ANTHROPOGENIC INFLUENCE ON SYMBIOTIC INTERACTIONS OF AUSTRALIAN SEA LIONS (*Neophoca cinerea*)

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"Nevertheless, the broader community of disease investigators and health care professionals has largely pursued a separatist approach for human, domestic animal, and wildlife rather than embracing the periodically proposed concept of "one medicine." We especially need to embrace this concept as the human population increases because there will be more contact, direct and indirect, among humans, domestic animals, and wildlife. An "Ecology for a Crowded Planet" will be an even more pressing concern, and that includes increasing our understanding of disease ecology, especially that of the zoonoses."

- *Dr. Milton Friend*

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Summary

Interactions between humans, domestic animals and wildlife populations present an interface for the dissemination of microorganisms. The spread of disease-causing microorganisms can have serious impacts to the health and population dynamics of vulnerable marine wildlife species, particularly those that aggregate in high-density colonies. Therefore, understanding microorganism dissemination at this interface is crucial for the long-term conservation of endangered marine species. Genetic characterization of indicator microorganisms provides a means with which to monitor dissemination of microorganisms of anthropogenic origin. Currently, accurate inferences detailing microorganism transmission routes to wildlife populations at this interface are limited by the low availability of genomic data. I used molecular tools to investigate potential anthropogenic impacts on an endangered endemic marine mammal, the Australian sea lion (*Neophoca cinerea*), a species whose contemporary disease ecology is largely unstudied. The sea lions' natural habitat encompasses coastal islands experiencing constant human visitation through to almost completely isolated islands, making the species ideal for the assessment of organism dissemination from anthropogenic sources. Through protozoal and microbial monitoring, I aimed to determine the establishment of terrestrial and human originated organisms in wild versus captive Australian sea lions. Screening for protozoan parasites identified *Giardia duodenalis* genotypes prominent in human infection in greater frequency in sea lions within close proximity to human settlements compared to more isolated colonies. *Cryptosporidium* was not detected in sea lion populations. Microbial monitoring indicated similar findings, with the presence of *E. coli* harbouring integron-borne antibiotic resistance mechanisms commonly identified in clinical settings, significantly greater in captive animals. Finally, the application of next-generation

sequencing indicated a high level of dissimilarity of microbiota community structure and membership between wild and captive animals. The genetic tools and target indicators described in this thesis can be applied to determine the dissemination of microorganisms from anthropogenic sources in most marine wildlife taxa. Through the identification of target indicators, we can bridge knowledge gaps in microorganism movement, and, as a result, better inform long-term conservation management strategies aimed at reducing anthropogenic impacts to sensitive marine mammal populations.

Declaration

The work described in this thesis was carried out in the Marsupial Research Laboratory at Macquarie University. All work described in this thesis is original and has not been submitted either whole or substantial part, for a higher degree at any other institution.

I certify that this is an original piece of research composed by myself. Any assistance received for research work and thesis preparation has been appropriately acknowledged.

In addition, I certify that all information sources and literature references are appropriately cited and indicated in the thesis.

Tiffany Claire Delport

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Chapter status and declarations

Chapter status

Chapter	Title	Status
Two	*Giardia duodenalis* and *Cryptosporidium* occurrence in Australian sea lions (*Neophoca cinerea*) exposed to varied levels of human interaction.	Published. International Journal of Parasitology: Parasites and Wildlife 3, 269-275.
Three	Molecular detection of antibiotic resistance determinants in *Escherichia coli* isolated from the endangered Australian sea lion *Neophoca cinerea*.	In press. Journal of Wildlife Diseases 51, in press.
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Chapter declarations

Chapter 2: *Giardia duodenalis* and *Cryptosporidium* occurrence in Australian sea lions (*Neophoca cinerea*) exposed to varied levels of human interaction.

I performed all aspects required for this paper including laboratory work and data analysis. Amy Asher assisted in the development of β-giardin PCR primers and was instrumental in optimizing PCR protocols. I wrote the manuscript with the help of my supervisors, Dr. Michelle Power and Prof. Robert Harcourt. Dr. Linda Beaumont, Dr. Koa Webster and Amy Asher provided feedback on this paper. Dr. Linda Beaumont assisted with the

generation of maps used to illustrate wild sea lion colonies and Dr. Koa Webster provided consultation on statistical analyses.

Chapter 3: Molecular detection of antibiotic resistance determinants in *Escherichia coli* isolated from the endangered Australian sea lion *Neophoca cinerea*.

I performed all aspects required for this paper including laboratory work and data analysis. I wrote the manuscript with the help of my supervisors, Prof. Robert Harcourt and Dr. Michelle Power. Dr. Linda Beaumont kindly provided feedback on the manuscript and Dr. Koa Webster provided consultation on statistical analyses.

Chapter 4: Comparison of the gut microbial communities of wild and captive Australian sea lions (*Neophoca cinerea*).

I performed preparation laboratory work for sample submission to the Ramaciotti Centre for Genomics at the University of New South Wales. MiSeq sequencing was performed by Jason Koval from the University of New South Wales. Dr. Sasha Tetu and I jointly performed data analyses. I wrote the manuscript with feedback from Dr. Michelle Power, Prof. Robert Harcourt and Dr. Sasha Tetu, and Dr. Koa Webster provided consultation on statistical analyses.

Australian sea lion faecal sample collection:

Faecal samples were collected during previous years of fieldwork carried out by members of the Marine Mammal Research Group at Macquarie University. All individuals involved have been recognized in chapter acknowledgements.

I, with the assistance of my supervisors Dr. Michelle Power and Prof. Robert Harcourt, organized the acquisition of faecal samples from animals housed at captive facilities and resident staff members at each facility collected faecal samples during routine animal husbandry.

Ethics and funding declaration

All work carried out for this thesis was conducted under the authorization of appropriate ethics and scientific collection permits.

This work was performed under approval from the Macquarie University Biosafety Committee. Biosafety approval: 5201100524 (LAB).

Faecal sampling was conducted with permission from the Department for Environment and Heritage, South Australia (permit Z25675), DEH SA Wildlife Ethics Committee (44/2008) and the Department of Environment and Conservation, Western Australia (DEC WA License SF007255 and SF008193). Grants were provided to Robert Harcourt from the Australian Marine Mammal Centre, Department of Environment, Heritage South Australia and West Australia, Water and the Arts through the Commonwealth Environment Research Fund and funds from Macquarie University (0809/27, 09/38, 0809/12). Funding was also provided to Tiffany Delport and Michelle Power from Macquarie University Department of Biological Sciences postgraduate project support.

Faecal samples were opportunistically acquired from wildlife parks for parasite and microorganism testing. Samples were collected as part of routine animal husbandry by collaborators and subsequently provided for testing. The provision of such samples for this research conforms to the requirements of the *Australian Code of practice for the care and use of animals for scientific purposes* (NH&MRC 7th Ed. 2004) and does not require animal ethics approval.

Personal Acknowledgements

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Finally, I would like to extend my thanks to my laboratory colleagues, Amy Asher and Matthew Lott. I am most grateful for their assistance and support over the last four years.

Extended summary

Interactions between humans, domestic animals and wildlife populations provide opportunities for the dissemination of terrestrial microorganisms to marine wildlife species. As anthropogenic contamination of the natural environment with disease-causing microorganisms can have serious impacts on animal health and population dynamics, it is important that we develop an in-depth understanding of potential anthropogenic impacts to sensitive marine mammal populations.

Currently, our understanding of microorganism movement at the terrestrial-marine interface is limited by dependence on culture-based screening techniques and low availability of genetic data from indicator microorganisms. The application of molecular tools can be used to bridge this existing gap in knowledge and provide a means to monitor the establishment of non-endemic microorganisms in marine mammals. I used molecular tools to investigate the establishment of human- and terrestrial-derived protozoa and microbes, in wild and captive populations of the Australian sea lion (*Neophoca cinerea*), a species whose contemporary disease ecology is largely unstudied.

The Australian sea lion is an endangered Australian endemic, the total population estimated at fewer than 15,000 animals (Shaughnessy et al., 2011). Their population is dispersed over greater than 76 small breeding colonies from Houtman Abrolhos in Western Australia (WA) to The Pages in South Australia (SA) (Shaughnessy et al., 2011). The sea lions' distribution is broad, encompassing both isolated islands and coastal beaches impacted by human visitation. In addition, as an Australian tourist icon, some sea lion colonies are exposed to constant habitat disturbance, with visitation to SA colonies, such as Seal Bay on Kangaroo Island, exceeding 150,000 tourists annually (Goldsworthy et al.,

2007). The habitat range and varied level of exposure to anthropogenic impacts makes this species an important candidate for assessing the dissemination of microorganisms from anthropogenic sources to semi-aquatic animals.

Protozoan markers, *Giardia* and *Cryptosporidium*, were used to detect the presence of human and terrestrial genotypes in Australian sea lions. Comparative analysis of wild and captive populations indicated a significantly higher presence of *Giardia duodenalis* (*G. duodenalis*) in captive animals and colonies distributed within close proximity (<25 km) to coastal settlements. Molecular characterization identified *G. duodenalis* assemblages AI and B, genotypes associated with terrestrial mammals and humans, in sea lion populations. *Cryptosporidium* was not detected. This study demonstrates the importance of multilocus typing when determining parasite origin, so that accurate inferences on transmission routes to sea lion populations may be drawn and appropriate conservation management strategies implemented.

Microbial markers, *E. coli* and integron-borne antibiotic resistance mechanisms were used to assess the establishment of anthropogenic-derived microbes in gut microbiota of sea lions exposed to human interaction. Comparative analysis of faecal culture from wild and captive individuals found that *E. coli* occurrence was high in faecal coliforms isolated from captive sea lions, however, *E. coli* was not a dominant member of wild sea lion gut microbiota. *E. coli* harbouring mobile genetic elements (class I integrons) conferring resistance to streptomycin-spectinomycin and trimethoprim were present in captive animals only. The detection of these integrons, which are commonly found in human clinical cases, indicates that conditions experienced in captivity may influence establishment in faecal coliforms of the sea lion. The findings from this study provide insight into the mechanisms facilitating antibiotic resistance in captive animals and are a

useful indicator of the potential source of dissemination to Australian sea lions.

Next-generation sequencing was used to define the gut microbiome of Australian sea lions and to compare the microbial communities of wild and captive populations. The gut microbiome of both wild and captive Australian sea lion populations were dominated by 5 bacterial phyla; *Firmicutes, Proteobacteria, Bacteroidetes, Actinobacteria* and *Fusobacteria.* Comparative analysis revealed the phylum *Firmicutes* as dominant in both wild and captive sea lions, while *Proteobacteria* contributed more to composition of the captive gut microbiome. Furthermore, the microbiome of wild seal lions had a richer diversity than captive animals. Differences in the biological environment may provide opportunity for unique microbial establishment in the gut and drive dissimilarity. This study highlights the importance of understanding the impact the biological environment has on gut microbial composition and is highly informative to long-term management of vulnerable marine mammal species.

This investigation demonstrates the importance of using culture-independent screening techniques when assessing microorganism movement from anthropogenic sources to Australian marine mammal populations. The genetic techniques and indicator microorganisms used here can be applied to improving understanding of terrestrial microorganism dissemination to most marine wildlife species. However, a multilocus approach and genetic typing at lower taxonomic levels are required to draw more accurate inferences on the origin of non-endemic microorganisms in marine wildlife. Ongoing protozoal and microbial monitoring in marine mammals will bridge existing knowledge gaps in microorganism flow at the human: domestic animal: wildlife interface and inform long-term conservation management strategies aimed at reducing anthropogenic impacts to vulnerable marine populations.

Chapter 1

Literature review

Microorganism spread at the human: domestic animal: wildlife interface

The human: domestic animal: wildlife interface

The movement of microorganisms between humans, domestic animals and wildlife is well documented. As the human population continues to grow and encroach into previously remote areas, there are increasing interactions between humans and wildlife. These interactions provide new opportunities for the introduction of human and terrestrial microorganisms to wildlife populations (eg. Bogomolni et al., 2008; Daszak et al., 2000) (Figure 1). Most recently, transfer of serious disease-causing microorganisms from human habitation to marine wildlife populations has signalled that these pathways need urgent attention (Baily et al., 2015; Miller et al., 2002).

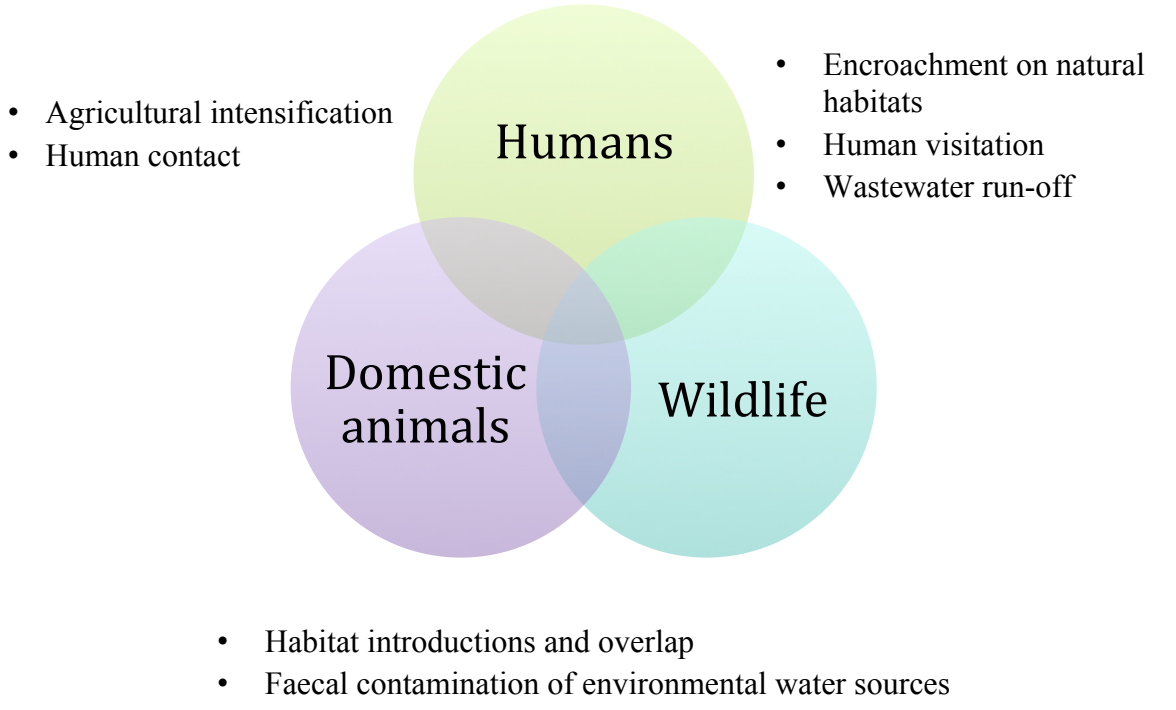

Figure 1. The human: domestic animal: wildlife interface.
Anthropogenic impacts to natural environments and habitation overlap with terrestrial species provide increased opportunities for the dissemination of non-endemic microorganisms to marine wildlife.

Marine mammals may be exposed to terrestrial microorganisms either through anthropogenic impact to the natural environment or from co-habitation with terrestrial animals (Daszak et al., 2000; Fenwick et al., 2004; Lasek-Nesselquist et al., 2010; Nelson et al., 2008). Anthropogenic contamination of the coastal environment significantly increases the connectivity of terrestrial and marine ecosystems, resulting in increased opportunities for the dissemination of non-endemic microorganisms to marine mammals (Baquero et al., 2008; Gaydos and Miller, 2008; Skurnik et al., 2006). Marine mammals, especially seal and sea lion populations, inhabit coastal islands and mainland shores at critical stages in their lifecycle. As such, many species are exposed to high levels of anthropogenic contamination through visitation to beaches frequented by humans, exposure to wastewater run-off or from human visitation to breeding colonies and haul-outs (Baily et al., 2015; Fenwick et al., 2004). The presence of microorganisms of terrestrial origin, including parasitic protozoa and antibiotic resistant bacteria, are becoming increasingly more common in marine mammals and appear to largely originate from faecal contamination of fresh and wastewater run-off (eg. Deng et al., 2000; Stoddard et al., 2005). While their presence indicates human impact, it is difficult to draw inferences on potential origin and transmission pathways from presence data alone.

Co-habitation and visitation (tourism) by terrestrial species facilitates the transmission of microorganisms from inland anthropogenic sources to marine mammal populations. Marine vertebrates, including flying vectors such as gulls, act as reservoirs of both terrestrial and marine protozoa and bacteria (Lasek-Nesselquist et al., 2010; Literak et al., 2010). The herring gull (*Larus argentatus*), in particular, has been associated with the dissemination of human and terrestrial wildlife protozoan parasites to marine mammals including grey (*Halichoerus grypus*) and harbor seals (*Phoca vitulina*) (Lasek-Nesselquist et al., 2010). Herring gulls often come in contact with untreated wastewater and other

terrestrial sources of enteric pathogens such as landfills, accumulating antimicrobial resistant faecal bacteria, which they may disseminate to coastal marine populations (Nelson et al., 2008). Vectors, combined with the high degree of connectivity between marine mammal populations, may increase the risk of pathogens from terrestrial sources establishing in sensitive pinniped populations (Lasek-Nesselquist et al., 2010; McCallum et al., 2003).

Rapid dissemination of microorganisms between pinnipeds

The geographic proximity of colonies to coastal settlements and social nature of pinniped species potentially increases transmission of bacterial and protozoal pathogens. Seals, fur seals and sea lions have a tendency to aggregate in high-density groups on coastal islands or the mainland when breeding, moulting and resting (Kirkman et al., 2007). Species including the Cape fur seal (*Arctocephalus pusillus pusillus*) and Australian fur seal (*Arctocephalus pusillus doriferus*) frequently comprise colonies exceeding 1,000 animals (Kirkman et al., 2007). Even geographically isolated pinniped populations such as the southern elephant seal (*Mirounga leonina*) aggregate in numbers exceeding 1,000 animals per colony (McCann, 1980). Such high-density habitation has been identified as a primary pathway for increased microbial transmission between individuals within the same colony (Lombardo, 2008).

Wide dispersal of disease-causing microorganisms in the marine environment poses serious concerns for the longevity of endangered marine mammal species. Historically, the dissemination of microorganisms to pinniped species has resulted in significant morbidity and mortality events (c.f. Gulland and Hall, 2007) (Table 1). Most notably, endemic scale morbilivirus infection with canine distemper virus resulted in mass mortality of 80-

100,000 Baikal seals (*Phoca siberica*) between 1987 and 1988 (Grachev et al., 1989). More recently, outbreaks of phocine distemper virus in harbor seals have had similar impacts to canine distemper, resulting in mortality of greater than 25,000 animals (Jensen et al., 2002). Large-scale bacterial disease events, such as a bacterial pneumonia outbreak in New Zealand sea lions (*Phocarctos hookeri*) in 1998 resulted in a reduction of greater than 33% of pup production in a single breeding season (c.f. Castinel et al., 2007; Wilkinson et al., 2006). Most recently, infection with an unknown bacterium, hypothesized as a novel *Streptococcus* species, caused rapid mortality of ~200-350 male sub-Antarctic fur seals (*Arctocephalus tropicalis*) over a two week period (De Bruyn et al., 2008). While parasitic infection has not been associated with major mortality events, high parasite loads have been associated with poor body condition and pup mortality in northern fur seals (*Callorhinus ursinus*) and New Zealand sea lions (Acevedo-Whitehouse et al., 2009; Castinel et al., 2004; Lyons et al., 1997). As the dissemination of pathogenic microorganisms has such severe consequences for pinniped populations, it is important that target indicators associated with pinniped disease are monitored to prevent mass mortality events and inform conservation management strategies for sensitive species.

