) and long (weeks to months) periods. Moreover, penguins occur on some of the world's most isolated oceanic islands, and penguin-associated ticks (hereafter 'penguin ticks') are found at almost all of these locations (Murray & Vestjens, 1967; Brooke, 1985), raising the question of whether non-permanent, terrestrial parasites such as penguin ticks can survive long trips at-sea with their hosts. What is known of penguin tick dispersal thus far has primarily used genetic structure as a measure of dispersal (McCoy et al., 2005; McCoy et al., 2012; Moon, Banks & Fraser, 2015). Although useful for inferring connectivity, such work does not directly reveal whether ticks might possess the physiological capability to survive oceanic trips of durations relevant to among-penguin colony dispersal.

Dispersal with an aquatic host arguably represents the most extreme test of the physiological limits of a terrestrial ectoparasite (Murray & Nicholls, 1965; Murray, Smith & Soucek, 1965; Murray & Vestjens, 1967). Indeed, ticks have been considered unlikely to survive extensive aquatic movements owing to limitations of attachment duration (Dietrich, Gómez–Díaz & McCoy, 2011) and physiology (Pugh, 1997). Nonetheless, evidence of genetic connectivity between penguin tick colonies in the isolated Southern Ocean Crozet Archipelago, along the Western Antarctic Peninsula, and along the east coast of Australia (McCoy et al., 2005; McCoy et al., 2012; Moon, Banks & Fraser, 2015), and some genetic evidence for host-species (or genus) specificity (McCoy et al., 2005), indicate that successful oceanic dispersal may be occurring. Direct observations also suggest that ticks may be taken out to sea by penguins (Mangin et al., 2003).

At least some characteristics of adult ticks suggest that they may be capable of surviving marine conditions. First, they are tracheate arthropods, capable of closing their spiracles for extended periods (exhibiting discontinuous gas exchange – DGE) (Lighton, Fielden & Rechav, 1993). Ticks also exhibit extremely low metabolic rates (as little as ca. 10% that of insects and spiders) (Lighton & Fielden, 1995), and can survive longer than any other arthropod without food or water (Needham & Teel, 1991; Lighton & Fielden, 1995). Finally, a growing body of evidence suggests that ticks are capable of surviving in fresh water for days to weeks depending on the species (Murray & Vestjens, 1967; Fielden et al., 2011; Giannelli, Dantas–Torres & Otranto, 2012; Sá–Hungaro et al., 2014). Whether these characteristics might enable ticks to survive at-sea and regularly at depth has not been explored, although some morphological assessments have suggested that the latter is unlikely (Pugh, 1997).

In terms of underwater survival, two major zones where penguin ticks attach present different challenges – in the auditory meatus (the inner ear) (Stedt, 2009) and on the head, body and legs (Gauthier–Clerc, 1998) (Fig. 1). While ticks attached to the body, head or legs would need to withstand anoxic conditions, seawater exposure and the increased pressure at penguin dive depths, ticks in the auditory meatus would be most at risk from long-term exposure to high host body temperature and the depletion of body stores that might result in detached, non-feeding individuals.

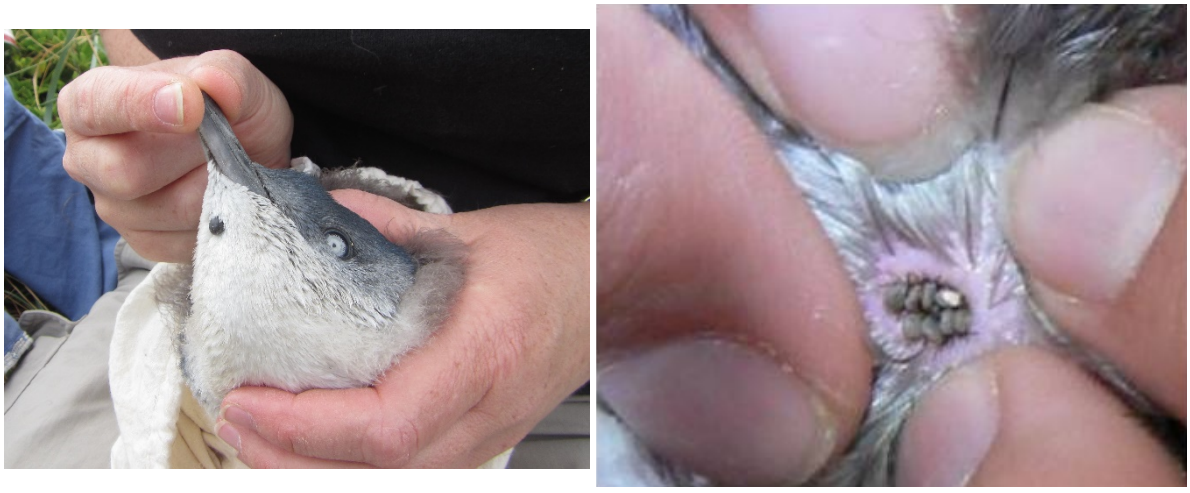

Figure 1. Images of a female adult tick attached under the bill of a little penguin (K.L. Moon, 2013), and several nymphal ticks attached to the auditory meatus of a little penguin in New Zealand (Van Rensburg, 2010).

Here, we used the little penguin (*Eudyptula novaehollandiae*) (Grosser et al., 2015) – hard tick (*Ixodes* species) system to investigate how the physiological tolerances of a terrestrial parasite with limited independent dispersal may facilitate or restrict movements with a predominantly marine host. First, in relation to ticks transported externally, we examined individual survival in seawater and at depth for all life stages except larvae. We also determined the duration of spiracle closure using flow-through respirometry. We assumed that attached ticks would not face food-resource related challenges. Then, for ticks transported in the ears, we assumed that ticks could either be attached, or detached and non-feeding. Flow-through respirometry was used to estimate metabolic rate to understand the likely resource requirements of both groups across all life stages (except larvae). For both fed and unfed animals we assumed that in the absence of re-attachment to the host, survival would be determined by the relationship between resource stores (lipid content) and metabolic rate (Irwin & Lee, 2000).

3.3 Methods

3.3.1 Animal collection and maintenance

A total of 572 individuals of an undescribed, morphologically cryptic *Ixodes* tick species (potentially representing more than a single species) was collected at Phillip Island Nature Reserve, Australia (38°31'S, 145°09'E). Individuals were taken preferentially from nest boxes, but were also directly removed from hosts that were being handled by management staff. Live ticks were washed with deionised water, dried with tissue paper and sorted by life cycle stages (adult males and females and nymphs), and by whether they were fed or unfed (where applicable), resulting in five groups: fed adult females, unfed adult females, unfed adult males, fed nymphs and unfed nymphs. Larvae were not represented due to their small size, making sampling problematic. Groups were placed in small specimen jars 1/5 filled with charcoal-tainted gypsum plaster that had been saturated with de-ionised water. Tissue moistened with deionised water was fastened across the specimen jar opening. Specimen jars were placed inside a 4 L container kept humid with deionised water-soaked tissue and placed in Sanyo MIR-154-PE incubator (Sanyo Electric Co. Ltd. Osaka. Japan) maintained at 7 °C on a 12:12 light-dark cycle.

3.3.2 Survival at sea

To examine survival at-sea, and at the pressures associated with the dive depths recorded for little penguins (up to 66.7 m, see Supplementary Text S1, and Table S1), an at-sea experiment, 1 km off the coast of Phillip Island (Fig. S1), was conducted from a small commercial fishing boat. Three groups of starved or fed, sexed adult ticks (one control, two experimental) (Table S2) were placed in mesh bags within perforated 100 ml plastic

sample jars. These jars were then attached to a light, weighted, marine rope, demarcated at 10 m intervals. In the first experiment one of the experimental groups was lowered to 40 m below the surface for one minute, with the control group held at 0.5 m. Survival was then assessed. Because all ticks survived, the two groups were then lowered to their respective depths for 1 h. Survival was again determined for both groups, and individuals were then placed back into two separate containers, and held at 19°C in the boat to recover. They were then returned to the laboratory some 7 h later to the laboratory conditions as above. Survival was re-assessed 24 h later in both groups. In the second experiment, the second experimental group was lowered to 60 m and again returned to the surface and individuals assessed for survival after 1 minute. All ticks survived, thus the group was lowered back to 60 m and held there for 30 minutes. Survival was assessed as above both immediately and after 24 h. A generalised linear model (binomial family, logit link), implemented in R v. 3.2.1 (R Core Team, 2014), was used to examine the effects of depth (control, 40 m, 60 m), sex and starvation level (fed and unfed) on survival.

Two further experiments were used to assess whether ticks can survive the diving behaviour exhibited by breeding little penguins. Daily foraging trip duration in little penguins is 12-18 hours, depending on the season (Gales, Williams & Ritz, 1990; Ropert–Coudert et al., 2006). An initial experiment exposed 17 adult ticks (15 females, two males) to 48 h submergence in perforated Eppendorf tubes, in simulated seawater (35 g of NaCl dissolved into 1 L of de-ionised water (Sagar Gawande et al., 2017)) in a 4 l tub held at 15 ± 1°C within a controlled temperature (CT) room. Little penguins are often found foraging in waters of 15-16°C (Ropert–Coudert, Chiaradia & Kato, 2006). A second experiment

was undertaken to simulate the effects of repeated submergence and exposure, typical of penguin foraging, rather than a 48 h submergence. Little penguin dive durations are, on average, 30 s, with a maximum recorded dive of 120 s (Gales, Williams & Ritz, 1990; Ropert–Coudert et al., 2003; Preston et al., 2008). A group of 20 ticks (3 adult males and 17 adult females) was exposed to 30 s submersion durations in simulated seawater at 15 ± 1°C. This experiment lasted a total of 36 minutes. After each exposure, survival was assessed. The experiment was then repeated using a 120 s interval. Survival was assessed after each exposure. In this case the trial lasted 4 h. Proportional survival was calculated for each of these experiments. Because survival was close to 100% in all cases, no further analyses were undertaken.

3.3.3 Gas exchange

The submergence experiments suggested that the adult ticks are able to close their spiracles for extended periods. Based on information from other ixodid ticks (Lighton, Fielden & Rechav, 1993; Fielden & Lighton, 1996) this should be the case. To verify spiracle closure for the species examined here, two approaches were used. First, gas exchange patterns were examined using flow-through respirometry at a range of temperatures because previous work on a range of groups has shown that temperature has a profound influence on gas exchange patterns (Chown & Nicolson, 2004; Contreras & Bradley, 2010; Heinrich & Bradley, 2014). Carbon dioxide production (VCO_2) was measured using a Li-Cor 7000 CO_2/H_2O infrared differential gas analyser (LICOR, Lincon, USA), sampling at a rate of 0.5 Hz, attached to a Sable Systems International (SSI) flow through respirometry system (SSI, www.sablesys.com, Las Vegas, USA)

(Supplementary Fig. S2). Air was pumped through three scrubber columns (1 soda lime, 1 silica gel, 1 1/3 Drierite, 2/3 Soda Lime) creating a dry, CO_2-free airstream. The flow rate was then set to 100 or 300 ml/min (depending on the volume of the animal chamber) using a Sierra SideTrack 840 series mass flow valve (Sierra Instruments, Monterey, USA) controlled by a MFC2 mass flow controller (SSI). The airstream was directed to the A-cell of the Li-Cor 7000, and then into a MUX-2 intelligent multiplexer (SSI) housing eight chambers (either 10 or 30 ml depending on the size of the animal). The MUX-2 was programmed to sequentially measure each chamber using Expedata (SSI). Once passing through the MUX-2, the air-stream was directed to the Li-Cor 7000 B-cell. Data acquisition was via Expedata. To reduce potential detrimental effects of desiccation, animals were flushed with a humidified air flow (approximately 82% relative humidity) between measurements. This was achieved using a second compressed gas flow, which was again scrubbed of CO_2 and H_2O vapour and directed to a Sierra mass flow valve with a flow rate of 35 ml/min. This air flow was then bubbled through a saturated potassium chloride (KCl) solution and directed to the flush input of the MUX-2. Four identical versions of the above respirometry set-up were constructed inside a Panasonic MLR-352H-PE Climate Chamber (Panasonic Healthcare Co., Ltd, Sakata, Japan) which provided temperature control for the assays. VCO_2 was measured at up to 10 set point temperatures randomised in the order 20°C, 10°C, 15°C, 30°C, 25°C, 35°C, 40°C, 42.5°C, 45°C and 47.5°C. Temperature was recorded using a type-T thermocouple (Omega Engineering Inc., Stamford, USA) and a TC-2000 (SSI), and data was recorded using Expedata. All trials were completed under dark conditions to reduce the activity of the animals. Activity of the ticks was measured using AD-2 activity detectors (SSI). For the

experiment, 34 fed adult females, 27 unfed adult females, 34 fed nymphs, 34 unfed adult males, and 25 unfed nymphs and were examined (Supplementary Table S2). Each animal was weighed before and after each temperature measurement using a Metter Toledo XP2U microbalance (Metter Toledo, Greifensee, Switzerland).

Data correction and extraction were performed using Expedata. Data were initially corrected for flow rate in a push system (Lighton, 2008), nearest neighbour smoothed and drift corrected using baseline data collected every 30 minutes for a period of 5 minutes. Gas exchange patterns were then identified for each individual following Marais et al., (2005). For animals showing DGE (typically only unfed adults and nymphs at 15°C and 20°C), for each DGE cycle the mean duration and mean VCO_2 of a total cycle, and the mean duration, emission volume and VCO_2 for each of the open phase (O) and joint closed (C) and flutter (F) phases (CF phases), were extracted. The flutter and closed phases were jointly considered because the F-phase may commence before CO_2 release is detected (Wobschall & Hetz, 2004; Groenewald, Chown & Terblanche, 2014). Typically, two cycles were analysed per individual to obtain a mean value for each trait at each temperature. Individuals were then used as independent data points per temperature. For continuous gas exchange (CGE), only mean VCO_2 was calculated across the full period of recording (fed ticks and ticks measured from 25°C onwards, though some DGE was observed in ticks at 25°C). Mean VCO_2 was estimated to determine variation with temperature, life cycle stage and starvation level (see below for rationale).

The second approach used to verify spiracle closure was flow-through normoxic-anoxic-normoxic (NAN) respirometry (Lighton & Fielden, 1996). If the spiracles are closed, the introduction of anoxic air during the CF phase should have no effect because oxygen will not leave the animal by diffusion either through closed spiracles or once the spiracles are partially opened for the F phase. By contrast, rapid outward oxygen diffusion during anoxia would be expected if spiracles were not closed, resulting in a large CO_2 burst (Lighton & Fielden, 1996). Ten unfed adult female ticks were each placed within a single 12 ml animal chamber housed inside an AD-2 activity detector (SSI) (see Supplementary Table S2). A Li-Cor 7000 was used to measure VCO_2 at a sampling rate of 0.5 Hz. Flow rate was set at 100 ml/min using a Sierra mass flow valve and the airstream was scrubbed of residual CO_2 and H_2O vapour using the same scrubber system used in the metabolic rate assays. Animals were held in normoxic air at 20°C until they exhibited DGE, at which time data recording commenced. Once the animal had entered into a closed phase (indicated by low VCO_2) for a period of 5 minutes, the airstream was switched to anoxic air for a period of 5 minutes, and then back to normoxic air for a further 15 minutes. Data recording and extraction were as above. Gas exchange traces were then adjudicated visually for the effects of anoxic air (Lighton & Fielden, 1996; Klok, Mercer & Chown, 2002).

3.3.4 Metabolic rate and resource depletion

For ticks that were assumed to be either attached or detached in the auditory meatus, two assessments were made. First, metabolic rate data collected in the gas exchange trials were used to assess the likely temperature at which animals are stressed by determining the

temperature at which metabolic rate declines after a maximum value (Pörtner, 2001). These effects were assessed for nymphs and fed and unfed adults, distinguished by sex. Mean VCO_2 was calculated as above for the metabolic rate experiments. The effects of temperature, mass, activity and life cycle stage and starvation level were examined using a linear mixed effects model (individual identity was included as a random factor in the model) as implemented in the lme4 package (Bates et al., 2015) in R. The temperature of maximum metabolic rate was assessed by inspection rather than by model fitting. Survival of ticks was also determined after each of the experimental treatments, providing a further indication of temperature-related mortality. Survival was compared among the life cycle stages and starvation levels using a generalized linear model (binomial distribution, logit link). LT50 values were calculated for each group from individual fitted models using the MASS package (Ripley et al., 2013) (as recommended by Crawley 2013).

Second, we assumed that attached ticks would not be resource limited, but that this might be the case for individuals that had detached within the auditory meatus. Here, we used metabolic rate information at 40°C, because the average body temperature of a penguin at-sea is 39.2 ± 0.5°C (Stahel & Nicol, 1982), to estimate the time taken to consume lipid resources of detached ticks. Lipid contents for nymphal *Ixodes scapularis* ticks have been found to be approximately 11% post-moult and 3.2% following 38 weeks of starvation (Pool et al., 2017). Thus, lipid contents were estimated for fed and detached ticks using these values. VCO_2 was converted to Watts assuming a respiratory quotient of 0.7 (Kleiber, 1961) and an energy content of 9.0 $kcal/g^{-1}$ (or approximately 37 kJ) for lipids based on the Atwater general factor system (Ferreira et al., 2015). Using the metabolic

rate and mass of each individual at 40°C, time taken to consume all lipid resources was then estimated based on 3.2% and 11% of total mass being lipid and figures were averaged for each life cycle stage and starvation level.

3.4 Results

3.4.1 Depth tolerance

All but one tick (an unfed adult male) of the 149 tested, survived for 1 h at 40 m, and all ticks survived for 30 minutes at 60 m. Neither depth, sex, nor starvation level affected survival (Table 1). Of the 17 unfed adult ticks submerged for 48 h, only a single female died. All animals survived ten replicates of 30 seconds repeated submergence, and all but one female tick survived ten replicates of 120 seconds repeated submergence.

Table 1. Results of the linear model examining relationships between survival during the depth experiment and depth, sex (male or female) and starvation level.

Factor	Estimate	Standard Error	z	*p*
Intercept	42.447	19232.503	0.002	0.998
Depth (40m)	-19.758	10352.178	-0.002	0.998
Depth (60m)	-0.330	14167.064	0.000	1.000
Sex	-19.620	7666.978	-0.003	0.998
Starvation level	-0.504	17930.536	0.000	1.000
Residual deviance/*df* = 7.205/142				

3.4.2 Gas exchange

Discontinuous gas exchange was typical of unfed individuals, most commonly at 15°C and 20°C, while several fed individuals also exhibited this gas exchange pattern (Table 2; Fig. 2). DGE CF phase durations varied between 51 and 1492 s, with mean values of between 185 ± 286 s to 437 ± 266 s depending on life cycle stage (Table 3). For adults, the mean duration of spiracle closure was 33 9 ± 237 s (n = 41) (Table 3).

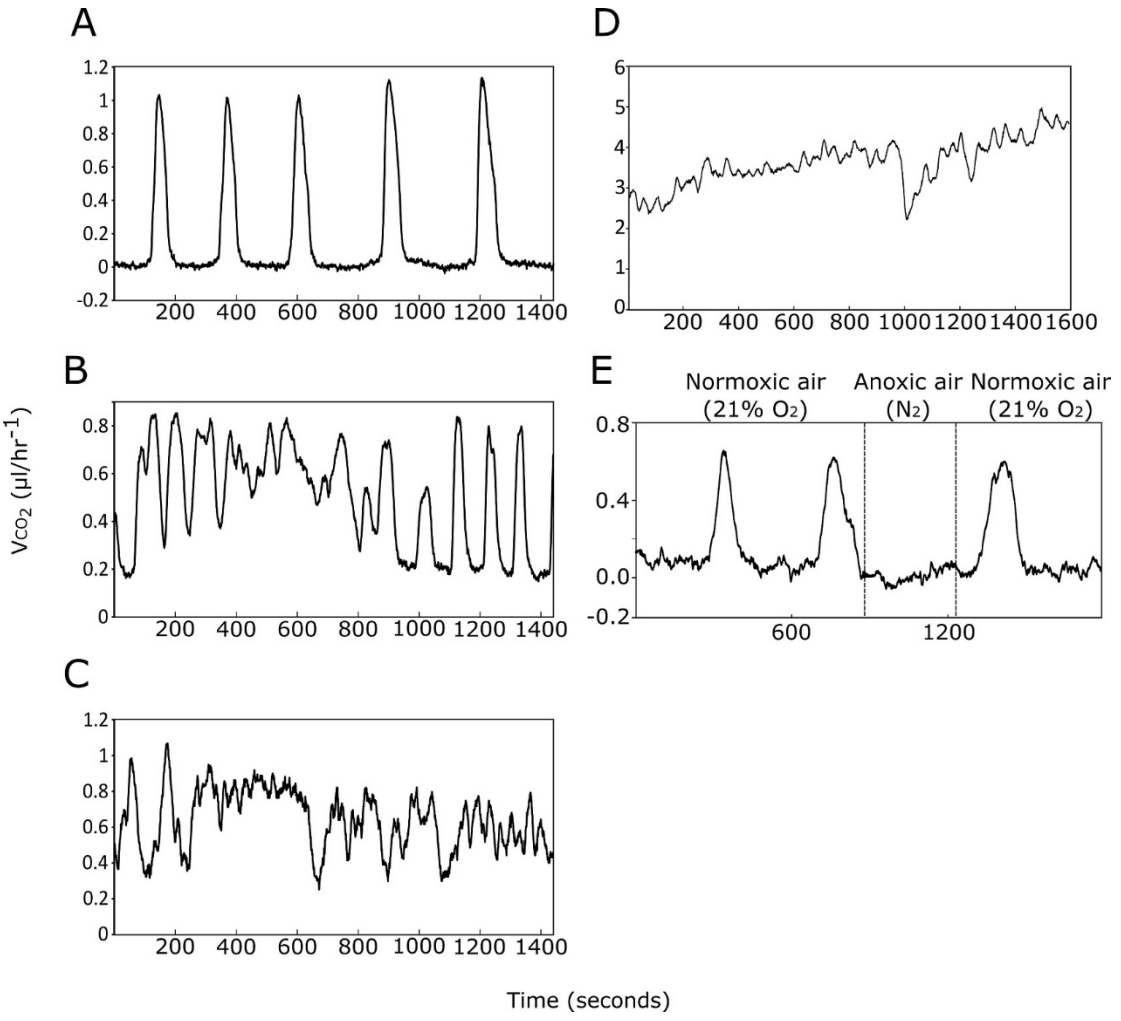

Figure 2. Example VCO_2 traces for an unfed adult female tick at A) 20°C, B) 30°C and C) 40°C, D) a fed adult female tick at 20°C, and E) an example VCO_2 trace of an unfed adult female tick during normoxic-anoxic-normoxic (NAN) respirometry tests. The dashed lines indicate where airflow flow was changed from normoxic (21% O_2, balance N_2) to anoxic (99.7% N_2) and then back to normoxic conditions.

Table 2. Proportions of ticks exhibiting CGE, DGE or mixed (showing elements of both CGE and DGE) (see Fig. 2 for example traces) during the metabolic rate experiment, including sample sizes for each temperature. Proportions are given as percentages of ticks measured, for each experimental temperature by life cycle stage and starvation level.

Temperature (°C)	Life cycle stage and starvation level	Gas exchange pattern (% of individuals)			N
		CGE	Mixed	DGE	
10	Fed adult female	82.4	11.8	5.9	34
	Unfed adult female	7.4	14.8	77.8	27
	Fed nymph	97.1	2.9	0.0	34
	Unfed adult male	17.6	23.5	58.8	30
	Unfed nymph	52.0	0.0	48.0	25
15	Fed adult female	76.5	5.9	17.6	34
	Unfed adult female	11.1	18.5	70.4	27
	Fed nymph	85.3	11.8	2.9	34
	Unfed adult male	14.7	14.7	70.6	34
	Unfed nymph	68.0	12.0	20.0	25
20	Fed adult female	64.7	23.5	11.8	34
	Unfed adult female	7.4	18.5	74.1	27
	Fed nymph	94.1	5.9	0.0	23
	Unfed adult male	14.7	38.2	47.1	34

	Unfed nymph	64.0	12.0	24.0	23
25	Fed adult female	76.5	5.9	17.6	34
	Unfed adult female	11.1	77.8	11.1	27
	Fed nymph	97.1	2.9	0.0	34
	Unfed adult male	67.6	32.4	0.0	34
	Unfed nymph	100.0	0.0	0.0	23
30	Fed adult female	100.0	0.0	0.0	34
	Unfed adult female	100.0	0.0	0.0	27
	Fed nymph	100.0	0.0	0.0	34
	Unfed adult male	100.0	0.0	0.0	34
	Unfed nymph	100.0	0.0	0.0	25
35	Fed adult female	100.0	0.0	0.0	32
	Unfed adult female	100.0	0.0	0.0	27
	Fed nymph	100.0	0.0	0.0	33
	Unfed adult male	100.0	0.0	0.0	34
	Unfed nymph	100.0	0.0	0.0	25
40	Fed adult female	100.0	0.0	0.0	32
	Unfed adult female	100.0	0.0	0.0	26

	Fed nymph	100.0	0.0	0.0	33
	Unfed adult male	100.0	0.0	0.0	34
	Unfed nymph	100.0	0.0	0.0	21
42.5	Fed adult female	100.0	0.0	0.0	32
	Unfed adult female	100.0	0.0	0.0	25
	Fed nymph	100.0	0.0	0.0	25
	Unfed adult male	100.0	0.0	0.0	33
	Unfed nymph	100.0	0.0	0.0	14
45	Fed adult female	100.0	0.0	0.0	30
	Unfed adult female	100.0	0.0	0.0	22
	Fed nymph	100.0	0.0	0.0	4
	Unfed adult male	100.0	0.0	0.0	14
47.5	Fed adult female	100.0	0.0	0.0	3
	Unfed adult female	100.0	0.0	0.0	2
	Unfed adult male	100.0	0.0	0.0	1

Table 3. Mean DGE phase data for male and female adults and nymphs exhibiting DGE at 15 and 20°C, including mean and maximum metabolic rate (μl/hr^{-1}) and the mean duration (in seconds) of the open (O) phase and the closed and flutter (CF) phase at 15 and 20°C. Mean and maximum metabolic rate (μl/hr^{-1}) and the average duration (in seconds) of the CF and O phases are also given for adults and nymphs averaged across both temperatures.