Table 1. High impact bacterial, viral and parasitic disease events in seal and sea lion species (1955-2007).
Historically, outbreaks of viral disease have had significant impacts on marine mammal population dynamics and health, resulting in near extinction of several pinniped species.

Pathogen	Pinniped species	Impact	Reference
Bacterial			
Klebsiella pneumoniae	*Phocarctos hookeri*	Mortality >33% pup population (2002) and >21% (2003)	c.f. Castinel et al. (2007)
Leptospirosis	*Zalophus californianus*	Low-level mortality ~1,191 animals (1984-2004)	Dierauf et al. (1985) c.f. Greig et al., (2005) Raverty et al. (2005)
Pseudomonas aeruginosa	*Phoca vitulina*	Mortality ~90 animals (1997)	c.f. Gulland and Hall (2007)
Unkn. bacterium	*Phocarctos hookeri* *Arctocephalus tropicalis*	Mortality >60% pup population, unkn. adults (1998) Mortality ~250-300 adult males (2007)	c.f. Greig et al., (2005) De Bruyn et al. (2008)
Calcivirus			
San Miguel sea lion virus	*Zalophus californianus*	Mortality ~20% pup population (1968-1971)	Gilmartin et al. (1976)
Morbilivirus			
Canine distemper virus	*Lobodon carcinophaga* *Phoca caspica* *Phoca siberica*	Mass mortality ~85% *L. carcinophaga* (1955) Mortality >10,000 animals *P. caspica* (2000) Mass mortality 80,000-100,000 animals *P. siberica* (1987-1988)	c.f. Gulland and Hall (2007) Kennedy et al. (2000) Grachev et al. (1989)
Phocine distemper virus	*Phoca vitulina*	Mass mortality >18,000 animals (1988) Mass mortality >25,000 animals (2002)	Dietz et al. (1989) Jensen et al. (2002)
Parasite			
Hookworm *Uncinaria sp.*	*Callorhinus ursinus* *Otaria flavescens* *Neophoca cinerea* *Phocarctos hookeri* *Zalophus californianus*	Associated with pup morbidity and mortality.	Acevedo-Whitehouse et al. (2009) Berón-Vera et al. (2004) Castinel et al. (2004) Lyons et al. (1997) Marcus et al. (2014a and 2014b)

Parasitic protozoa as indicators of transmission at the human: domestic animal: wildlife interface

Protozoan parasites are a common cause of enteric disease of humans, domestic animals, livestock and wildlife. Two genera, *Giardia* and *Cryptosporidium*, are the most common cause of gastrointestinal infections arising from protozoa in mammalian populations worldwide (Xiao and Fayer, 2008). Both genera contain zoonotic members that are often disseminated between humans and other vertebrate species (c.f. Ryan and Cacciò, 2013; Ryan et al., 2014). Environmental contamination with faecal matter from humans and other terrestrial vertebrates is recognized as a primary pathway for dissemination of protozoa to marine wildlife populations (Xiao and Fayer, 2008). As *Cryptosporidium* and *Giardia* species have specific host occurrence, these genera are potentially useful protozoan markers of anthropogenic impact. The ongoing application of molecular tools in identifying protozoan species infecting marine mammal populations will provide valuable insight into potential dissemination pathways of these two parasites from human and terrestrial sources to endangered species (Benton et al., 2014).

Giardia

Introduction to Giardia

Giardia species are binucleate flagellated protozoan parasites in the phylum Metamonada (Adam, 2001). *Giardia* infects a broad range of vertebrates including mammalian, amphibian and avian wildlife species (c.f. Ryan and Cacciò, 2013). In humans, infections are typically self-limiting, characterized by diarrhoea, malabsorption and weight loss, although asymptomatic cases are commonly reported (Cacciò and Ryan, 2008). In contrast, very little is known of the effect *Giardia* has on marine wildlife species.

Giardia has a simple lifecycle consisting of two stages, the trophozoite, which replicates in the host gastrointestinal tract and the cyst, which is the infective stage (Figure 2).

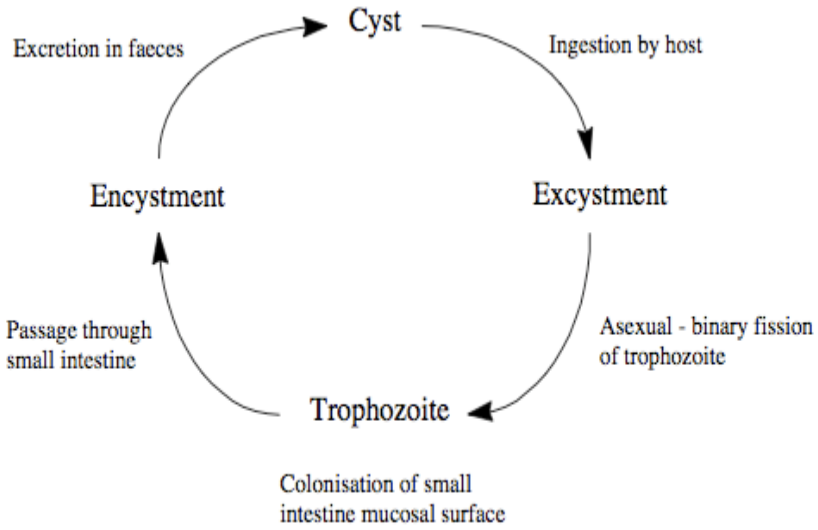

Figure 2. Lifecycle of *Giardia duodenalis.*
Giardia duodenalis has a simple lifecycle, with only one stage, the cyst, occurring outside the host. Once cysts are excreted by an infected host they can be transmitted via ingestion of contaminated environmental sources (Source: Monis and Thompson, 2003).

Cysts are transmitted either via ingestion of contaminated food and water sources, or by faecal-oral route contact (Gardner and Hill, 2001). Cysts become infective when passed in faeces and are environmentally robust, capable of surviving months in the natural environment (Erickson and Ortega, 2006). In cool, moist environments such as rivers and seawater, cysts may remain infective for greater than 65 days (Erickson and Ortega, 2006). Consequent accumulation of cysts in the natural environment may lead to contamination of naturally occurring food and drinking water sources of many marine species (c.f. Feng and Xiao, 2011).

Giardia taxonomy

Traditionally, delineation of *Giardia* species was based on host occurrence and morphology of the trophozoite (Adam, 2001). Currently, the taxonomy recognizes 6 distinctive species of *Giardia*; *G. agilis* the primary form existing in amphibians, *G. duodenalis* common in most mammals; including humans and their livestock, *G. muris* and *G. microti* in rodents and a further two species *G. ardeae* and *G. psittaci* described in birds (Table 2).

Table 2. Currently recognized species of *Giardia*.
The taxonomy currently recognizes six species of *Giardia*. While most *Giardia* species have a specific vertebrate host range, *Giardia duodenalis* infects a variety of mammalian taxa.

Giardia species	**Main host(s)**	**Reference**
G. agilis	Amphibians	Filice (1952)
G. ardeae	Birds	Erlandsen et al. (1990)
G. duodenalis	Mammals	Filice (1952)
G. microti	Rodents	Feely (1988)
G. muris	Rodents	Filice (1952)
G. psittaci	Birds	Erlandsen and Bemrick (1987)

Unlike other *Giardia* species, *G. duodenalis* infects a broad range of mammalian hosts including humans and wildlife taxa, raising concerns for zoonotic transmission (c.f. Feng and Xiao, 2011).

Giardia duodenalis

Giardia duodenalis infects a broad range of mammalian species including humans, domestic animals and wildlife. Molecular characterization has shown that *G. duodenalis* is a multi-species complex, defined by unique polymorphisms at the DNA level (Monis et al., 2003). Multilocus analyses have identified eight *G. duodenalis* assemblages; assemblages A and B predominantly associated with human infection, assemblages C and D the primary form in dogs, assemblage E in domestic ruminants, assemblages F and G from cats and rodents respectively, and assemblage H identified in seals (c.f. Feng and Xiao, 2011; Lasek-Nesselquist et al., 2010) (Table 3).

Among the currently identified assemblages of *G. duodenalis*, assemblages A and B have the broadest host range and have been identified in humans, domestic animals and livestock, and diverse mammalian wildlife species (c.f. Feng and Xiao, 2011). Multilocus testing has identified further genetic complexity and host specificity within assemblages A and B. Assemblage A is composed of four closely related genetic subtypes (I-IV), where sub-assemblages AI and AII are of human origin, while sub-assemblages AIII and AIV originated in domestic and hoofed wildlife species (c.f. Cacciò and Ryan, 2008). Similarly, assemblage B is further categorized into genetic sub-assemblages (I-IV), where sub-assemblages BIII and BIV arose from human infections, and sub-assemblages BI and BII are thought to have originated from non-human primate and canine hosts (c.f. Ryan and Cacciò, 2013).

Table 3. *Giardia duodenalis* species complex.
There are currently eight recognized *G. duodenalis* assemblages infecting mammalian taxa. While all assemblages have a specific host range, assemblages A and B have been described in diverse mammalian species.

Assemblage	Strain	Host(s)	Reference
A	I	Human	c.f. Feng and Xiao (2011)
	II	Human	
	III	Cat	
	IV	Alpaca, cat, guinea pig	
B	I	Dog, siamang	
	II	Marmoset	
	III	Human	
	IV	Human	
C	I-III	Dog	
D	-	Dog	
E	I-III	Cattle, hoofed livestock	
F	-	Cat	
G	-	Rat	
H	-	Seal	Lasek-Nesselquist et al. (2010)

Human and terrestrial genotypes of Giardia duodenalis in marine taxa

Giardia duodenalis assemblages A and B have enzoonotic strains that are often disseminated between humans and mammalian species (c.f. Ryan and Cacciò, 2013). Assemblages AI and AII, both human genotypes, have been detected in marine mammals including the common dolphin (*Delphinis delphis*) and harbor porpoise (*Phocoena phocoena*) (Lasek-Nesselquist et al., 2008). While subtyping analyses are not frequently performed for marine wildlife, the assemblage B genotype has been identified in marine populations including the common thresher shark (*Alopias vulpinus*), Risso's dolphin (*Grampus griseus*) and Atlantic white-sided dolphin (*Lagenorhynchus acutus*) (Lasek-Nesselquist et al., 2008).

Giardia presence in pinniped populations from the Northern Hemisphere is well defined, however, little is known about prevalence in Australasian marine mammals. *Giardia duodenalis* is currently the only *Giardia* species identified in pinniped populations, although the prevalence and distribution of specific haplotypes remain undefined (Lasek-Nesselquist et al., 2010). Of the eight existing assemblages of *G. duodenalis,* six have been described in seal and sea lion populations (Table 4).

Table 4. *Giardia duodenalis* assemblages identified in pinniped populations. *G. duodenalis* assemblages A and B are common in geographically disparate seal and sea lion species. The presence of human and terrestrial genotypes implicates anthropogenic introduction of *Giardia* to marine pinnipeds.

Assemblage	**Pinniped species**	**Reference**
A	*Crystophora cristata* *Halichoerus grypus* *Pagophilus groenlandicus* *Phoca vitulina vitulina*	Appelbee et al. (2010) Lasek-Nesselquist et al. (2010)
B	*Halichoerus grypus* *Pagophilus groenlandicus* *Phoca vitulina richardsi* *Phoca vitulina vitulina*	Appelbee et al. (2010) Gaydos and Miller (2008) Lasek-Nesselquist et al. (2010)
C	*Phoca vitulina richardsi*	Gaydos and Miller (2008)
D	*Phoca vitulina richardsi*	Gaydos and Miller (2008)
F	*Halichoerus grypus** *Phoca vitulina*	Bogomolni et al. (2008)
H	*Halichoerus grypus*	Lasek-Nesselquist et al. (2010)
Giardia sp. cysts	*Phoca hispida* *Zalophus californianus*	Olson et al. (1997) Deng et al. (2000)

*Sequence similar to strain.

Giardia duodenalis assemblages A and B are commonly reported in pinniped species including grey, harp (*Pagophilus groenlandicus*) and Eastern Atlantic harbor seals

(Appelbee et al., 2010; Lasek-Nesselquist et al., 2010). Assemblages C, D and F have been detected in low frequency in harbor seals and a strain similar to assemblage F found in grey seals (Bogomolni et al., 2008; Gaydos and Miller, 2008). Finally, assemblage H, a novel seal genotype, has been identified in grey seal populations (Lasek-Nesselquist et al., 2010). Molecular characterization of *Giardia duodenalis* sub-assemblages in wild seals is under-investigated, however, a single study has reported the AI genotype in harp seals, and both AI and AII genotypes in grey seals (Lasek-Nesselquist et al., 2010). The presence of human and terrestrial *Giardia duodenalis* genotypes suggests anthropogenic introduction to marine mammals. While exact transmission routes to marine mammals are undefined, shared coastal habitation and visitation of human-impacted beaches have been identified as significant contributing factors to the presence of *G. duodenalis* in wild pinniped species (Gaydos and Miller, 2008; Lasek-Nesselquist et al., 2010).

Cryptosporidium

Introduction to Cryptosporidium

Cryptosporidium is an intracellular protozoan parasite belonging to the phylum Apicomplexa (Fayer et al., 2000). *Cryptosporidium* has been described in a variety of vertebrate hosts including mammalian, avian and reptilian wildlife species (c.f. Ryan et al., 2014). Infections are more commonly reported in young animals, and characterized by self-limiting symptoms including diarrhoea, dehydration and weight loss, or are asymptomatic in nature (c.f. Ramirez et al., 2004). While the impact of infection has been documented for domestic animals and commercial livestock, the effect on marine mammals is unknown.

Cryptosporidium has a complex lifecycle with only one stage, the oocyst, occurring outside the host (Figure 3). Oocysts are transmitted via the faecal-oral route or through the ingestion of contaminated food and water sources (Xiao et al., 2004).

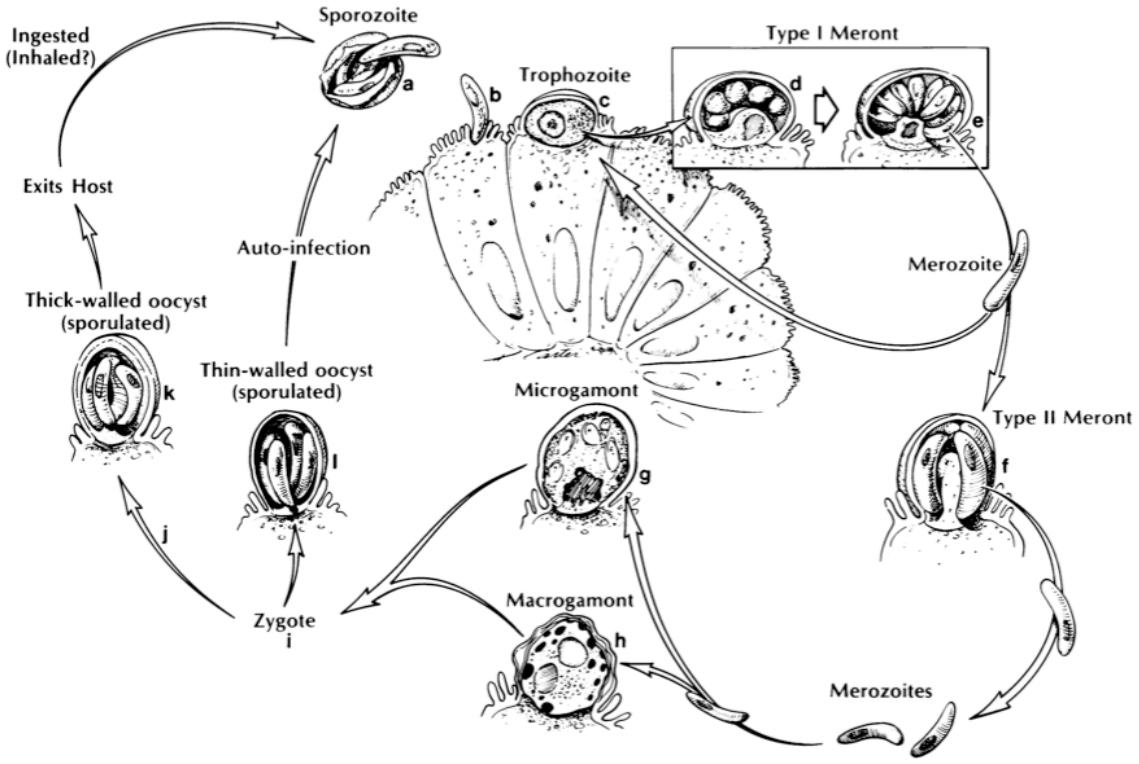

Figure 3. *Cryptosporidium parvum* lifecycle.
The *Cryptosporidium* lifecycle begins when oocycts are excreted in faeces of an infected host. Oocysts are then transmitted to a new host via the ingestion of contaminated food and environmental sources (Source: Current and Garcia, 1991).

Upon ingestion of oocysts, sporozoites are released and penetrate the epithelial surface of the gut wall (Thompson, 2004). Once the sporozoite differentiates into a trophozoite, asexual and sexual stages of reproduction occur, producing thin- and thick-walled oocysts. While thin-walled oocysts cause autoinfection in the host, thick-walled oocysts are expelled in faeces, where they accumulate in the natural environment and can be

transmitted to new host groups (Thompson, 2004). Oocysts are small, buoyant and environmentally robust, capable of surviving extended periods of time in cool, wet environments (Fayer, 2004). In the ocean and coastal waters, oocysts can remain infective for up to a year, contributing to widespread dispersal and increased risk of dissemination to marine mammal populations (Tamburrini and Pozio, 1999).

Cryptosporidium taxonomy

Traditionally, *Cryptosporidium* species were classified according to host specificity and site of infection (Fayer, 2010). The application of molecular tools has better enabled identification of unique species that have morphologically similar features (Appelbee et al., 2005). The current taxonomy considers 26 species valid based on morphological, biological and molecular characterization analyses, of which, twenty species are described as originating in mammalian taxa (c.f. Ryan et al., 2014) (Table 5). Among the currently accepted *Cryptosporidium* species*, C. hominis* is the primary form existing in humans and the most common species identified in clinical cases (Leoni et al., 2006; c.f. Xiao et al., 2004).

The application of molecular tools has also enabled the identification of zoonotic strains within the *Cryptosporidium* genus, of which, *C. parvum* is considered to have the most diverse host range (c.f. Xiao, 2010). While *C. parvum* is common in commercial livestock such as cattle and sheep, it is frequently identified in humans, domestic animals and diverse mammalian wildlife species (c.f. Xiao, 2010). *Cryptosporidium* species common in domestic pets, *C. felis* and *C. canis* from cat and dog respectively, are also frequently identified in humans and wildlife species (c.f. Xiao, 2010). To a lesser extent, infections with *C. muris* and *C. ubiquitum*, the primary form in rodents and hoofed

domestic ruminants, have also been reported in humans (c.f. Ryan et al., 2014). While many diverse species cause infection in humans, the frequent reporting of *Cryptosporidium* species associated with livestock and domestic animals in clinical cases is a major zoonotic disease concern (c.f. Ryan et al., 2014).

Table 5. *Cryptosporidium species* infecting mammalian taxa.
Of the currently accepted *Cryptosporidium* species, twenty have been identified in diverse mammalian taxa. While most species have specific host range, domestic animals and livestock have been identified as a reservoir for human *Cryptosporidium* infection.

Cryptosporidium species	**Main host(s)**	**Reference**
C. andersoni	Cattle	Lindsay et al. (2000)
C. bovis	Cattle	Fayer et al. (2005)
C. canis	Dog	Fayer et al. (2001)
C. cuniculus	Rabbit	Inman and Takeuchi (1979)
C. erinacei	Hedgehog, horse	Kváč et al. (2014)
C. fayeri	Kangaroo	Ryan et al. (2008)
C. felis	Cat	Iseki (1979)
C. hominis	Human	Morgan-Ryan et al. (2002)
C. macropodum	Kangaroo	Power and Ryan (2008)
C. meleagrdis	Human, turkey	Slavin (1955)
C. muris	Rodents	Tyzzer (1907)
C. parvum	Ruminants	Tyzzer (1912)
C. ryanae	Cattle	Fayer et al. (2008)
C. scrofarum	Pig	Kváč et al. (2013)
C. suis	Pig	Ryan et al. (2004)
C. tyzzeri	Rodents	Tyzzer (1912)
C. ubiquitum	Ruminants	c.f. Fayer (2010)
C. viatorum	Human	Elwin et al. (2012)
C. wrairi	Guinea pig	Vetterling et al. (1971)
C. xiaoi	Sheep, goats	c.f. Fayer (2010)

Compared to other protozoan parasites such as *Giardia*, *Cryptosporidium* is reported at low frequency in marine mammal populations (c.f. Appelbee et al., 2005; Rengifo-Herrera et al., 2011). *Cryptosporidium hominis* is the most commonly identified species in clinical cases and thought to be host-specific (c.f. Xiao, 2010). Infection with *C. hominis* has been identified in a terminally-ill dugong from Australia (*Dugong dugon*) (Hill et al., 1997).

The presence of *Cryptosporidium* in Northern Hemisphere and Antarctic seals is well defined, however, little is known regarding the incidence and specific genotypes present in Australasian pinnipeds (Fayer et al., 2000). Of the 20 currently accepted *Cryptosporidium species* infecting humans and terrestrial mammals, two have been described in pinniped populations (Table 6). Infections with *C. parvum* have been identified in ringed seals (*Phoca hispida*) and California sea lions (*Zalophus californianus*), while *C. muris* is described in ringed seals only (Deng et al., 2000; Hughes-Hanks et al., 2005). The presence of livestock and rodent genotypes suggest that parasites are disseminated from terrestrial sources to marine mammal populations. While the exact transmission routes are undefined, faecal contamination of natural water sources by wildlife species and run-off from commercial dairy farms have been suggested as significant contributing factors to the dispersal of *Cryptosporidium* to marine populations (Deng et al., 2000).

More recently, novel genotypes genetically similar to *C. canis* and *C. felis* have been identified in harbor, harp and hooded seals, while a skunk-like genotype has been detected in southern elephant seals (Bass et al., 2012; Bogomolni et al., 2008; Rengifo-Herrera et al., 2013).

Table 6. *Cryptosporidium species* identified in pinniped populations.
Of the currently recognized *Cryptosporidium* species, *Cryptosporidium muris* and *Cryptosporidium parvum* have been identified in pinniped populations. Genetic analyses have also identified several novel genotypes in 7 seal and sea lion species.

Cryptosporidium sp.	**Pinniped species**	**Reference**
C. muris	*Phoca hispida*	Santin et al. (2005)
C. parvum	*Phoca hispida* *Zalophus californianus*	Deng et al. (2000) Hughes-Hanks et al. (2005)
C. sp. seal 1	*Phoca hispida*	Santin et al. (2005)
C. sp. seal 2		
C. sp	*Cystophora cristata** *Halichoerus grypus* *Leptonychotes weddellii* *Pagophilus groenlandicus** *Phoca hispida* *Phoca vitulina**	Bass et al. (2012) Bogomolni et al. (2008) Dixon et al. (2008) Rengifo-Herrera et al. (2013)
C. skunk-like genotype	*Mirounga leonina*	Rengifo-Herrera et al. (2011) Rengifo-Herrera et al. (2013)

*Sequence similar to *C. canis* and *C. felis.*

Multilocus testing has identified further genetically distinct *Cryptosporidium* species, seal genotypes 1 and 2 in ringed seals, and novel species in Weddell (*Leptonychotes weddellii*) and grey seals (Bass et al., 2012; Bogomolni et al., 2008; Dixon et al., 2008; Santin et al., 2005). As the host-specificity of these novel genotypes is yet to be determined, infections may present downstream concerns for zoonotic transmission to humans sharing coastal environments with marine mammals, and highlights the importance of identifying genotypes present in pinniped species (Santin et al., 2005).

Current knowledge gap

There exists a current gap in our knowledge of *G. duodenalis* and *Cryptosporidium* genotypes present in Australasian pinniped populations, contributing to uncertainty over dispersal pathways within the marine ecosystem. As human and terrestrial mammal genotypes are increasingly observed in marine mammals, further investigation and genetic typing is required. Identification of *Cryptosporidium* and *Giardia* genotypes present in pinnipeds will contribute to our understanding of protozoa host range and the potential for transmission of terrestrial and human genotypes to marine mammals.

Microbial indicators of transmission at the human: domestic animal: wildlife interface

The occurrence of bacteria harbouring antibiotic resistance determinants is becoming more commonly observed in marine wildlife (eg. Bogomolni et al., 2008; Radhouani et al., 2014; Stoddard et al., 2008). In species not previously exposed to the selective pressures of antibiotics, this is a strong indicator of microbial flow at the terrestrial-marine interface (Bogomolni et al., 2008). Proximity to humans and water sources contaminated by faecal pollution is recognized as a primary pathway for the dissemination of antimicrobial resistant bacteria to marine mammals (eg. Baquero et al., 2008; Lockwood et al., 2006). Analyzing genetic characteristics of faecal indicator bacteria, such as *Escherichia coli*, can be used to provide an indication of environmental contamination with human and terrestrial mammal faecal matter (Gordon, 2004). Culture-independent analyses, such as molecular mapping of integrons, are a useful indicator of anthropogenic contamination as they often accumulate genes conferring antibiotic resistance common in human and domestic animal treatment (Gillings et al., 2014). Further microbial characterization using next-generation sequencing technologies can be used to provide novel insight into microbial diversity and enhance our understanding of gene flow at the terrestrial-marine interface (Nelson et al., 2013).

Escherichia coli

Taxonomy

Escherichia coli are gram-negative, facultatively anaerobic bacteria from the Enterobacteriaceae family (Eisenstein and Zaleznik, 2000). *E. coli* is a common enteric symbiont in vertebrates, including humans and mammalian wildlife species (Souza et al., 1999). While most enteric strains are commensal, pathogenic strains are a primary cause of intestinal and extra-intestinal disease (Eisenstein and Zaleznik, 2000; Selander et al., 1987).

E. coli strains can be assigned to four major phylogenetic groups; A, B1, B2 and D, each differing in phenotypic characteristics including the distribution of virulence genes (Gordon, 2004; Johnson et al., 2001). Of the four phylotype groups, phylotype B2 is characterized by an increased presence of extra-intestinal virulence factors and demonstrates the strongest resistance to antibiotics (Johnson et al., 2001; Skurnik et al., 2005).

E. coli in pinniped gut microbiota

The identification of *E. coli* in marine mammals is primarily limited to recovery and isolation using culture-based techniques. *E. coli* is often absent in cultures recovered from wild pinnipeds including harbor seals, California sea lions and northern elephant seals (Johnson et al., 1998). In contrast, *E. coli* is often the most frequently identified faecal coliform of captive pinnipeds including grey, harp and harbor seals (Greig et al., 2014; Wallace et al., 2013). These findings suggest that *E. coli* may not be a dominant

member of natural gut microbiota in pinniped populations. As such, monitoring the establishment of *E. coli* in gut microbiota of captive marine mammals may be a useful indicator of microbe dissemination from non-endemic sources.

Antibiotic resistance as an indicator of agricultural and anthropogenic impact on marine ecosystems

Introduction to antibiotic resistance

The persistent use of antimicrobial agents in human and animal health care has been a major selective force increasing the spread of antibiotic resistance mechanisms in naturally occurring ecosystems and wildlife (Gillings et al., 2008; Partridge et al., 2009). As a result, many strains of bacteria have become resistant to previously successful antimicrobial agents. The mechanisms involved in clinical resistance cases are diverse and well described (c.f. Partridge et al., 2009). In addition to naturally acquired resistance, bacterium can develop resistance via spontaneous genetic mutation and the acquisition of resistance genes from other bacterial species (c.f. Wright, 2005). First, spontaneous genetic mutation of bacterium can result in the development of numerous adaptive physiological changes such as; enzymatic production to reduce the efficacy of antibiotics and functional modification of channels responsible for efflux from the cell (c.f. Wright, 2005). Secondly, bacteria can acquire resistance genes via conjugation (the transfer of genetic material between cells) and horizontal gene transfer between bacterial species, furthering the dissemination of resistance genes (c.f. Wright, 2005).

The mobility of DNA elements encoding antibiotic resistance has facilitated rapid spread

to the natural environment (Gillings et al., 2008). Bacteria harbouring resistance from human and terrestrial sources can enter marine ecosystems via faecal contamination of water sources and exposure to untreated wastewater run-off (Baquero et al., 2008; Reinthaler et al., 2003). Microbes isolated from over-flow of sewerage treatment facilities are resistant to many clinical antimicrobials including; ampicillin, piperacillin, trimethoprim and tetracycline, facilitating dissemination between populations at the terrestrial-marine interface (Reinthaler et al., 2003).

Identification of antibiotic resistance profile

Traditionally, identification of antimicrobial resistance is determined using culture-based, antibiotic sensitivity screening (Bogomolni et al., 2008; Stoddard et al., 2008). While culture-based screening enables the determination of resistance to selected clinical antimicrobials, it limits the ability to identify unique genetic mechanisms driving resistance (Stoddard et al., 2008; Stokes et al., 2001). The application of molecular tools has provided remarkable insights into antimicrobial resistance acquisition and expression (Gillings et al., 2008; Partridge et al., 2009).

Integron-mediated antibiotic resistance

Integrons are genetic elements that enable the capture and recombination of antibiotic resistant genes. Three classes of integrons (class I, II and III) have been described, defined by the type of integrase they possess. For the purpose of this review, class I integrons are described as they are most commonly identified in clinical cases and marine wildlife populations (eg. McIntosh et al., 2008; Partridge et al., 2009). Class I integrons possess a variable region bound by 5' and 3' conserved regions. The 5' region is

composed of an integrase gene (*int*), a recombination site (*attI*) and an adjacent promoter (P_c), while the 3' region consists of an ethidium bromide resistance locus (*qacE*Δ), a sulfamide resistance gene (*sul*) and an open reading frame (*ORF*) (Collis et al., 1998; Gillings et al., 2009) (Figure 4).

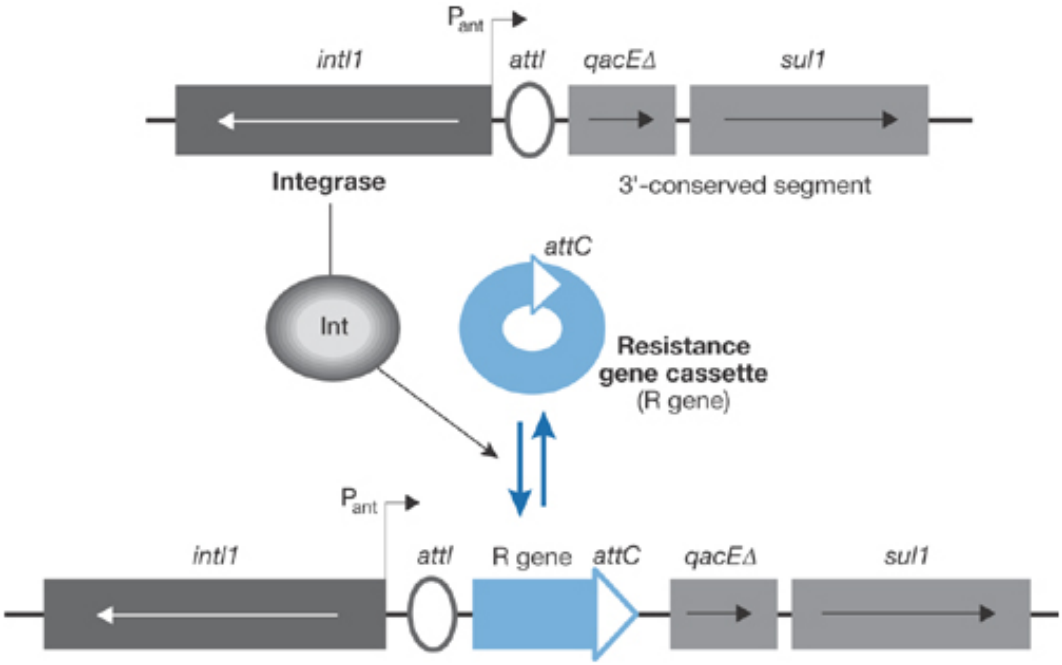

Figure 4. The integron mechanism of antibiotic resistance gene cassette acquisition. The integron integrase (*int*) acts as a promoter for the integration of gene cassettes. The *int* catalyses site specific recombination between *attI* and *attC* sites, allowing antibiotic resistance genes to be integrated into integron structure (Source: Davies, 2007).

Recombination sites on integrons enable the capture of gene cassettes, most commonly captured are those comprised of antibiotic resistance genes (Gillings et al., 2008). Gene cassettes are circular, non-replicating DNA molecules composed of a coding region and a recombination site known as *attC* or the 59-base element located at the 3' region of the gene (Collis and Hall, 1992). The integron integrase recognizes the 59-base element and the *attC* site inserts the cassette into the integron recombination site (*attI*). Once inserted into the integron, the cassette is bound by the *attI* site on the 5' conserved region and by the *attC* on the 3' region (Collis and Hall, 1992) (Figure 4).

The site of gene cassette insertion plays a key role in the level of antibiotic resistance expression (Collis and Hall, 1992; Stokes and Hall, 1989). Antibiotic resistance is strongest when gene cassettes are inserted closer to a P_c and the effect lessened when the gene is inserted further downstream (Collis and Hall, 1992). In addition, the number of single-base changes at the recombination site has an impact on antibiotic resistance expression, where changes occurring closer to the recombination site have a stronger effect than those at the opposite end of the 59-base element (Stokes and Hall, 1989). Furthermore, integrons are capable of capturing numerous gene cassettes, increasing resistance to multiple antimicrobial agents (Partridge et al., 2009).

Genetic characteristics of resistance genes in marine taxa

To date, the genetic characteristics of integrons present in antimicrobial resistant bacteria of marine mammals remains unexplored. However, the presence of resistance genes in aquatic vertebrates, including avian and fish species, is well documented (Dolejska et al., 2009; Nawaz et al., 2009; Sato et al., 2009). Genes encoding resistance to common clinical antimicrobials have been identified in several species exposed to anthropogenic impact in their natural habitats including the black-headed gull (*Larus ridibundus*), lesser flamingo (*Phoeniconaias minor*) and channel catfish (*Ictalurus punctatus*) (Dolejska et al., 2009; Nawaz et al., 2009; Sato et al., 2009).

Current observations of antimicrobial resistance in pinnipeds are mostly limited to the use of traditional culture-based screening and sensitivity techniques, as a result, the genetic characteristics facilitating establishment in gut microbiota remain unclear (eg. Bogomolni et al., 2008; Lockwood et al., 2006). Culture-based screening of faecal

coliforms has identified resistance to greater than 10 common clinical antimicrobials (eg. González et al., 2011; Stoddard et al., 2008) (Table 7).

Table 7. Antimicrobial resistance in faecal coliforms isolated from pinniped species. Antimicrobial sensitivity screening has identified resistance to greater than 10 common clinical antimicrobials in faecal coliforms of 8 pinniped species.

Pinniped species	Antimicrobial resistance	Reference
Arctocephalus gazella	No significant resistance.	Palmgren et al. (2000)
Crystophora cristata	Augmentin, ampicillin, amoxicillin-clavulanic acid, carbenicillin, cefiofur, cephalothin, chloramphenicol, enrofloxacin, tetracycline, ticarcillin	Rose et al. (2009) Stoddard et al. (2005) Glad et al. (2010)
Halichoerus grypus	Ampicillin	Glad et al. (2010)
Mirounga angustirostris	Ampicillin, cefazolin, chloramphenicol, clindamycin, lincomycin, tetracycline, trimentin	Johnson et al. (1998) Stoddard et al. (2008)
Otaria flavescens	Clarithromycin, levofloxacin, metronidazole, trovafloxacin	González et al. (2011)
Pagophilus groenlandicus	Amikacin, ceftazidime, gentamycin, tobramycin	Rose et al. (2009)
Phoca vitulina	Amikacin, ampicillin, cephalosporins, clindamycin, chloramphenicol, ciprofloxacin, gentamicin, kanamycin, lincomycin, penicillins, streptomycin, sulphonamides, tetracycline, trimethoprim	Johnson et al. (1998) Lockwood et al. (2006) Samadpour et al. (2005)
Zalophus californianus	Chloramphenicol, clindamycin, lincomycin, tetracycline	Johnson et al. (1998)

In pinniped populations exposed to anthropogenic impact from coastal settlements and untreated wastewater, the prevalence of faecal bacteria harbouring resistance to multiple antimicrobial classes is increasing (Stoddard et al., 2005; Wallace et al., 2013).

Periodical observation of stranded harbor, harp and grey seals from the Northwest Atlantic between 2004-2010, indicated significant increases of *E. coli* antimicrobial resistance across numerous classes, where proximity to human settlements and faecal pollution of the marine ecosystem were associated with increased establishment in marine mammal faecal culture (Wallace et al., 2013).