Life cycle stage and starvation level	Temperature (°C)	Mean metabolic rate (μl/hr^{-1} ± SD)	Maximum metabolic rate (μl/hr^{-1} ± SD)	Duration (s ± SD)	N
O phase					
Unfed adult female	15	0.287 ± 0.166	0.569 ± 0.329	277 ± 59	5
	20	0.491 ± 0.206	0.869 ± 0.250	233 ± 93	11
Unfed adult male	15	0.308 ± 0.132	0.606 ± 0.249	268 ± 169	9
	20	0.395 ± 0.184	0.722 ± 0.322	257 ± 81	16
Unfed nymph	15	0.037 ± 0.018	0.065 ± 0.024	182 ± 38	3
	20	0.125 ± 0.141	0.232 ± 0.280	153 ± 60	7
Adult		0.386 ± 0.190	0.714 ± 0.305	256 ± 109	41
Nymph		0.101 ± 0.127	0.187 ± 0.250	161 ± 56	10
CF phase					
Unfed adult female	15	0.067 ± 0.057	0.135 ± 0.089	437 ± 266	5

	20	0.130 ± 0.222	0.181 ± 0.223	344 ± 166	11
Unfed adult male	15	0.072 ± 0.119	0.184 ± 0.282	247 ± 163	9
	20	0.054 ± 0.051	0.116 ± 0.074	372 ± 295	16
Unfed nymph	15	0.012 ± 0.013	0.024 ± 0.016	398 ± 277	3
	20	0.024 ± 0.028	0.050 ± 0.035	185 ± 286	7
Adult		0.079 ± 0.132	0.152 ± 0.189	339 ± 237	41
Nymph		0.021 ± 0.025	0.043 ± 0.033	240 ± 295	10

In the NAN respirometry trial, the 10 unfed adult female ticks all showed DGE. No peak in CO_2 was observed for the duration of the exposure to anoxic air for nine out of the 10 tested individuals (Fig. 2E). Open DGE phases were then observed once the chambers were switched back to normoxic air flow. Unfed adult female ticks therefore exhibited a trace consistent with an ability to fully close their spiracles.

3.4.3 Metabolic rate and resource depletion

Metabolic rate was significantly influenced by temperature, mass, activity, life cycle stage and starvation level (Fig. 3; Table 4). The peak metabolic rate was 40, 42.5, 42.5, and 42.5°C respectively, for fed adult females, unfed adult females, unfed adult males, fed nymphs and unfed nymphs, (Fig. 3), while in the case of the unfed nymphs no clear peak in metabolic rate with temperature was found, although the highest value recorded was at 40°C. Survival during the metabolic rate experiment was significantly influenced by temperature, life cycle stage, and starvation level, but did not differ significantly between fed and unfed adult females (Fig. 4; Table 5). The optimum and LT50 temperatures for all life cycle stages and starvation levels exceeded little penguin body temperature (39.2 ± 0.5°C) (Stahel & Nicol, 1982), except the LT50 for unfed nymphs (Table 5).

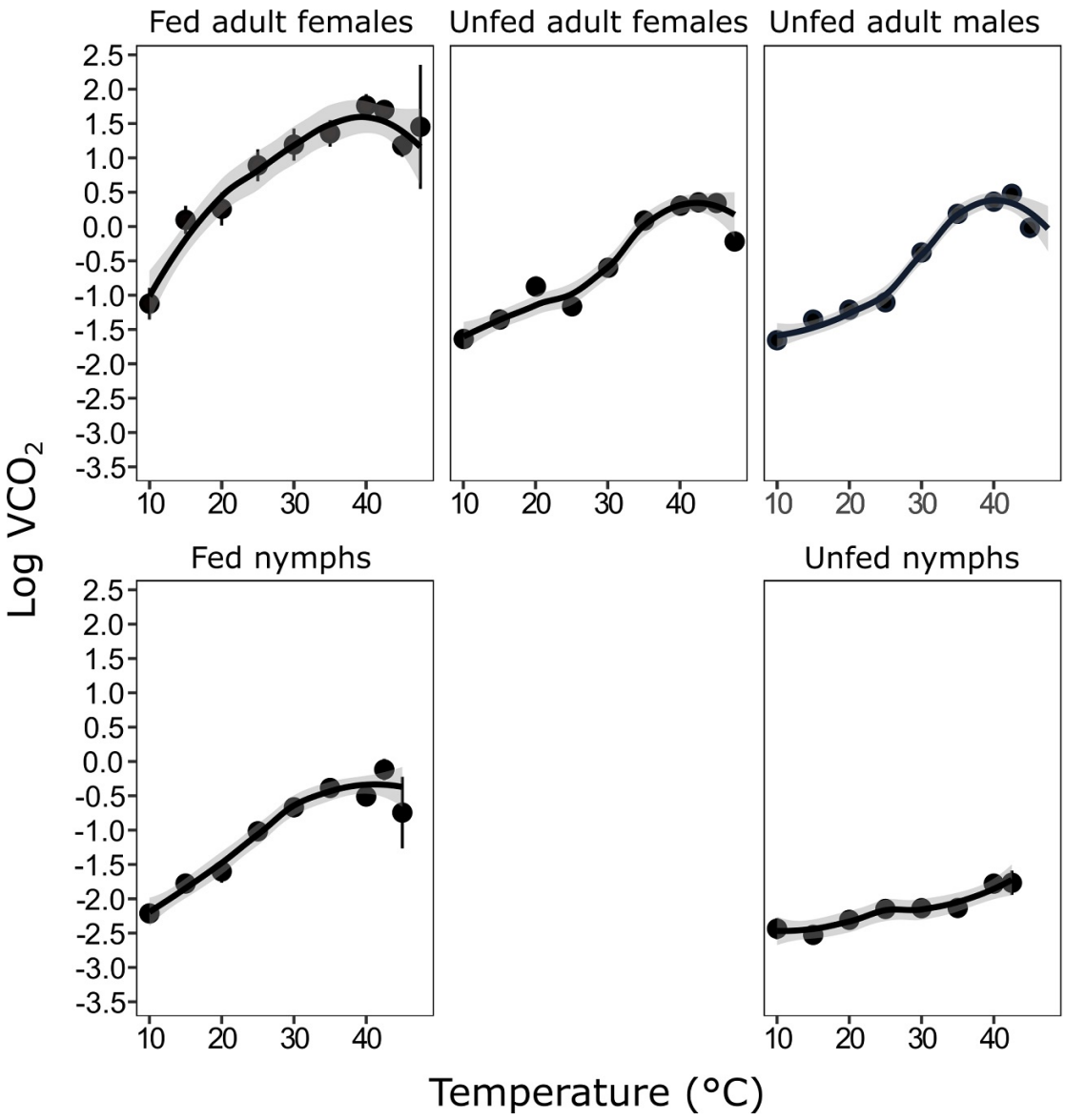

Figure 3. Metabolic rate – temperature relationships for little penguin ticks by life cycle stage and starvation level. Black bars indicate standard error, and grey shading represents the 95% confidence interval.

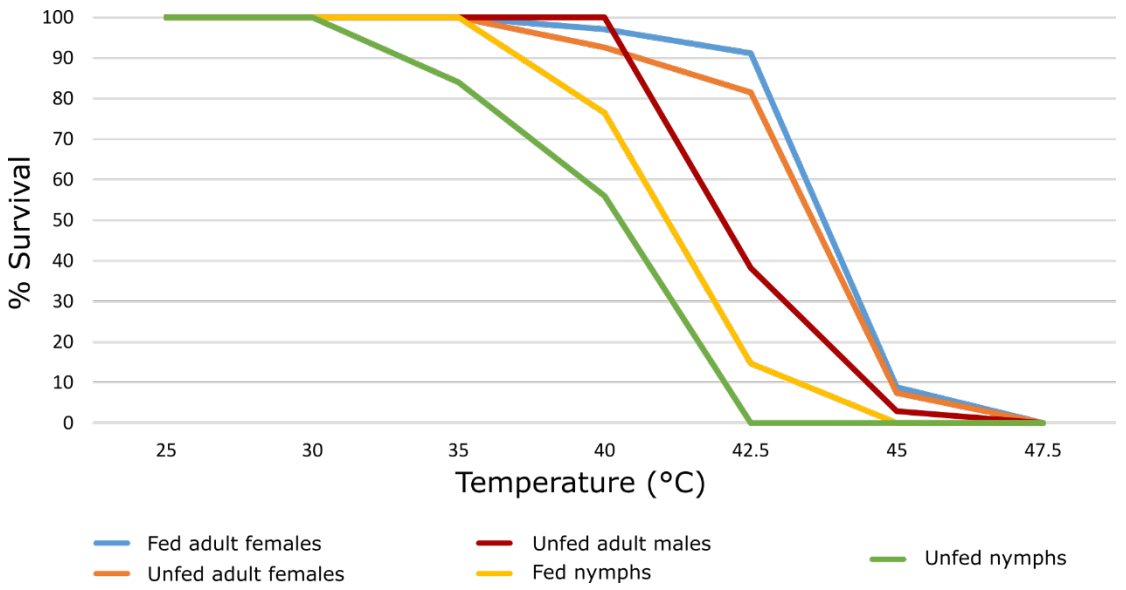

Figure 4. Survival rates of little penguin ticks at experimental temperatures during the metabolic rate experiment by life cycle stage and starvation level.

Table 4. Outcome of the linear mixed effects model investigating relationships between metabolic rate, and temperature, mass, activity, and life cycle stage and starvation level.

Factor	*Df*	F	*p*
Temperature	*1*	*1519.870*	*0.000*
Mass	*1*	*113.524*	*0.000*
Activity	*1*	*55.278*	*0.000*
Life cycle stage and starvation level	*4*	*11.095*	*0.000*

Table 5. Outcome of the linear model investigating relationships between survival during the metabolic rate experiment, and temperature, life cycle stage and starvation level. LT50 values (in °C) are provided for each life cycle stage and starvation level.

Factor	Estimate	Standard error	z	*p*
Intercept	*50.852*	*4.287*	*11.861*	*0.000*
Temperature	*-1.164*	*0.098*	*-11.886*	*0.000*
Unfed adult female	-0.466	0.447	-1.042	0.297
Fed nymph	*-3.138*	*0.493*	*-6.363*	*0.000*
Unfed adult male	*-1.627*	*0.435*	*-3.740*	*0.000*
Unfed nymph	*-4.802*	*0.592*	*-8.117*	*0.000*
Residual deviance/*df* = 323.460/1688				

LT50 values (±SD)	
Fed adult female	43.7 ± 0.2
Unfed adult female	43.3 ± 0.3
Fed nymph	41.0 ± 0.3
Unfed adult male	42.3 ± 0.2
Unfed nymph	39.0 ± 0.5

Estimates of survival time from lipid content indicate that detached, unfed nymphs and adults, with a lipid content of 3.2%, would face resource depletion within one week of exposure to penguin body temperature (Table 6). If lipid content is 11% to begin with, however, all stages except unfed nymphs are capable of surviving for at least 2 weeks at body temperatures typical of adult penguins.

Table 6. Starvation assessments of all life cycle stage and starvation levels, given body temperatures of little penguins at-sea (Stahel & Nicol, 1982), given a fixed lipid content of 3.2% and 11% (Pool et al., 2017).

Life cycle stage and starvation level	Sample size	Time until death given 3.2% lipid (days)	Time until death given 11% lipid (days)
Fed adult female	32	7	22
Unfed adult female	26	4	15
Fed nymph	33	20	67
Unfed adult male	34	5	18
Unfed nymph	21	3	11

3.5 Discussion

By contrast with some previous proposals that ticks are unlikely to survive transport at-sea (e.g. Pugh 1997), the outcomes of the experiments conducted here suggest that little penguin ticks could be resilient to the conditions faced during aquatic dispersal with their hosts. Despite no clear adaptations to marine environments, the ticks were able to survive anoxic conditions, repeated and extended exposure to seawater, and the effects of

pressures associated with depths typical of little penguin dives (Bethge et al., 1997; Ropert–Coudert, Chiaradia & Kato, 2006). Survival is, however, influenced by life stage and starvation level, and the body site where ticks might be found. Typically, penguin ticks attach either to partially sheltered sites (notably the auditory meatus) (Stedt, 2009) or fully external sites (head, body or legs) (Gauthier–Clerc, 1998; Mangin et al., 2003) (Fig. 1). Thus, these sites should be treated separately.

3.5.1 Ticks on the body surface

On the body, despite the layer of air penguins can trap under their feathers (Murray, 1967), penguin ticks are typically too large to remain fully within this layer (Fig. 1). Thus they are unable to make use of this aerial layer as is the case in some other ectoparasites of marine birds and mammals (Murray, 1967; Leonardi & Lazzari, 2014). In consequence, ticks attached to the external body surface must be able to survive both the pressures of depth and the problems associated with gas exchange in water or only occasional exposure to air. Both the gas exchange patterns and the NAN respirometry indicate that the penguin ticks investigated here can shut their spiracles. The duration of spiracle closure during DGE at 15 and at 20°C (215-381 s) certainly exceeds the dive duration of little penguins (less than 120 s) (Table S1). However, gas exchange at the end of a dive would be plausible only for ticks on the host's head, which would breach the surface of the water after each dive. Ticks parasitising other penguin species are most commonly found attached to the head and neck (Gauthier–Clerc, 1998), which would make survival using gas exchange with air plausible. Ectoparasites of other marine air-breathers, including pinnipeds and sea otters, show a similar pattern, likely for similar reasons (Dunlap, Piper

& Keyes, 1976; Fay & Furman, 1982; Pugh, 1996; Izdebska & Fryderyk, 2008; Izdebska & Rolbiecki, 2010; Alonso–Farré, D'Silva & Gestal, 2012). Ticks attached to the body or legs, however, would need to be able to rely on some other mechanism for survival.

Much evidence indicates that ticks can survive submersion in fresh water, for extended periods of up to 15 days (although there are reports of survival underwater several months) (MacLeod, 1935; Murray, Smith & Soucek, 1965; Murray & Vestjens, 1967; Smith, 1973; Paula et al., 2000; Carroll, 2003; Louzada & Daemon, 2003; Barrett et al., 2009; Fielden et al., 2011; Giannelli, Dantas–Torres & Otranto, 2012; Müeller, 2012; Sá–Hungaro et al., 2014). We found that little penguin ticks are also capable of surviving for at least 48 h submerged in seawater. Given the short durations of spiracle closure relative to a 48 h period, and the frequent absence of DGE in fed individuals, survival suggests that the penguin ticks we investigated might be exchanging gasses within a sea water medium. Survival of penguin ticks under water is thought to be facilitated by their spiracle which functions as a plastron (Woolley, 1972; Fielden et al., 2011). When a tick is submerged, air becomes trapped in the spiracular plate which then acts as a physical gill, with oxygen diffusing into the trapped air, allowing the tick to respire (Hinton, 1970; Fielden et al., 2011). Given the extended duration or survival to submersion we recorded, such gas exchange may be taking place. Irrespective, the current results demonstrate that both fed and unfed ticks can withstand submergence times well in excess of the duration of little penguin foraging trips in the breeding season (approximately 12-18 hours) when they are most likely to be exploited by ticks (Gales, Williams & Ritz, 1990; Bethge et al., 1997; Ropert–Coudert et al., 2006; Hoskins et al., 2008). However, whether plastron

respiration would remain effective during longer over-winter trips at-sea (weeks to months) (Gales, Williams & Ritz, 1990; McCutcheon et al., 2011) remains unknown.

One challenge of a 12-18 h period at-sea, including the considerable dive depths during foraging (average of ~10 m, maximum of 69 m, Table S1), is the pressures associated with such depth. Previously, it has been proposed that Antarctic penguin ticks (*I. uriae*) might be unable to survive exposure to even modest pressure, due to the air-water interface spanning the spiracular plate imploding at a depth of 12 m (1.2 atm) and the failure of the valve that closes the spiracle at 20 m (Pugh, 1997). Given their biological similarities (Heath, 2006), *I. uriae* and little penguin ticks would be expected to exhibit the same limitations. By contrast, the current outcomes demonstrate that little penguin ticks are capable of surviving depths of 40 and 60 m (the equivalent of around 3.9 and 5.8 atm, respectively). These depths were maintained for an hour and half an hour respectively, which is well in excess of the dive limits of the little penguin (under two minutes: see Supplementary Table S1). Pressure associated with little penguin dives is therefore unlikely to present a challenge to tick survival during dispersal.

3.5.2 Ticks in the auditory meatus

Investigations of tick loads in several mammal hosts have suggested that the ear may be one of the preferred attachment sites (Randolph, 1975; Nilsson, 1981; Fourie, Horak & Van Zyl, 1991; Matthee, Meltzer & Horak, 1997; Warwick et al., 2016). A study of adult magellanic penguins (*Spheniscus magellanicus*) in southern Chile found 99% of their ticks (*Ixodes uriae*) attached to the auditory meatus (or ear) (Stedt, 2009), while previous

studies of *I. uriae* have found them mainly on the head, neck and feet of penguins (Gauthier–Clerc, 1998; Mangin et al., 2003). There may also be differences in cooperative grooming among penguin species; tick attachment location has been linked to the avoidance of host grooming in other tick species (Randolph, 1975; Wilhelmsson et al., 2013). The tissue and muscle structures surrounding the external ear canal of penguins suggests that it closes during submergence (Sadé et al., 2008). This characteristic is also common in diving marine mammals, and probably evolved to protect the inner ear from pressure and seawater inundation during dives (Sadé et al., 2008). In penguins, the ear canal is also protected by a layer of waterproof feathers (Stahel, Gales & Burrell, 1987). Attachment inside the ear of a penguin may thus offer the dual benefit of protection from desiccation on land, and survival when the host is at-sea (Stedt, 2009).

The auditory meatus might represent a warm, stable environment for tick feeding, but could be metabolically costly. Ticks tend to attach to seabirds on relatively warm parts of the body (Karpovich, 1970; Lee & Baust, 1987). Ectoparasites of marine hosts in cold regions similarly attach to warm areas to ensure their blood meal may continue while at-sea (Murray, Smith & Soucek, 1965). High host body temperature can, however, present a challenge to underwater survival for ectoparasites (e.g. elephant seal lice) (Murray & Nicholls, 1965), and body temperatures of little penguins are significantly raised at-sea (*ca.* 39.2 ± 0.5°C: (Stahel & Nicol, 1982).

Our results suggest little penguin ticks could withstand considerable host-environment temperatures, albeit with increased metabolic rate. At 40°C the estimated metabolic rate

for fed little penguin ticks is 8.13 ± 0.68 $\mu l/hr^{-1}$, over four times that of fed ticks at normal little penguin foraging water temperatures (Ropert–Coudert, Chiaradia & Kato, 2006) of around 15°C (1.87 ± 0.77 $\mu l/hr^{-1}$). Increases in tick metabolic rate because of host body heat and feeding will result in a rapid use of resources and could potentially limit the survival of ticks. Our results indicate, however, that adult and nymphal little penguin ticks are capable of surviving these conditions. If lipids make up around 11% of a tick, all life cycle stages and starvation levels except unfed nymphs have the resources to survive at penguin body temperature for at least two weeks, whereas calculations based on 3.2% lipid content suggest almost all ticks may die of starvation within a week (Table 6). Presumably, however, ticks attached to the inner ear would be feeding, offering a constant source of energy and water, which could improve survival rates.

3.5.3 Implications for dispersal

Overall, the current results suggest that little penguin ticks are capable of dispersal with their hosts at-sea. While survival duration is likely to differ among sites of location of the ticks, it is clear that two days of survival, including at dives of up to 60 m, is plausible for those little penguin ticks attached externally. Whether ticks attached to the external zones of the penguin could survive exposure to seawater for longer than two days is not clear, but those attached in the auditory meatus are likely to be able to survive for the duration of their blood meal. Perhaps the remaining factor that might limit long-distance dispersal of ticks with penguins is therefore the duration of feeding attachment.

Attachment durations vary considerably among tick species and are unknown for little penguin ticks. In seabird ticks, durations vary depending on host species, tick life cycle stage (Finney & Elston, 1999), and attachment location (Barton, Harris & Wanless, 1995). If one assumes that the maximum attachment duration of little penguin ticks is similar to *I. uriae* (9-13 days) (Eveleigh & Threlfall, 1974; Barton, Harris & Wanless, 1995; Finney & Elston, 1999), it is possible to predict potential dispersal range on their hosts. Depending on host swim speed (with a mean of 1.8 m s^{-1} and a maximum of 3.3 m s^{-1}) (Bethge et al., 1997), a tick could travel 1400–2566 km in nine days and 2022–3707 km in 13 days with a little penguin (see Fig. 5). Penguin hosts therefore have the potential to facilitate movement of ticks among Australian colonies, particularly during prospecting activities when they visit non-natal sites (Danchin, 1992; Boulinier et al., 2016) (see Fig. 5). Likewise, trans-Tasman movements of little penguins, though considered rare (Peucker, Dann & Burridge, 2009; Grosser et al., 2015), have happened in the past (Moon, Banks & Fraser, 2015) and could facilitate movements of little penguin ticks between eastern Australian (*Eudyptula novaehollandiae*) and New Zealand (*E. novaehollandiae* and *E. minor*) colonies (Grosser et al., 2015) (Fig. 5).

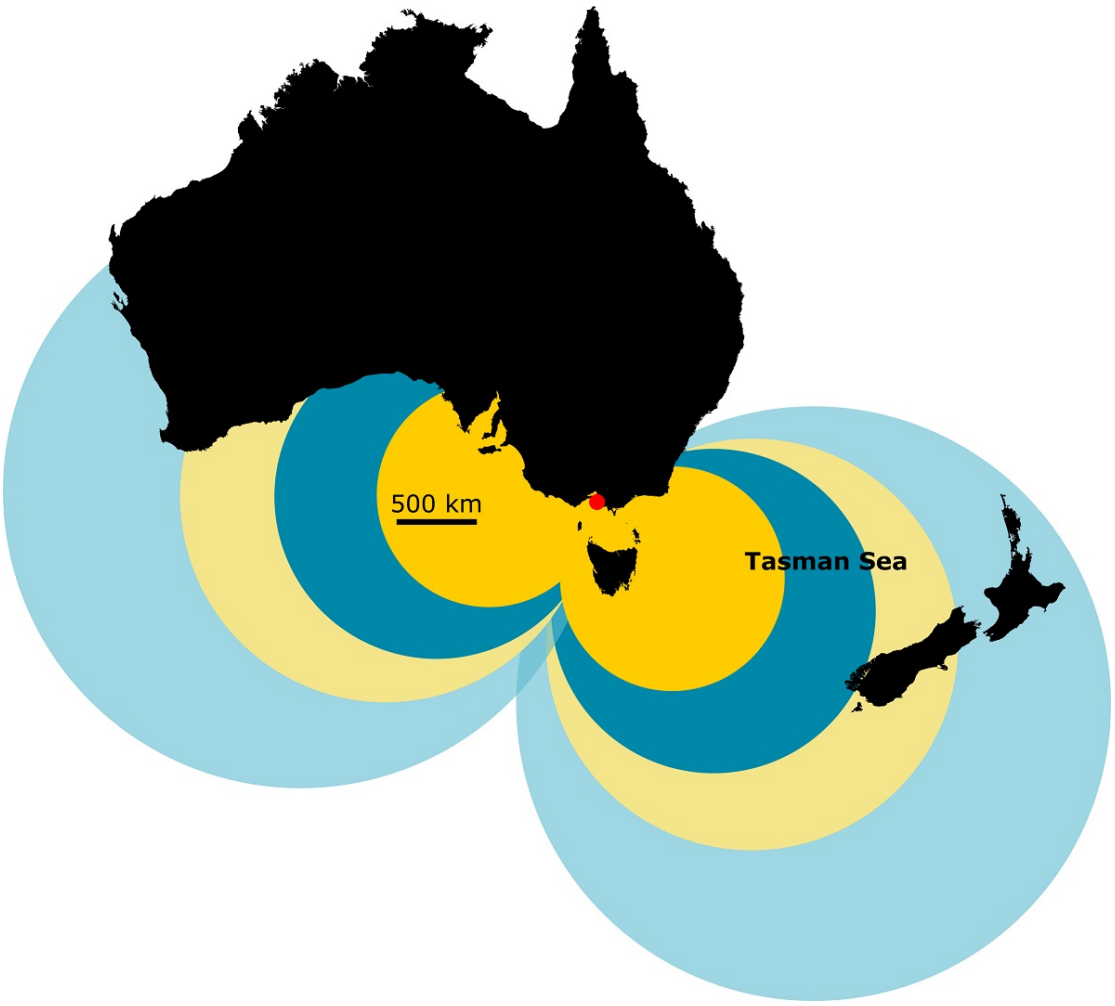

Figure 5. Theoretical dispersal range of ticks associated with little penguins at Phillip Island (red dot) given average (darker circles) and maximum (lighter circles) swim speeds, and 9-day (yellow circles) and 13-day (blue circles) attachment durations.

In conclusion, our work demonstrates that little penguin ticks have the physiological capability to overcome the conditions they are likely to encounter whether attached externally, or either unattached or attached in the auditory meatus of the little penguin. Given that several penguin tick species belong to the genus *Ixodes* (Murray & Vestjens, 1967; Moon, Banks & Fraser, 2015), dispersal with their penguin hosts to new sites seems plausible for this group of ectoparasites. Thus, penguins might readily facilitate the

dispersal of their ticks, with the latter perhaps not relying fully on dispersal by other seabird species (as has previously been suggested: McCoy et al. 2012; McCoy et al. 2005). These outcomes indicate that penguin ticks may readily keep pace with the changing distributions of their hosts that are now being recorded, especially in the Antarctic (Clucas et al., 2014), and, importantly, may readily facilitate disease spread were an infected individual to be moved to a new site. The latter has important management implications given the importance of tick-borne disease not only for wildlife (Jones & Shellam, 1999; Vanstreels, Braga & Catao–Dias, 2016), but also for human health (Gauthier-Clerc et al. 1999; see also Chapter 7).

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3.8 Supplementary material

3.8.1 Supplementary text

Text S1. *Dive data analyses of little penguins*

Dive data from numerous little penguin colonies across Australia and New Zealand was collected and assessed (see Supplementary Table S2). Bathymetry of the foraging area, prey availability, fledgling success at the colony, the position of thermoclines, availability of light and water currents are all major factors determining whether a penguin will dive to depth (Ropert–Coudert et al., 2003; Ropert–Coudert, Chiaradia & Kato, 2006; Ropert–Coudert et al., 2006; Chiaradia et al., 2007; Hoskins et al., 2008). In theory, a penguin of average weight (1.2 kg) can dive to 70 m (Wilson, 1995). However, dives to and over 60 m are exceedingly rare, occurring in far less than 1% of dives recorded (see Supplementary Table S2). Instead, individuals appear to dive most commonly to a depth of 30 m or less (Gales, Williams & Ritz, 1990; Bethge et al., 1997; Ropert–Coudert et al., 2003; Kato et al., 2006; Ropert–Coudert, Chiaradia & Kato, 2006; Ropert–Coudert et al., 2006) though dives to 40 m are relatively frequent at Phillip Island (S. Sanchez, unpub. data). The deepest dive ever recorded for a little penguin was 69 m (Montague, 1985) but the method (capillary depth gauge) has a large margin of error (5%) and so may be unreliable. The next deepest dive recorded was 66.7 m (Ropert–Coudert, Chiaradia & Kato, 2006), and, although it was an outlier, was measured using a more reliable method (accelerometer). More recent unpublished data also support these figures (S. Sanchez, unpub. data).

3.8.2 Figures and tables

Figure S1. Bathymetry of the sea surrounding Phillip Island, Victoria, showing average depths. Black circle indicates approximate location of depth experiment.