Current knowledge gap

The presence of bacteria harbouring antibiotic resistance in Australasian pinniped populations is currently undefined. As bacteria resistant to common human and animal health care antimicrobials are increasingly reported in marine mammal populations, further investigation is required to determine; first, the presence of antibiotic resistance in faecal coliforms and secondly, the genetic characteristics of bacteria facilitating dissemination. Identification of the aforementioned factors will further contribute to our understanding of microbial dissemination from anthropogenic sources, assisting the development of effective management and treatment protocols for marine mammals.

Improving characterization of microbial communities

Introduction to next-generation sequencing technology

Recent advances in DNA sequencing technologies have dramatically improved our ability to characterize microbial communities (c.f. Shendure and Ji, 2008). The application of these technologies enables rapid processing of millions of sequence reads in a single run, with high raw base accuracy, allowing novel insights into microbial diversity, limited using culture-dependent screening techniques (c.f. Morozova and Marra, 2008; Zhang et al., 2011). The generation of sequence reads from fragment 'libraries' reduces potential cloning bias issues that result from conventional vector-based cloning, providing a more accurate picture of microbial prevalence and abundance within a sample (Grada and Weinbrecht, 2013). The use of next-generation sequencing technologies in microbial ecology has provided profound insight relative to bacterial diversity, enabling more accurate inferences to be drawn on factors contributing to gut microbial community structure.

Next-generation insights in marine mammal microbiota

Next-generation sequencing technologies have been applied to advancing understanding of the influence biological- and community-related differences have on gut microbial community composition of Australian fur and Weddell seal populations (Banks et al., 2014; Smith et al., 2013). Further comparative microbial analyses have been used to draw inferences on factors influencing gut microbiota of wild and captive leopard (*Hydrurga leptonyx*) and southern elephant seals, where exposures in the external environment were identified as a primary factor driving gut microbiome dissimilarity

(Nelson et al., 2013). The sensitivity of next-generation techniques has also enabled the identification of genes encoding ampicillin and tetracycline resistance in grey and hooded seals respectively (Glad et al., 2010). The low prevalence, less than 0.001% of sequences obtained, would most likely have been unidentified using culture-dependent screening techniques (Glad et al., 2010).

Marine mammal gut microbiota, and the factors influencing community structure and membership are understudied. As gut microbes play an important role in the development and health of mammalian species, and anthropogenic disturbance to wildlife populations is likely to increase, understanding factors influencing gut microbial composition may prove crucial for managing the impact of microorganism dissemination at the terrestrial-marine interface (Stappenbeck et al., 2002; Tremaroli and Bäckhed, 2012; Wikoff et al., 2009). Understanding factors influencing gut microbial communities, and identifying the occurrence of microbial indicators of anthropogenic impact, will better inform current knowledge gaps regarding the dissemination of terrestrial microorganisms to vulnerable Australasian pinnipeds.

Host study species

The Australian sea lion is an endangered species, inhabiting coastal islands and mainland shores of South and Western Australia. While many colonies are remote, a number of small colonies exist on the mainland and are within close proximity to human impacted beaches. Additionally, as a tourist icon some colonies, such as Seal Bay, Kangaroo Island, experience habitat disturbance from tourist visitation.

Taxonomy and description

The Australian sea lion (family Otariidae) is the only seal species endemic to Australia. The Otariidae family is comprised of 16 species from 7 genera, commonly referred to as sea lions or fur seals. Otariids are distinguished from relatives, the Phociidae, by the presence of small external pinnae or ears (c.f. Shaughnessy, 1999).

Adult males are described as large (weight range= 61-300 kg, length= ~250 cm), typically chocolate brown and characterized by a cream-yellow crown extending from the eye to the back of the head (King and Marlow, 1979; Walker and Ling, 1981). Adult females are traditionally smaller (mean body weight= 77 kg, ~181 cm) and coloured silver-grey dorsally, fading to pale yellow ventrally (King and Marlow, 1979; Walker and Ling, 1981) (Figure 5).

Conservation status

The Australian sea lion is considered an endangered species on the IUCN Redlist of Threatened species (Endangered A2bd+3d ver 3.1) (Goldsworthy and Gales, 2008). The global population is small and genetically fragmented, comprised of fewer than 15,000

animals, with the only site monitored long-term, Kangaroo Island, South Australia, showing continual population decline (McIntosh et al., 2012).

Figure 5. The Australian sea lion.
Adult male Australian sea lions (rear) are differentiated from adult females (front right) by increased body size and the appearance of a cream-yellow crown extending behind the head. Pups of both genders (front left) are typically small and chocolate-brown in colour (Source: Robert G. Harcourt).

Distribution and habitat

Australian sea lions are dispersed over 76 small breeding colonies from the Houtman Abrolhos in Western Australia (WA) to The Pages in South Australia (SA) (Shaughnessy et al., 2011). The geographic range extends beyond 2,700 km of Australian coastline, encompassing major metropolitan areas (Adelaide, SA and Perth, WA) and large

rural centres, as well as protected environmental sites. The majority of the population occurs on coastal islands of South Australia (~70%), while a number of smaller colonies (~30%) are dispersed along the west Australian coastline (Gales et al., 1994). Historically, sea lions were more abundant on the Western Australia coast, their range including islands near the Albany region, however this region is no longer inhabited due to unregulated exploitation during the 18th and 19th centuries (Gales et al., 1994) (Figure 6).

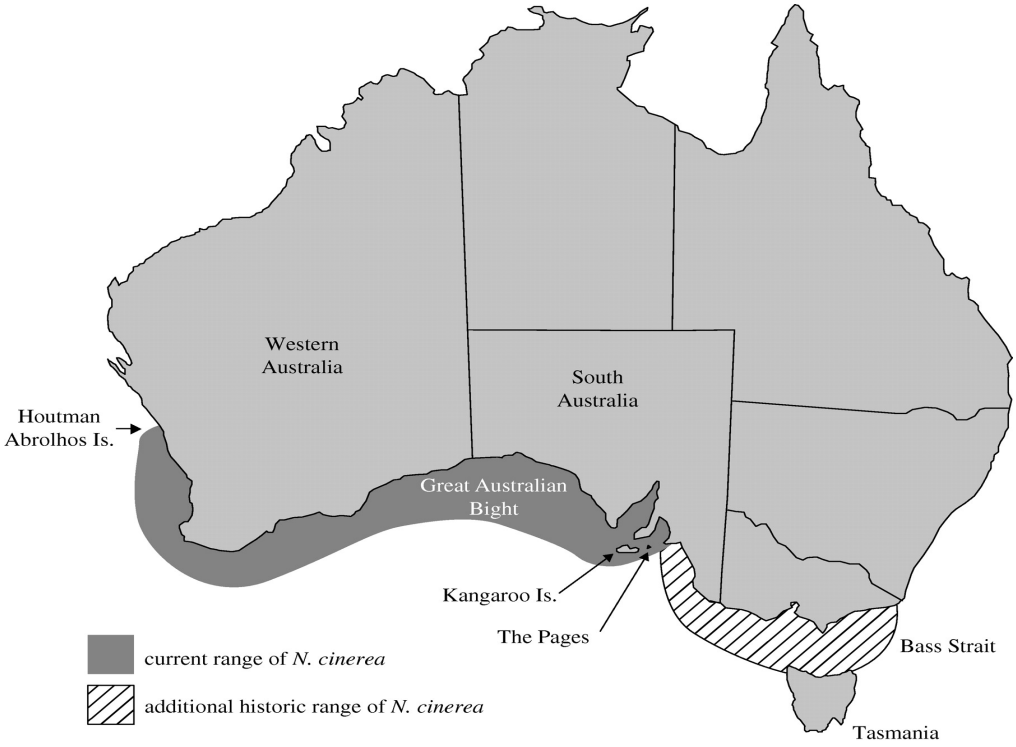

Figure 6. Australian sea lion distribution.
Current distribution of Australian sea lion colonies and historical range, now uninhabited due to unregulated exploitation during the sealing era (Source: Campbell et al., 2008).

While habitat preference is wide, sea lions are most commonly sited hauling-out on the sheltered side of coastal islands. The terrestrial habitat is generally characterized by shallow, protected rock pools in which both pups and adults rest (Gales et al., 1994).

Coastal beaches are also frequently visited by sea lions for breeding and moulting, and when seeking protection from predators or poor weather (Gales et al., 1994). The marine habitat encompasses shallow-waters over the continental shelf, where sea lions forage and seek refuge during periods of colony disturbance (Gales et al., 1994).

Ecology

While sea lion distribution is broad, a tendency for localized foraging by both males and females is common (Lowther et al., 2012; Lowther et al., 2013b). Sea lions are opportunistic foragers of shallow on-shelf water, feeding on a diverse range of prey including octopus, teleost fish, cuttlefish, squid, rays, small sharks, penguins, and small crustaceans, such as rock lobster (Gibbs et al., 2011; McIntosh et al., 2007).

The Australian sea lion demonstrates the strongest natal philopatry of any mammalian taxa (Campbell et al., 2008; Lowther et al., 2012). Movement of adults is incredibly limited compared to other species; females have been observed no greater than approximately ~60 km from natal site, while males have been sited at distances up to approximately ~200 km (Campbell et al., 2008). Strong site fidelity and limited interchange of animals between breeding colonies is reflected by genetic distinctiveness between colonies, even those within close geographic range from each other (Ahonen et al., 2013; Campbell et al., 2008). The vulnerability of sea lions and high degree of genetic kinship within the population poses serious concern for local extinctions from disease spread events and anthropogenic impact (Lowther et al., 2012).

Exposure to anthropogenic impact

The sea lion is an Australian tourist icon attracting high levels of human visitation to several South Australian colonies. Seal Bay on Kangaroo Island is the only long-term monitored visitation site, and tourism has dramatically increased from 112,000 in 1996 to upwards of 150,000 tourists annually in 2007 (Goldsworthy et al., 2007). At Seal Bay and in other areas, tourists are able to walk on the beach while viewing sea lions. While care is taken not to interfere with the natural behaviour of the sea lion, animals demonstrate an increased state of vigilance during human visitation and have been observed retreating from sites with high human disturbance (Orsini and Newsome, 2005). Such habitat disturbance has significant impact on the breeding capacity of sea lions (Campbell et al., 2008). Displacement of sea lions from breeding sites during breeding season has been associated with increased pup mortality, further reducing population size of the vulnerable pinniped (Campbell et al., 2008; Gales et al., 1994).

While most sea lion colonies are on coastal islands, there exist a number of small colonies on the Australian mainland within close proximity to human settlements and local wastewater run-off (Goldsworthy et al., 2007). Sea lions may be indirectly exposed to pathogenic or non-endemic microorganisms through the ingestion of seawater contaminated by wastewater and behaviours such as hauling-out on human impacted beaches (Fenwick et al., 2004; Gales et al., 1994). Exposure to contaminated seawater may present a pathway for the dissemination of terrestrial and non-endemic microorganisms, increasing disease risk to the Australian sea lion (Bogomolni et al., 2008; Goldsworthy et al., 2007).

Current knowledge gap

The disease ecology of the Australian sea lion is largely understudied. Given the vulnerability of sea lions, frequent habitat disturbance and exposure to microorganisms from contaminated environmental sources poses significant concerns for potential disease transmission from anthropogenic sources (Bogomolni et al., 2008; Fayer et al., 2000). Understanding the presence and dissemination of non-endemic microorganisms to the sea lion is crucial for long-term conservation strategy.

Thesis aims

The dissemination of non-endemic, disease-causing microorganisms to marine wildlife can seriously impact population health and dynamics. Understanding microorganism dissemination from anthropogenic sources is therefore crucial for the long-term conservation of endangered marine species. Genetic characterization of indicator microorganisms provides a means with which to monitor the extent of transmission from terrestrial sources to marine mammals. As many protozoal and microbial strains have specific host occurrence, they provide a useful indicator of anthropogenic impact to marine mammals. To assess the dissemination of terrestrial microorganisms into the Australian marine environment, the Australian sea lion was chosen as the study host species. As an Australian endemic, the sea lion population is widely dispersed across coastal islands, both those experiencing high levels of human disturbance and some more isolated habitats. This makes the sea lion an ideal species to assess the impact of anthropogenic impact on the dispersal of microorganisms at the human: domestic animal: wildlife interface.

Specifically, the primary aim of this work was to apply molecular tools to assess the presence of protozoa and microbial microorganisms characteristic of terrestrial taxa in an endangered endemic, the Australian sea lion. This goal was achieved through three key objectives targeted to:

i. Determine the presence of human and terrestrial genotypes of *Giardia* and *Cryptosporidium* in Australian sea lions exposed to varied levels of human interaction (Chapter two).

ii. Assess the occurrence of *E. coli* and integron-borne antibiotic resistance mechanisms in the gut microbiota of sea lions and determine if colony exposure to anthropogenic impact and captivity influence presence (Chapter three).

iii. Identify bacterial communities contributing to Australian sea lion gut microbiota and determine potential patterns of composition in wild colonies and captive sea lion populations (Chapter four).

Chapter 2

Title

Giardia duodenalis and *Cryptosporidium* occurrence in Australian sea lions (*Neophoca cinerea*) exposed to varied levels of human interaction.

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Abstract

Giardia and *Cryptosporidium* are amongst the most common protozoan parasites identified as causing enteric disease in pinnipeds. A number of *Giardia* assemblages and *Cryptosporidium* species and genotypes are common in humans and terrestrial mammals and have also been identified in marine mammals. To investigate the occurrence of these parasites in an endangered marine mammal, the Australian sea lion (*Neophoca cinerea*), genomic DNA was extracted from faecal samples collected from wild populations (n= 271) in Southern and Western Australia and three Australian captive populations (n= 19). These were screened using PCR targeting the 18S rRNA of *Giardia* and *Cryptosporidium*. *Giardia duodenalis* was detected in 28 wild sea lions and in seven captive individuals. Successful sequencing of the 18S rRNA gene assigned 27 *Giardia* isolates to assemblage B and one to assemblage A, both assemblages commonly found in humans. Subsequent screening at the *gdh* and β-giardin loci resulted in amplification of only one of the 35 18S rRNA positive samples at the β-giardin locus. Sequencing at the β-giardin locus assigned the assemblage B 18S rRNA confirmed isolate to assemblage AI. The geographic distribution of sea lion populations sampled in relation to human settlements indicated that *Giardia* presence in sea lions was highest in populations less than 25 km from humans. *Cryptosporidium* was not detected by PCR screening in either wild colonies or captive sea lion populations. These data suggest that the presence of *G. duodenalis* in the endangered Australian sea lion is likely the result of dispersal from human sources. Multilocus molecular analyses are essential for the determination of *G. duodenalis* assemblages and subsequent inferences on transmission routes to endangered marine mammal populations.

Introduction

Protozoan parasites are a primary cause of morbidity and mortality in terrestrial and marine mammal populations (Hughes-Hanks et al., 2005; Ryan et al., 2014). Two genera, *Cryptosporidium* and *Giardia*, are amongst the most common organisms identified as causing enteric disease in pinniped species (eg. Appelbee et al., 2010; Bass et al., 2012; Dixon et al., 2008; Olson et al., 1997). Currently, 26 *Cryptosporidium* species are considered valid and over 40 genotypes or cryptic species have been identified (c.f. Ryan et al., 2014). Several *Cryptosporidium* species present in humans and terrestrial mammals have been identified in marine mammals, including *C. parvum, C. muris* and *C. hominis* (eg. Appelbee, 2005; Deng et al., 2000; Santin et al., 2005). In addition, *Cryptosporidium* seal genotypes 1 and 2, thought to be specific to marine mammals, have been identified in the ringed seal (*Phoca hispida*), and seal genotype 2 in the harbor seal (*Phoca vitulina*) and grey seal (*Halichoerus grypus*) (Bogomolni et al., 2008; Dixon et al., 2008; Santin et al., 2005). More recently, novel genotypes of *Cryptosporidium* have been described in the southern elephant seal (*Mirounga leonina*), harp seal (*Pagophilus groenlandicus*) and Weddell seal (*Leptonychotes weddellii*) (Bass et al., 2012; Rengifo-Herrera et al., 2011; Rengifo-Herrera et al., 2013).

Of the six *Giardia* species, *Giardia duodenalis* has the broadest host range (c.f. Feng and Xiao, 2011). Molecular characterization of *G. duodenalis* has revealed significant genetic diversity, an indicator of complexity within this species. Accordingly, *G. duodenalis* is divided into assemblages A to H (c.f. Feng and Xiao, 2011), with assemblages A and B being the most diverse with at least four sub-assemblage types (I-IV) (c.f. Monis and Thompson, 2003). Assemblages A and B are human infective and have also been documented in wildlife and domestic animals (c.f. Feng and Xiao, 2011).

Assemblages C and D are found in dogs, assemblages E, F and G in domestic ruminants, cats and rodents respectively, while assemblage H has been described in seals (c.f. Cacciò et al., 2005; Lasek-Nesselquist et al., 2010).

Infections of *G. duodenalis* are reported with a greater frequency in marine mammals than *Cryptosporidium*. *Giardia* assemblages A and B are the most commonly identified assemblages in pinniped species (eg. Appelbee et al., 2005; Lasek-Nesselquist et al., 2008). Assemblages C and D have been described in harbor seals (*Phoca vitulina*) and the novel seal genotype H identified in grey seals (Gaydos and Miller, 2008; Lasek-Nesselquist et al., 2010). The presence of human host specific *Giardia* assemblages in marine mammals may be an indication of human impacts on the marine environment, which poses potential concerns for conservation of endangered pinniped species.

The Australian sea lion (*Neophoca cinerea*) is one of the rarest seals in the world, with a total population of less than 15,000 (Shaughnessy et al., 2011). Colonies of this endangered pinniped are distributed on coastal islands and the mainland of Western and South Australia, many within close proximity to human settlements (Goldsworthy and Gales, 2008; Goldsworthy et al., 2007). As a tourist icon, some South Australian colonies, in particular Seal Bay on Kangaroo Island, experience frequent human visitation and habitat disturbance. Interactions with, and proximity to, humans and wastewater run-off increases the likelihood of transmission of *Giardia* and *Cryptosporidium* from humans and domestic animals to seal populations. The aim of this study was to detect and characterize *Giardia* and *Cryptosporidium* in wild and captive Australian sea lions within a phylogenetic framework, and to determine if proximity to human settlements was related to parasite detection. We hypothesized that if transmitted through human influences, protozoal strains would be more likely to be detected in seal colonies in close proximity to

human settlements and would be similar to those found in domestic animals and human populations.

Materials and Methods

Sample collection

Fresh wild faecal samples (n= 271) were collected over a range of seasons during a 2 year period from coastal and island colonies in Western Australia (Figure 7A) and South Australia (Figure 7B and 7C). For captive animals, freshly passed faecal samples (n= 19) were collected from animal housing over a period of 4 months from the resident populations held at Dolphin Marine Magic and Taronga Zoo, New South Wales, and Sea World, Queensland. Faecal samples were transported to the laboratory and stored at 4° C until processing for genomic DNA extraction.

DNA extraction

Genomic DNA was extracted from sea lion faecal samples (n= 290) using the ISOLATE Fecal DNA Kit (Bioline, Sydney, Australia). Faecal samples (approximately ~150 mg) were aliquoted into lysis bead tubes and DNA extraction performed as per the manufacturers protocol. Eluted DNA was stored at -20° C until further analysis.

PCR screening for characterization of Giardia duodenalis

The presence of *Giardia* isolates was determined using the protocol targeting the 18S rRNA gene described in Hopkins et al. (1997) and Read et al. (2004).

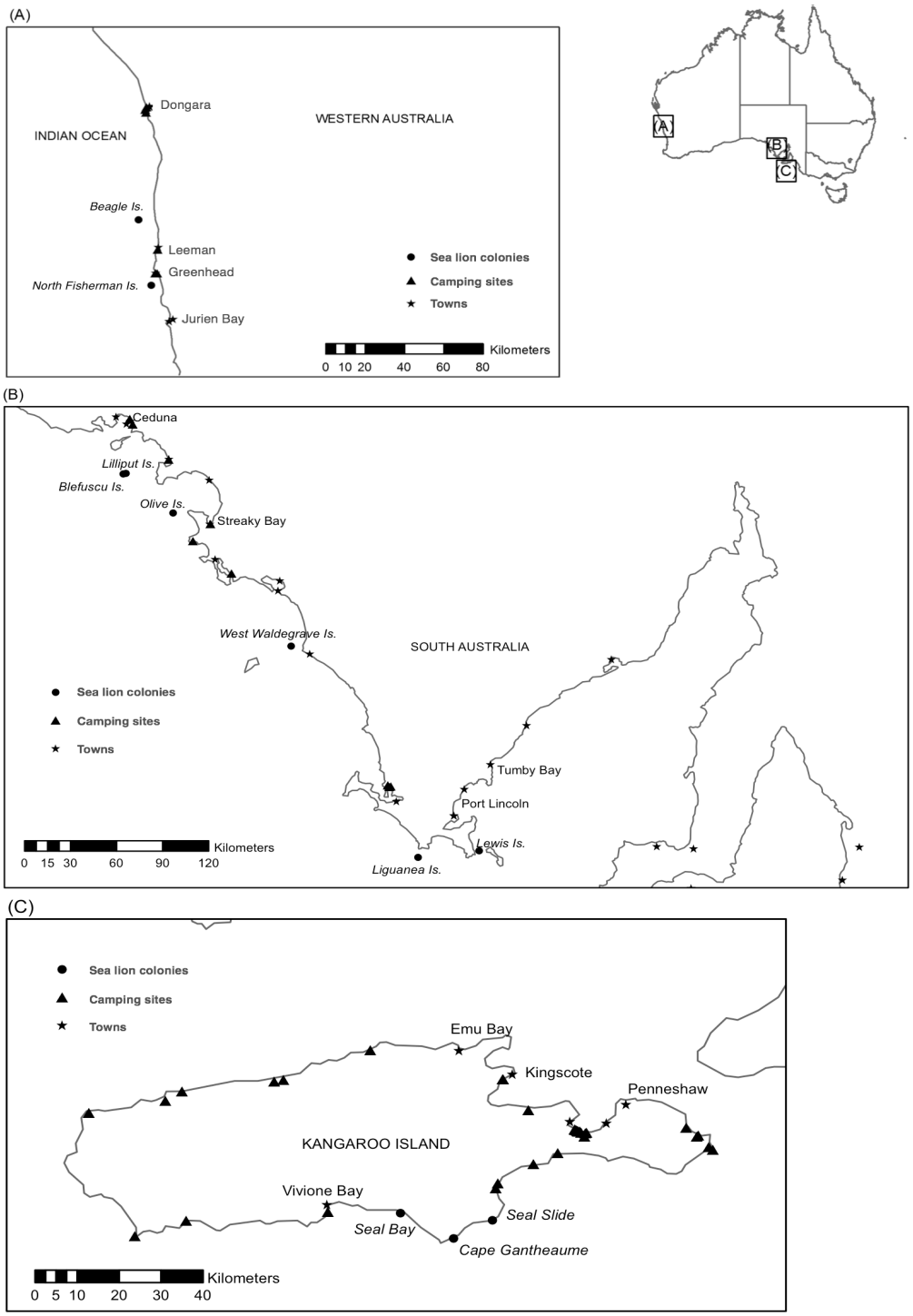

Figure 7. (A) Western Australia sampling locations. Faecal samples were collected from Beagle and North Fisherman Islands. Coastal settlements and human impacted camping locations within close proximity to sea lion colonies are indicated. **(B)** South Australia sampling locations. Faecal samples were collected from South Australia colonies; Blefuscu, Lewis, Liguanea, Lilliput, Olive and West Waldegrave Islands. **(C)** South Australia sampling locations: Kangaroo Island. Three colonies were sampled from Kangaroo Island including Cape Gantheaume, Seal Bay and Seal Slide.

Nested PCR using the primers RH11/RH4LM in the primary reaction and GiAR18SeR/GiAR18SiR in the secondary reaction were used to amplify a ~175 bp fragment of the 18S rRNA gene. Primary and secondary reactions (25 μL) were prepared using the GC-RICH PCR system (Roche Diagnostics, Indianapolis, IN) (Asher et al., 2012). Thermocycling for both the primary and secondary reactions were performed using the conditions described by Hopkins et al. (1997).

To characterize *Giardia* assemblages, 18S rRNA positive isolates (n= 35) were screened at the *gdh* and β-giardin loci. For *gdh* amplification the previously described semi-nested protocol of Read et al. (2004) was used. Primary and secondary reactions (25 μL) were prepared using the GC-RICH PCR system (Roche Diagnostics) and the primer set GdheF/GdhiR in the primary reaction and primers GdhiF/GdhiR in the secondary. Cycling was performed at denaturation for 2 min at 94° C, 1 min at 56° C, 2 min at 72° C; 35 cycles at 94° C for 30s, 56° C for 20s and 72° C for 45s; and a final extension step at 72° C for 7 min (Read et al., 2004). Where samples failed to produce an amplicon (n= 35), template DNA was increased from 1 to 2.5 μL in the primary reaction and secondary reactions prepared using identical PCR chemistry, with 2.5 μL of primary PCR product. Amplification of the β-giardin locus was achieved following the nested protocol described by Cacciò et al. (2002) and Lalle et al. (2005). Primary reactions (25 μL) were prepared using 1.5 mM MgCl2, 200 μM dNTPs, 200 nM of each primer G7/G759, 1U of DNA polymerase Tth Plus (Fisher Biotec, Wembley, Australia) and 2 μL template DNA and the cycling conditions used by Cacciò et al. (2002). Secondary reactions (25 μL) were prepared using PCR chemistry identical to the primary reactions, with 2 μL of primary PCR product and following the thermocycling conditions described by Lalle et al. (2005). PCR was performed with the internal primers described by Lalle et al. (2005) with a slight modification to the internal forward primer (GAA CGA GAT CGA GGT CCG) after β-

giardin sequence comparisons available on the NCBI GenBank database (http://www.ncbi.nlm.gov/genbank/index.html) showed a 4 bp difference between *Giardia* sequences and the internal forward primer sequence.

A spike analysis using DNA extracted from an existing *Giardia* laboratory trophozoite isolate was performed on 18S rRNA positive samples that failed to amplify at the *gdh* and β-giardin loci.

PCR screening for characterization of Cryptosporidium sp.

Screening for *Cryptosporidium* was conducted using a nested PCR protocol targeting the small subunit 18S rRNA (Xiao, 1999). RedHot Taq (Thermo Scientific, Scoresby, Australia) was used for reactions and all conditions were as described by Xiao et al. (2000). To confirm that the absence of *Cryptosporidium* was not the result of faecal inhibitors impairing DNA amplification, all samples (n= 290) were spiked with *Cryptosporidium parvum* DNA from an existing laboratory isolate (Waldron et al., 2011) and PCR screening repeated as described above.

All PCRs were performed in an Eppendorf Mastercycler (Eppendorf, North Ryde, Australia). PCR products (8 µL) were resolved by agarose gel electrophoresis (2% w/v, 110 V for 30 min) in TBE (Tris, boric acid, EDTA pH8) with 2 µL SYBR safe (Invitrogen, Mulgrave, Australia) using a HyperLadder II DNA marker (Bioline) to estimate amplicon size.

DNA sequencing and phylogenetic analyses

To identify *Giardia* assemblages the 18S rRNA products (n= 35) from GiAR18SeR/GiaR18SiR secondary reaction were purified for sequencing using the QIAquick PCR Purification Kit (Qiagen, Melbourne, Australia) and sequenced in the forward direction using the internal primer GiAR18SeR and in the reverse direction using GiAR18SiR. To identify *Giardia* sub-assemblage the β-giardin product (n= 1) was purified and sequenced in the forward and reverse direction using the secondary β-giardin PCR primers. All sequencing was performed by Macrogen Inc. (Seoul, Korea) on a 3130x1 genetic analyser (Applied Biosystems, Foster City, California) using the standard run protocol for a 50 cm, 16 capillary array using a Big Dye terminator kit (Applied Biosystems).

Forward and reverse sequences (18S rRNA and β-giardin) were checked manually and trimmed in GeneiousPRO version 5.0.3 (Biomatters Ltd, Auckland, New Zealand) and a single contiguous sequence (contig) was assembled for each sample. BlastN sequence searches were performed to assign contiguous 18S rRNA and β-giardin sequences to an assemblage. To allow for assemblage identification sequences were analyzed within a phylogenetic framework. Representative 18S rRNA sequences for *Giardia* assemblages A to G were obtained from the NCBI GenBank database using accession numbers AF199446, AF199447, AF199449, AF199443, AF199448, AF199444 and AF199450 for 18S rRNA analyses (Wielinga and Thompson, 2007). 18S rRNA and β-giardin sequences representing assemblage H were not available on GenBank and could not be included in analyses. Representative β-giardin sequences for *Giardia* assemblages AI-III to G were obtained from the NCBI GenBank database using accession numbers X85958, AY072724, FJ971410, AY072727, AY545646, AY545647, DQ116608, AY647264 and EU769221

(Kosuwin et al., 2010; Lalle et al., 2005; Lebbad et al., 2010; Wielinga and Thompson, 2007). Contiguous 18S rRNA and β-giardin sequences generated in this study were aligned to GenBank sequences using ClustalW (Thompson et al., 1994) in MEGA version 6.0 (Tamura et al., 2013). For phylogenetic analyses, nucleotide substitution models were tested for maximum likelihood in MEGA6 (Tamura et al., 2013). Akaike Information Criterion corrected (AICc) values were used to determine the optimal parameters. Phylogenetic trees were constructed for 18S rRNA and β-giardin sequences using maximum likelihood (Tamura 3-parameter distance model with the uniform distribution parameter) and bootstrap analysis (1000 replicates) and compared to existing assemblages (Kosuwin et al., 2010; Lalle et al., 2005; Lebbad et al., 2010; Wielinga and Thompson, 2007). 18S rRNA sequences generated in this study have been submitted to The European Nucleotide Archive (ENA) under the accession numbers LN610171-LN610198. The β-giardin sequence generated in this study has been submitted to GenBank under accession number KM497498.

Mapping and statistical analyses

Maps illustrating the locations of wild sea lion populations sampled and proximity of towns and camping grounds were developed using ArcGIS version 10.0 (ESRI, 2010).

A Pearson's χ^2 test was used to identify differences in the occurrence of *Giardia duodenalis* between wild and captive populations. For the wild populations only, a generalized linear model (GLM) with a binomial probability distribution was used to examine the effect of sea lion colony distance from human settlements and sampling season on presence/absence of *Giardia*. For this analysis, colonies were grouped into three distance-from-settlement categories: < 25 km, 26-69 km and > 70 km (Table 8).

Differences in occurrence between distance categories were determined using a Tukey's post-hoc test.

Results

Giardia detection and species identification

Screening of genomic DNA using a *Giardia* specific 18S rRNA protocol resulted in the detection of *Giardia* in 28 samples from wild sea lions (10.3%) and in seven samples from captive sea lions (36.8%). There was a significant difference in *Giardia* presence between wild and captive individuals (χ^2 = 11.758, df = 1, p= <0.001). In wild colonies, the distance from human settlement had a significant effect on the presence or absence of *Giardia* (Wald χ^2 = 39.078, df = 2, p= <0.001). Colonies less than 25 km from human settlements had a higher occurrence of *Giardia* than colonies more than 26 km away (Table 8). There was no effect of sampling season on *Giardia* presence (Wald χ^2 = 6.112, df = 3, p= 0.106).

DNA sequences were obtained for 28 of the 35 18S rRNA positive samples. BlastN search identified 27 sequences as belonging to *Giardia duodenalis* assemblage B and one belonging to assemblage A. Analysis using the phylogenetic framework clustered all samples from wild sea lions (n= 24) and three samples from captive animals within a clade that also contained the assemblage B reference sequence from GenBank (Figure 8). One captive sample clustered within a clade containing the reference sequences from GenBank for assemblages A, E and F. Analysis of clustalW alignment showed a 2 bp polymorphism between the assemblages with the captive sample most closely aligned to assemblage A.

Alignment of the sample to representative sequences showed that the sample was 100% identical to assemblage A but not E or F.

Table 8. Australian sea lion colony groupings and analysis of *G. duodenalis* presence. Wild sea lion colony distance-from-settlement categories. Differences in occurrence between distance categories were determined using a Tukey's post-hoc test.

Distance category (km)	Colonies in category	Mean occurrence of *G. duodenalis* (%)	Total number of samples
< 25	Beagle Island Cape Gantheaume North Fisherman Island Seal Bay Seal Slide	23.8[a]	80
26-69	Blefuscu Island Lewis Island Liguanea Island Lilliput Island	5.8[b]	120
>70	Olive Island West Waldegrave Island	2.8[b]	71

[a, b] Significant difference in *Giardia duodenalis* presence between groupings.

18S rRNA positive samples (n= 35) failed to amplify at the *gdh* locus. Representative samples all produced a *gdh* amplicon when spiked with *Giardia* isolate DNA. The β-giardin locus amplified in one of 35 18S rRNA positive samples identified as assemblage B. A DNA sequence was obtained for the β-giardin positive sample and a BlastN search identified the sequence as belonging to *Giardia duodenalis* assemblage AI. The inferred phylogeny placed the sample within a clade that also contained the assemblage AI reference sequence from GenBank. All samples spiked with *Giardia* positive DNA produced an amplicon when screened using β-giardin PCR.

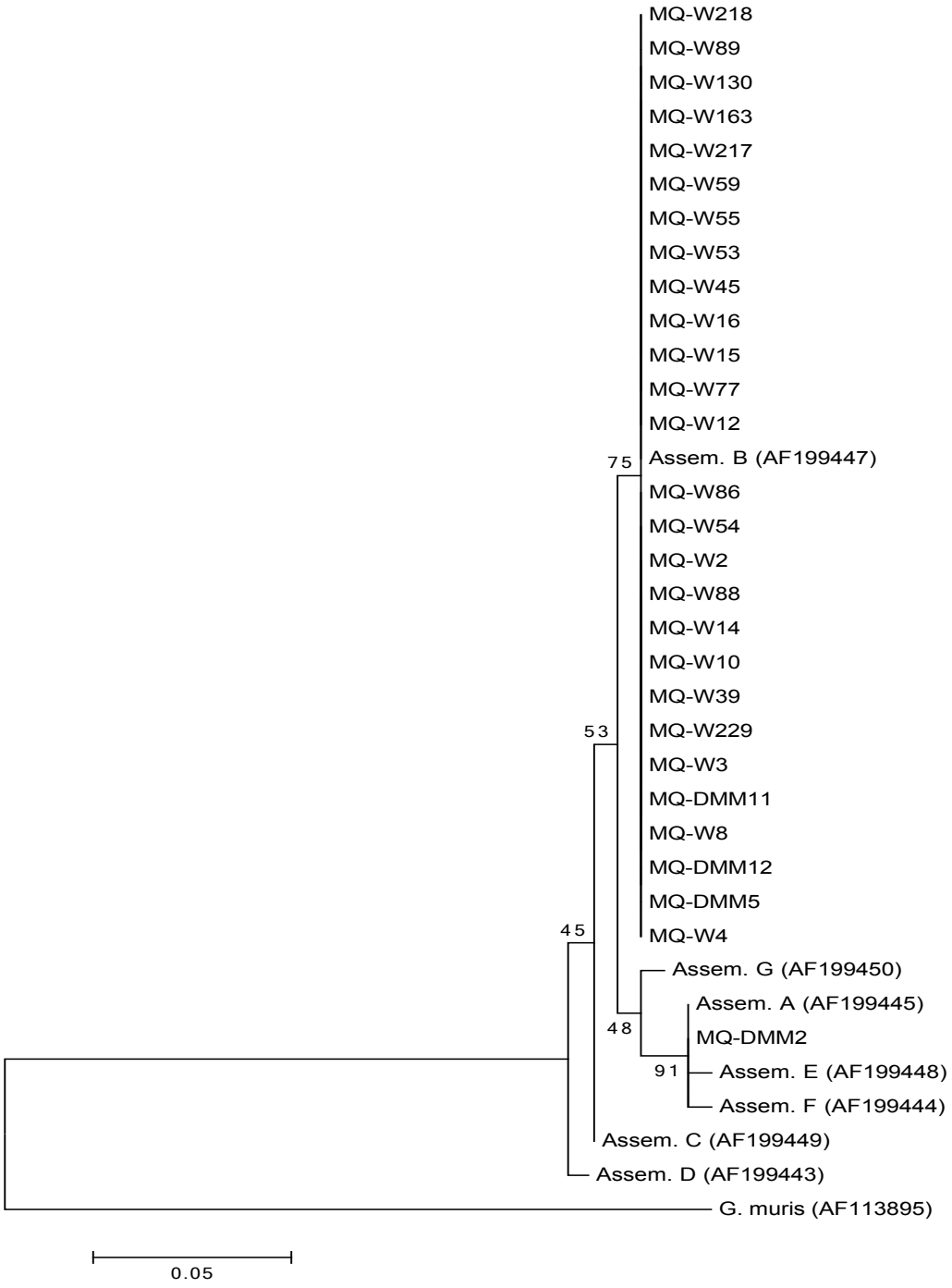

Figure 8. *Giardia duodenalis* 18S rRNA phylogenetic tree.

Phylogenetic analysis of *Giardia duodenalis* positive samples was performed using a fragment of the 18S rRNA gene. Analysis within the phylogenetic framework placed sea lion samples within the assemblage B (n= 27) and assemblage A clades (n= 1). Branch values indicate percent bootstrapping using 1,000 replicates.

Cryptosporidium screening

Cryptosporidium was not detected in any of the faecal samples (n= 290). The purified genomic DNA were deemed PCR competent using DNA spike analysis with all 290 samples generating an amplicon when screened using 18S rRNA PCR.