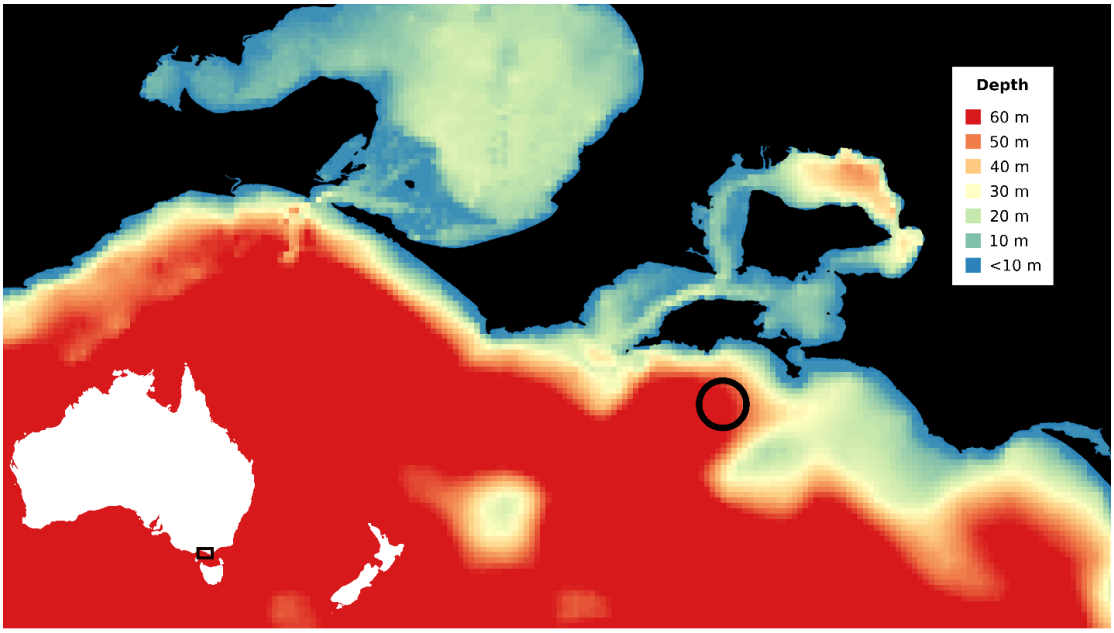

Figure S2. Schematic of metabolic rate assay setup.

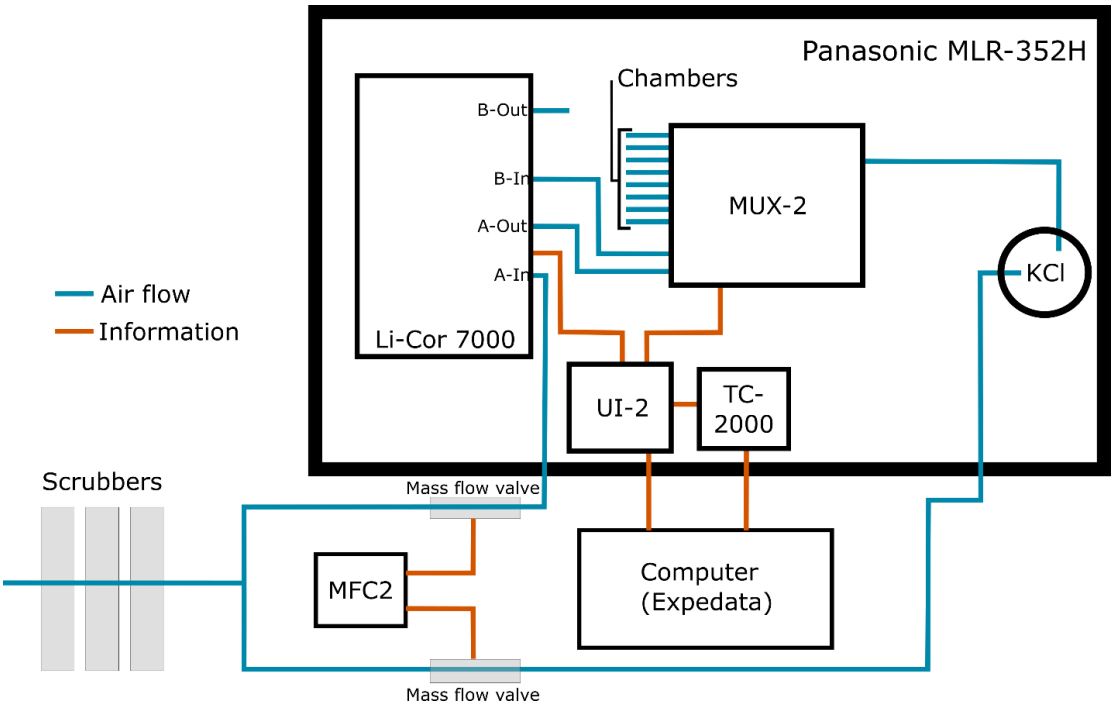

Table S1. Little penguin dive depth data.

Reference	Sample size	Mean dive duration (sec)	Max dive duration (sec)	Mean dive depth (m)	Max dive depth (m)	Dives to 60m	Total dives recorded	% of dives ≥ 60 m
(Montague, 1985)	32	30	-	30	69	2	32	6.25%
(Gales, Williams & Ritz, 1990)*	2	27.5	-	2.1 ± 3.14	50	0	1 035	0
(Bethge et al., 1997)	8	21.3	88	3.4	27.4	0	6 025	0
(Ropert–Coudert et al., 2003)^	6	-	<120	1.9, 8.1	<20	0	12 637	0
(Ropert–Coudert, Chiaradia & Kato, 2006)	38	-	90	-	66.7	1	42 028	0.002%

(Ropert–Coudert et al., 2006)	4	31.3-46.7	-	8.9-12.9	<25	0	2 064	0
(Kato et al., 2006)	4	37.2 ± 5.7	87	10.4 ± 1.85	22.1	0	2 121	0
(Watanuki et al., 2006)	5	-	-	5-8	23	0	4 931	0
(Chiaradia et al., 2007)	38	-	-	5-13	55	0	53 071	0
(Hoskins et al., 2008)	27	8-40	92	4.0-15.6	50.7	0	-	0
(Wiebkin, 2012)	9	36.4-38.6	-	12.0-13.4	47.5	0	5 116	0
(Preston et al., 2008)	10	28.51 ± 3.8	79	8.4 ± 1.8	26.5	0	32 690	0
(Zimmer et al., 2011)	19	7.73-10.83	-	7.72-10.57	<25	0	-	0

*Differences were seen in March vs December (deeper dives in March)

^Using the deeper diving birds for average depths, but full dataset for number of dives measured

Studies where maximum dive depth were not reported (e.g. (Pelletier et al., 2014)) have been excluded

Table S2. Sample sizes for each experiment by life cycle stage.

Experiment	Sample sizes by life cycle stage					TOTAL
	Fed adult females	Unfed adult females	Adult males	Fed nymphs	Unfed nymphs	
Metabolic rate assays*	35 (34)	27 (27)	39 (34)	46 (34)	42 (25)	189 (154)
Normoxic–anoxic–normoxic (NAN) respirometry		10				10
Depth tolerance	20	82	45			147
Seawater tolerance (extended survival)		15	2			17
Seawater tolerance (repeated submergence survival)		17	3			20

*The data from some individuals was not used as the individual died, moulted or laid eggs during the experiment. Numbers in brackets indicate final sample sizes, following the removal of unusable data.

Chapter Four

Penguin ectoparasite panmixia suggests frequent host movement within a colony

Phillip Island penguin colony, taken by Katherine L Moon.

This chapter is now published as:

Moon, K., P. Dann, S. Chown, A. McGaughran, C. Fraser, Penguin ectoparasite panmixia suggests extensive host movement within a colony. *The Auk* **135**(3):657-668.

4.1 Abstract

Parasite population structure can be used to infer fine-scale dispersal in host species. Many penguin species form large social colonies, and are highly philopatric, returning to the same nest or burrow, along the same route, after each trip to sea. Within a colony, however, the local abundance, physical similarity and nocturnal habits of penguins hinder the observation of fine-scale movements. To determine the extent of movement and interaction of penguins within colonies, a genotyping by sequencing (GBS) approach was used to study the fine-scale structure of ticks – which depend on host movements for dispersal – exploiting the largest little penguin (*Eudyptula novaehollandiae*) colony in Australia (Phillip Island, Victoria). No barriers to tick gene flow were identified, and we infer that extensive and frequent penguin movement occurs throughout the colony. Our findings support the hypothesis that some penguin species are highly gregarious, socialising widely within colonies despite strong nest-site philopatry.

4.2 Introduction

Penguins are primarily monogamous and philopatric, often mating with the same partner and returning each season to the same burrow or patch Croxall (1984); (Reilly and Cullen 1981; Williams 1995), usually along established routes. Such traits are generally believed to be common among many seabird species, and their spatially and temporally explicit behaviour has been thought to restrict the movements of their associated parasites (McCoy et al. 1999; but see McCoy et al. 2003a). Indeed, inbreeding hotspots have been inferred at the sub-colony level in king penguins (*Aptenodytes patagonicus*) (Cristofari et al. 2015) suggesting there is potential for structure to develop within penguin colonies. However, mate and site fidelity vary among penguin species (see Appendix table 2 in Croxall and Davis 1999), and among seabirds more generally (Coulson 2016). Research also suggests there may be considerable movement of penguins within their colonies, for example to engage in promiscuous behaviour (e.g. little penguin: Reilly and Cullen 1981; Chiaradia 1999; Adélie penguin (*Pygoscelis adeliae*): Hunter et al. 1995; Humboldt penguin (*Spheniscus humboldti*): Schwartz et al. 1999; royal penguin (*Eudyptes schlegeli*): St Clair et al. 1995).

Gregarious behaviours occur at night in some penguin species, making them particularly hard to observe and quantify. Although transponder tags have been used to study penguin movements to and from colonies (e.g. king penguins: (Le Bohec et al. 2008) and little penguins: (Hoskins et al. 2008; McCutcheon et al. 2011)), tracking stations on land are usually limited to key communal pathways (e.g. McCutcheon et al.

2011), and are therefore not ideal for studying fine-scale penguin movements within a colony. Such information is, however, useful both for understanding colony dynamics (e.g., Cristofari et al. 2015), and for local colony management (Chiaradia 1999; Reilly and Cullen 1981; Reilly and Cullen 1983).

Parasite dispersal is often largely dependent on host movement (Esch and Fernández 2013). Most parasites cannot move far on their own, and rely on transport with hosts even at the scale of metres (Esch and Fernández 2013; Falco and Fish 1991). Some host movements, such as those associated with social or feeding behaviours rather than mating behaviour, can be difficult to observe directly and will leave no genetic signal. Parasite distributions and rates of gene flow can, however, be used to infer such host movements. The use of parasites to clarify host biology, biogeography (sometimes referred to as the 'von Ihering method'), dispersal, and population structure is well established (reviewed in Esch and Fernández 2013), particularly for fish. In some cases, parasite genetic structure has been used to better effect than host genetic structure in identifying host origins (Criscione et al. 2006). Cryptic aspects of host movements can thus be inferred from associated parasite population structure.

Penguins are exploited by obligate ectoparasites when they come ashore to breed and moult. Ticks (Acari: Ixodida) are common seabird ectoparasites (Boulinier and Danchin 1996) that have direct negative impacts on the health of their hosts, such as causing delays in chick development and even death (review in Dietrich et al. 2011). Ticks are also likely to have an impact on host behaviour and population dynamics

(such as demography, decision to disperse, breeding success, and nest and colony desertion) (Boulinier and Danchin 1996; Cristofari et al. 2015), but these interactions remain largely unstudied, particularly for penguins. With warming temperatures already resulting in increased feeding by penguin ticks in some regions (Benoit et al. 2009), understanding how host interactions influence tick transmission is becoming increasingly important.

Seabird ticks have the capacity to show fine-scale, within-colony genetic structure. For example, a study of the most common seabird tick (*Ixodes uriae*) and a seabird host (the kittiwake: *Rissa tridactyla*) found that aggregation of ticks at the among-nest scale (Boulinier et al. 1996) was partly reflected in fine-scale genetic structure of the ticks (McCoy et al. 2003b).

We set out to test the extent of penguin movements across a colony via a genomic study of ticks (*Ixodes* spp.) taken from little penguins (*Eudyptula novaehollandiae*, previously *E. minor*: Grosser et al. 2015) from Phillip Island in southern Australia. Australian little penguins are predominantly parasitised by two tick species; *I. eudyptidis* and *I. kohlsi* (Roberts 1970). Although some minor morphological characteristics were originally proposed to distinguish the two species (Roberts 1970), these have since been shown not to correspond with genetic differences (Moon et al. 2015), and identification of *I. eudyptidis* versus *I. kohlsi* is thus not currently possible without genetic analysis. The life history of the two little penguin-associated *Ixodes* species (hereafter little penguin ticks) has not been investigated but is thought to be

similar to the widespread seabird tick *I. uriae* (Heath 2006), with three active life stages; larvae, nymphs and adults (see Fig. 1). Moult to each stage requires a blood meal, and the full life cycle can takes roughly three to four years to complete in *I. uriae*, but depends on host availability and may be considerably faster in temperate regions (Frenot et al. 2001). Following a blood meal, the ticks take shelter at the nest of the penguin to moult or reproduce. Available information suggests that active movement in *Ixodes* ticks may be limited to a few metres (Carroll and Schmidtmann 1996; Falco and Fish 1991). Nonetheless, we hypothesised that little penguin ticks would show little or no genetic structuring within a penguin colony, as social interactions among hosts are suspected to be high and could thus facilitate tick panmixia. We used a GBS (Elshire et al. 2011) approach to analyse over 100,000 genomic single nucleotide polymorphisms (SNPs) from penguin ticks from across the little penguin colony at Phillip Island, to assess fine-scale (metres to kilometres) population structure.

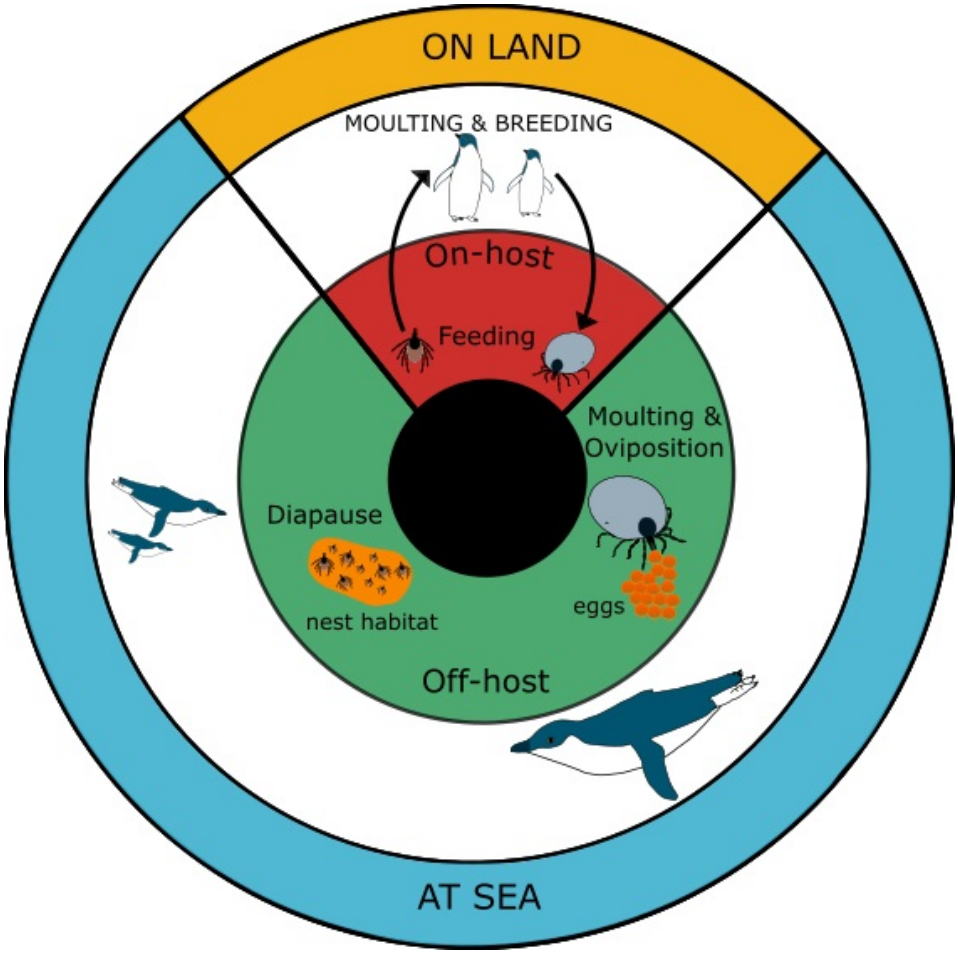

Figure 1. Relationship between penguin and Ixodidae tick life cycles. Considerable variation exists between timings of on-host and off-host phases, and will depend on whether penguins return to breeding sites during non-breeding seasons (such as in little penguins: Reilly and Cullen, 1981). Availability of hosts dictates tick life cycle length and opportunities for movement. The figure depicts a standard penguin life cycle, but in little penguins at Phillip Island there is the potential for the on-host phase to continue year-round, speeding up the tick life cycle. Figure and legend modified from Dietrich et al. (2011).

4.3 Methods

4.3.1 Study site

Phillip Island in Victoria, Australia (100 ha^2, 38.4833° S, 145.2333° E) contains the largest breeding colony of little penguins in the world with approximately 31,000 breeding birds (Sutherland and Dann 2014). Penguins have been nesting at Phillip Island for at least as long as human records extend. Females and males exhibit generally high breeding site and mate fidelity (often returning to the same burrow annually) and, like many penguin species, share responsibilities for incubation and provisioning of chicks (Reilly and Cullen 1981).

4.3.2 Sampling

Ixodidae ticks were collected from seven discrete breeding sites across Phillip Island (see Fig. 2). Soft ticks (Argasidae, lacking a scutum) were rare and were therefore not sampled. Sites at Phillip Island were chosen to represent clumped distributions of nest burrows, with penguins from each area known by park managers to take distinct routes to the sea, and inferred to have different foraging associations (see Supplementary Fig. S1). Sites were separated by areas of habitat that were unsuitable for ticks (without hosts or shelter) and which exceeded the limits of active movement previously recorded for the *Ixodes* genus (Falco and Fish 1991).

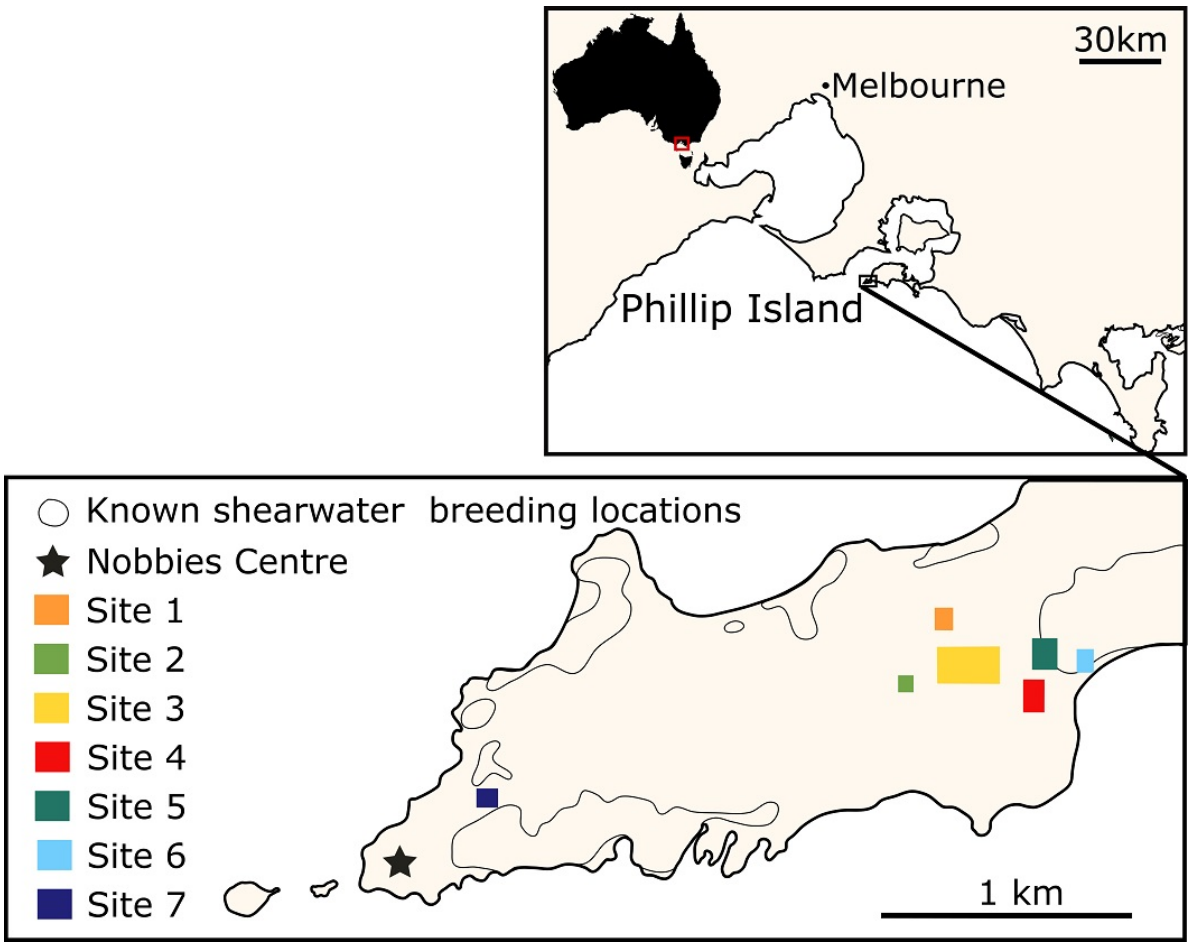

Figure 2. Map of study sites at Phillip Island in Victoria, Australia.

Sampling was undertaken in November of 2014, and was conducted as per methods outlined in Moon et al. (2015). During the breeding season, ticks were taken from the nest environment, either inside burrows or nest boxes of the little penguins. Forceps were also used to remove ticks directly from chicks at the site. In total, 174 ticks were obtained from 117 burrows or nest boxes. Upon collection, ticks were immediately placed in 96% ethanol for preservation. Because blue tongue lizards (*Tiliqua nigrolutea*) are commonly found moving among penguin burrows on Phillip Island, 15 ticks were also obtained from these lizards to enable testing of tick host-specificity.

4.3.3 DNA extraction

Extractions were undertaken as per the Qiagen (Qiagen, Valencia, CA) QIAamp DNA Micro Kit Protocol: Isolation of Genomic DNA from Tissues. Between 4-8 tick legs were removed from adult- and nymph-stage specimens, with remaining tissue kept as a voucher for morphological analysis. Unfed nymphs and any larval specimens were extracted whole, due to their small size and lack of blood meal. The tick tissue was placed in liquid nitrogen immediately following removal from the body. Sterilised micropestles were used to grind tissue before adding the Buffer ATL and proteinase K. Extractions were incubated overnight at 56 °C and eluted in 50 µl of elution buffer.

4.3.4 Genetic sequencing

4.3.4.1 COI amplification

Because we were interested in intra- rather than inter-specific differences, and because *I. kohlsi* and *I. eudyptidis* cannot readily be distinguished without genetic analysis, we first sequenced all samples from Australia for mitochondrial COI as a 'barcoding' (species delineation) tool. PCR amplifications were carried out in 25 µl volumes, each containing 2.5 µl of DNA, 10 x buffer Q solution, 0.8 mM of dNTPs, 1.5 mM $MgCl_2$ and 1 U of EconoTaq DNA Polymerase (Lucigen Corporation, Middleton, Wisconsin, United States of America) and 0.5 µM each of PCR primers LCOI490 and HCO2198 (Folmer et al. 1994). Amplification was performed in an Eppendorf Mastercycler (EP Gradient S, Eppendorf, Hamberg Germany) using the following profile: 94°C for 2 minutes; 40 cycles of 15 s at 94°C, 30 s at 45°C, 1 min at 72°C, followed by a final 4

min extension at 72°C. Resulting PCR products were quantified and sequenced by Macrogen Inc. Standard Sequencing Service (Guman-sugan, Korea). Geneious 6.1.6 (available at http://www.geneious.com) (Kearse et al. 2012) was used to process, align and check the sequence data.

4.3.4.2 Genotyping by sequencing library preparation

Library preparations for GBS were carried out as per Elshire et al. (2011) with the following alterations: DNA extractions were transferred to a 96-well plate and dried using a vacuum centrifuge at 45°C, before being re-suspended in 15 µl of MilliQ H_2O. A uniquely barcoded PstI adapter (2.25 ng) was added to each sample to enable pooling of samples for sequencing (Elshire et al. 2011). DNA digestion was undertaken using Pst1-HF (New England Biolabs, Ipswich, MA) in 10 x NEBuffer 4 (New England Biolabs, Ipswich, MA), with a 2-hour incubation at 37°C. Adapter ligation was performed with T4 DNA Ligase and 10 x ligation buffer (New England Biolabs, Ipswich, MA), with incubation at 16°C for 90 minutes and 80°C for 30 minutes. Purification was performed with a Qiagen (Qiagen, Valencia, CA) MinElute 96-well PCR Purification Kit, with elution in 25 µl of 1 x TE Buffer. PCRs were undertaken in 50 µl volumes, each containing 10 µl of purified DNA product, 25 µl of 1 x MyTaq^{TM} HS Master Mix (Bioline), 13 µl of MilliQ H_2O, and 1 µM each of forward and reverse PCR primer (forward: 5'AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATC*T and reverse: 5'CAAGCAGAAGACGGCATACGAGATCGGTCTCGGCATTCCTGCTGAACCG

CTCTTCCGATC*T, where * indicates phosphorothioation) (see Elshire et al. 2011). PCRs were carried out in an Eppendorf Mastercycler Nexus under the following conditions: 72°C for 5 min, 95°C for 60 s, and 24 cycles of 95°C for 30 s, 65°C for 30 s, and 72°C for 30 s, and a final extension step at 72°C for 5 min. A LabChip GXII (Caliper Life Sciences) was used to assess DNA concentrations before samples were pooled (containing 20 ng of DNA per sample). Gel electrophoresis was conducted on a 1.5% agarose gel to achieve size fractionation. A 100-bp range (from 400-500 bp fragments) was selected for paired-end sequencing, which was carried out on two lanes of an Illumina HiSeq 2500. Sequencing was undertaken by the Bimolecular Resource Facility in the John Curtain School of Medical Research at the Australian National University.

4.3.5 Analysis

4.3.5.1 COI data

Maximum Likelihood (ML) phylogenetic analyses were carried out on the COI data using PhyML 3.0 (Guindon et al. 2010) with evolutionary model parameters as estimated by the Akaike Information Criterion (AIC) of jModeltest2 (Darriba et al. 2012). Outgroups were included to root trees (see Supplementary Table S1 for a list of GenBank Accession numbers), and included sequences from a study previously undertaken at the site (Moon et al. 2015). ML analyses were performed with a GTR + I + Γ model (as selected by jModeltest2; base frequencies A = 0.3235, C = 0.1704, G = 0.1036, T = 0.4026, gamma shape parameter: 0.7030; proportion of invariant sites: 0.471). While a TPM1uf+I+Γ model was originally selected by jModeltest2, PhyML

3.0 does not support this model, so GTR was implemented instead, but with I and Γ parameters as estimated by jModeltest2. Support for each node was assessed by bootstrapping, with heuristic analysis of 1,000 replicate data sets.

Bayesian phylogenetic analysis was subsequently carried out using MrBayes (Huelsenbeck and Ronquist 2001) to confirm ML analyses, and incorporated the same outgroups used in the ML analyses (see Supplementary Table S1). Markov Chain Monte Carlo (MCMC) searches were executed with a total of four chains of 5,000,000 generations, with trees samples every 100 generations, and the first 10,000 trees discarded as burn-in. Convergence was confirmed using the MrBayes output and Tracer 1.6 (Rambaut et al. 2014). ESS values, which were all well above 200 (>900), were used to confirm convergence as well as examining the MCMC trace.