Discussion

In this study we examined the occurrence of *Giardia* and *Cryptosporidium* in the endangered Australian sea lion. *Giardia duodenalis* assemblage B, commonly found in humans and terrestrial mammals, was detected in wild and captive sea lion populations and *G. duodenalis* assemblage A was detected in a captive animal. Screening for *Cryptosporidium* failed to identify this parasite in any of the samples. Infections of *Cryptosporidium* are commonly reported at lower frequencies than *Giardia* in marine mammal populations (eg. Appelbee et al., 2005; Gaydos and Miller, 2008; Hueffer et al., 2011; Lasek-Nesselquist et al., 2008). Of the eight *Giardia duodenalis* assemblages A and B are the most commonly identified in wild seal and sea lion populations (eg. Appelbee et al., 2010; Lasek-Nesselquist et al., 2010). Our findings indicate that assemblage B is the most common assemblage detected in wild and captive sea lions while assemblage A occurs at low frequency. The host range of assemblages A and B are broad including domestic animals, livestock and humans (c.f. Monis et al., 1999; Monis and Thompson, 2003). Infections with assemblages A and B are very common in human cases but based on the absence of subtype characterization, it is difficult to assess the association between parasite transmission and humans.

The presence of *G. duodenalis* assemblages AI and B in this sea lion species is a strong indicator of the spread of parasites from terrestrial mammals to the marine environment. *Giardia duodenalis* is reported in higher frequencies in seal and sea lion species distributed within close proximity to human settlements and wastewater run-off localities (Appelbee et al., 2010; Gaydos and Miller, 2008). The presence of *G. duodenalis* in seals visiting haul-out sites distributed near coastal settlements can be up to 5 times greater than individuals at more sparsely populated sites with limited human exposure (Dixon et al., 2008; Hughes-Hanks et al., 2005; Lasek-Nesselquist et al., 2010). Some Australian sea lion colonies are within close proximity to coastal settlements and experience high levels of human interaction as a major tourist icon (Gales et al., 1994; Rodger et al., 2011). Sea lion behaviour such as hauling-out on human impacted beaches increases the potential for exposure to parasites from terrestrial sources. Compared with the more isolated Australian sea lion colonies (> 70 km from human settlement), *Giardia* presence is significantly higher in colonies nearer (< 25 km) to human coastal settlements and those colonies that experience high human visitation. Australian sea lions have limited dispersal and a high degree of philopatry so impacts are likely to be localized (Lowther et al., 2012). Future observation of protozoan prevalence in South Australian (Seal Bay and Seal Slide on Kangaroo Island) and Western Australian colonies (Shoalwater Marine Park, Perth; North Fisherman Island, Jurien Bay and Recherche Archipelago, Esperance) is therefore essential for monitoring the spread of parasites and associated potential disease risks, and will assist in the development of conservation management strategies.

Giardia duodenalis presence is significantly greater in captive Australian sea lions (36.8%) than wild animals (10.3%) indicating that occurrence may be the result of atypical habitat interactions. Exposure to humans and interactions atypical to those within the natural habitat of sea lions may increase the risk of *Giardia* transmission within captive

environments (Beck et al., 2011). Captive mammals may be exposed to *Giardia* through human contact during hand feeding and touching by zoo visitors (Thompson et al., 2008). The captive facilities observed in this study have varying levels of visitor interaction programs with sea lions, some even include activities such as swimming with and touching the animals. While *Giardia* presence in captive marine mammal populations is rarely observed or indeed investigated, screening of *Giardia* in other captive mammal species with similar levels of human interactions would provide an indication of the extent of transmission in the captive environment. This in turn may reveal potential avenues of dispersal of *Giardia* in the captive environment, and by deduction, potential mitigation strategies for improved husbandry.

While the use of molecular tools has facilitated a greater understanding of protozoan origins and host specificity, we had limited success in accurately confirming *Giardia* species sub-assemblage across multiple loci. This poses significant biological implications for inferring host specificity and transmission of *Giardia*. We failed to amplify 18S rRNA positive isolates at the *gdh* and β-giardin loci. Difficulty in confirming positive 18S rRNA detection at the *gdh* locus has been observed in other marine and captive mammal studies. Failure to amplify at the *gdh* locus in samples from Pacific harbor seals and captive mammals was attributed to variation in sequences and failure of primers to anneal (Beck et al., 2011; Lasek-Nesselquist et al., 2010). While analyses at the 18S rRNA locus alone can enable assemblage identification, multilocus gene screening is required to determine *G. duodenalis* sub-assemblage and specific host origin.

Further, we were unable to consistently assign *G. duodenalis* assemblage across multiple loci for the one sample that amplified at β-giardin. Inconsistent assemblage identification across multiple loci has been observed in other marine mammal studies (Lasek-Nesselquist

et al., 2008; Lasek-Nesselquist et al., 2010). Failure to confirm genotype across multiple loci in samples from grey and Pacific harbor seals was attributed to target gene amplification biases, where assemblages A and B preferentially amplified at different loci, and the presence of mixed assemblage infection (Lasek-Nesselquist et al., 2010). Mixed infection of *G. duodenalis* assemblages A and B are commonly reported in human and marine mammal studies, although there is much debate about whether this is the result of infection by multiple isolates or the haplotype of a single isolate (Cacciò and Ryan 2008; Cacciò et al., 2008; Lasek-Nesselquist et al., 2008; Lasek-Nesselquist et al., 2010). While the occurrence of recombination between *G. duodenalis* assemblages A and B has been supported by several studies, the mechanisms involved remain unclear (Cooper et al., 2007; Cacciò and Sprong, 2010; Teodorvic et al., 2007). Consequently, due to limited amplification across multiple loci, we were unable to draw inferences on the potential for target gene amplification biases or the presence of mixed infection. These findings emphasize the need for multilocus molecular characterization to definitively assign *G. duodenalis* assemblages present in wild sea lion populations and determine the origin of parasite dispersal into the marine environment.

Increasing exposure to agricultural run-off and untreated wastewater represents new challenges for managing the dispersal of protozoan parasites into the marine ecosystem. The high occurrence of *Giardia* and similarity to *Giardia* species found in humans in both wild and captive sea lions warrants the need for further molecular investigation to identify the dispersal routes of parasites from terrestrial ecosystems into marine vertebrate populations.

Acknowledgements

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Chapter 3

Title

Molecular detection of antibiotic resistance determinants in *Escherichia coli* isolated from the endangered Australian sea lion *Neophoca cinerea*.

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Abstract

Greater interaction between humans and wildlife populations poses significant risks of anthropogenic impact to natural ecosystems, especially the marine environment. Understanding the spread of microorganisms at the marine interface is therefore important if we are to mitigate any adverse effects on marine wildlife. Here we investigate the establishment of *Escherichia coli* (*E. coli*) in the endangered Australian sea lion (*Neophoca cinerea*), by comparing faecal isolation from wild and captive sea lion populations. Faecal samples were collected from wild colonies March 2009 - September 2010 and from individuals housed in captive facilities March 2011 - May 2013. Molecular screening was undertaken to assign a phylotype to *E. coli* isolates and determine the presence of integrons, mobile genetic elements able to capture gene cassettes conferring resistance to antimicrobial agents common in faecal coliforms. Phylotyping identified group B2 as the most abundant phylotype in all *E. coli* isolates (n= 37) with groups A, B1 and D also identified. Integrons were not observed in *E. coli* (n= 21) isolated from wild sea lions but identified in *E. coli* from captive animals (n= 16) where class I integrases were detected in eight isolates. Sequencing of gene cassette arrays indicated the presence of genes conferring resistance to streptomycin-spectinomycin (*aadA1)* and trimethoprim (*dfrA17, dfrB4*). Class II integrases were not detected in the *E. coli* isolates. The frequent detection of *E. coli* with resistance genes commonly identified in human clinical cases in captive sea lions suggest that conditions experienced in captivity may contribute to establishment. Identification of antibiotic resistance in the microbiota of Australian sea lions provides crucial information for disease management. Our data will inform conservation management strategies and provide a mechanism to monitor microorganism dissemination to sensitive pinniped populations.

Introduction

Interactions at the human: domestic animal: wildlife interface have facilitated the movement of microorganisms from terrestrial sources to marine ecosystems (Halpern et al., 2008). In particular, the occurrence of antibiotic resistant bacteria is becoming more commonly observed in marine wildlife populations (Rose et al., 2009; Stoddard et al., 2008) indicating either colonization by terrestrial bacteria or integration of antibiotic resistance determinants within the microbiota of wildlife species.

The mobility of DNA elements encoding antibiotic resistance has been instrumental in the rapid spread of antibiotic resistance, both in a clinical setting and in naturally occurring ecosystems (Partridge et al., 2009). Mobile DNA elements termed integrons have been particularly important in the emergence of antibiotic resistance. Integrons have the ability to encode for integrase genes (*int*) that enable insertion of gene cassettes at a recombination site (*attI*) and subsequent expression at an adjacent promoter (P_c) (Collis et al., 1998; Hall and Collis, 1995). Integrons can be classified into three classes (I, II, and III) with class I being most common in clinical pathogens (Stokes and Hall, 1989). Class I integrons integrate within transposons or plasmids, a feature which has further facilitated their spread within and between species of bacteria (Gillings et al., 2008).

The presence of antimicrobial resistance determinants in coastal seawater and wastewater run-off is well documented (eg. Reinthaler et al., 2003; Schwartz et al., 2003). Exposure of wildlife species to untreated wastewater may present a pathway for transfer of human-derived bacteria, and the antibiotic resistance genes they carry, to wildlife populations (Pellegrini et al., 2009). Ultimately this may lead to colonization by

microorganisms atypical of the natural habitats of wildlife species (Power et al., 2013). Intriguingly, class I integrons reported in clinical pathogens are commonly identified in aquatic vertebrates including gulls, flamingo, carp, salmon, and catfish, all animals frequently exposed to anthropogenic impact on natural habitats (eg. Dolejska et al., 2009; McIntosh et al., 2008).

Culture-based screening and antibiotic sensitivity testing has identified resistance to greater than 10 antibiotics across six seal species (eg. Lockwood et al., 2006; Rose et al., 2009). However, these techniques are unable to identify specific resistance determinants and their origins and this knowledge gap limits our understanding the dispersal of antibiotic resistance in wild marine populations (Stokes et al., 2001).

The Australian sea lion (*Neophoca cinerea*) is an endangered marine mammal endemic to Australia. The total population is estimated at <15,000 animals, with the only site monitored in the long-term, Kangaroo Island, showing continual decline (McIntosh et al., 2012). Australian sea lions breed in at least 76 small, dispersed colonies on islands and some protected coves from Houtman Abrolhos in Western Australia (WA) to The Pages in South Australia (SA) (Shaughnessy et al., 2011). Their geographic range stretches across in excess of 2,700 km of coastline and encompasses two major cities of > 1 million people (Perth, WA and Adelaide, SA) and a number of large rural centres, as well as some very isolated sites. Although their distribution is broad, individual colonies show limited dispersal, localized foraging by both males and females and a high degree of population genetic structure (Lowther et al., 2012; Lowther et al., 2013b). Sea lions are indirectly exposed to terrestrial microorganisms through the ingestion of seawater contaminated with wastewater run-off, and via behaviours such as hauling-out on beaches utilized by humans (Gales et al., 1994). In addition, as a tourist icon, sea lion colonies in both WA and SA

receive high levels of human visitation, with visitor numbers to Seal Bay on Kangaroo Island exceeding 150,000 tourists annually (Goldsworthy et al., 2007). Such interactions significantly increase the risk of introducing atypical microbes to vulnerable wildlife populations (Daszak et al., 2000; Skurnik et al., 2006). We hypothesized that sea lions in captive environments and wild colonies exposed to increased anthropogenic impacts may have higher levels of *E. coli* carrying class I or class II integrons than isolated sea lion populations with limited disturbance. We screened isolates of *E. coli* from different sea lion populations using PCR for the presence of class I and class II integrons. Further, we examine the relationship between *E. coli* presence and phylotype distribution.

Materials and Methods

Faecal sample collection

Fresh Australian sea lion faecal samples (n= 271) were opportunistically collected over a 2 year period from 11 coastal and island colonies in South Australia and Western Australia (Table 9) taking note of wetness and sheen of samples. Recently passed faecal samples were collected from captive animals (n= 19) held at Dolphin Marine Magic and Taronga Zoo, NSW and Sea World, Queensland over a period of 4 months. Faecal samples were transported to the laboratory and stored at 4° C until processing.

Enrichment for E. coli

To obtain pure cultures of *E. coli* Chromocult agar plates (Merck, Darmstadt, England) were streak-inoculated with faeces using sterile swap applicators and incubated

at 37°C for 24 hrs (Finney et al., 2003). Following the absence of *E. coli* isolation from agar plates, MacConkey enrichment broth (2 mL) (Oxoid, Hamshire, England) was inoculated with faecal material (approximately 500 mg) and broth cultures incubated at 37° C for 24 hrs. Enriched broth cultures were then used to streak-inoculate Chromocult agar plates. *E. coli* positive colonies as indicated by dark-blue to violet growth were selected to establish pure cultures.

DNA extraction and PCR protocols

Genomic DNA was extracted from *E. coli* cultures using the Isolate Fecal DNA Kit (Bioline, Sydney, Australia) according to manufacturers protocol. PCR competency of extracted DNA was determined by 16S rRNA amplification using the universal eubacterial primers F27 and R1492 (Yeates and Gillings, 1998). Reactions were performed using the DNA polymerase *GoTaq® Green 2X Master Mix* (Promega, Madison, USA).

To assign *E. coli* isolates to a phylotype, DNA extracted from *E. coli* cultures (n= 37) were amplified according to Clermont et al. (2000) using *GoTaq® Green Master Mix* (Promega, Madison, USA). Phylotype groups were determined by the presence and absence of fragments associated with each of the ChuA (279 bp), YjaA (211 bp) and TSP (152 bp) primer pairs using gel electrophoresis (2 % *w/v*, 110 V for 30 min) in TBE (Tris, boric acid, EDTA pH8) with SYBR safe (Invitrogen, Mulgrave, Australia). Product size was approximated against a HyperLadderII DNA marker (Bioline, Sydney, Australia).

Positive controls representing *E. coli* phylotypes A, B1, B2 and D were used in all reactions (Power et al., 2005).

To screen for the presence of class I integrons an integrase (*intI1*) specific PCR was performed using the primers HS463a and HS464 (Gillings et al., 2008). HS463a and HS464 amplify an internal fragment of the class I integron integrase (*intI1*) gene producing a ~473 bp product. DNA polymerase *GoTaq® Colourless Master Mix* (Promega, Madison, USA) was used for amplification and cycling comprised initial denaturation for 3 min at 94° C; 35 cycles at 94° C for 30s, 60° C for 30s and 72° C for 1 min 30s; and a final extension step at 72° C for 5 min. A spiked analysis using DNA extracted from a class I integron positive laboratory strain was performed on negative samples.

Samples producing *intI1* amplicons were further amplified to determine gene cassette arrays using the primers HS458 and HS459 which amplify from the *attI1* region and the 3' conserved segment, a region spanning the gene cassette array (Stokes et al., 2006). PCR cycling conditions included initial denaturation at 94° C for 3 min; 35 cycles at 94° C for 30s, 60° C for 30s and 72° C for 1 min 30s; and a final extension step 72° C for 5 min. Amplicons will vary in size, depending on the number and types of gene cassettes that are present. *intI1* positive samples that failed to amplify using HS458 and HS459 were screened using the primers MRG284 and MRG285 (Gillings et al., 2009) using PCR conditions as described for HS458 and HS459 primers except for the annealing temperature and time which were increased to 65° C for 2 min.

To screen for class II integrons the integrase specific PCR for integrase 2 (*intI2*) was performed using the primers IntI2F and IntI2R (Mazel et al., 2000). IntI2F and IntI2R amplify an internal fragment of the class II integron integrase (*intI2*) gene. PCR was performed using *GoTaq® Colourless Master Mix* (Promega, Madison, USA) and PCR cycling performed as described above for *intI1* amplification. All reactions were resolved using agarose gel electrophoresis as described above.

DNA sequencing and analyses

Amplicons from HS463a/HS464 and HS458/HS459 were purified for sequencing using the QIAquick PCR Purification Kit (Qiagen, Melbourne, Australia). For cassette array determination overlapping sequence fragments were obtained using the primers HS458, HS459 and HS320 (Murray et al., 1988). All sequencing was performed by Macrogen Inc. (Seoul, Korea) on a 3130x1 genetic analyser (Applied Biosystems, Foster City, California) using the standard run protocol for a 50 cm, 16 capillary array using a Big Dye terminator kit (Applied Biosystems). Sequences generated from HS458, HS320 and HS459 were assembled using GeneiousPRO version 5.4.6 (Biomatters Ltd, Auckland, New Zealand) and a consensus sequence extracted. Sequences were annotated by hand after performing BlastN and BlastX searches (http://www.ncbi.nlm.nih.gov/BLAST) and open reading frames identified using BlastX comparisons, and the core sequences (GTTRRRY) and recombination site (*attC*) used to identify gene cassette arrays. GenBank flat files were generated for submission to GenBank using BankIt. Sequences generated from this study are lodged as GenBank accession numbers KP314737 - KP314740.

Statistical analyses

Fisher's exact test was used to test for differences between wild and captive populations in the prevalence and phylotype distribution of *E. coli* isolates, using the SISA Fisher's exact test calculator for up to 2×5 contingency tables (http://www.quantitativeskills.com/sisa/statistics/five2hlp.htm).

Results

E. coli culture and phylotyping

Isolation of *E. coli* using coliform selective media resulted in low yields, with isolation from 21 wild animal faecal samples (7.7%) compared to 16 captive individuals (84%) (Table 9). The yield of *E. coli* from captive and wild animals was significantly different (Fisher's exact test, two-sided p= <0.001). A low recovery of *E. coli* was reflected in initial direct streak plate isolation however subsequent enrichment using MacConkey broth and Chromocult agar increased coliform recovery rates by 34%.

In both captive and wild sea lion populations, the four phylotypes of *E. coli* isolates were not evenly distributed. The phylotype group distribution pattern was not significantly different between the two populations (Fisher's exact test, two-sided p= 0.590). In wild animal faecal samples, phylotype B2 was the most common (67% of all phylotypes isolated) (Table 9), followed by phylotype B1 and D (14% each), with phylotype A represented by a single *E. coli* isolate (4.8%). Phylotype frequency was similar for captive animal faecal samples with phylotype B2 represented by 9 isolates (56%), phyloptype A by 3 isolates (19%) and phylotypes B1 and D represented by 2 isolates (13% each).

Detection and characterization of class I and class II integrons

Genomic DNA was successfully extracted from the 37 *E. coli* isolates and DNA from all isolates was deemed PCR competent by 16S rRNA screening. Class I integrase screening (*intI1*) of DNA samples resulted in no amplicons in *E. coli* DNA from wild animals (n= 21). *intI1* amplicons were detected in 8 captive animal samples (Table 9).