4.3.5.2 Genotyping by sequencing data

Raw Illumina data were processed using the *Stacks* 1.35 pipeline (Catchen et al. 2013). Fragments were first demultiplexed using ligated barcodes and all sequences were trimmed to 93 bp. As the phylogenetic analysis showed two deeply divergent clades probably representing distinct species, *Stacks* (Catchen et al. 2013) was used to process the short-read sequences for each clade separately. In the absence of a reference genome, *de novo* assembly was employed, calling each component of the pipeline separately. Several sequential scripts were used to demultiplex and quality control the reads (process_radtags), stack homologous reads to build loci and call SNPs for each sample given the polymorphisms assayed by Illumina (ustacks), create

a catalogue of all loci (cstacks) and match the loci of each sample against the catalogue (sstacks). The process_radtags script discards a read if its quality drops below a 90% probability of being correct (a phred score of 10). The minimum depth of coverage required to create a stack in ustacks was set to 5, the maximum distance (in nucleotides) allowed between stacks was set to the default of 2, the maximum distance (in nucleotides) allowed to align secondary reads to primary stacks was set to 0, and the removal algorithm was enabled to remove highly repetitive stacks. In cstacks, the number of mismatches allowed between sample loci when building the catalogue was set to the default of 1. The populations *Stacks* script was then used to filter the data and export loci for downstream analyses. The minimum minor allele frequency required to process a nucleotide site at a locus was set to 0.01 and the minimum percentage of individuals required to process a locus was set to 20%, meaning each SNP had to be present in 20% of the individuals to be called. PGDSpider 2 (Lischer and Excoffier 2012) was used to convert all full data set FASTA files into BayeScan files. These files were then imported into BayeScan 2.1 (available at http://cmpg.unibe.ch/software/BayeScan/) to test for loci under selection. Output files from BayeScan were analysed with R 3.1.2 (R Core Team 2014) using the plot_R.r file provided with the BayeScan download. Using a false discovery rate of 0.01 (a 1% chance of a false positive), 99.96% of loci were assigned as neutral, thus none were removed from subsequent analysis.

Complete GBS datasets

The .plink file outputs of the full data set from the *Stacks* population script were used to generate Principle Components Analysis (PCA) plots for each clade. The data set for Clade B included 3,849 SNPs, whereas the data set for Clade A included 103,156 SNPs (see Table 1 for site representation). PCA is a model-free method that focuses on eigenvalue decomposition to visualise underlying population structure. fcgene 1.0.7 (Roshyara and Scholz 2014) was used to convert the .map and .ped plink output files from the populations script into eigensoft format. The convertif script from EIGENSOFT 6.1.2 (Price et al. 2006) was then used to convert the files to eigenstrat format. The SmartPCA script from the same package was used to record the number of SNPs present in each analysis, as well as the eigenvalue, Tracy-Widom Statistic (Patterson et al. 2006) and associated *p*-value for each principle component. R 3.1.2 (R Core Team 2014) was used to plot the SmartPCA output with each individual coloured by site.

Table 1. Number of penguin ticks representing each site in the mitochondrial and genomic analysis by clade.

Clade	Site 1	Site 2	Site 3	Site 4	Site 5	Site 6	Site 7
A	21	16	20	22	27	27	8
B				7			9

The .plink files from the *Stacks* populations script were also used to infer population structure with fastSTRUCTURE 1.0 (Raj et al. 2014), which employs a Bayesian model-based approach. Each value of *K* from *K*=1 to *K*=10 was run five times using the structure.py code. The choosek.py script was then used to choose the most likely

value of *K*. When the choosek script in fastSTRUCTURE is asked to find the true number of populations when underlying population structure is very weak, a range of values is given. Using the *K* value/s chosen by fastSTRUCTURE, Distruct 2 (available at http://www.crypticlineage.net/pages/distruct.html) was used to visualise population assignment/s.

Reduced GBS datasets

For population genetic analyses unable to cope with large SNP data sets, three subsets of 1,000 random SNPs were generated for each of the full data sets, using a simple grep command performed on the summary file produced by *Stacks*. Three separate replicates were used to ensure there was no bias. These reduced data sets were converted to nexus format using PGDSpider 2 (Lischer and Excoffier 2012), and made into .csv files. The .csv files were then imported into R 3.1.2 (R Core Team 2014) and adegenet 1.4-2 (Jombart 2008), Hierfstat 0.04-14 (Goudet 2005) and poppr 1.1.5 (Kamvar et al. 2014) packages were used to conduct basic population genetic calculations, as well as analyses of molecular variance (AMOVA), for each reduced data set to investigate the relative importance of within and among-site genetic variation. Significance ($p < 0.01$) of AMOVAs was appraised with 999 random permutations of the data.

4.4 Results

4.4.1 Species delineation

Genotyping by sequencing and COI data were obtained for a total of 15 blue tongue lizard ticks and 157 penguin ticks from Phillip Island (see Table 1). Both Maximum Likelihood and Bayesian analyses revealed two well-supported monophyletic clades (one common and one rare) for most of the penguin ticks (Supplementary Fig. S2), and seven individuals that grouped with the lizard ticks. Topologies were consistent between methods and large sequence divergences separated each group. Furthermore, the clades were consistent with those identified in a previous study of penguin ticks at the site (Moon et al. 2015) see Supplementary Table S1 for Genbank Accession numbers). Given the large divergence (13.7-17.3% uncorrected p distance: Moon et al. 2015), these clades are likely to represent the two major species that exploit little penguins (*Ixodes eudyptidis* and *I. kohlsi*).

An outgroup comparison with a *Bothriocroton* tick species (*Bothriocroton hydrosauri*) confirmed that the ticks from the blue tongue lizards were not from the same genus as those on penguins, but seven ticks taken from penguin burrows grouped with this reptile tick genus (Supplementary Fig. S2). These ticks were removed from the GBS analyses, which was carried out on the separate penguin tick clades to assess within-species structure. Henceforth, Clade A refers to the more common clade, comprising 141 ticks from all seven sites, while Clade B refers to the rarer clade, with 16 ticks with limited representation across two sites.

4.4.2 Population structure

4.4.2.1 Complete GBS data sets

SmartPCA analyses

SmartPCA analyses for the complete data sets of Clade A and Clade B from Phillip Island suggests there was no significant population structuring (Fig. 3 and Supplementary Table S2), though Clade B had limited representation (see Table 1). Analyses were conducted on the first 10 principal components (PCs). The first two PCs explain 23.85% (PC1: 13.45%, PC2: 10.40%) of the total variation in Clade A and 33.00% (PC1: 22.00%, PC2: 10.99%) of the total variation in Clade B (Fig. 3). Tracy-Widom statistics were non-significant for all but the first PC of both clades, a result consistent with absence of genetic structure. While some outliers were evident in the PCA plots, there is no geographic basis for the genetic variation in either clade, suggesting no geographic barriers exist to tick movement across the Phillip Island colony.

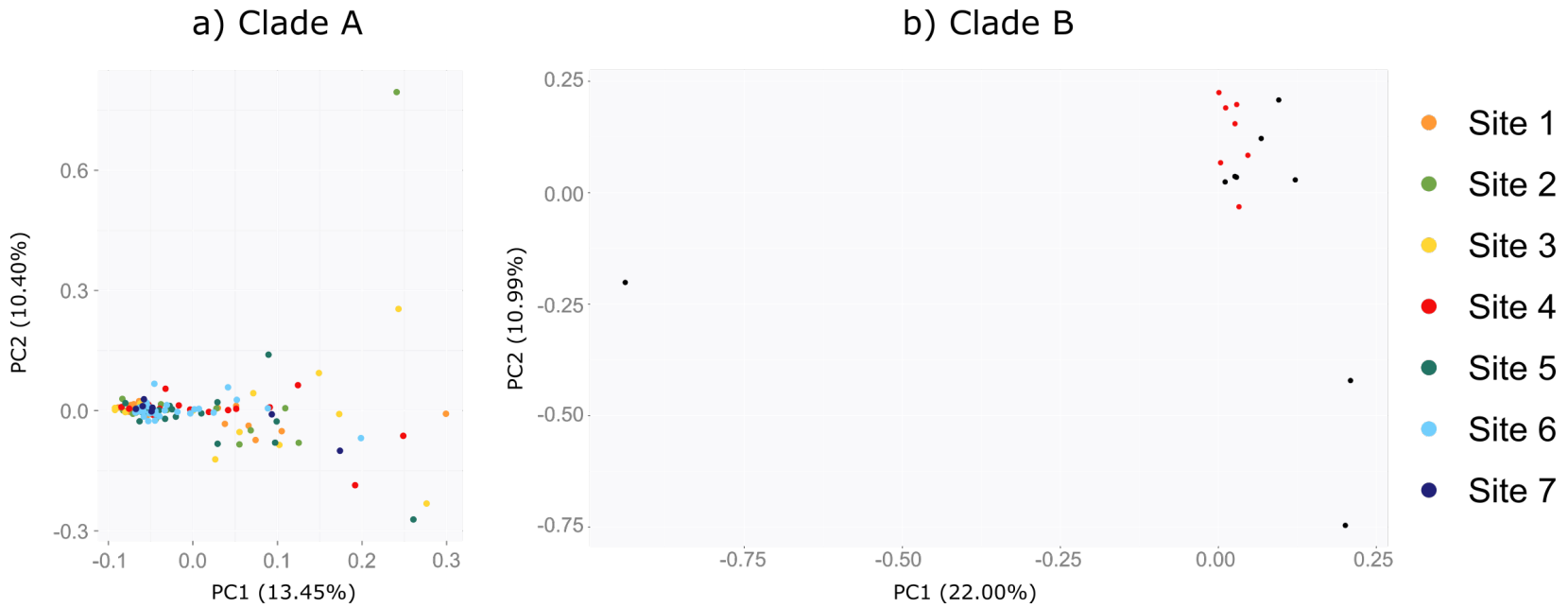

Figure 3. PCA plots of both a) Clade A and b) Clade B Phillip Island little penguin tick samples. Percentage of variation explained by each principal component (PC) is given in brackets. Sites are differentiated by colour. The absence of any clear clustering by site suggests there is little geographic genetic structure.

FastSTRUCTURE analyses

FastSTRUCTURE analyses of the complete data sets of both Clade A and Clade B also showed that individuals with similar ancestry were not geographically co-located, with one major population predominating (Fig. 4). FastSTRUCTURE analyses of Clade A from Phillip Island resulted in a model complexity of K=2-3 (Fig. 4a). When the likelihood of membership to each population given each expected value of K was plotted with distruct 2 (Fig. 4a), however, there was no clear genetic differentiation among sites. FastSTRUCTURE analyses of Clade B resulted in a model complexity of K=2, and the distruct 2 plot (Fig. 4b) shows that the two populations are completely intermixed at the two sites.

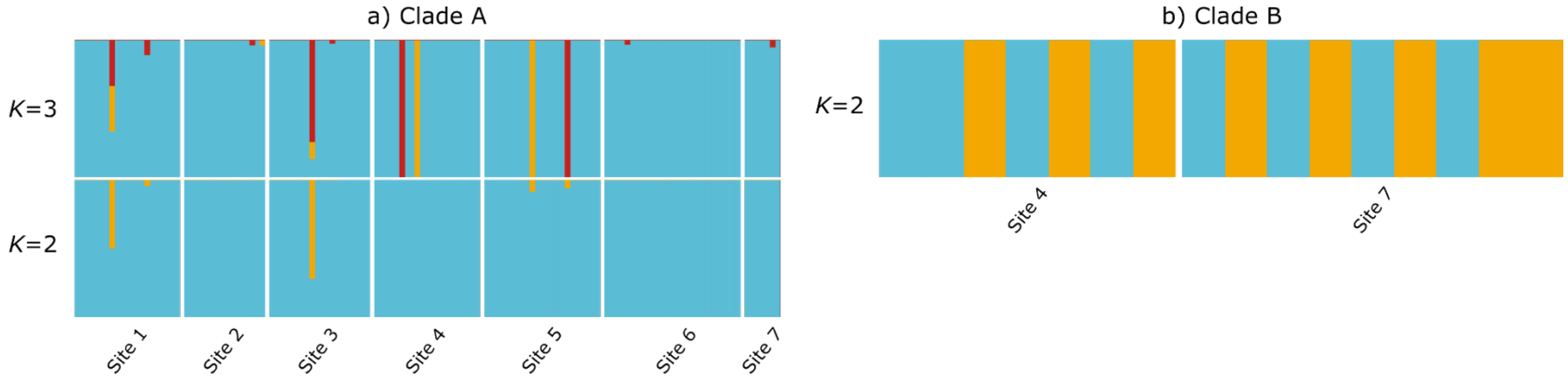

Figure 4. fastSTRUCTURE Distruct2 plots of both a) Clade A and b) Clade B Phillip Island penguin tick samples.

Reduced GBS data sets

Analyses of Molecular Variance (AMOVA) for all three of the 1,000 SNP reduced data sets of the Clade A samples from Phillip Island showed that genetic variation within sites (within populations and individuals) accounted for almost all genetic diversity (99.7-99.9%: see Table 2a), compared to variation among sites (0.1%-0.3%: see Table 2a and Supplementary Table S3). Consistent results from all three data sets suggests there is no linkage of the SNPs. Effect sizes were small (between 0.046-0.048: see Table 2a), and analyses of variation among populations were not significant ($p < 0.01$).

Table 2. AMOVA analysis results for 1,000 SNP reduced data sets of Phillip Island samples. No analyses were statistically significant ($p < 0.01$). Basic effect sizes (partial eta-squared: η^2) for variation among populations were calculated using the following equation:

$$\eta^2 = \frac{\text{Sum of Squares}_{\text{AMONG POPULATIONS}}}{\text{Sum of Squares}_{\text{AMONG POPULATIONS}} + \text{Sum of Squares}_{\text{WITHIN POPULATIONS}}}$$

According to Cohen (1969), effect sizes of $\eta^2 = 0.0099$ are considered small, 0.0588 moderate and 0.1379 are considered large (see discussion in Richardson, 2011).

a) Clade A

Replicate	% variation among pops	% variation within pops	% variation within individuals	*p*-value	Effect size (partial η^2)
1	0.334	92.526	7.140	0.179	0.048
2	0.270	93.223	6.507	0.209	0.047
3	0.101	92.571	7.328	0.318	0.046

b) Clade B

Replicate	% variation among pops	% variation within pops	% variation within individuals	*p*-value	Effect size (partial η^2)
1	7.368	68.544	24.088	0.034	0.109
2	8.167	68.458	23.375	0.012	0.114
3	7.963	69.406	22.631	0.018	0.113

AMOVA analyses of reduced data sets of Clade B from Phillip Island also indicated that genetic variation within sites accounted for most of the genetic diversity (92.0-92.6%: see Table 2b and Supplementary Table S3). Effect sizes were moderate (0.109-0.113: see Table 2b) and variation among populations was not significant ($p < 0.01$). Although all three data sets provided consistent results, ticks from Clade B were poorly represented in analyses.

4.5 Discussion

Ticks from little penguins at Phillip Island were found to be panmictic within the colony, supporting the hypothesis of no significant barriers to tick gene flow. Sites were separated by habitat that is unsuitable for ticks, suggesting the parasites would be reliant on hosts for fine-scale dispersal. The absence of tick population structure may therefore be driven by penguin chick behaviours (such as tendency to form loose crèches while waiting for food), prospecting behaviours (especially by failed and pre-breeding birds), promiscuous activity exhibited by breeding penguins, and moulting outside nesting areas. We infer that penguin movement within colonies can be both frequent and extensive, facilitating local dispersal of parasites. Host biology, breeding colony topology, and the parasite's host-specificity likely underpin these patterns, as discussed below. Our results also support the presence of two sympatric, but as yet cryptic, penguin tick species at Phillip Island, one being less common than the other.

4.5.1 Alternative host usage

Host-specificity is common in seabird ticks (e.g. McCoy et al. 2005), though may not occur as frequently in penguin ticks (McCoy et al. 2012). Consequently, secondary hosts such as sympatric seabirds or other animals present at a colony could be responsible for the movement of penguin ticks. At Phillip Island, for example, blue tongue lizards are a common visitor among and even within penguin burrows, and these lizards are often heavily infested with Ixodidae ticks. Our results, however, indicate that the ticks parasitising blue tongue lizards are distinct from those parasitising penguins. Ticks on blue tongue lizards were identified as *Bothriocroton* spp. by expert morphological examination (Allen Heath, TePapa Museum, Wellington) and this was supported by genetic analysis, which grouped the ticks into a genus distinct from the penguin ticks (Supplementary Fig. S2). Blue tongue lizards are thus not likely to be facilitating intra-colonial movement of penguin ticks.

Sympatric seabirds that have previously been found to share seabird tick species / lineages either occupied very similar ecological niches (such as between three penguin species in the Antarctic Peninsula: McCoy et al. 2012), or were phylogenetically closely related (such as between two species of *Eudyptes* penguin sampled at Possession and Kerguelen Islands: McCoy et al. 2005, or between sibling seabird species in the North Pacific: Dietrich et al. 2012). Little penguins do not share ecological niches nor phylogenetic similarities with any sympatric seabirds (short-tailed shearwaters: *Ardenna tenuirostris*; silver gulls: *Chroicocephalus novaehollandiae*; kelp gulls: *Larus dominicanus*; Pacific gulls: *L. pacificus*; and crested terns: *Thalasseus bergii*) at Phillip Island, and while shearwaters have been

recorded breeding close to the penguins (see Fig. 2), there were no shearwaters near our sampling locations. Thus, in the absence of any evidence for little penguin ticks using secondary hosts (such as shearwaters or blue tongue lizards) at our study sites, we infer that the genetic patterns observed are predominantly the result of penguin movements.

Our results are in-keeping with previous suggestions of considerable social interactions across penguin colonies, based on behavioural studies of individual species (e.g. Richdale, 1951). Nonetheless, as our findings are based on a single colony of a single penguin species, we cannot be sure the gregarious behaviours of little penguins are shared by other penguin species. Future studies should examine whether social interactions are as extensive in other penguin species and colonies, and whether these interactions also facilitate parasite transmission. Broader-scale studies and / or simulation analyses could also provide greater insights on the relative influence of other factors, such as tick effective population size, on patterns. However, with structure observed at scale of only metres in common ixodid tick species elsewhere (e.g., kittiwakes: McCoy *et al*, 2003a), and evidence that seabird ticks have very limited active dispersal capacity (Falco and Fish 1991), we infer that the absence of within-colony genetic structure in penguin ticks at Phillip Island is most probably the result of frequent, penguin-facilitated movement.

4.5.2 Chick associations

Because of their constant presence at colonies during the breeding season, penguin chicks are more likely than adults to be parasitised by ticks. Little penguin chicks tend to come out of burrows and nest boxes as evening approaches, gathering together in groups to await the return of their parents (Richdale 1951). Chicks may also inhabit burrows during the day with chicks from other broods, with up to eight chicks being found in one burrow during daytime checks at Phillip Island (P. Dann, personal observation). This clustering behaviour could facilitate transmission of ticks among burrows and regions. Furthermore, at sundown during the breeding season, returning adults associate in groups ('rafts') offshore before returning to their burrows en masse. Bold, hungry chicks will physically confront adults that are not their parents in the hope of gaining a meal. Physically confronting unrelated adults for food appears to be a trait common amongst penguins (Williams 1995), and can drive adults away from burrows following feeding (K.L. Moon, personal observation). Chick crèching behaviours have also been noted in many other species of penguin, including southern rockhopper (*Eudyptes chrysocome*), Gentoo (*Pygoscelis papua*), Adélie and yellow-eyed penguins (*Megadyptes antipodes*) (Richdale 1951; Williams 1995). In the absence of burrows, this behaviour likely protects chicks against predation (due to predator swamping) and harsh environmental conditions. Aggregation of little penguin chicks may therefore represent an opportunity for transfer of ticks between different burrows or nest sites.

4.5.3 Adult associations

Once fledged, some penguin species disperse far from natal sites (Reilly and Cullen 1981). During this time, pre-breeding penguins may return to natal colonies or disperse to other colonies to moult and walk through the site looking for future breeding locations (known as prospecting). Prospecting behaviours are thought to account for most seabird tick dispersal (Danchin 1992; Dietrich et al. 2011). Pre-breeding penguins are also known to associate in pairs at the colony during breeding season for a few years before their first clutch (arriving increasingly early leading up to their first season), in a behaviour known as 'keeping company' (Rowley 1983). During this time there is considerable interaction between individuals (Rowley 1983).

Mate and nest fidelity in most penguins are generally high, but vary considerably between species and between sexes, and may be much lower during years of low food availability (Croxall and Davis 1999; Williams and Rodwell 1992). Penguin divorce rates can be anywhere between <10% (macaroni: *Eudyptes chrysolophus*; Gentoo and magellanic: *Spheniscus magellanicus*) and <35% (rockhopper, little, chinstrap: *Pygoscelis antarctica*; African: *Spheniscus demersus*; yellow-eyed penguins), to >75% (emperor: *Aptenodytes forsteri* and king penguins) a year (Reilly and Cullen, 1981; and see Appendix Table 2 in Croxall and Davis, 1999). Searching for and acquiring new mates or nest sites may facilitate parasite dispersal due to increased interaction between adults on land. Little penguins have divorce rates of approximately 18% per year (Croxall and Davis 1999; Reilly and Cullen 1981). Little penguins are also unique in that they are the only penguin species capable of a double clutch, and have been

known to change mates between clutches in one season (Reilly and Cullen 1981) creating more opportunities for parasite transmission.

Investigations of burrow or nest box fidelity in little penguins suggest nest-swapping events also occur relatively commonly, though penguins often appear to remain nearby (Chiaradia 1999; Reilly and Cullen 1981). As in mate-changing, the prospecting behaviours associated with the dynamics of nest ownership may facilitate the movement of parasites across Phillip Island.

Several penguin species are also often involved in extra-pair activities. Little penguins have been recorded in burrows with birds that are neither their old nor new mate during the pre-egg period (before reuniting with partners), during the breeding season and during the moult (Chiaradia 1999; Reilly and Cullen 1981; Reilly and Cullen 1983). Penguins of both sexes have also been identified trying to copulate outside their partnership (called extra-pair copulation or EPC). EPC also occurs in Adélie penguins (Chiaradia 1999; Hunter et al. 1995), Humboldt penguins (*Spheniscus humboldti*) (Schwartz et al. 1999) and royal penguins (*Eudyptes schlegeli*: evidenced by extra-pair fertilisations: (St Clair et al. 1995)), and may occur in other species. This behaviour often occurs quickly, at night, in burrows, and does not always result in offspring (see example in Schwartz et al. 1999) making EPC difficult to observe (Chiaradia 1999) even using host genetic patterns.

The geography of a colony will also influence parasite transmission (see McCoy et al. 2003b for an example). Much like penguins, puffins move around colonies freely and even enter burrows during prospecting, while kittiwakes breed on cliff faces with limited access. In accordance with this behaviour, puffin ticks have been found to exhibit no significant genetic structure (in microsatellites) between populations separated by more than 1,000 km, but kittiwake ticks were structured over much smaller (metres) scales (McCoy et al. 2003a; McCoy et al. 2003b). Likewise, patchy availability of nest sites (surrounded by uninhabitable rock) was found to increase population structure in ticks because they require suitable shelter and microclimates (Benoit et al. 2007; McCoy et al. 2003b). Roughly one quarter of breeding birds have been found in burrows on any given night during off-breeding times at Phillip Island (Reilly and Cullen 1981). Year-round presence at the colony may also increase the opportunities for tick movement within colonies, especially as birds may not only shelter in their own burrows, but use others nearby (Reilly and Cullen 1981).

While much of the current seabird tick literature focuses on the barriers to gene flow imposed by host life history traits (McCoy et al. 1999; McCoy et al. 2003a; McCoy et al. 2003b), our results indicate that penguins' gregarious natures facilitate a considerable amount of tick movement within their colonies, reducing local (within colony) adaptation and genetic differentiation of ticks. Understanding the influence of host interactions on the transmission of potentially harmful parasites is of growing importance for penguin colonies, as they face increasing pressure from anthropogenic climate change across their range.

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4.8 Supplementary material

4.8.1 Figures and tables

Figure S1. Movements and associations of little penguins at Phillip Island. Arrows indicate routes adult penguins use to access their foraging sites at sea. Penguins from Sites 4, 5 and 6 feed in approximately the same location and come up on the same beach (Penguin Parade), but were considered by local managers to be unlikely to associate or move between the sites on land based on long term (>40 years) monitoring conducted at the sites. Penguins from Site 7 feed in an entirely different location to 4, 5 and 6. Sites 1, 2 and 3, though close, are made up of birds that feed in different locations, and come ashore on opposite sides of the island. All nesting locations were clumped and separated by areas with no penguin burrows.

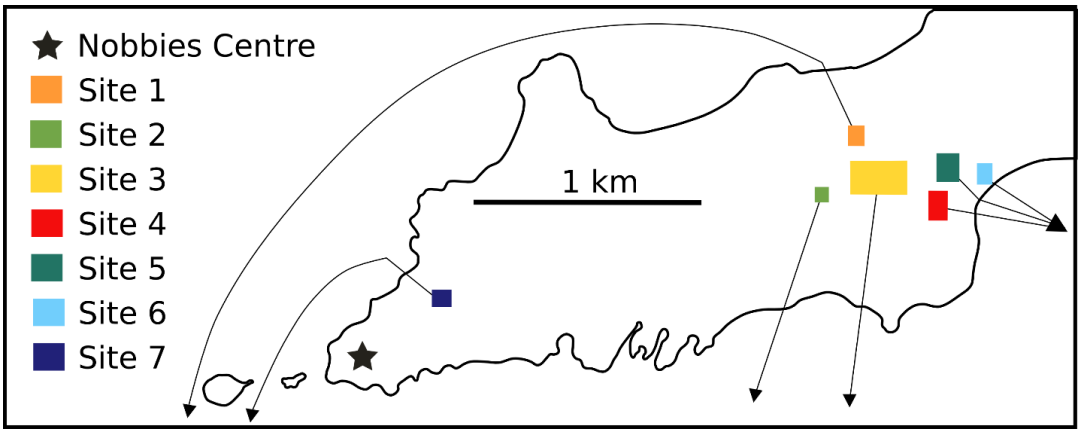

Figure S2. Maximum likelihood tree for COI of Phillip Island samples. Bootstrap values (as percentages in black) and Bayesian PP values (as probability values in blue) are shown. Pie charts indicate site representation in Clade A (likely to represent *Ixodes kohlsi*) and Clade B (likely to represent *Ixodes eudyptidis*), as well as in the penguin ticks that were found to group with the *Bothriocroton* genus. All blue tongue ticks also grouped with this genus. Outgroups are shown, and include specimens from a previous little penguin tick study (see Supplementary Table S1).

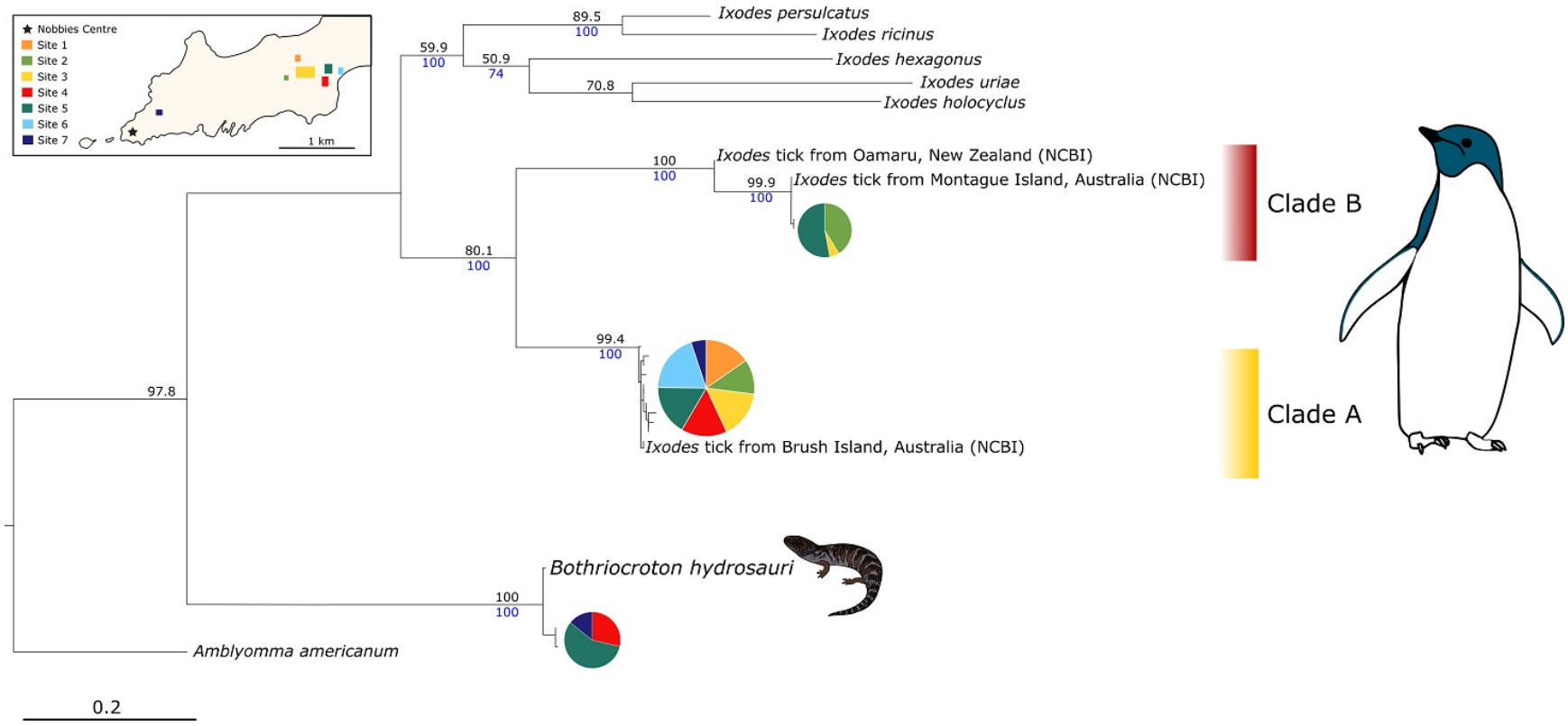
Nobbies Centre
Site 1
Site 2
Site 3
Site 4
Site 5
Site 6
Site 7
1 km
89.5
100
Ixodes persulcatus
Ixodes ricinus
59.9
100
50.9
74
Ixodes hexagonus
70.8
Ixodes uriae
Ixodes holocyclus
100
100
Ixodes tick from Oamaru, New Zealand (NCBI)
99.9
100
Ixodes tick from Montague Island, Australia (NCBI)
80.1
100
99.4
100
97.8
Ixodes tick from Brush Island, Australia (NCBI)
Clade B
Clade A
Bothriocroton hydrosauri
100
100
Amblyomma americanum
0.2

Table S1. NCBI accession numbers used in COI analysis

Species	NCBI accession number used
Ixodes sp. taken from Oamaru, New Zealand that grouped with the OAMA clade (see Moon *et al.,* 2015)- referred to as OAMA Clade Tick Oamaru in Supplementary Fig. S2.	KM488506.1
Ixodes sp. taken from Montague Island, Australia that grouped with the OAMA clade (see Moon *et al.,* 2015)- referred to as OAMA Clade Tick Montague Island in Supplementary Fig. S2.	KM488528.1
Ixodes sp. taken from Brush Island, Australia that grouped with the AUST clade (see Moon *et al.,* 2015)- referred to as AUST Clade Tick Brush Island in Supplementary Fig. S2.	KM488521.1
Bothriocroton hydrosauri (reptile tick)	FJ584422.1
Ixodes persulcatus	KU935457.1
Ixodes uriae	AB087746.1
Ixodes ricinus	KF197134.1
Ixodes hexagonus	AF081828.1
Ixodes holocyclus	AB075955.1

Amblyomma americanum	DQ168131.1

Table S2. Full results of the SmartPCA analysis results for a) Clade A and b) Clade B, including eigenvalues, Tracy-Widom statistics, *p*-values and percentage of variation explained for each of the first 10 principal components. Tracy-Widom (TW) *p*-values that were found to be significant ($p < 0.01$) are in **bold**.

a) Clade A

Principal Component	Eigenvalue	% Variance explained	Tracy-Widom statistic	TW *p*-value
1	**2.965374**	**13.45**	**13.195**	**0.000**
2	2.292497	10.40	-1.299	0.510
3	2.244089	10.18	-1.533	0.584
4	2.219468	10.07	-1.164	0.467
5	2.136483	9.69	-2.395	0.826
6	2.122988	9.63	-1.806	0.669
7	2.068336	9.38	-2.359	0.818
8	2.051460	9.31	-1.878	0.690
9	1.998486	9.07	-2.441	0.836
10	1.946686	8.83	-3.043	0.936

b) Clade B

Principal Component	Eigenvalue	% Variance explained	Tracy-Widom statistic	TW *p*-value
1	**2.634424**	**22.00**	**3.763**	**0.000**
2	1.316330	10.99	-1.928	0.705
3	1.196618	9.99	-2.714	0.889
4	1.167010	9.75	-2.175	0.773
5	1.079770	9.02	-2.570	0.863
6	1.054757	8.81	-1.938	0.708
7	0.988992	8.26	NA	NA
8	0.957386	8.00	NA	NA
9	0.811002	6.77	NA	NA
10	0.766475	6.40	NA	NA

Table S3. Basic statistics for each reduced dataset replicate for a) Clade A and b) Clade B.

a) Clade A

Replicate	Ho	Hs	Ht	Dst	Htp	Dstp	Fst	Fstp	Fis	Dest
1	0.028	0.392	0.393	0.001	0.393	0.002	0.003	0.004	0.929	0.003
2	0.025	0.392	0.393	0.001	0.393	0.001	0.003	0.003	0.935	0.002
3	0.029	0.396	0.397	0.001	0.397	0.001	0.002	0.002	0.927	0.002

b) Clade B

Replicate	Ho	Hs	Ht	Dst	Htp	Dstp	Fst	Fstp	Fis	Dest
1	0.107	0.408	0.424	0.017	0.441	0.034	0.040	0.076	0.739	0.057
2	0.102	0.398	0.417	0.018	0.435	0.037	0.044	0.084	0.744	0.061
3	0.100	0.406	0.424	0.018	0.442	0.036	0.043	0.082	0.753	0.061

Where:

Ho Observed heterozygosity

Hs Mean gene diversities within a population

Ht Overall gene diversity

Dst Gene diversity among samples (Dst=Ht-Hs)

Htp Corrected overall gene diversity

Dstp Corrected gene diversity among samples

Fst Fixation index

Fstp Corrected Fst

Fis	Calculated following Nei (1987) per overall loci
Dest	Measure of population differentiation as defined by Jost (2008)

Chapter Five

Phylogeographic patterns similar in penguins and their ectoparasites

The author removing a late stage chick from a penguin burrow on Brush Island in New South Wales. Taken by Ceridwen Fraser.

This chapter has been formatted for submission to the *Journal of Biogeography*.

5.1 Abstract

Aim

There is increasing recognition that long-distance dispersal has had a major influence on global biogeographic patterns. Commensalistic, mutualistic and parasitic relationships often involve organisms with somewhat differing life histories, however, which could present challenges for concerted dispersal and lead to discordant biogeographies among partners. Penguins, for example, are exploited by terrestrial ectoparasites (including ticks) when they come ashore to breed. Recent phylogenetic and physiological studies suggest that penguin ticks may be capable of surviving short periods at sea with their hosts, but their capacity to survive longer voyages is not known. We here aimed to assess whether phylogeographic patterns in penguins and their ticks indicate that the terrestrial parasites are able to disperse long distances at sea with their swimming hosts.

Location

Southern Australia and New Zealand.

Methods

We conducted a broad-scale genomic assessment of little penguin (*Eudyptula minor* and *E. novaehollandiae*) ticks (*Ixodes eudyptidis* and *I. kohlsi*) from across their hosts' ranges in Australia and New Zealand. Using Genotyping-by-Sequencing, we generated SNP data sets from ticks from 14 penguin colonies, and analysed phylogeographic structure. We included ticks from sympatric flighted seabirds to test for host-specificity.

Results

We resolved two distinct lineages of *Ixodes* from little penguins, with one restricted to Australia, and the other found throughout New Zealand and in low numbers at some Australian sites. Both lineages exhibited phylogeographic structure consistent with patterns observed in their hosts, with some evidence of occasional dispersal, including across the Tasman Sea between Australia and New Zealand. Ticks from sympatric shearwaters were genetically distinct to those collected from little penguins.

Main conclusions

Some terrestrial ectoparasites associated with aquatically-dispersing hosts have apparently evolved the capacity to survive oceanic voyages: little penguin ticks appear capable of surviving considerable aquatic, and even trans-oceanic, dispersal events with their hosts.

5.2 Introduction

Oceanic dispersal is emerging as an important mechanism underpinning Southern Hemisphere biodiversity patterns (McGlone, 2005; Sanmartín and Ronquist, 2004; Waters, 2008a). Phylogeographic analyses of plants, invertebrates, bats, birds, fish, and marine animals, for example, indicate that post-Gondwanan (< 80 Ma) trans-oceanic movement between Australia and New Zealand has not been uncommon (Sanmartín and Ronquist, 2004; Wallis and Trewick, 2009). Dispersal has been in both directions, but movement against the prevailing eastward winds has been more frequent in animals than plants (Sanmartín and Ronquist, 2004). Long-distance dispersal has likewise played a critical role in structuring biodiversity across many other parts of the largely-oceanic Southern Hemisphere, including postglacial recolonisation of high-latitude regions such as the sub-Antarctic islands and south-western South America (Fraser et al., 2012; Gillespie et al., 2012; Moon et al., 2017). At a smaller-scale, Trans-Tasman movements have usually been too rare to maintain gene flow (Pratt et al., 2008; Wagstaff et al., 2002; Waters et al., 2000), but have facilitated important colonisation events (Wallis and Trewick, 2009). Indeed, a large proportion of the New Zealand biota has been inferred to have dispersed to the region long after the break-up of Gondwana (Wallis and Trewick, 2009).

Even organisms with poor intrinsic dispersal capacity (e.g., those unable to fly or swim) can sometimes disperse considerable distances via transport with more dispersive species. For example, entire communities of sedentary coastal invertebrates have been shown to raft hundreds of kilometres at sea with buoyant kelp (Fraser et al.,

2011). When dispersal of one species is dependent on another, these species might be expected to show similar phylogeographic structure, as observed for two species of crustaceans associated with rafting kelp (Nikula et al., 2010). Parasites often rely entirely on hosts for dispersal (Esch and Fernández, 2013), and although there have as yet been few comparative host-parasite phylogeographic studies, these have generally found that parasites show more phylogeographic structure than their hosts (Criscione et al., 2006; McCoy et al., 2005a; Nieberding et al., 2004), perhaps partly due to faster mutation rates in parasites (Page et al., 1998), and to dispersal opportunities being a subset of those of the hosts. Parasite phylogeographic research thus has the capacity to augment our knowledge of processes (e.g., oceanographic and ecological factors) influencing dispersal of their hosts (McCoy et al., 2005a; Nieberding et al., 2004), and can shed light on coevolutionary interactions (Gandon et al., 1996; Gandon and Michalakis, 2002).

A wide range of marine and intertidal species are genetically distinct to the east and west of Bass Strait in southern Australia, which was the site of a landbridge connecting the mainland to Tasmania until ~13,000 years ago (Jones, 1977; Jones, 1995; Fig. 1). These taxa include *Durvillaea* kelp (Fraser et al., 2009), Cirrhitoid fish (Burridge, 2000), *Nerita* snails (Waters, 2008b), and *Patiriella* and *Coscinasterias* sea-stars (Waters et al., 2004; Waters and Roy, 2003). Density-dependent processes have been proposed to maintain structure across such biogeographic breaks once the dispersal barrier is removed and contact among zones reinitiatated (Waters et al., 2013). Intriguingly, protistan algal pathogens in southeastern Australia appear to show

the same biogeographic structure as their kelp hosts (Blake et al., 2017), with an east-west split across Bass Strait, indicating that parasite phylogeography can indeed mirror that of the host. Co-diversification – whereby parasite and host diversification occur in tandem – is an important process driving parasite evolution (Morand et al., 2015) including seabird ectoparasites (Paterson et al., 2000), but may be less common in penguin ectoparasites (Banks et al., 2006). In order for co-diversification to occur, the parasite must be able to track host movements, by surviving host-associated dispersal. In the case of kelp pathogens, both host and parasite are marine, and so linked dispersal is unlikely to be problematic. However, some parasites that occupy primarily terrestrial environments have biotic associations with other species that are either terrestrial or marine. Penguins, for example, are parasitised by terrestrial ectoparasites (e.g. fleas, lice and ticks), yet their movements are almost entirely in an aquatic environment. Have these ectoparasites evolved the capacity to survive trips at sea with the penguins (in which case we would expect similar phylogeographic structure in the parasites as in the penguins), or do they have a more limited dispersal capacity (in which case we would expect far greater structure in the parasites than the penguins)?

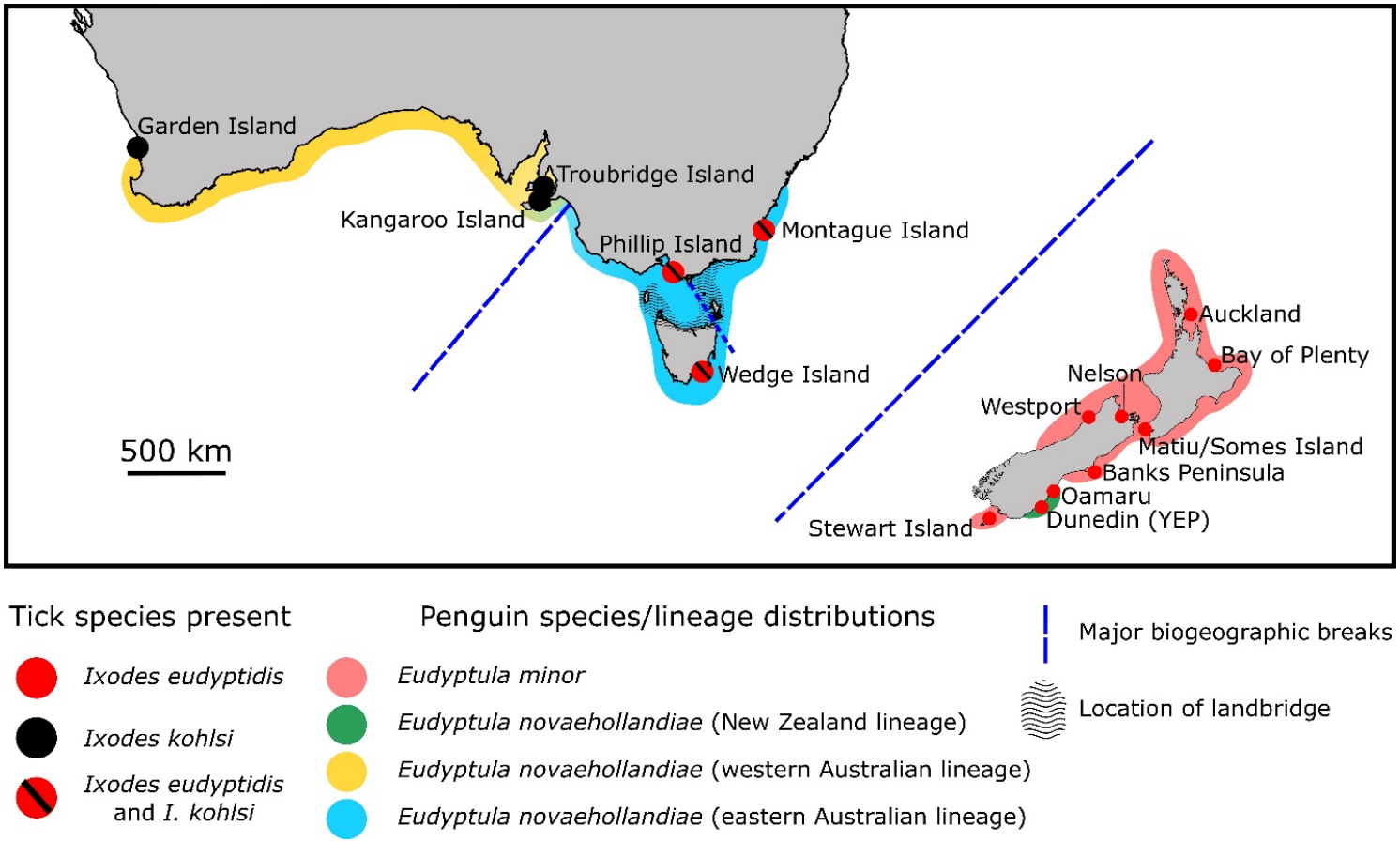

Figure 1. Figure indicating the major biogeographic breaks across Australia and New Zealand, identified in a number of phylogeographic studies of diverse taxa, as well as the landbridge that connected Tasmania to the mainland of Australia until ~13 000 years ago (Waters, 2008b). Little penguin species / lineages (Banks et al., 2002; Burridge et al., 2015; Grosser et al., 2015) and sample sites are also shown, with symbols representing the *Ixodes* tick species present at each sampled colony.

Little penguins (*Eudyptula* spp.) are native to southern Australia and New Zealand, with one species present in Australia and in some parts of the south of New Zealand (*Eudyptula novaehollandiae*), and the other restricted to New Zealand (*E. minor*) (Grosser et al., 2015) (Fig. 1). There is therefore an important, although not absolute, biogeographic break in little penguins across the Tasman Sea. Phylogenetic studies have inferred an ancient New Zealand origin for the little penguin, followed by a late Pleistocene colonisation of Australia (at the onset of cooling) and a recent secondary colonisation of southern New Zealand (Banks et al., 2002; Peucker et al., 2009). Its history is therefore consistent with multiple but infrequent trans-Tasman dispersal events, as inferred for diverse other taxa including weta insects (Pratt et al., 2008) and *Nothofagus* trees (Cook and Crisp, 2005; Manos, 1997). Within Australia, the little penguin shows genetic homogeneity across large (>1000 km) scales, except for a zone of high genetic structure (populations differentiated at the scale of 10s of km) between Troubridge and Granite Island colonies in South Australia (Burridge et al., 2015; Overeem et al., 2008). The zone may represent the location of a founding event or a bottleneck (and subsequent expansion), or secondary contact of two distinct lineages (see review in Burridge et al., 2015). The lineages are largely consistent with an east/west divide, the cause of which remains largely speculative, but which could be the result of a biogeographic break during recent glacial maxima (Burridge et al., 2015).

Little penguins are parasitised by two morphologically cryptic *Ixodes* ticks (hereafter little penguin ticks) when they come ashore to breed and moult (Fig. 1). Penguin ticks

are non-permanent terrestrial ectoparasites, with no obvious adaptations to oceanic conditions, and so dispersal in association with their host is thought to present a challenge (Dietrich et al., 2011; Pugh, 1997). Little penguin ticks are nonetheless present across the entire range of their hosts in Australia and New Zealand. If little penguin ticks are not host-species specific, their dispersal could be facilitated by flighted seabird hosts. However, genetic studies from penguin ticks in other regions indicate they usually have host-specific races/lineages (McCoy et al., 2012; McCoy et al., 2005b), and although some little penguin ticks have been recorded on non-penguin hosts (Roberts, 1970), the description of a new *Ixodes* species on seabirds in Australasia (Heath and Palma, 2017) suggests these records could be the result of poorly-resolved taxonomy. Dispersal of little penguin ticks is therefore probably dependent on their swimming hosts. Although a recent physiological study of little penguin ticks suggests that they could be capable of surviving short periods of immersion in seawater (Chapter 3), whether they could survive long journeys at sea is not known.

We here carried out broad-scale phylogenomic assessments of little penguin ticks from across their range. Given the repeated evolution of host-associated races in other seabird ticks (McCoy et al., 2005b), we hypothesised that little penguin ticks would not occur on sympatric flighted seabirds. Based on physiological analyses suggesting little penguin ticks can survive some time at sea (Chapter 3), which would facilitate co-diversification, we also hypothesised that they would show similar

phylogeographic structure to their hosts, supporting their capacity to survive lengthy ocean trips.

5.3 Materials and methods

5.3.1 Study sites and sampling

In order to evaluate whether penguin ticks were moving at sea with their hosts, it was important to first test whether little penguin ticks were shared with sympatric flighted seabirds. Therefore, 20 ticks from short-tailed shearwaters and 40 ticks from little penguins were removed directly from hosts at an intermingled colony on Wedge Island in Tasmania, Australia (43.1352° S, 147.6722° E), and preserved for genetic analysis using methods described in Chapter 4. Short-tailed shearwaters are a highly migratory flighted seabird species and have been recorded as an alternate host for little penguin ticks (Roberts, 1970). The Wedge Island colony is unique in that it represents one of the only known locations where shearwaters and little penguins live in true sympatry. Although several little penguin colonies at other locations (e.g. Phillip Island, Australia) are in close proximity to shearwater colonies, the two species share a single completely intermixed colony at Wedge Island and are often found in each other's company in the vegetation covering the burrows (K. L. Moon, pers. obs.).

For phylogenomic analyses, ticks were obtained from 14 little penguin colonies (including Wedge Island) throughout the entire range of the two species in Australia and New Zealand (with the exception of the Chatham Islands: see Fig. 1). Australian sites include Garden Island in Western Australia (32.2043° S, 115.6776° E),

Troubridge Island (35.1180° S, 137.8276° E) and Kangaroo Island in South Australia (35.7752° S, 137.2142° E), Phillip Island in Victoria (38.4899° S, 145.2038° E), Montague Island in New South Wales (36.2510° S, 150.2270° E) and Wedge Island in Tasmania. Sites in New Zealand include Stewart Island (46.9973° S, 167.8372° E), Oamaru (45.0975° S, 170.9704° E), Banks Peninsula (43.7500° S, 173.0000° E), Westport (41.7545° S, 171.6059° E) and Nelson (41.2706° S, 173.2840° E) on the South Island, and Matiu/Somes Island (41.2582° S, 174.8659° E), Bay of Plenty (37.6893° S, 177.1423° E) and Auckland (36.8485° S, 174.7633° E) on the North Island.

Field collections were undertaken between November 2014 and November 2016. Sampling was conducted as per Chapter 4, and yielded 328 ticks from 10 sites (see Table 1 for sample sizes). Ticks from Phillip Island comprised the same samples used in a previous study (Chapter 4). No ticks were found at Penguin Island (32.3057° S, 115.6906° E) in Western Australia, despite extensive searches of over 20 burrows and birds, and although the colony at Garden Island – only 6.5 km away from Penguin Island – had ticks (K.L. Moon, pers. obs.). An additional 19 preserved little penguin tick samples representing a further four little penguin colonies were collected from the insect collection at Te Papa Museum and from Massey University, New Zealand. A single tick from a yellow-eyed penguin (*Megadyptes antipodes*), collected in Dunedin, was also taken from Te Papa Museum and included in the genetic analyses, to assess whether little penguin ticks exploit other penguin species within their range. DNA extractions were undertaken as per Chapter 4.

Table 1. Sample sizes from each colony, with the number of samples yielding data for genomic analysis given in brackets. Where applicable, samples used in the *I. eudyptidis* analyses are shown first, followed by the number used in the *I. kohlsi* analysis.

Location		*N*
Australia	Phillip Island	50 (12, 31)
	Montague Island	22 (1, 15)
	Wedge Island	30 (1, 26)
	Kangaroo Island	14 (13)
	Troubridge Island	23 (22)
	Garden Island	27 (23)
New Zealand	Auckland	2 (1)
	Bay of Plenty	13 (6)

	Matiu/Somes Island	40 (39)
	Nelson	1 (1)
	Westport	40 (37)
	Banks Peninsula	35 (35)
	Oamaru	47 (40)
	Dunedin	1 (1)
	Stewart Island	3 (2)

5.3.2 Mitochondrial genetic sequencing

Because there are two, morphologically-cryptic species of little penguin tick in Australia (*I. kohsli* and *I. eudyptidis*; see Fig. 1) , we first analysed a number of samples from Australian colonies (Phillip Island, Montague Island, Troubridge Island, Kangaroo Island, and Garden Island) for mitochondrial COI to delineate the species. Methods are described in Chapter 4, except that sequencing was undertaken by the Genetic Analysis Services at the University of Otago.

5.3.3 Genotyping by sequencing library preparation

Library preparations for GBS were carried out as per Chapter 4 but with the following alterations: post-ligation PCRs were performed in two sets of 25 µl volumes, each containing 5 µl of purified DNA product, 12.5 µl of 1 x MyTaq^{TM} HS Master Mix (Bioline), 6.5 µl of MilliQ H_2O, and 0.5 µM each of forward and reverse PCR primer (see Elshire et al.., 2011). The products from the two PCRs were then combined. Following quantitation and pooling, a 200-bp range (400-600 bp fragments) was excised for paired-end sequencing, carried out on a single lane of an Illumina HiSeq 2500 undertaken by the Biomolecular Resource Facility in the John Curtin School of Medical Research at the Australian National University.

5.3.4 Analysis

COI data

Maximum likelihood (ML) and Bayesian phylogenetic analyses were undertaken as described in Chapter 4 (including the same outgroups), and included samples from Chapter 4 (representing Phillip Island) and Moon et al. (2015).

Genotyping by sequencing data

Raw Illumina data were processed using the *Stacks* (Catchen et al., 2013) pipeline as described in Chapter 4 with the following alterations and specifications (see Supplementary Text S1 for example of code used): all fragments were trimmed to 68 bp during demultiplexing, the minimum depth of coverage required to create a stack was set to the default of 2, the maximum distance (in nucleotides) allowed between stacks was set to the default of 2, the maximum distance (in nucleotides) allowed to align secondary reads to primary stacks was set to 0 in ustacks, and the number of mismatches allowed between sample loci when building the catalogue was set to the default of 1 in cstacks. Following sstacks, rxstacks was used to correct genotype and haplotype calls made by cstacks and sstacks, before cstacks and sstacks were then rerun on the output from rxstacks. In rxstacks, the minimum log likelihood required to keep a catalog locus was set to -15.0, the proportion of loci in a population that must be confounded relative to the catalog locus was set to 0.25, and the prune haplotype algorithm was enabled to prune out non-biological haplotypes considered unlikely to occur in the population. The populations *Stacks* script was then used to filter the data and export loci for downstream analyses. The minimum minor allele frequency required to process a nucleotide site at a locus was set to 0.1, the minimum stack depth required for individuals at a locus was set to 5, and the minimum percentage of

individuals required to process a locus was set to 50%, meaning each SNP had to be present in at least 50% of the individuals to be called. A python script was then used to prune samples that had >95% missing data (see Supplementary Material). Loci were tested for selection as described in Chapter 4.