Table 9. *E. coli* isolate yields and phylogenetic groupings from Australian sea lion colonies and captive facilities.

Colony	Geographic co-ordinates [a]	Sample size (no. samples)	*E. coli* isolates (no. cultured)	*E. coli* phylotype[b]	Gene cassette array
South Australia					
Blefuscu Island	32° 28' 15" S, 133° 37' 39" E	37	6	*A* (1) *B2* (5)	
Cape Gantheaume	36° 00' 37" S, 137° 28' 28" E	7	-	-	
Lewis Island	34° 57' 25" S, 136° 01' 54" E	21	1	*B2* (1)	
Liguanea Island	34° 59' 54" S, 135° 37' 11" E	24	-	-	
Lilliput Island	32° 28' 01" S, 133° 38' 38" E	38	3	*B2* (2) *D* (1)	
Olive Island	32° 43' 25" S, 133° 57' 53" E	62	5	*B1* (2) *B2* (2) *D* (1)	
Seal Bay	35° 59' 55" S, 137° 21' 45" E	38	3	*B1* (1) *B2* (2)	
Seal Slide	36° 03' 43" S, 137° 29' 36" E	5	1	*B2* (1)	
West Waldegrave Island	33° 35' 46" S, 134° 45' 41" E	9	-	-	
Western Australia					
Beagle Island	29° 48' 23" S, 114° 52' 37" E	15	1	*B2* (1)	
North Fisherman Island	30° 07' 47" S, 114° 56' 38" E	15	1	*D* (1)	
Captive					
Dolphin Marine Magic		12	10	*A* (2) *B2* (7) *D* (1)	 |*aadA1*|*qacEΔ*| (1) |*dfrB4*|*qacEΔ*| (2)
Sea World		5	3	*A* (1) *B1* (2) *B2* (1)	 *aadA*|*dfrA17* (1) |*qacEΔ*| (1)
Taronga Zoo		2	2	*B2* (1) *D* (1)	

[a] Geographic coordinates determined using Google Earth satellite mapping database (2010).
[b] Phylogenetic groups were determined by multilocus enzyme electrophoresis.

Class II integrons were not detected in DNA samples from wild or captive sea lion populations. All DNA samples tested positive when spiked for class I and class II integron screening, confirming PCR competency for these targets. DNA sequencing and BlastN searches confirmed that *intI1* amplicons (n= 8) represented class I integrase. Gene cassette arrays amplified in 7 of the 8 *intI1* positive isolates. Successful DNA sequencing identified 4 constructs; |*aadA1*|*qacEΔ*| in 1 sample (MQ-DMM3), |*dfrB4*|*qacEΔ*| in 2 samples (MQ-DMM5 and MQ-DMM10) and an empty cassette |*qacEΔ*| in one sample (MQ-S5). A mixed cassette array containing partial *dfrA17* and *aadA* genes was identified in one sample (MQ-S4). The *aadA1*, *dfrB4* and empty gene cassette constructs were identified in *E. coli* strains phylotyped B2, while the mixed gene cassette array was identified in a strain phylotyped as B1.

Discussion

In this study we examined the occurrence of *Escherichia coli* harbouring antimicrobial resistance genes in the endangered Australian sea lion. While isolation of *E. coli* from wild sea lion populations was limited, *E. coli* isolates were frequently identified in captive sea lions. *E. coli* isolation was significantly lower in wild Australian sea lions (7.7%) compared to captive animals (84%) suggesting that *E. coli* may not be a dominant member of natural gut microbial communities. The low occurrence of *E. coli* in wild Australian sea lions is similar to findings from other pinniped populations including harbor seals (*Phoca vitulina*), California sea lions (*Zalophus californianus*) and northern elephant seals (*Mirounga angustirostris*) where *E. coli* isolates were undetected in isolated faecal coliforms (Johnson et al., 1998). In contrast, *E. coli* was frequently identified in captive Australian sea lions indicating that the increased presence may be influenced by conditions

experienced in captivity. Dissimilarities in gut microbiota abundance and richness between wild and captive animals have been observed in leopard seals (*Hydrurga leptonyx*) where variation was attributed to dietary changes experienced in captivity (Nelson et al., 2013). In captivity, Australian sea lions are fed fish with a limited range in terms of both species and size that have been processed through fish markets, while wild sea lions have a broad diet primarily benthic prey including octopus, cuttlefish, squid and lobster from inshore to the shelf edge (Gibbs et al., 2011; McIntosh et al., 2007). The introduction of microbes from unnatural prey may be a contributing factor to variations in microbial diversity observed in captivity. However, as the core gut microbial community structure of the Australian sea lion remains undefined, further molecular characterization is required to draw inferences on the impact of captivity on microbial prevalence.

Identification of *E. coli* phylotypes prominent in Australian sea lions has revealed the potential for *E. coli* harboring antibiotic resistance determinants to penetrate the animals gut biota. Of the four *E. coli* phylotype groups (A, B1, B2, D), phylotype B2 has the highest presence of extra-intestinal virulence factors and has been suggested to have the greatest resistance to antibiotics (eg. Johnson et al., 2001; Skurnik et al., 2005). Typically phylotype B2 is dominant in *E. coli* strains isolated from human and omnivorous terrestrial Australian mammals, while B1 is the most abundant phylotype in carnivorous species (Escobar-Páramo et al., 2006; Gordon and Cowling, 2003). In the case of the Australian sea lion, B2 is the most abundant phylotype representing 67% of isolates from wild populations and 56% in captive individuals. The dominance of phylotype B2 may increase the risk of transfer of antibiotic resistance genes from the surrounding environment to endangered species and warrants further investigation. Further genetic characterization assessing B2 phylotype strain variation and virulence carriage in sea lion

isolates would provide insight into the potential for establishment of antimicrobial resistance genes in wild animals.

Class I integrons containing diverse gene cassettes were detected in *E. coli* strains identified as B1 and B2 from captive sea lions. Class II integrons were not detected in either wild or captive populations. One of the genes present in the class I integrons (*aadA1*) is commonly found in both environmental and human clinical strains and encodes resistance to streptomycin-spectinomycin (Partridge et al., 2009). The second and third genes (*dfrA17* and *dfrB4*) identified in *E. coli* from captive seals confer resistance to trimethoprim (Partridge et al., 2009). The presence of class I integrons with gene cassette arrays similar to those commonly found in human clinical cases provide a useful indicator of potential microbial flow through the captive environment. Here we only found integrons in captive animals, indicating that the presence of resistance genes in *E. coli* from captive sea lions may result from conditions experienced in captivity and the dissemination of human-derived microbes at this interface. Similar findings have been reported in northern elephant seals (*Mirounga angustirostris*) introduced to rehabilitation centres where time elapsed in captivity and veterinary treatment significantly increased resistance profiles of gastrointestinal *E. coli* (Stoddard et al., 2009). These findings suggest that exposure to captive environments plays a major role in the establishment of antimicrobial resistance genes in captive animals (Sidjabat et al., 2006; Skurnik et al., 2006). Future observation including molecular characterization of samples from the animal housing and enclosure water may provide useful insight into factors driving selection of antibiotic resistance genes in gut microbiota.

The use of antimicrobial agents in companion animal and wildlife veterinary care may also contribute to increasing the proportion of enteric bacteria harbouring antibiotic

resistance determinants (Sidjabat et al., 2006; Smith et al., 2002) presenting two specific issues. First, the persistent use of antibiotics may limit the ability to treat bacterial infection and increase the incidence of infectious disease in marine mammals (Allen et al., 2010; McEwen and Fedorka-Cray, 2002). Secondly, selection for antibiotic resistant bacteria provides a reservoir of resistance that may potentially be transferred to microbiota of other captive animals (Stoddard et al., 2009). Greater understanding of microbial flow through captive environments is therefore essential for determining the potential for antibiotic resistance gene dissemination in wildlife species.

While resistance determinants were not detected in wild populations, the potential risk for antimicrobial movement to sea lion colonies exists and is clearly possible given our findings in captive sea lions. Although most Australian sea lion colonies are found in isolated areas or on coastal islands where unlicensed visits are prohibited, some South Australian colonies, in particular Seal Bay, Kangaroo Island, experience high levels of human tourist activity and visitation (Gales et al., 1994; Shaughnessy, 1999). Several haul-outs (non breeding sites) in WA are also subject to very high levels of tourist visitation (Orsini et al., 2006). The high frequency of such interactions within the natural habitat of sea lions increases the potential risk for exposure to foreign microorganisms and consequent colonization in sea lion coliforms (Allen et al., 2010; Skurnik et al., 2006).

The human: domestic animal: wildlife interface represents an area of emerging disease, zoonoses, and public health concern. For endangered endemics such as the Australian sea lion, understanding the ecology of their microbiota will provide insight into microbial dissemination routes. Given the vulnerability of pinnipeds and other colonially breeding animals to high rates of disease transfer, this is an area of research requiring further pursuit (Härkönen et al., 2006; Lynch et al., 2011). If we are to mitigate the effects

of adverse microbial transfer, we must first identify mechanisms of dispersal of atypical microbes, the methods outlined in this paper are a first step along this path.

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Chapter 4

Title

Comparison of the gut microbial communities of wild and captive Australian sea lions (*Neophoca cinerea*).

Authors

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Abstract

Marine mammals harbour diverse gut microbial communities. Gut microbiota play an important role in the maintenance of mammalian metabolism and immune system regulation and disturbance to this community can have adverse impacts on animal health. To better understand the composition of microorganisms contributing to the health of an endangered marine mammal, the Australian sea lion (*Neophoca cinerea*), faecal bacterial communities of sea lions from 11 wild colonies in Southern and Western Australia and three Australian captive populations were surveyed and compared. Sea lion gut microbial communities comprised five dominant bacterial phyla including *Firmicutes*, *Proteobacteria*, *Bacteroidetes*, *Actinobacteria* and *Fusobacteria*. The phylum *Firmicutes* was dominant in both wild (76.4±4.73%) and captive animals (61.4±10.8%) while *Proteobacteria* contributed more to captive (29.3±11.5%) than wild gut communities (10.6±3.43%). The gut microbiota of wild and captive sea lions differed in overall community membership and community structure, with wild animals showing a greater diversity of taxa (H= 2.98, E_H= 0.629) compared to captive animals (H= 2.89, E_H= 0.688). Three colonies from South Australia and one from West Australia possessed gut microbial communities whose composition was dissimilar to other wild colonies. Differences in the biological environment and foraging site fidelity may provide varying opportunity for unique microbial establishment in the gut and drive colony dissimilarity. As anthropogenic disturbance to marine mammals is likely to increase, understanding the potential for such disturbances to impact gut microbial community composition, and subsequently impact animal health, may aid management of these vulnerable species.

Introduction

The mammalian gastrointestinal tract is home to a diverse array of microbial species. Microbial gut communities have co-evolved with mammalian hosts to form mutualistic relationships beneficial to animal health (Bäckhed et al., 2005; Ley et al., 2008). Gut microbiota play a vital role in daily regulatory functions of the host including; the maintenance of metabolic processes, immune defense and intestinal tissue maturation and health (Stappenbeck et al., 2002; Tremaroli and Bäckhed, 2012; Wikoff et al., 2009). Complex microbial interactions also assist in the functional capacity of the gut through the digestion of food and the absorption of nutrients and minerals (Hooper et al., 2002).

The composition of mammalian gut microbiota is determined by factors unique to host genotype and the variable community which has colonized each individual (Benson et al., 2010). The microbiota of marine mammals is largely dominated by three phyla; *Firmicutes, Bacteroidetes* and to a lesser extent *Actinobacteria* (Banks et al., 2014; Lavery et al., 2012; Nelson et al., 2013; Smith et al., 2013). Representatives of the phylum *Firmicutes* predominate in the gut of many pinniped species including the Australian fur seal (*Arctocephalus pusillus doriferus*), leopard seal (*Hydrurga leptonyx*) southern elephant seal (*Mirounga leonina*) and Weddell seal (*Leptonychotes weddellii*) (Banks et al., 2014; Nelson et al., 2013; Smith et al., 2013). The predominance of *Firmicutes* in microbiota of endothermic mammals is often associated with layering of body fat to assist thermoregulation in the cool ocean environment (Bäckhed et al., 2004; Pabst et al., 1999). Members of *Bacteroidetes* and *Actinobacteria* phyla account for a smaller proportion of the gut community in wild pinnipeds and consist of many commensal bacterial species (Banks et al., 2014; Lavery et al., 2012; Nelson et al., 2013).

Pinniped gut microbial diversity may be facilitated by behavioural traits and environmental exposures. Many pinniped species, including sea lions, are colonial breeders, aggregating at high densities and thereby increasing the potential for microbial transfer between individuals (Harcourt, 1992; McCann, 1980; Shaughnessy et al., 2005). In wild pinnipeds the gut microbial composition is also likely to be influenced by diet composition, interactions with other marine mammal species and seabirds, and naturally occurring marine bacteria (Nelson et al., 2008; Nelson et al., 2013). In captive pinnipeds the composition of gut microbiota is influenced by their limited and carefully controlled diet and habitat, as well as potential interactions with a different set of foreign animals (Delport et al., 2015; Nelson et al., 2013). Additionally, wild mammals newly introduced to captive environments often experience physiological changes in hormonal production, thermoregulation and metabolic rate, subsequently influencing the microbial profile of gut communities (Fanson et al., 2010; Hooper et al., 2002; Rangel-Negin et al., 2009). These effects may be further exacerbated by the routine administration of antibiotics during veterinary care which may also profoundly affect gut microbial communities (Stoddard et al., 2009). Given the sensitivity of many marine mammal populations to the spread of microbes between individuals further work needs to be undertaken to identify the effect of external environment on gut microbiota composition.

The Australian sea lion (*Neophoca cinerea*) is an endangered otariid endemic to Australia, with a total population estimated at fewer than 15,000 animals (Shaughnessy et al., 2011). The population is dispersed over approximately 76 small island colonies and protected mainland coves from The Pages, South Australia (SA) to Houtman Abrolhos, Western Australia (WA) (Shaughnessy et al., 2011). The geographic range of the Australian sea lion extends over 2,700 km of Australian coastline, with some colonies situated <25 km from high-density metropolitan areas and more isolated colonies located

farther than 100 km from the nearest coastal settlement. Despite broad population distribution, both male and female sea lions from individual colonies exhibit strong natal-site philopatry and a tendency for localized foraging (Lowther et al., 2012; Lowther et al., 2013b).

The distribution of sea lions along the coastline and Australian mainland brings them into contact with humans and habitats influenced by terrestrial processes. As a tourist icon, some SA and WA sea lion colonies also experience high levels of human visitation at close proximity where people may walk within metres of wild sea lions. For example, the number of visitors to Seal Bay, Kangaroo Island, SA, exceeds 150,000 annually (Goldsworthy et al., 2007). Microbial monitoring of colonies experiencing high anthropogenic disturbance will inform on the impacts to Australian sea lions and potentially assist long-term conservation management.

The aim of this study was to examine the gut microbial communities of Australian sea lions to determine patterns of composition in wild and captive sea lion populations. We hypothesized that sea lions from different colonies may have different gut microbiota composition due to dissimilarity in their biological environment and diet-related microbial exposures. In order to better understand the gut bacterial communities of wild sea lions, the faecal microbiota of sea lions from geographically disparate colonies were examined and compared.

Materials and methods

Sample collection

Australian sea lion faecal samples (n= 271) were freshly collected over a period of 2 years (March 2009 - September 2010) from 11 coastal and island colonies in Southern and Western Australia (Figure 9A-C). Freshly passed faecal samples were collected from captive sea lions (n= 19) over a period of 2 years (March 2011- May 2013) from the resident populations held at Dolphin Marine Magic and Taronga Zoo, New South Wales, and Sea World, Queensland. Faecal samples were transported to the laboratory and stored at 4° C until processing for genomic DNA extraction.

DNA isolation and sub-sampling

Total genomic DNA was extracted from wild (n= 55) and captive (n= 19) sea lion faecal samples (approximately ~150 mg) using the ISOLATE Fecal DNA Kit (Bioline, Sydney, Australia) and extraction performed as per the manufacturers protocol (Table 10). Extracted sea lion DNA (n= 74) was quantified using Qubit® 2.0 Fluorometer (Invitrogen, Life Technologies) and stored at -20° C until further analysis.

16S rRNA gene PCR and sequencing

PCR amplification and sequencing of the 16S rRNA gene was conducted by the Ramaciotti Centre for Genomics at the University of New South Wales (Sydney, Australia). PCR was performed using the bacterial universal forward primer 27F and reverse primer 519R producing a ~530 bp fragment spanning the hyper-variable regions

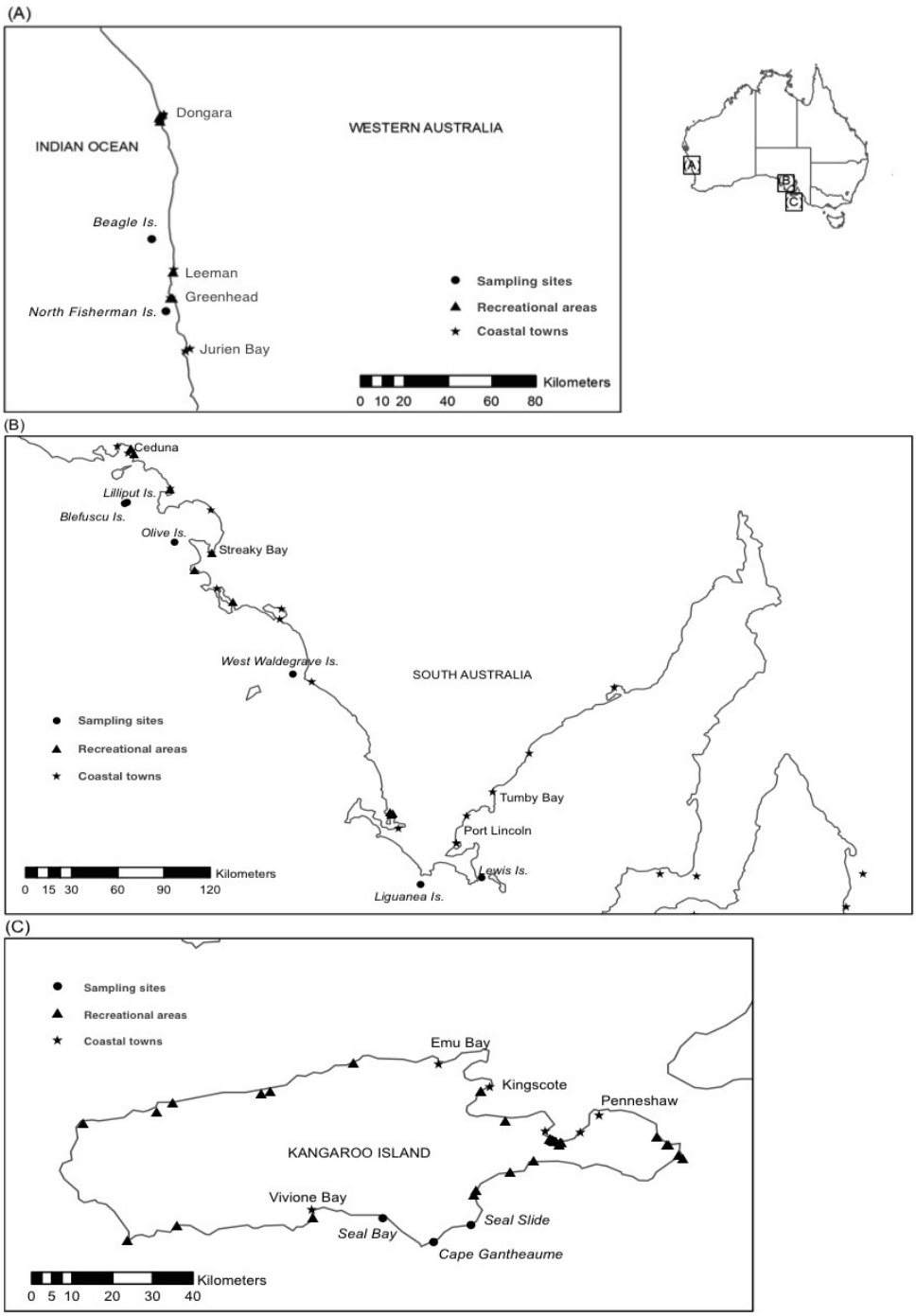

Figure 9. Sea lion sampling locations in **(A)** Western Australia, **(B)** South Australia (mainland) and **(C)** Kangaroo Island, South Australia.