The .plink file outputs from the *Stacks* population script were used for Principle Components Analysis (PCA) (Patterson et al., 2006; Price et al., 2006; R Core Team, 2014; Roshyara and Scholz, 2014) and fastSTRUCTURE (Raj et al., 2014) for each species independently as per Chapter 4. IQ-TREE (Nguyen et al., 2014) was used to infer unrooted phylogenetic trees for each species using ML analyses. The –m MPF flag was enabled so that IQ-TREE would firstly identify the optimal model of evolution, based on the Akaike Information Criterion (AIC) score, corrected AIC score and Bayesian Information Criterion (BIC) score, and would then subsequently perform the analysis with the selected model. 1000 bootstraps were used to assess node support, and trees were visualised using FigTree v.1.4.3 (Rambaut, 2009).

5.4 Results

5.4.1 Species identification

COI data were obtained for a total of 8 ticks from Kangaroo Island (SA), 5 ticks from Troubridge Island (SA), and 6 ticks from Garden Island (WA). These data were analysed with sequences from 24 ticks from Phillip Island (sequenced during Chapter 4), and previously-used outgroups (see Supplementary Table S2 in Chapter 4 for GenBank accession numbers). Two deeply divergent clades were identified using both

ML and Bayesian analyses, and these were consistent with the clades found in Moon et al. (2015) and in Chapter 4 (see Supplementary Fig. S1). Unrooted phylogenomic trees (IQ-TREE analyses) were then used to classify the remaining individuals from Australia into these two clades, using the placement of COI-barcoded individuals as a guide. The two clades likely represent the two little penguin species, with one present across New Zealand and in small numbers in eastern Australia (henceforth *I. eudyptidis*), and the other present across Australia (henceforth *I. kohlsi*).

5.4.2 Host-species specificity

Following quality control and filtering of genomic data, 4,726 SNPs remained from 25 little penguins ticks and 18 short-tailed shearwater ticks from Wedge Island. Both the fastSTRUCTURE and IQ-TREE analyses of the little penguin and shearwater ticks provide strong evidence for host-species specificity (see Fig. 2). A model complexity of K=1-2 was identified, and the distruct2 plot of K=2 shows that the two populations are almost entirely delineated by host species. IQ-TREE chose the K3Pu+F+R2 model of substitution and this model was subsequently used with the following rate parameters: A-C: 1.000 A-G: 3.196 A-T: 0.632 C-G: 0.632 C-T: 3.196 G-T: 1.000, and base frequencies: A: 0.217 C: 0.280 G: 0.281 T: 0.222. IQ-TREE analyses further support host-species specificity, with two well-supported tick clades that correspond to ticks from the two different host species (see Fig. 2). The PCA plot comparing little penguin ticks with short-tailed shearwater ticks was not particularly informative (see Supplementary Fig. S2).

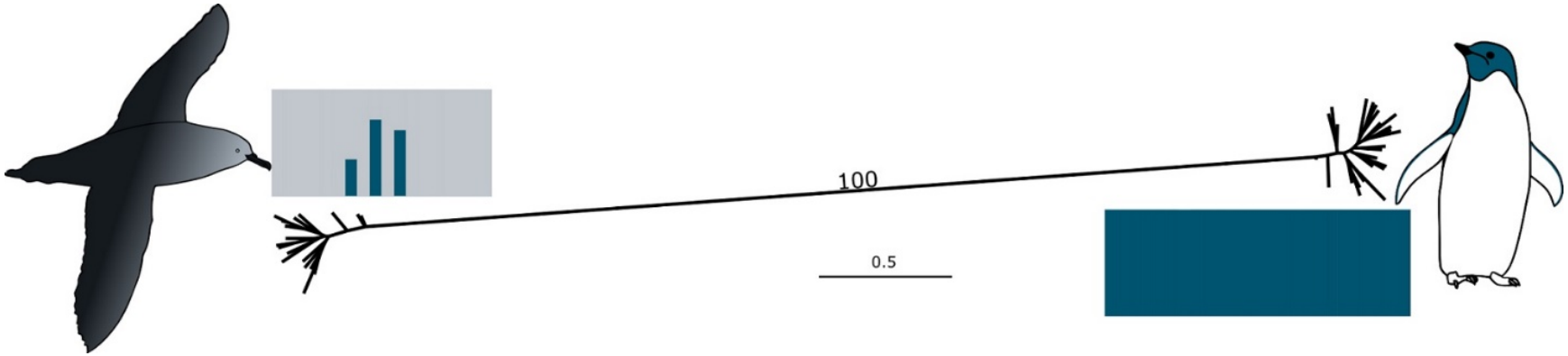

Figure 2. Genomic results from little penguins and short-tailed shearwaters, including a fastSTRUCTURE distruct2 plot (*K*=2), and IQ-TREE results (see Supplementary Fig. S2 for PCA plot).

5.4.3 Genomic structure

PCA analyses

Following quality control and filtering of genomic data, a total of 60,412 SNPs were retained from 176 *I. eudyptidis* ticks, and 7,196 SNPs were retained from 130 *I. kohlsi* ticks (see Table 1 for site representation) across Australia and New Zealand. PCA plots provide evidence for population structuring in both little penguin tick species, but movement between some colonies is evident. In *I. eudyptidis*, the PCA plot was strongly skewed by differences between Australian and New Zealand colonies (Supplementary Fig. S3). A second PCA analysis was therefore performed without the Australian ticks (Fig. 3). When the Australian ticks were removed, Tracy-Widom statistics were significant for all of the first 10 principle components (Supplementary Table S1), and the PCA indicated similarities among tick colonies on the east coast of the South Island in New Zealand (particularly Banks Peninsula and Oamaru) (Fig. 3). The yellow-eyed penguin tick grouped with little penguin ticks from the same area.

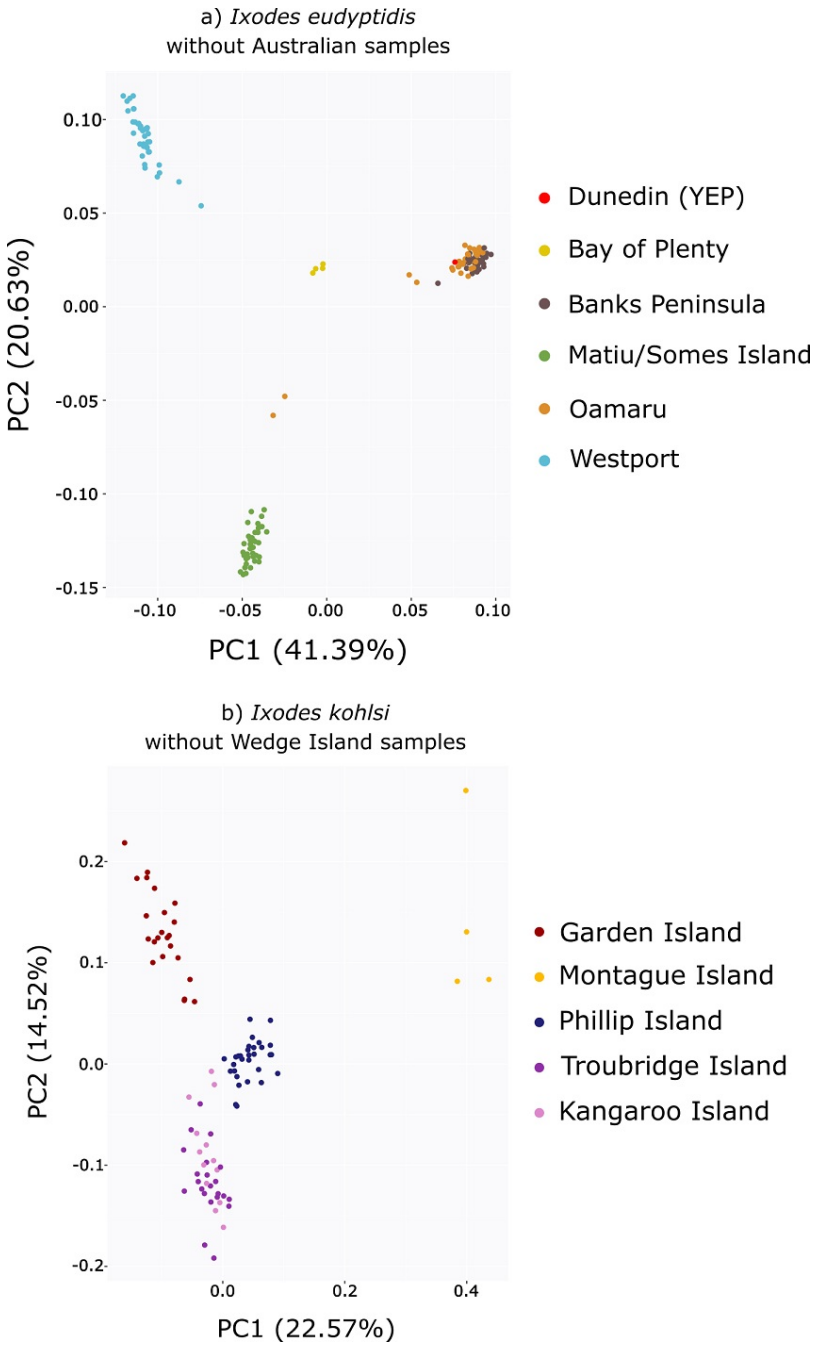

Figure 3. PCA plots of both a) *Ixodes eudyptidis* and b) *I. kohlsi* little penguin tick samples from Australia and New Zealand. Percentage of variation explained by each PC is given in parentheses. Sites are differentiated by colour, and 'YEP' identifies the yellow-eyed penguin sample.

The *I. kohlsi* PCA plot was skewed by differences between mainland colonies and the Wedge Island (Tasmania) colony (Supplementary Fig. S3), so a second analysis was performed without this site, to allow differences among mainland colonies to be examined (Fig. 3). In this second analysis, Tracy-Widom statistics were significant for all of the first 10 principle components (Supplementary Table S1) and all sites appeared distinct except the two South Australian colonies, which were intermixed (Fig. 3).

fastSTRUCTURE analyses

FastSTRUCTURE analyses of *I. eudyptidis* suggested a model complexity of *K*=6-7. The distruct2 plots show population structure among colonies, but also suggest some movement between colonies is occurring (Fig. 4). In particular, Oamaru and Bay of Plenty ticks were diverse, and population assignments reflected ancestry in other populations (particularly Banks Peninsula). Inferred population membership suggested both Stewart Island and Nelson ticks may also have a mixed ancestry, but sample sizes were too small to confirm gene flow. A single *I. eudyptidis* tick from Wedge Island (Tasmania, Australia) grouped with New Zealand ticks (North Island or Oamaru), rather than with the Australian *I. eudyptidis* tick population. The yellow-eyed penguin tick from Dunedin grouped with the Oamaru population.

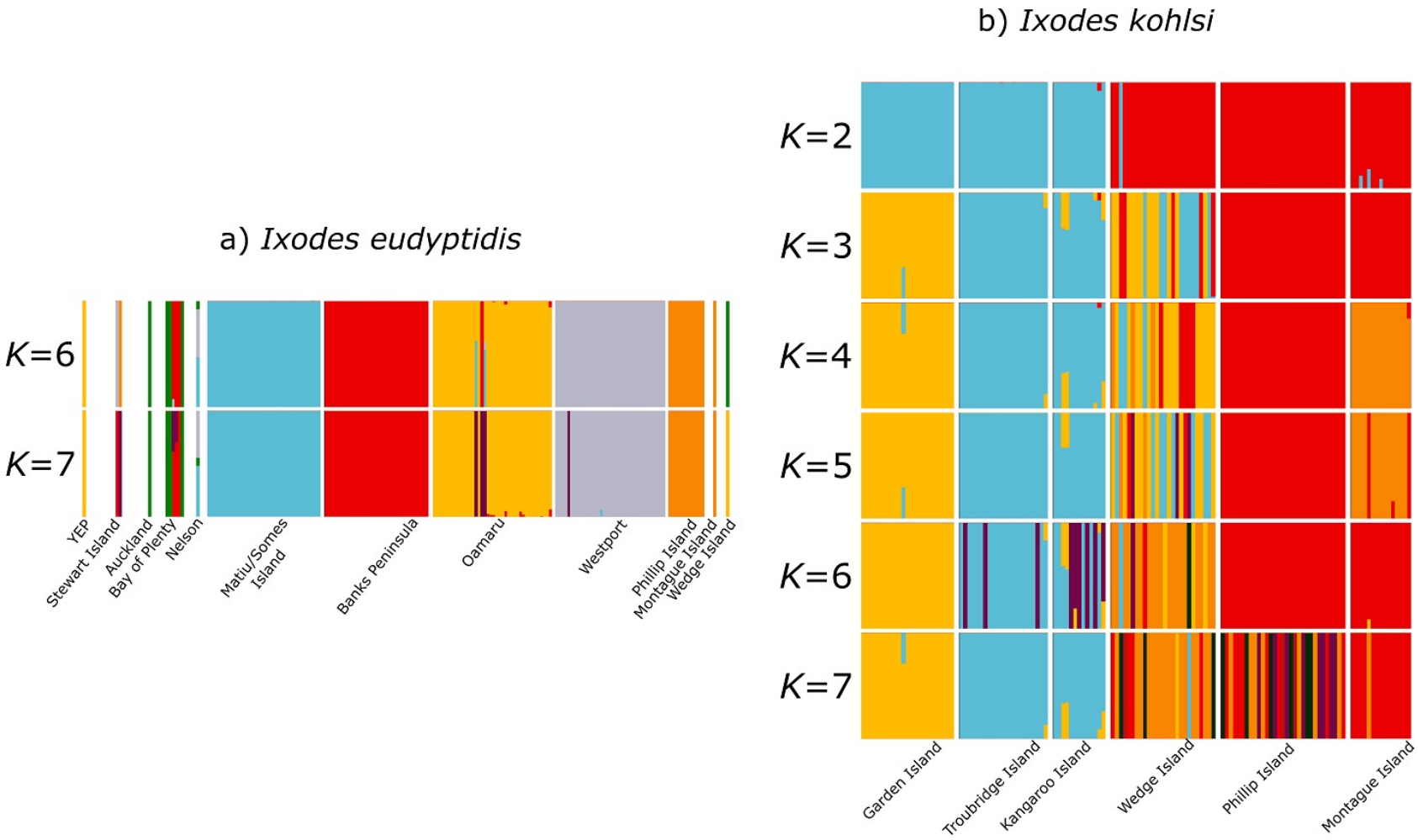

Figure 4. fastSTRUCTURE distruct2 plots of both a) *Ixodes eudyptidis* and b) *I. kohlsi* little penguin tick samples.

FastSTRUCTURE analyses of *I. kohlsi* inferred a model complexity of *K*=2-7. Distruct2 plots suggest a division between ticks from eastern coast colonies (Phillip Island, Montague Island and Wedge Island) and more western colonies (Troubridge Island, Kangaroo Island and Garden Island), with little movement inferred between them, although Wedge Island appears to have mixed ancestry with some possible genetic input from western areas (see Fig. 4). Movement also appears to be restricted between Garden Island and the South Australian colonies (at *K*>2), but the two South Australian colonies are not genetically distinct.

IQ-TREE analyses

For the putative *I. eudyptidis* species, the K3Pu+F+R5 model of substitution was chosen by IQ-TREE and subsequently used with the following rate parameters: A-C: 1.000 A-G: 4.772 A-T: 0.911 C-G: 0.911 C-T: 4.772 G-T: 1.000, and base frequencies: A: 0.225 C: 0.280 G: 0.274 T: 0.221. The K3Pu+F+I+G4 model of substitution was chosen by IQ-TREE and subsequently used for the putative *I. kohlsi* species with the following rate parameters: A-C: 1.000 A-G: 2.867 A-T: 0.791 C-G: 0.791 C-T: 2.867 G-T: 1.000, base frequencies: A: 0.226 C: 0.271 G: 0.268 T: 0.235, proportion of invariable sites: 0.050, and gamma shape alpha parameter: 2.298. The phylogenetic tree for *I. eudyptidis* provided further support for the differentiation of Australian and New Zealand ticks (see Fig. 5). In accordance with fastSTRUCTURE analyses, the single Wedge Island tick grouped with the New Zealand ticks suggesting recent trans-Tasman movement. Within New Zealand, IQ-TREE analysis suggests there has been some – but limited – recent movement among colonies, with a single

tick from the Bay of Plenty grouping with Banks Peninsula ticks, and three ticks from Oamaru more closely related to North Island ticks than to others from the eastern South Island.

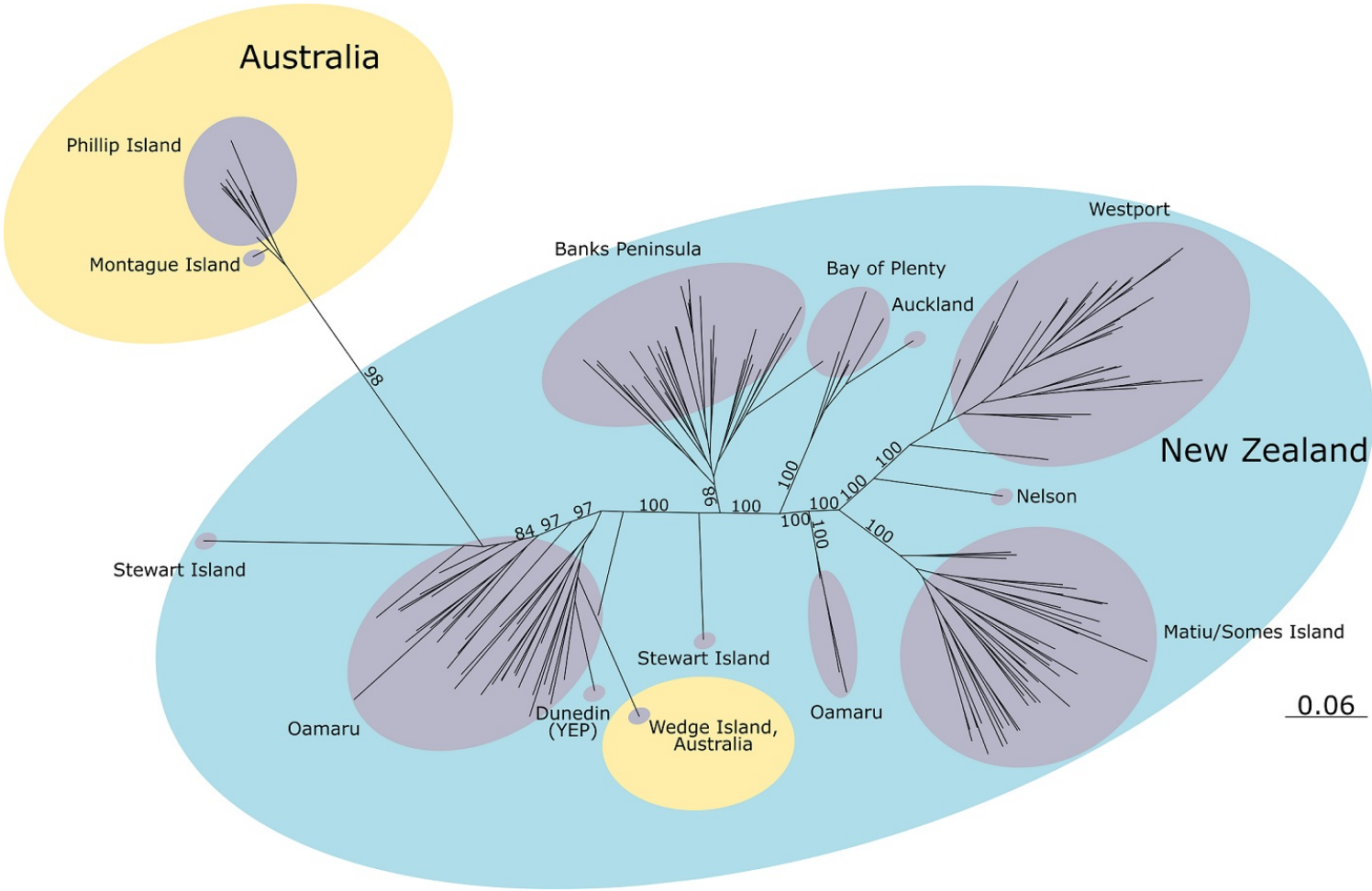

Figure 5. IQ-TREE results from *Ixodes eudyptidis* little penguin tick samples.

The tree for *I. kohlsi* indicated a division between colonies on the east coast of Australia and those to the west (Fig. 6). The eastern colonies – including Wedge Island (Tasmania) – were not well differentiated. Ticks from the two South Australian colonies formed a single clade.

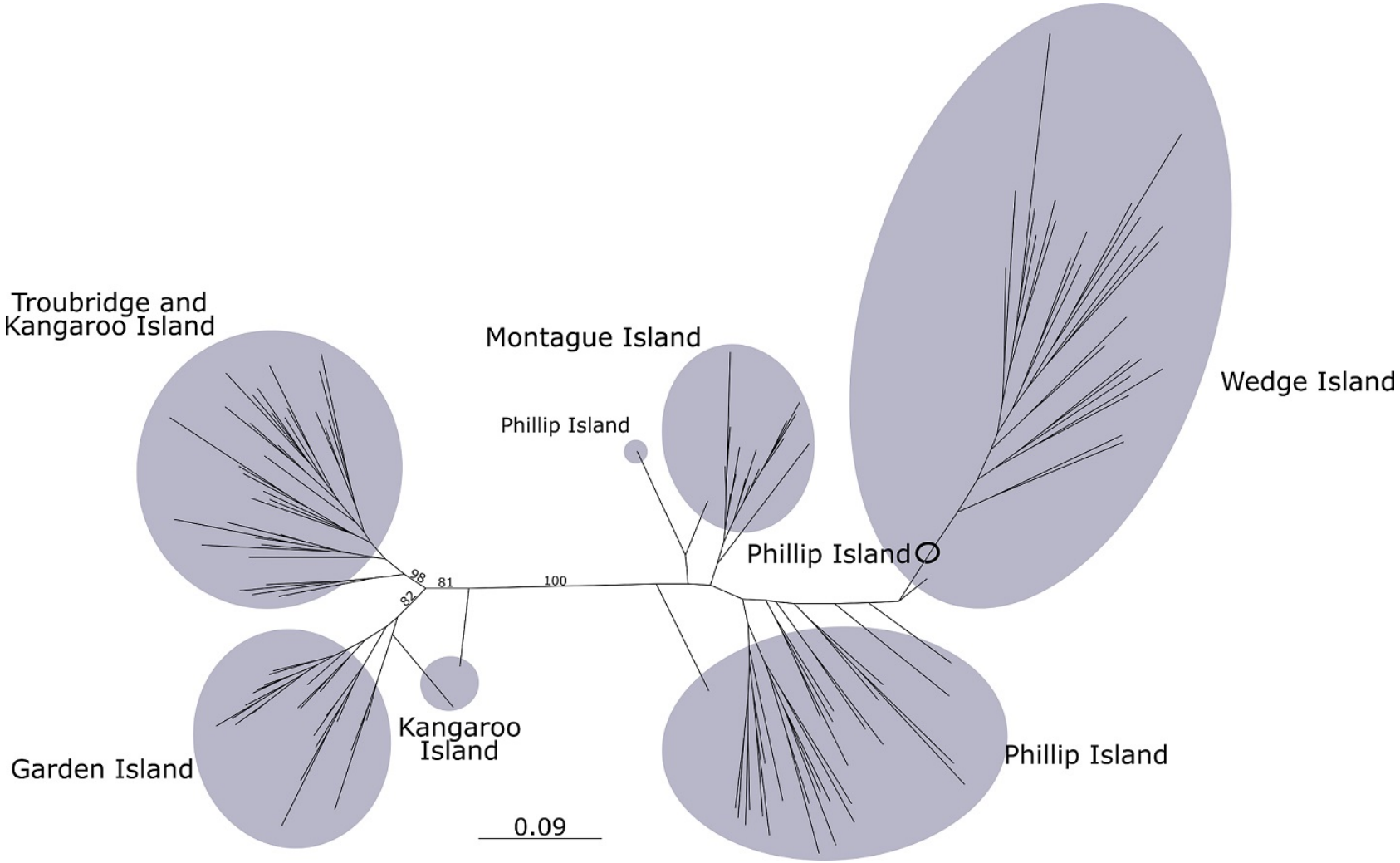

Figure 6. IQ-TREE results from Ixodes kohlsi little penguin tick samples.

5.5 Discussion

Our results support our hypotheses that little penguin ticks are host-species specific, and that they are capable of considerable aquatic dispersal in association with their hosts. These results are consistent with previous fine-scale genetic studies (McCoy et al., 2012; McCoy et al., 2005b; Moon et al., 2015), and a recent study of little penguin tick physiological tolerances (Chapter 3), but is the first to reveal penguin-associated tick movement between landmasses separated by thousands of kilometres of open ocean. Although the ticks showed greater phylogeographic structure than their hosts – which may be due, in part, to the higher-resolution markers used in this study – major biogeographic breaks were consistent for both hosts and parasites. We discuss our results in terms of their implications for biogeography and penguin conservation.

5.5.1 Species identification

The genetic division indicating two divergent clades (one found throughout New Zealand and in low numbers on the east coast of Australia, and the other from all colonies across Australia: see Supplementary Fig. S1) is consistent with Moon et al. (2015) and Chapter 4, and likely represents the two cryptic little penguin *Ixodes* species. Species identities were assigned based on geographic patterns. *Ixodes eudyptidis* is found in both Australia and New Zealand and is often thought to be the most common tick parasitising little penguins in Australia (Roberts, 1970). Contrary to these records, however, it was the rare clade that grouped genetically with New Zealand ticks, suggesting *I. kohlsi* (which has not previously been recorded from New Zealand) is currently the more common species in Australia. In addition, our

mitochondrial and genomic data provides the first evidence that *I. kohlsi* is present in western Australian colonies. *Ixodes kohlsi* has never been recorded in Western Australia, and penguins in the west were thought to be exploited by *I. eudyptidis* (Heath and Palma, 2017; Roberts, 1970).

5.5.2 Host-species specificity in little penguin ticks

Despite records of little penguin ticks exploiting short-tailed shearwaters (Roberts, 1970), our results suggest that the two hosts do not commonly share ticks even when they share a colony. Though penguin ticks were compared with a single sympatric seabird species at a single colony, the short-tailed shearwater is commonly found in sympatry with little penguins and therefore represents an ideal test. Australian and New Zealand seabird tick taxonomy is poorly resolved (Heath and Palma, 2017), and so host records cannot be relied upon to resolve host range (McCoy et al., 2013). Our results support a recent taxonomic revision that has described a new tick species (*I. laridis*) on flighted seabirds in New Zealand and Australia, which was erroneously recorded as *I. eudyptidis* (Heath and Palma, 2017). *Ixodes laridis* is known to occur on birds in Tasmania, and may be the species present on Wedge Island short-tailed shearwaters.

Previous seabird tick studies have shown that host specificity can evolve between penguins and flighted birds (e.g. albatross and shags) when they breed in sympatry (McCoy et al., 2012). However, specificity was not evident between three penguin species in the western Antarctic Peninsula (McCoy et al., 2012), nor among two

related species of penguin in the Crozet Archipelago (McCoy et al., 2005b), nor among the two recently split little penguin species (this study), suggesting that ticks might readily be shared among penguin species. Because the two little penguin species rarely share a colony (except at Oamaru), it seems unlikely that *I. eudyptidis* would form host races, but this warrants further investigation. The present study also suggests – although based on a single specimen – that New Zealand penguin species (little and yellow-eyed penguins) may share ticks. Our results nonetheless suggest that little penguin ticks do not exploit flighted seabirds. Phylogeographic patterns of penguin ticks must therefore result from penguin movements.

5.5.3 Contemporary movement

Our genomic analyses indicate that movement of little penguin ticks may be mediated by the distance between colonies, and the quality of a colony. For example, Kangaroo and Troubridge Islands in South Australia are only separated by ~60 km of open ocean, and were found to share a single, panmictic tick population. Movement between these colonies is well-supported by the physiological capabilities of little penguin ticks, and, based on average swimming speeds of little penguins (1.8 m/sec: Bethge et al., 1997), dispersal between them could be achieved by a swimming penguin in under an hour (Chapter 3). Likewise, population assignments and phylogenetic analyses of the ticks at Oamaru (New Zealand), Wedge Island (Tasmania) and Phillip Island (Victoria) colonies also provide evidence for immigration. Phillip Island and Oamaru represent the largest and most reproductively successful colonies in Australia and New Zealand (Agnew et al., 2014; Chiaradia et

al., 2007; Perriman and Steen, 2000; Sutherland and Dann, 2014) and Wedge Island is exhibiting considerable growth (~17% per annum) (Vertigan, 2010). A previous study of seabird ticks found that the extent of gene flow into colonies may be partially explained by quality, as larger, more productive colonies will attract more prospecting birds, facilitating long-distance gene flow in ticks (McCoy et al., 2003). Increased immigration of penguins into Oamaru, Wedge Island and Phillip Island due to their high quality may explain the phylogeographic patterns, and confirms that prospecting activities in seabirds translates into effective dispersal of their ectoparasites (Danchin, 1992; McCoy et al., 2005a).

5.5.4 Biogeography

Within Australia, phylogeographic patterns of *I. kohlsi* reflected a similar phylogenetic split (see Figs. 4 and 6) to that of their hosts (Burridge et al., 2015; Overeem et al., 2008). If host phylogenetic structure was related to a past bottleneck in the South Australian colonies (as suggested in Burridge et al., 2015), reduced genetic diversity would be expected in the ticks, but there was no evidence for this. Our results could, however, support the theory of secondary contact of isolated eastern and western penguin (and associated tick) lineages resulting from the historical closure of the Bass Strait via the Bassian Isthmus, as inferred for other phylogeographic studies of marine animals from the region (see Fig. 1) (Burridge, 2000; Fraser et al., 2009; Waters, 2008b; Waters et al., 2004; Waters and Roy, 2003).

Rare trans-Tasman movements of little penguins have been inferred based on phylogeographic patterns (Grosser et al., 2015; Peucker et al., 2009), and these occasional host movements have been suggested to have facilitated tick dispersal between Australia and New Zealand (Heath and Palma, 2017; Moon et al., 2015). Our genomic data support these hypotheses, providing evidence for host-associated penguin tick movements between Australia and New Zealand in both directions. In addition, we found that a single *I. eudyptidis* tick from Wedge Island (Tasmania, Australia) grouped genetically with ticks from New Zealand in multiple analyses (see Fig. 4 and 5), suggesting a recent dispersal event from New Zealand to Australia. However, as for the host – and a number of other organisms (Pratt et al., 2008; Wallis and Trewick, 2009; Waters et al., 2000) – trans-Tasman movements have not been frequent enough to maintain gene flow, resulting in divergence.

5.5.5 Movement of terrestrial parasites with aquatically-dispersing hosts

A small number of terrestrial ectoparasites were able to remain associated with host groups whose ancestors returned to the oceans, but very little is known of their dispersal capacity in association with their swimming hosts. These groups are almost entirely restricted to hosts that have maintained close contact with land (e.g. pinnipeds, sea otters and seabirds), because they still rely on terrestrial environments for reproduction and transmission (Raga et al., 2009). Some avoid marine conditions via microhabitat, for example the sucking lice of penguins which inhabit the layer of trapped air under the feathers of its host while it is at sea (Murray, 1967). As a result, the lice have been able to maintain considerable genetic contact across their range

(Banks et al., 2006). Previous studies of terrestrial ectoparasites that are exposed to marine conditions have focussed on the sucking lice of seals and the river otter (Echinophthiriidae) and have only described parasite loads, transmission dynamics within colonies, preferred attachment locations, and morphological adaptations to marine conditions (Kim, 1971; Kim, 1975; Kim and Emerson, 1974; Leonardi and Lazzari, 2014; Leonardi and Palma, 2013; Murray and Nicholls, 1965; Murray et al., 1965). The present study therefore represents the first to characterise the dispersal capacity of a terrestrial ectoparasite exposed to marine conditions on an aquatically-dispersing host (but see components of McCoy et al. 2005, 2012). However, such movements may be restricted by host life history, including philopatry (Moon et al., 2017).

Dispersal of little penguin ticks was found to be linked to their hosts, despite their aquatic habit. Most notably, biogeographic breaks were observed in both ticks and their penguins between the east and west of Australia, and between Australia and New Zealand. These breaks are consistent with studies of some other organisms (see Fig. 1), and highlight the importance of climatic cycles and occasional trans-Tasman dispersal in the evolution of biodiversity in the region. The correspondence of phylogenetic structure in little penguins and their host-specific ticks also provides some of the first direct evidence that terrestrial ectoparasites exploiting semi-aquatic hosts are capable of long-distance aquatic movements.

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5.7 Supplementary material

5.7.1 Supplementary text

Text S1. Example scripts for Stacks and python script used to prune samples with <5% missing data. Developed in collaboration with Ang McGaughran and Cameron Jack.

Ustacks

ustacks -t fastq -f ./ustacks_input/samplename.fq -o ./ustacks_out -i 1 -d -r -m 2 -M 2 -N 0 -p 8 ;

Cstacks

cstacks -b 1 -o cstacks_out -s ./cstacks_input/samplename

Sstacks

sstacks -b 1 -c ./sstacks_input/batch_1 -s ./sstacks_input/samplename -o ./sstacks_out- p 8;

Rxstacks

rxstacks -b 1 -P ./sstacks_out/ -o ./sstacks_out_corr/ --conf_lim 0.25 --prune_haplo --lnl_lim -15.0 -t 12

Populations

populations -b 1 -P populations_input -M popmap -t 16 --min_maf 0.1 -m 5 -r 0.5 --phylip_var

Then I would create a whitelist of SNPs using

python make_whitelist.py --whitelist runname.whitelist –sumstats ./populations_input/batch_1.sumstats.tsv

Where make_whitelist.py:

```
#!/usr/bin/env python
import argparse
"""
   Reads the a Stacks Populations sumstats.csv file for a batch
   and outputs a SNP whitelist for use with an individual based
   Populations list
"""
def main():
   parser = argparse.ArgumentParser()
   parser.add_argument('--sumstats', required=True, help='Path to sumstats.tsv file')
   parser.add_argument('--whitelist', required=True, help='Path to output whitelist
file')
   args = parser.parse_args()
   loci_columns = set()
   with open(args.sumstats, 'r') as f:
      for line in f:
         if line.startswith('#'):
            continue  # header line
         cols = line.strip().split('\t')
         locus = cols[1]
         column = cols[4]
         loci_columns.add(tuple([locus, column]))
```

```
    with open(args.whitelist, 'w') as out:
        for (l, c) in loci_columns:
            out.write(l + '\t' + c + '\n')
if __name__ == '__main__':
    main()
```

Then I would rerun populations using the whitelist and individual names:

populations -b 1 -P populations_input -M popmap_indiv -t 16 --phylip_var -W runname.whitelist

Then I would run the following python script to the prune samples:

```
#!/usr/bin/env python3

import sys
import argparse
import numpy
from matplotlib import pyplot as plt
from math import log10



def read_phy(args):
    """
        Read the input .phy file. Each line becomes a tuple of sample and loci
```

```
    Return list of tuples, number of samples, number of loci
    """
    sample_loci = []
    with open(args.phy, 'r') as f:
        for i, line in enumerate(f):
            if i == 0:
                continue  # header
            sample = line[0:10]
            nucs = line.strip()[10:]
            #print (sample, nucs)
            sample_loci.append(tuple([sample, nucs]))
    return sample_loci, i, len(nucs)


def remove_uninformed_samples(args, sample_loci):
    """
        Uninformed samples are a major problem as they can end up appearing
        closely related to many potentially unrelated samples.
    """
    kept_sample_loci = []
    discarded = 0
    for sample, nucs in sample_loci:
        total_positions = len(nucs)
```

```
        count_a = nucs.count('a') + nucs.count('A')
        count_c = nucs.count('c') + nucs.count('C')
        count_g = nucs.count('g') + nucs.count('G')
        count_t = nucs.count('t') + nucs.count('T')
        count_r = nucs.count('r') + nucs.count('R')
        count_y = nucs.count('y') + nucs.count('Y')
        count_s = nucs.count('s') + nucs.count('S')
        count_w = nucs.count('w') + nucs.count('W')
        count_k = nucs.count('k') + nucs.count('K')
        count_m = nucs.count('m') + nucs.count('M')
        count_n = nucs.count('n') + nucs.count('N')
        count_homs = count_a + count_c + count_g + count_t
        count_hets = count_r + count_y + count_s + count_w +\
                     count_k + count_m
        count_other = total_positions - count_homs - count_hets - count_n
        info_prop = (count_homs + count_hets + count_other) / total_positions
        if info_prop > args.cutoff:
            kept_sample_loci.append(tuple([sample, nucs]))
        else:
            discarded += 1
    return kept_sample_loci, discarded
```

```
def remove_constant_sites(args, sample_loci):
    """
        After removing samples, we may create a situation where now some
        sites are no longer variable. These constant loci must be removed
        for IQ-Tree won't run.
    """
    variant_loci_indices = set()
    num_invariant = 0
    sample, nucs = sample_loci[0]
    num_loci = len(nucs)
    print ('... evaluating loci for invariant/common sites')
    for i in range(num_loci):
        # get all bases at this locus, ignore Ns
        locus_bases = set()
        for sample, nucs in sample_loci:
            if (nucs[i].lower()) != 'n':
                locus_bases.add(nucs[i].lower())
        # keep this locus if we have more than one base
        if len(locus_bases) > 1:
            if args.noambig:
                ambig_bases = set(['r','y','w','s','k','m'])
                overlap = ambig_bases.intersection(locus_bases)
                if len(overlap) == 0:
```

```
                variant_loci_indices.add(i)
            else:
                num_invariant += 1
        else:
            variant_loci_indices.add(i)
    else:
        num_invariant += 1

print('... building new sample loci table')
kept_sample_loci = []
for sample, nucs in sample_loci:
    kept_nucs = []
    for i, locus in enumerate(nucs):
        if i in variant_loci_indices:
            kept_nucs.append(locus)
    kept_sample_loci.append(tuple([sample, ''.join(kept_nucs)]))
return kept_sample_loci, num_invariant
def write_phy(args, sample_loci):
    """
    Write sample names and SNP info back to PHYLIP format.
    This format has 10 fixed characters for sample name,
    padded with blank spaces, followed hard by the SNP bases.
    """
```

```
    with open(args.keep, 'w') as out:
        # calculate header first
        num_samples = len(sample_loci)
        num_bases = len(sample_loci[0][1])
        out.write(str(num_samples) + '    ' + str(num_bases) + '\n')
        # now write out the entries
        for sample, loci in sample_loci:
            out.write(sample + loci + '\n')
def main():
    """
        Remove samples with less than --cutoff proportion of informative loci
        There may now exist a number of sites that are invariant, remove these
        Repeat this process till there is no further change
    """
    parser = argparse.ArgumentParser()
    parser.add_argument('--phy', required=True, help='Path to input phylip file')
    parser.add_argument('--keep', required=True, help='Path to output phylip file')
    parser.add_argument('--cutoff', type=float, default=0.05,
            help='Min informative SNP proportion per sample, default=0.05')
    parser.add_argument('--plots', action='store_true',
            help='Enable information content histograms')
    parser.add_argument('--noambig', action='store_true', help='Ambiguous base '+\
            'codes fail with some phylogenetic models. This removes loci '+\
```

```
        'containing ambiguous base codes')
args = parser.parse_args()
print('Reading', args.phy)
sample_loci, pre_samples, pre_loci = read_phy(args)
print(pre_samples, 'samples and', pre_loci, 'loci found')


bad_samples = 1  # this is required as there is no do..while loop in python
invariant_loci = 1  # as above
# this loop always converges to 0
while bad_samples > 0 or invariant_loci > 0:
    sample_loci, bad_samples = remove_uninformed_samples(args, sample_loci)
    print('Removed', bad_samples, 'uninformed samples')
    sample_loci, invariant_loci = remove_constant_sites(args, sample_loci)
    print('Removed', invariant_loci, 'invariant loci')
post_samples = len(sample_loci)
post_loci = 0
if post_samples > 0:
    post_loci = len(sample_loci[0][1])
print('Creating filtered PHYLIP file', args.keep, 'with', post_samples,
      'samples, and', post_loci, 'bases')
write_phy(args, sample_loci)
sys.exit(0)
info_props = []
```

```
low_info = 0
cutoff = 0.05
kept_lines = []
with open(args.phy, 'r') as f:
    for i, line in enumerate(f):
        if i == 0:
            continue  # header
        sample = line[0:10]
        if args.spacing:
            nucs = line.strip()[11:].lower()
        else:
            nucs = line.strip()[10:].lower()
        total_positions = len(nucs)
        count_a = nucs.count('a')
        count_c = nucs.count('c')
        count_g = nucs.count('g')
        count_t = nucs.count('t')
        count_r = nucs.count('r')
        count_y = nucs.count('y')
        count_s = nucs.count('s')
        count_w = nucs.count('w')
        count_k = nucs.count('k')
        count_m = nucs.count('m')
```

```
    count_n = nucs.count('n')
    count_homs = count_a + count_c + count_g + count_t
    count_hets = count_r + count_y + count_s + count_w +\
                 count_k + count_m
    count_other = total_positions - count_homs - count_hets - count_n
    info_prop = (count_homs + count_hets) / total_positions
    info_props.append(info_prop)
    if info_prop < cutoff:
       low_info += 1
    else:
       kept_lines.append(line)
    print(sample, 'Homs', count_homs, 'Hets', count_hets, 'Other',
       count_other, 'Missing', count_n, 'Prop. informative', info_prop)

print('Average information', numpy.mean(info_props))
print('Number of samples with info less than', cutoff, 'is',
     low_info, 'out of', i+1, 'samples')
if args.plots:
    plt.hist(info_props, bins=15)
    plt.show()
    info_props_log10 = [log10(a) for a in info_props]
    plt.hist(info_props_log10, bins=15)
    plt.show()
```

```
    with open(args.keep, 'w') as out:
        out.write(str(i-low_info) + '    ' + str(total_positions) + '\n')
        for line in kept_lines:
            out.write(line)


if __name__ == '__main__':
    main()
```

5.7.2 Figures and tables

Figure S1. Maximum likelihood tree for COI of Australian and New Zealand tick samples, with inferred species identifications based on distributions. Bootstrap values (as percentages in black) and Bayesian PP values (as probability values in blue) are shown. *Ixodes* outgroups are shown (see Supplementary Material Table S1 of Chapter 4).

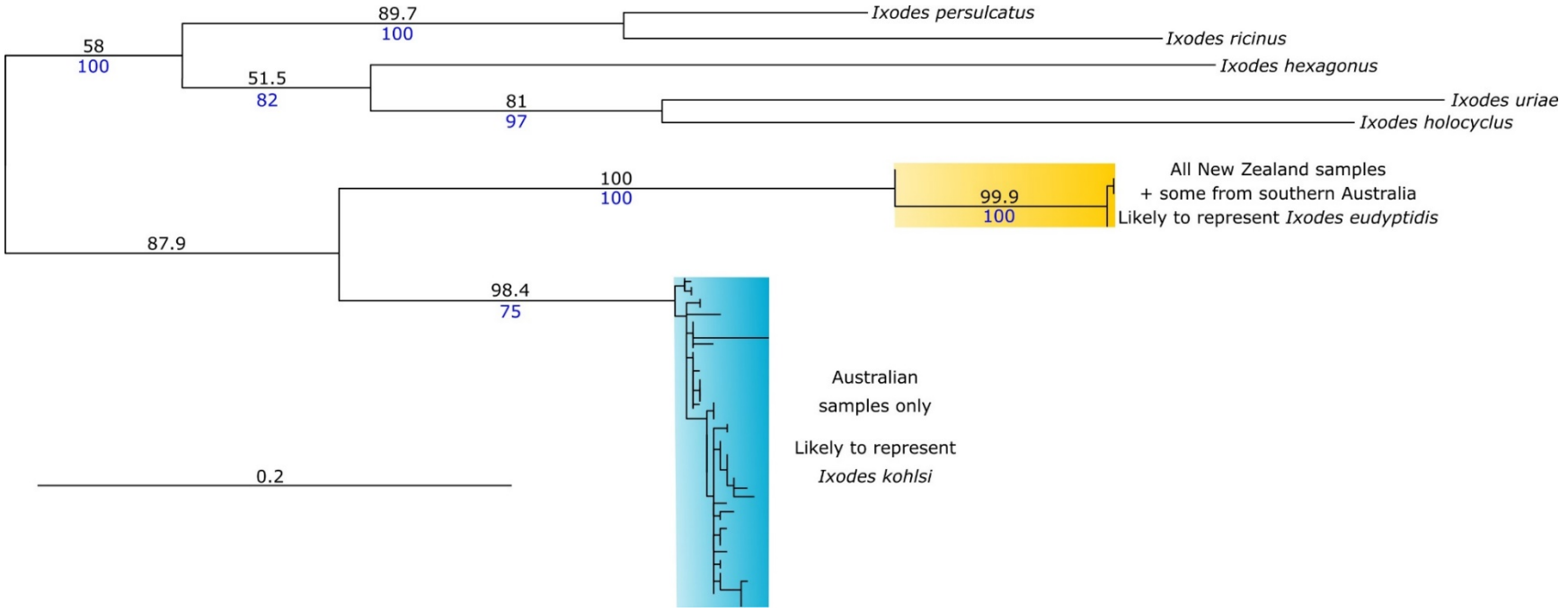


Figure S2. PCA plot of little penguin versus short-tailed shearwater ticks, with host species differentiated by colour. Percentage of variation explained by each PC is given in parentheses.

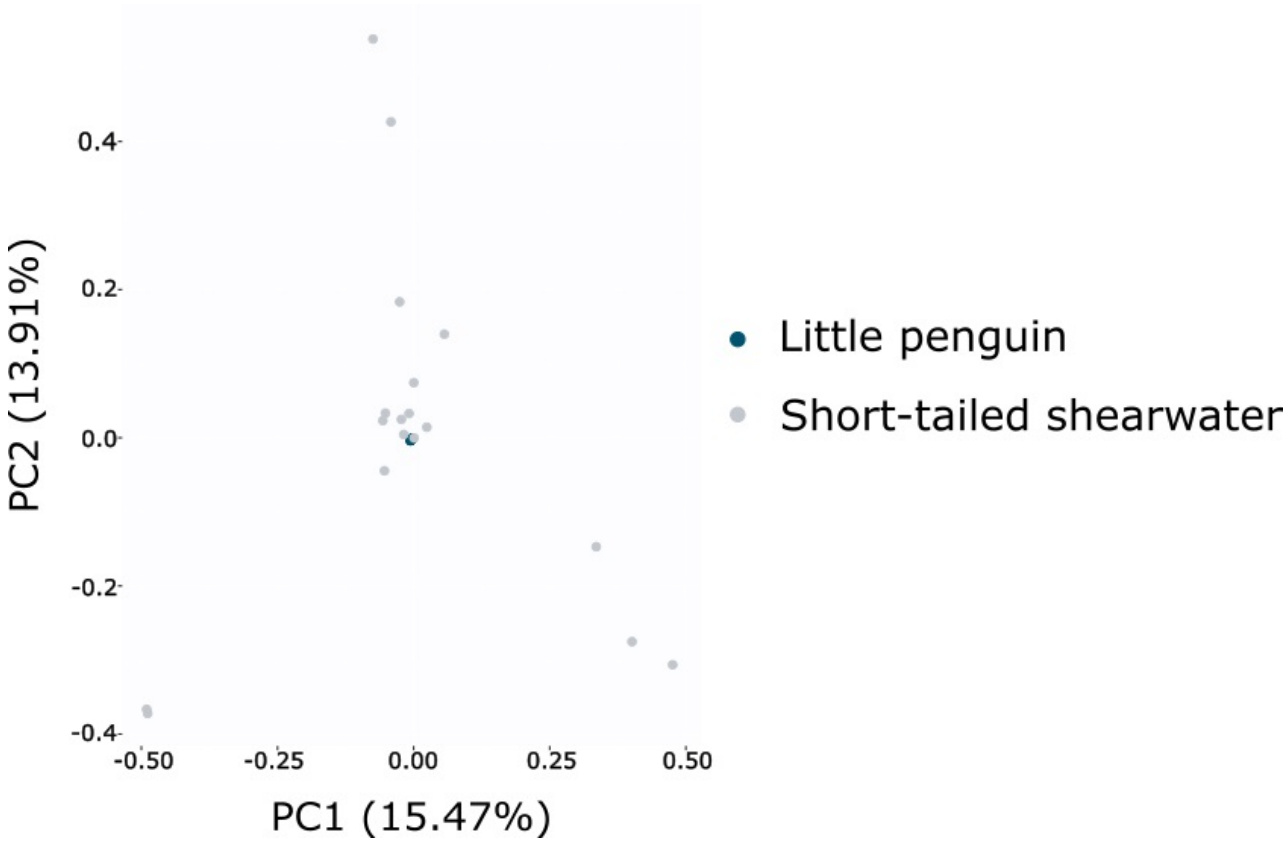
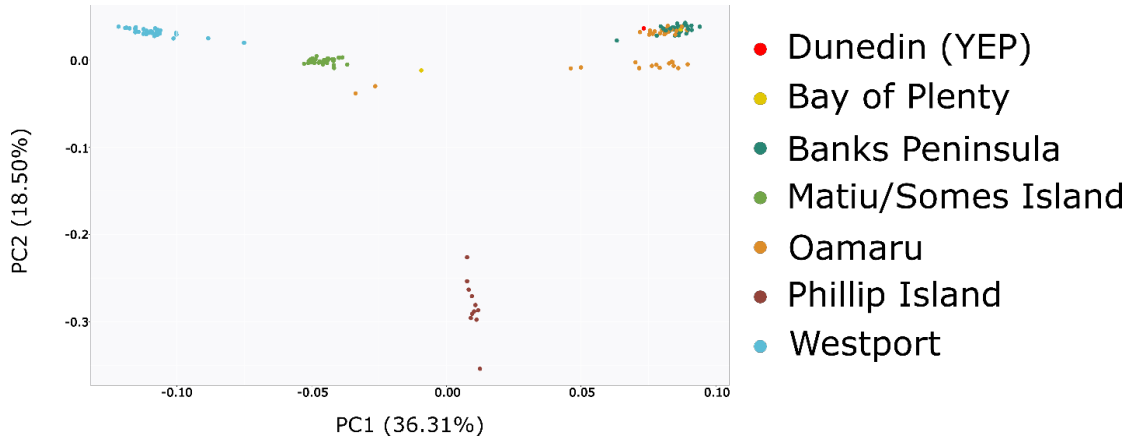


Figure S3. PCA plots of a) *Ixodes eudyptidis* before the removal of Australian samples, and b) *I. kohlsi* with before the removal of Wedge Island samples. Percentage of variation explained by each PC is given in parentheses. Sites are differentiated by colour, and 'YEP' identifies the yellow-eyed penguin sample.

a)



b)

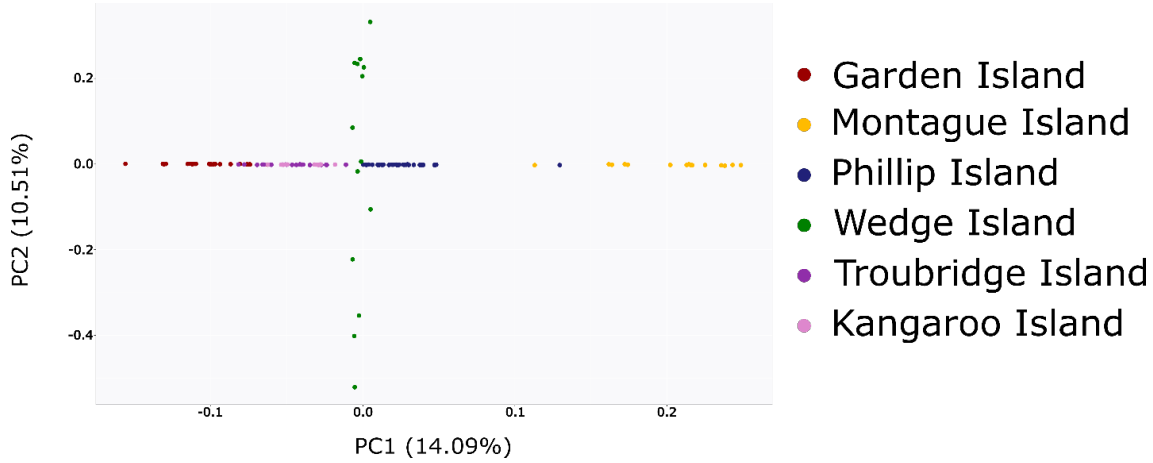


Table S1. Full results of the SmartPCA analysis results for a) *Ixodes eudyptidis* and b) *I. kohlsi*, including eigenvalues, Tracy-Widom statistics, *p*-values and percentage of variation explained for each of the first 10 principal components. All Tracy-Widom (TW) *p*-values were significant ($p < 0.01$).

a)

Principle component	Eigenvalue	% Variation explained	Tracy-Widom Statistic	TW *p*-value
1	19.97788	41.39	48.274	0.000
2	9.957069	20.63	76.002	0.000
3	5.348337	11.08	90.958	0.000
4	2.792169	5.78	56.952	0.000
5	2.527294	5.24	59.534	0.000
6	1.798169	3.73	28.206	0.000
7	1.727489	3.58	28.054	0.000
8	1.415426	2.93	8.96	0.000
9	1.397139	2.89	9.346	0.000
10	1.325182	2.75	5.39	0.000

b)

Principle component	Eigenvalue	% Variation explained	Tracy-Widom Statistic	TW *p*-value
1	5.959575	22.57	36.957	0.000
2	3.833628	14.52	29.31	0.000
3	2.853378	10.80	19.827	0.000
4	2.702959	10.23	22.647	0.000
5	2.409734	9.12	21.316	0.000
6	2.108403	7.98	17.316	0.000
7	1.847161	6.99	11.849	0.000
8	1.601541	6.06	4.445	0.000
9	1.581916	5.99	5.46	0.000
10	1.511521	5.72	4.156	0.000

Chapter Six

Local, but not long-distance dispersal of ticks between two sub-Antarctic islands

King penguins at Volunteer Point, on the Falkland Islands. Taken by Katherine L Moon, January 2016.

This chapter has been formatted for submission to *Ecography* as a *Brevia* article.

6.1 Article

The sub-Antarctic, generally comprising small islands separated by several thousands of open ocean, represents an excellent model system for testing hypotheses about long-distance dispersal and connectivity. Despite the large distances among many sub-Antarctic islands, molecular studies indicate that dispersal has played, and continues to play, a key role in structuring biodiversity patterns in the region (Moon et al. 2017). Nonetheless, natural dispersal mechanisms in the sub-Antarctic are changing (Moon et al. 2017), and some of the region's most iconic taxa – including penguins (Clucas et al. 2014) – are already shifting their distributions in response to these changes. How such changes will affect ecosystem structure will depend partly on the capacity of biotic interactions to be maintained with changing distributions, for example through concerted long-distance dispersal of symbionts.

Penguins, like any other seabird, are parasitised by ticks (*Ixodes uriae*) when they come ashore to breed, and high tick loads can negatively impact penguin breeding success (Mangin et al. 2003). As ticks rely on hosts for dispersal, analysis of population structure in ticks can be used to help infer host movement (Chapter 3). We analysed 73,533 SNPs from *I. uriae* ticks collected from rockhopper (*Eudyptes chrysocome*) colonies, and from nearby grey-headed (*Thalassarche chrysostoma*) and light-mantled albatross (*Phoebetria palpebrat*) colonies, on Marion Island (southern Indian Ocean); and from rockhopper, king (*Aptenodytes patagonicus*) and gentoo penguin (*Pygoscelis papua*) colonies across the Falkland Islands (southern Atlantic Ocean) (Fig. 1). Based on previous findings (McCoy et al. 2005, 2012), we expected

that ticks on penguins would be genetically distinct from those on flighted birds, but shared between penguin host species that were sympatric and shared breeding characteristics. We hypothesised, however, that movement between distant colonies in the sub-Antarctic – islands separated by thousands of kilometres of ocean – would present a challenge to the penguins and their ticks, resulting in phylogeographic structure among sites.

Whereas ticks from the two Marion Island albatross species represented a single intermixed population, ticks from nearby rockhopper penguins (~12 km from the albatross colony) were genetically distinct, supporting specificity among ticks on flighted seabirds versus those on penguins. King, gentoo and rockhopper penguins on the east coast of the Falkland Islands shared a single intermixed population of ticks. Rockhopper ticks from the western Falkland Islands population were genetically distinct from those on the east, but some movement among western and eastern colonies was evident. Rockhopper ticks on Marion Island were genetically distinct to those on the Falkland Islands (Fig. 1; Supplementary material).

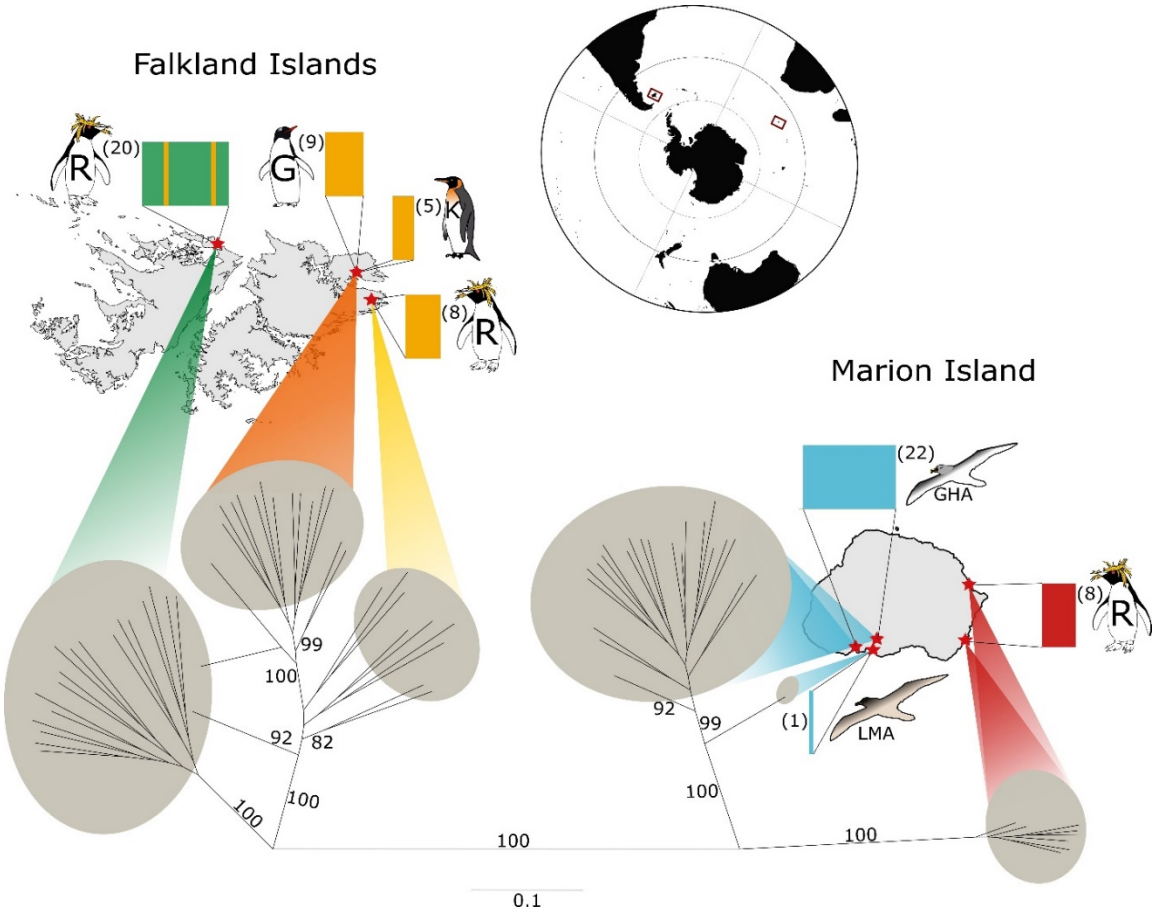


Figure 1. Sample sites across the Falkland Islands and Marion Island. Host species are depicted as images; R = rockhopper, G = gentoo; K = king penguin; LMA = light-mantled albatross; GHA = grey-headed albatross. Sample sizes for each site are shown in parentheses. Coloured plots depict the results of fastSTRUCTURE analyses, and the IQ-TREE phylogeny (including bootstrap values) is also shown.

Our genomic results indicate that penguin ticks can move among penguin colonies within islands, but that – based on differences between Marion and Falkland Island rockhopper ticks – movements of ticks between sub-Antarctic islands are restricted. Although rockhopper penguins are capable of dispersing between Marion and the Falkland Islands (separated by > 6000 km), phylogenetic studies suggest they rarely make the voyage (Banks et al. 2006). Oceanographic features – such as the sub-Antarctic Front – might act as an effective barrier to rockhopper movement as is the case for other species (Vianna et al. 2017). Despite evidence for shared ticks among rockhopper and king penguins (this study) and for considerable movement of king penguins between colonies across the sub-Antarctic region (Clucas et al. 2016), penguin ticks do not appear to be dispersing between Marion and the Falkland Islands. Our results therefore suggest that either host movements between the islands are restricted, or that penguin ticks cannot survive the journey. Furthermore, although genetic studies of grey-headed albatross suggest frequent movement around the region (Burg and Croxall 2001), we infer that host-specificity of *I. uriae* lineages (McCoy et al. 2005, 2012; this study) limits the capacity of penguin ticks to disperse with flying hosts.

High-resolution genomic data have greatly improved our ability to track dispersal events and infer population connectivity. In fragmented regions such as the sub-Antarctic, where long-distance dispersal underpins historic distributions and will define the course of ecosystem change, we can use genomics to better understand challenges to biotic interactions. Wider sampling in future genomic studies, including

more host species and colonies, are now required to clarify movements across the region.

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6.3 Supplementary material

6.3.1 Methods

Sampling

Ixodes uriae ticks were collected from under rocks across two discrete rockhopper penguin colonies at the Murrell (east Falkland Islands) and two sites across a small area of Pebble Island (west Falkland Islands), and from gentoo and king penguin sites at Volunteer Point (east Falkland Islands) (see Fig. 1), in January of 2015 by Katherine Moon. Upon collection, the ticks were immediately placed in 96% ethanol for genomic analysis. Ticks were also removed directly from rockhopper penguins, grey-headed albatross and light-mantled albatross on Marion Island (see Fig. 1) in April of 2016 by Dr. Ralph Vanstreels. In July 2016, the legs of the ticks were removed, placed in 100% ethanol and sent to Katherine Moon for genomic analysis.

DNA extraction and genomic analyses

Extractions were undertaken as per the method outlined in Chapter 3, except that ticks from Marion Island already had their legs removed. Library preparations and genomic analyses were undertaken as outlined in Chapter 3, except that the rxstacks script in *Stacks* was used to correct genotype and haplotype calls (minimum log likelihood = -15.0, proportion of loci = 0.25, prune haplotype algorithm enabled), and a python script was used to remove samples that had >95% missing data.

6.3.2 Figures and tables

Figure S1. PCA plot of *Ixodes uriae* ticks from Marion Island (M), and the east Falkland Islands (EF) and west Falkland Islands (WF). Percentage of variation explained by each PC is given in brackets, and sites and hosts are differentiated by colour.

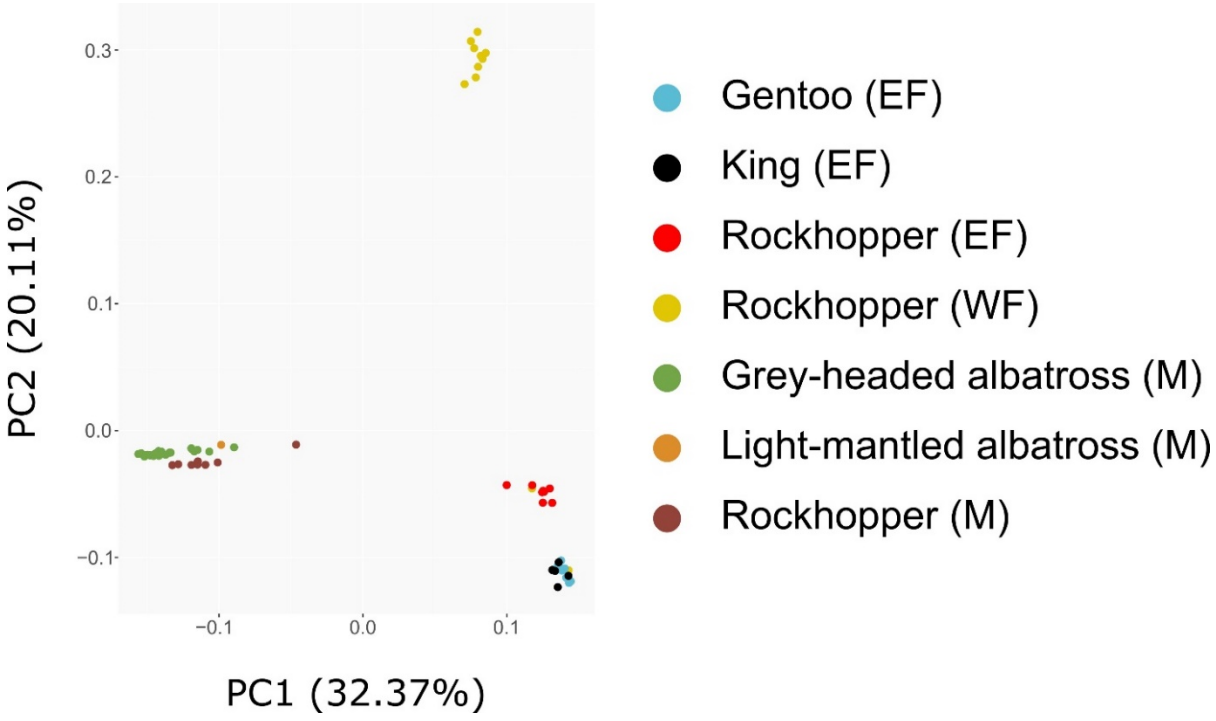

Table S1. Full results of the PCA analysis including eigenvalues, Tracy-Widom (TW) statistics, *p*-values and percentage of variation explained for each of the first 10 principal components (PCs). Tracy-Widom *p*-values that were found to be significant ($p < 0.01$) are in **bold**.

Principle component	Eigenvalue	% Variation explained	Tracy-Widom Statistic	TW *p*-value
1	19.97788	41.39	48.274	0.000
2	9.957069	20.63	76.002	0.000
3	5.348337	11.08	90.958	0.000
4	2.792169	5.78	56.952	0.000
5	2.527294	5.24	59.534	0.000
6	1.798169	3.73	28.206	0.000
7	1.727489	3.58	28.054	0.000
8	1.415426	2.93	8.96	0.000
9	1.397139	2.89	9.346	0.000
10	1.325182	2.75	5.39	0.000

Chapter Seven

Australian penguin ticks screened for novel *Borrelia* species

Image depicting the struggle between humans and tick disease vectors. Available at http://www.lymenet.de.

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7.1 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.

7.2 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 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.

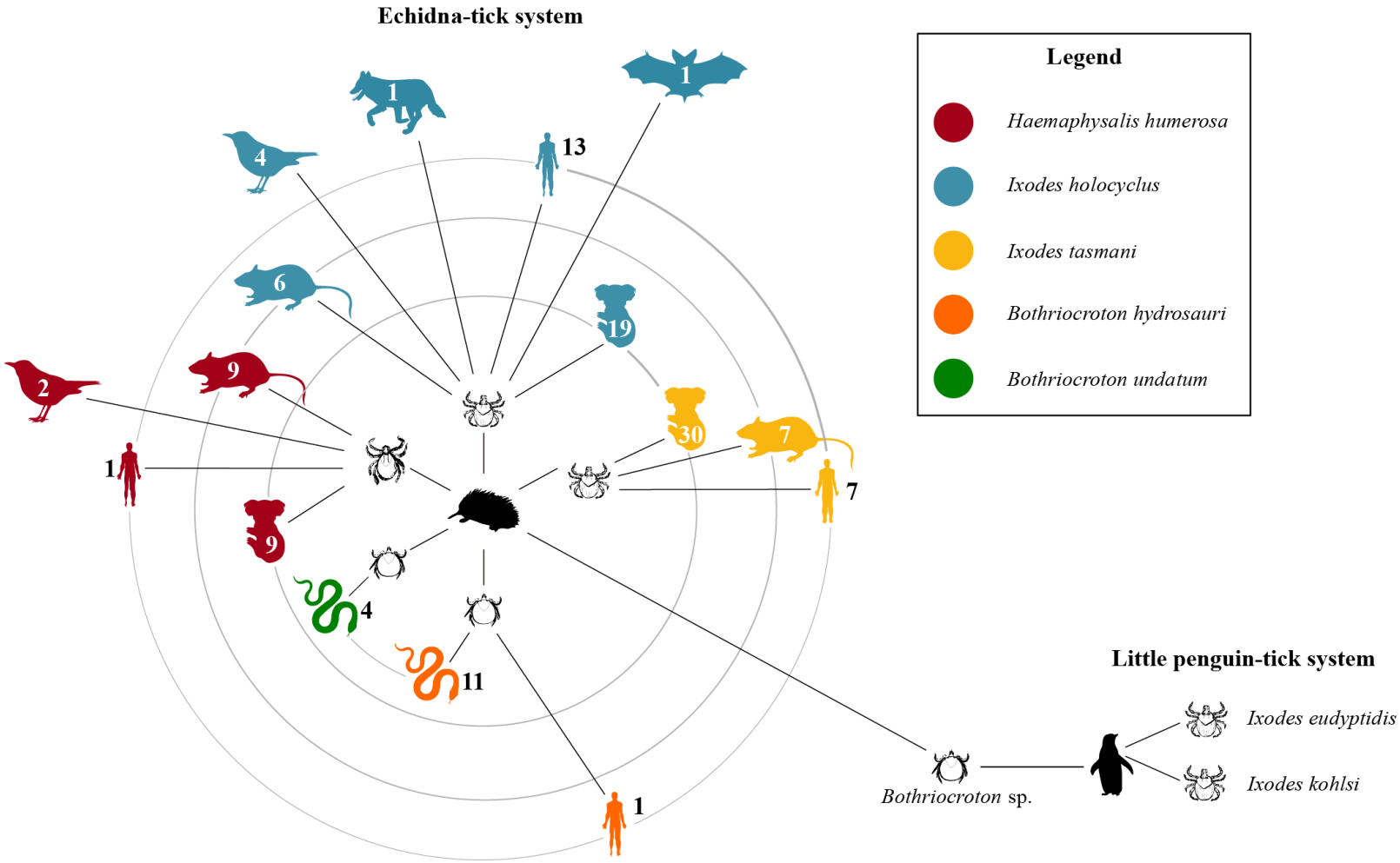
Echidna-tick system
Legend
Haemaphysalis humerosa
Ixodes holocyclus
Ixodes tasmani
Bothriocroton hydrosauri
Bothriocroton undatum
1
1
4
13
6
19
2
9
30
7
1
7
9
4
11
1
Little penguin-tick system
Ixodes eudyptidis
Ixodes kohlsi
Bothriocroton sp.

Figure 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.

7.3 Materials and methods

7.3.1 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.

7.3.2 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/).

Table 1. Primers used for *Borrelia*-specific nested PCR assays, including annealing temperature (AT), expected product size (EP) and primer reference (PR).

Gene	Primer	Sequence (5' – 3')	Annealing temperature (°C)	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		

7.4 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.

7.5 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.

7.6 Acknowledgements

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Chapter Eight

General discussion

Rockhopper penguins at the Murrell in the Falkland Islands. Taken by Katherine L Moon, January 2016.

8.1 Thesis conclusions

This thesis has explored the importance of dispersal in an isolated model region, and in an unusual host-parasite model system. As a body of work, it provides a number of new insights – particularly for conservation – and raises new questions for future phylogeographic research. Chapter 2 represents an up-to-date, comprehensive appraisal of dispersal and connectivity literature from the sub-Antarctic, with implications for both the way species are investigated and the way they are managed in the region. I established that that dispersal has been, and continues to be, an integral driver of sub-Antarctic biodiversity, but that the true extent of dispersal is often misinterpreted based on traditional biogeographic techniques. Just as we begin to understand how dispersal works in the sub-Antarctic, however, it is clear that conditions are changing, with implications for all communities. Interestingly, I found that biological vectors (e.g. algal rafts, and swimming or flying vertebrates) are dispersing a wide range of non-motile taxa across the region, in some cases connecting populations that are separated by thousands of kilometers of open ocean.

Building on this, I tested the ability of penguin ticks to survive immersion in water (Chapter 3). I found that, contrary to claims of limited survival, penguin ticks exhibit considerable physiological tolerances that would likely confer survival during marine dispersal. In order to test whether ticks were, in fact, moving with their hosts, I then used genomic data to investigate gene flow across various scales; within a colony (Chapter 4), across the range of a penguin-tick system (Chapter 5), and between two very distant sub-Antarctic islands (Chapter 6). I showed that there are no barriers to little penguin tick

movement within a colony (Chapter 4), and that occasional movements among distant colonies across Australia and New Zealand are evident – inferred to the result of penguin movements rather than those associated with secondary hosts, based on host-species specificity analysis – but are too sporadic to maintain gene flow (Chapter 5). Sub-Antarctic penguin ticks were also host-group specific, with different lineages on penguins *vs* flighted seabirds, but I found no evidence for movement of penguin ticks between sub-Antarctic islands separated by > 6000km (Chapter 6), suggesting that dispersal is restricted over very large scales.

I found no evidence for *Borrelia* bacteria in little penguin ticks, despite the presence of a common co-infection (*Babesia*) in the host. Nonetheless, this negative result does not conclusively demonstrate absence of *Borrelia* in the little penguin-tick system, and – furthermore – *Borrelia* represents only one of the many potential pathogens that little penguin ticks may carry (Cunningham *et al*, 1993; Vanstreels *et al*, 2016). Further research is needed to understand to potential relevance of penguin ticks for wildlife and human health.

8.2 Implications for sub-Antarctic biodiversity

My second chapter used the sub-Antarctic as a model system to study dispersal, but the results from my review have major implications for conservation in the region. There has been a tendency to think of the sub-Antarctic, and greater Antarctic, as a series of pristine, isolated environments. My review provides a more complex appraisal, with a spectrum of responses from long-distance movement and even connectivity across the region, to

cryptic speciation and subdivision at fine scales. These are important results, because the extent of gene flow in a species – which is incredibly variable in the region – will influence adaptation, diversification, and the potential for movement in response to changing environmental conditions. For example, penguin dispersal into areas of habitat that have opened due to climate change have been a major factor defining expansions in the region (though some species are now benefiting more than others: Clucas *et al*, 2014). While a number of iconic or economically important species have now been investigated, information is limited for other taxa, and future studies should focus on building a more comprehensive understanding of sub-Antarctic dispersal dynamics, particularly using high-resolution genomic markers that can show fine-scale patterns.

Baselines for the influence of dispersal mechanisms are also important because these mechanisms are already changing (Gillett and Thompson, 2003; Thompson and Solomon, 2002) with impacts on sub-Antarctic biota (Weimerskirch *et al*, 2012). Understanding the mechanisms and patterns of dispersal will help us to predict what environmental changes will mean for the biodiversity of the region. For example, the growing influence of humans in the sub-Antarctic is already facilitating the movements of some species (Frenot *et al*, 2005; Hughes and Convey, 2010). In particular, the preferential movement of cold-adapted species into and between areas in this region (Chown *et al*, 2012; Lee and Chown, 2009; Whinam *et al*, 2005) is of particular interest, because these organisms are the most likely to overcome climatic barriers to establishment (Chown *et al*, 2012). Genetic studies of invasive species in the sub-Antarctic are severely lacking (but see Piertney et al., 2016), however, and future research should aim to clarify the extent and

mechanism of movement in these species, to inform future management decisions. Understanding the common pathways for introduction, using genomic data, is the next step to managing further alteration to these systems.

8.3 Genomics and the third wave of biogeography

Predictions of the extent of dispersal in an organism based on observations of movement, species distributions or life history should be interpreted with caution. Inferences are particularly problematic for organisms that can use others (biological vectors) to assist dispersal, as their movement will be influenced by behavioral constraints on vector movement (McCoy *et al*, 1999; McCoy *et al*, 2003), the effectiveness of vector movement (Fraser *et al*, 2015; Waters *et al*, 2013) and the manner of vector movement (this thesis). Further complicating inferences of dispersal, single or few gene phylogeographic studies can also be misleading as a result of biased inheritance (e.g. mitochondrial markers) or different rates of mutation. There are now a number of examples of studies where genomic methods were capable of picking up more fine-scale genetic structure than traditional techniques, for example in kelp (Fraser *et al,* 2009 vs Fraser *et al,* 2016). Following this trend, I found considerably more intricate structure in little penguin ticks using genomic techniques (Chapters 4, 5), than using traditional phylogeographic methods (mitochondrial and nuclear markers: Moon *et al*, 2015). Although this year only marks the 30th birthday of phylogeography, this shift towards genomics is already offering a much deeper insight into phylogeographic processes.

8.4 Terrestrial parasite, aquatic host

My thesis investigated biogeographic patterns in an unusual and fascinating host-parasite model system. Although host-associated dispersal has begun to receive attention from a phylogenetic perspective, almost nothing is known about what happens when an organism must withstand the dual requirements of marine and terrestrial environments in order to disperse with its host. My thesis has begun to answer some of these questions. I have shown that even ectoparasites who do not appear to have any adaptations for marine life can be capable of surviving oceanic conditions long enough to be moved considerable distances by their hosts. The ancestors of present day penguin ticks must have been able to overcome this considerable compatibility filter, which is believed to have caused the extinction of almost entire groups of terrestrial parasites in other hosts that returned to the oceans (e.g. the helminth communities of cetaceans: Anzar *et al*, 1994; Anzar *et al*, 2001; Balbuena and Raga, 1993). However, my results also raise a number of biogeographic questions. Firstly, it is unclear whether other terrestrial ectoparasites disperse at sea with aquatically dispersing hosts, as no other groups have yet been investigated via phylogeographic analyses. For example, despite evidence for marine adaptations in Echinophthiriidae lice, phylogenetic analyses have not been used to assess whether these parasites move effectively, or over long-distances, with their hosts. Furthermore, it is likely that terrestrial parasites that exploit aquatic hosts with stronger links to land may have more opportunities to transmit than those on almost entirely pelagic hosts (Aznar *et al*, 2001; Raga *et al*, 2009), but this remains unconfirmed with phylogenetic data. There is growing recognition that dispersal plays a critical role in global biogeography, and in species' responses to environmental change (see Chapter 1). The capacity of parasites to move with their hosts – and indeed of any organism to use a biological vector to disperse

through an inhospitable medium – is thus important to understand as we attempt to deal with shifting species distributions.

8.5 Conservation biogeography

The first biogeographers documented and speculated on human-mediated changes to biodiversity. Von Humboldt noted human alterations to landscapes in Latin America, while Darwin commented on the dire implications of species introductions when he toured the Galapagos, and more generally of declines in nature, in the mid-1800s (Lomolino, 2010). Even Wallace, who could not really be considered a proto-conservationist (Smith and Beccaloni, 2010), commented on the disappearance of biota (Lomolino, 2010). Biogeography plays an important role in species management. Around a decade ago, and out of a growing need for informed management decisions, conservation biogeography was born and continues to apply biogeographic theory to current threats (Ladle and Whittaker, 2011; Laurance, 2008; Lomolino, 2004; Opdam and Wascher, 2004; Richardson and Whittaker, 2010). Spatially explicit conservation management practices are evident in regions such as the greater Antarctic, where a review of traditional biogeographic studies has led to the determination of 15 Antarctic Conservation Biogeographic Regions that are being used to inform management (Terauds *et al*, 2012). Determination of regionalisation and connectivity is, however, only as good as the baseline data used, and my thesis posits that traditional techniques often underestimate the importance of dispersal in ongoing evolutionary processes. As a result, future studies should use high resolution genomic data to clarify connectivity across regions, particularly to assist conservation in increasingly fragmented systems.

8.6 Conclusion

This body of research provides consistent, multidisciplinary evidence for the importance of oceanic dispersal, even for organisms thought to be ill-equipped for voyages at sea. Many sub-Antarctic species incapable of active dispersal are nonetheless moving around the region, and penguin ticks appear to be capable of moving at sea despite having no clear adaptations to marine conditions. Genomic data is emerging as an incredibly useful resource in phylogeographic studies, answering questions on finer scales, for more individuals, than previously possible. Our world may be changing, but every day our ability to identify, monitor and compare these changes grows. Understanding the fundamental importance of dispersal for all living things, at all scales, is a pivotal step towards being able to predict and manage changes that will inevitably alter the world's biodiversity.

8.7 References

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Appendix One

Chown SL, Clarke A, Fraser CI, Cary SC, Moon KL, McGeoch MA (2015). The changing form of Antarctic biodiversity. *Nature* **522**(7557):431-438.