In West Australia, sea lion faecal samples were collected from colonies on Beagle and North Fisherman Islands. Colonies sampled in South Australia included; Blefuscu, Lewis, Liguanea, Lilliput, Olive and West Waldegrave Islands, and three colonies from Kangaroo Island; Cape Gantheaume, Seal Bay and Seal Slide. Coastal settlements and recreational locations within close proximity to sea lion colonies are indicated.

V1-V3 (Caporaso et al., 2012; Lane 1991). Reverse primers contained a MiSeq adaptor sequence, 12-base "barcode" and the universal primer sequence, as described by Caporaso et al. (2012). PCR reactions (25 µL) were prepared using 200 nM dNTPs, 2.5 mM MgCl2, 500 nM of each primer (27F/519R), 1x IMMOLASE Immobuffer (Bioline, Australia), 1U of IMMOLASE DNA polymerase (Bioline, Australia) and 1 µL template DNA. Thermocycling was performed as follows; activation for 10 min at 95° C; 35 cycles at 94° C for 30s, 55° C for 10s and 72° C for 45s; and a final extension step at 72° C for 10 min. PCR products were purified using the AMpure XP purification kit (Beckman Coulter, Australia) following the manufacturers protocol.

To assess the integrity of total RNA, each sample was quantified on an Agilent Bioanalyzer RNA Nano 6000 chip. Samples that amplified poorly (n= 16) were excluded from further analyses. Post integrity assessment, sequencing was carried out on a MiSeq sequencer (Illumina) yielding 250 bp paired-end reads.

Computational analyses

MiSeq forward and reverse reads were merged into single contiguous sequences with the mergepairs tool in USEARCH version 7.0 (Edgar, 2010). Quantitative Insights Into Microbial Ecology (QIIME) version 1.8.0 (Caporaso et al., 2010) was used for all subsequent sequence analysis unless otherwise noted. Sequences were filtered for quality using the default settings and a further 15 samples were removed from subsequent analysis. A total of 9.8 million reads were obtained from the remaining samples (n= 43). These were clustered into operational taxonomic units (OTUs) using a closed-reference OTU picking protocol at a 97% sequencing identity level using UCLUST (Edgar, 2010) against the August 2013 release of Greengenes, core dataset 18_3 (DeSantis et al., 2006).

OTUs at very low abundance, <0.0005% of the total number of sequences, were filtered out following the QIIME default settings. Each library was sub-sampled to an even sequencing depth of 10,000 reads per sample to mitigate biases arising from different depths of sequence across all samples. Alpha and beta diversity analyses were conducted on rarefied data.

Mapping and statistical analyses

Maps illustrating wild Australian sea lion colonies sampled were developed using ArcGIS version 10.0 (ESRI, 2010).

Relative abundances of genera in wild and captive samples were compared after selection of a random subset of 10 wild samples, equivalent to the number of successfully amplified captive samples. Shared OTUs were compared to assess similarities in community structure. OTUs were defined as 'shared' between wild and captive populations when the taxon was present in at least 50% of the animals within that population. Variation in gut community richness of wild and captive sea lion populations was determined using the Shannon-Weiner index at the genus level. Differences in community structure (relative microbial abundance) of core phyla between wild and captive populations were determined using the Mann-Whitney U test in IBM SPSS Statistics version 20.0 for Mac. Phylum abundances were compared across individual animals within wild and captive groups and the standard deviation (SD) used to give an indication of inter-individual variation. To determine taxa driving dissimilarity of gut microbial communities between groups at the family and genus levels, Bray-Curtis SIMPER (similarity percentages procedure) analysis was performed in PAST 3.0.1 (Hammer et al., 2001).

Table 10. Description of samples used in this study.

Australian sea lion faecal samples (n= 5) were randomly selected from each wild colony (n= 11) and all samples obtained from captive individuals (n= 19) for PCR amplification of the 16S rRNA gene.

Collection site	Sea lion faecal samples (Samples successfully amplified)	Collection date	Sample name
South Australia			
Blefuscu Is.	5 (4)	8 Jul 2009	A27, A28, A29, A31
Lewis Is.	5 (4)	11 Feb 2010	A74, A78, A79, A80
Liguanea Is.	5 (2)	5 May 2009	A90, A93
Lilliput Is.	5 (4)	22 Jun 2009	A120, A122, A123, A124
Olive Is.	5 (3)	1 Sept 2010	A187, A188, A189
West Waldegrave Is.	5 (2)	21 May 2009	A235, A243
South Australia: Kangaroo Is.			
Cape Gantheaume	5 (3)	18 Mar 2009	A40, A41, A44
Seal Bay	5 (2)	16 Mar 2009	A203, A206
Seal Slide	5 (4)	23 Jul 2009	A230, A231, A232, A233
Western Australia			
Beagle Is.	5 (2)	16 Mar 2010	A12, A15
North Fisherman Is.	5 (3)	18 Mar 2009	A50, A51, A52
Captive			
Dolphin Marine Magic (NSW)	12 (5)	24 Mar 2011	P7, P9, P10, P11, P12
Sea World (QLD)	5 (3)	22 May, 4 April, 17 April 2013	S1, S4, S6
Taronga Zoo (NSW)	2 (2)	2 May 2013	T1, T2

Taxa contributing to dissimilarity were defined as 'unique' if present in only one population group, wild or captive. To determine taxa driving dissimilarity of wild colony gut microbiota to the overall 'wild' group, phylum abundance was compared as described above and SIMPER analysis performed at the phylum and family levels. To determine differences in OTU richness of colony groups, a mean OTU count of contributing genera was calculated and individual colonies compared.

Sequences generated in this study were submitted to MG-RAST as the project titled 'Australian sea lion faecal collection' under reference identification numbers 4629998.3-4630040.3 (https://metagenomics.anl.gov/linkin.cgi?project=13457).

Results

16S rRNA gene hyper-variable region V1-V3 sequencing of sea lion faecal microbiota

Following all quality filtering steps in QIIME, our dataset constituted a total of 4,993,234 bacterial sequences spanning the hyper-variable V1-V3 region of the 16S rRNA gene from wild (n= 33) and captive (n= 10) sea lion faecal samples (mean 116,122, n= 43). Analyses performed on rarified data sub-sampled to 10,000 reads per sample clustered sequences into 309 OTUs from 7 bacterial phyla: *Firmicutes, Proteobacteria, Bacteroidetes, Actinobacteria, Fusobacteria, Chloroflexi* and *Cyanobacteria.*

Taxonomic differences in gut microbial community composition between wild and captive sea lions

The gut microbiota of wild and captive sea lions differed in overall community

membership and community structure. Based on analysis of an equivalent subset, the gut microbial community of wild sea lions possessed a greater number of OTUs (n= 85) than captive animals (n= 61). Twenty OTUs were shared between population groups with the greatest number of shared OTUs in the phylum *Firmicutes* followed by *Proteobacteria* (Figure 10). Wild sea lions had a greater diversity of taxa than captive animals (H= 2.98, E_H= 0.629), however the taxa were more equitably distributed between captive individuals (H= 2.89, E_H= 0.688).

Community structure: phylum level

The bacterial gut communities of wild and captive sea lions differed in the relative abundance of phyla present (Figure 11). Wild sea lions possessed a higher average relative abundance of the phylum *Firmicutes*, *Bacteroidetes* and *Actinobacteria* within their gut bacterial community than captive sea lions (Table 11), however these differences were not significant (Mann-Whitney U test, p= >0.05). Similarly, although the phylum *Proteobacteria* contributed more to the gut bacterial community of captive animals than wild (Table 11), this difference was not statistically significant (Mann-Whitney U test, p= >0.05). The phylum *Fusobacteria* contributed significantly more to the gut bacterial community composition of captive than wild animals (Mann-Whitney U statistic= 93.5, p= 0.04), with average relative abundances of 2.29±0.663% and 0.505±0.0753% respectively (Table 11). Bacterial phyla *Chloroflexi* and *Cyanobacteria* were observed in very low abundance (0.0139±0.0108% and 0.0118±0.0112% respectively) in wild animals only.

Table 11. The relative abundance of dominant bacterial phyla within the gut microbial communities of wild and captive sea lions.
Classification is based on 16S rRNA sequences from the hyper-variable V1-V3 region.

	Mean relative abundance in habitat (%) (SD) [Minimum-Maximum]	
Phylum	**Wild**	**Captive**
Firmicutes	76.4 (27.2) [11.5-98.8]	61.4 (34.0) [7.23-99.6]
Proteobacteria	10.6 (19.7) [0.18-79.4]	29.3 (36.2) [0.17-90.4]
Bacteroidetes	9.53 (15.7) [0.01-55.8]	5.28 (8.67) [0.01-29.1]
Actinobacteria	2.95 (5.37) [0-26.8]	1.77 (2.03) [0.02-5.51]
Fusobacteria	0.505 (0.433) [0.03-1.71]	2.29 (2.10) [0.19-5.97]
Chloroflexi	0.0139 (0.0621) [0-0.34]	-
Cyanobacteria	0.0118 (0.0644) [0-0.37]	-

Inter-individual variation in phyla abundance differed between sea lions from wild and captive habitats. Comparison of standard deviations of the two most dominant phyla, *Firmicutes* and *Proteobacteria,* showed that there was less inter-individual variation among wild sea lions compared with captive animals (Table 11). Comparison of remaining phyla abundances, *Bacterioidetes* and *Actinobacteria,* showed that inter-individual variation was lower in captive animals, while *Fusobacteria* was lower in wild animals (Table 11).

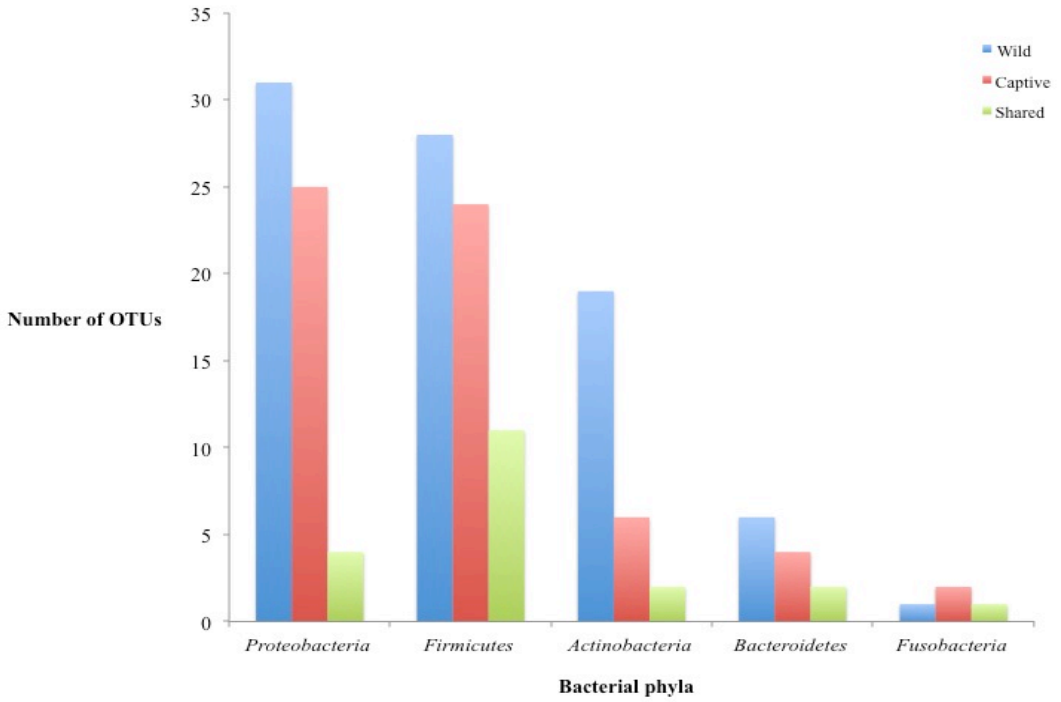

Figure 10. Comparison of OTU distribution of wild and captive faecal microbiota. OTU counts were performed to determine the abundance of taxa in each phylum group. OTUs were considered 'shared' when present in 50% of the individuals from both populations.

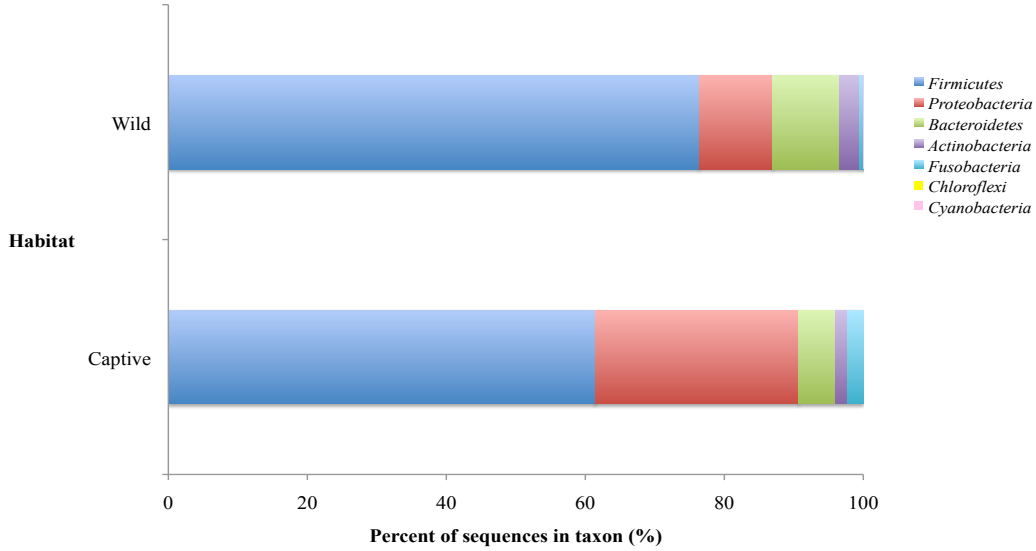

Figure 11. Relative abundance of bacterial phyla of wild and captive sea lion gut microbiota.

The *Firmicutes* phylum was dominant in gut microbiota of both wild (76.4±4.73%) and captive animals (61.4±10.8%). *Proteobacteria* formed a greater contribution to captive (29.3±11.5%) than to wild gut communities (10.6±3.43%).

Of the bacterial families observed (n= 67) there were 19 families that contributed equal to or more than 1% to the dissimilarity of wild and captive populations (SIMPER Overall average dissimilarity= 73.4) (Table 12).

Table 12. SIMPER analysis of dissimilarity between wild and captive gut microbiota. SIMPER analysis representing families contributing more than 1% to the average dissimilarity of wild and captive animal gut microbial communities.

Taxon*	**Average dissimilarity**[a]	**Contribution**[b]	**Wild**[c] **Mean abundance**	**Captive**[d] **Mean abundance**
Clostridiaceae	14	19.1	34.8	19.2
Ruminococcaceae	8	10.9	16.7	12.9
Pseudoalteromonadacea	5.11	6.96	0.09E-06	10.2
Peptostreptococcaceae	4.81	6.56	2.91	8.26
Enterobacteriaceae	4.45	6.07	0.0127	8.91
Clostridales f. unclas.	3.91	5.33	3.03	8.4
Planococcacceae	3.77	5.15	7.55	0.034
Carnobacteriaceae	3.59	4.89	7.15	0.187
Peptococcaceae	3.32	4.53	1.82E-05	6.65
Moraxellaceae	3.26	4.45	2.47	4.69
Rikenellaceae	2.70	3.69	3.77	2.91
Lachnospiraceae	2.34	3.19	2.92	4.21
Bacteroidaceae	2.13	2.91	4.35	1.24
Xanthomonadaceae	1.86	2.53	3.71	0
Pseudomonadaceae	1.64	2.23	0.3	3.16
Coriobacteriaceae	1.25	1.70	1.66	1.70
Fusobacteriaceae	1.02	1.40	0.505	2.29
Porphyromonadaceae	0.761	1.04	1.06	0.507
Campylobacteraceae	0.744	1.01	0.751	1.04
		Overall average dissimilarity: 73.4		

* Family.
[a] Bray-Curtis average dissimilarity between wild and captive groups.
[b] Contribution to dissimilarity between wild and captive groups, expressed as %.
[c,d] Mean abundance expressed as %.

Characteristic OTUs from bacterial families Clostridiaceae and Ruminococcaceae were more abundant in wild (34.8±4.98% and 16.7±2.98% respectively) than captive animals (19.2± 4.77% and 12.9±4.24% respectively) and contributed most to the average dissimilarity between groups (SIMPER contribution= 19.1 and 10.9% respectively) (Table 12). In captive animals, higher average abundances of characteristic OTUs from Pseudoalteromonadacea (10.2±6.55), Peptostreptococcaceae (8.27±6.66) and Enterobacteriaceae (8.91±7.71) families contributed most greatly to gut microbiota dissimilarity (SIMPER cumulative contribution= 19.6%) (Table 12). Characteristic OTUs from the Xanthomonadaceae family (3.71±2.21%) were unique to wild animal microbiota (Table 12).

Differences in gut microbial community composition between wild sea lion colonies

The microbial gut communities of most individuals from wild sea lion colonies showed similar patterns of abundance at the phylum level, which we refer to here as the typical wild profile (Figure 12). Individuals from three colonies from South Australia: Lilliput, Olive and West Waldegrave Islands, and one West Australian colony; North Fisherman Island, had notable differences in the distribution of certain phyla abundance from this typical profile (Table 13).

Lilliput Island sea lion microbial communities contained a higher relative abundance of OTUs from the phylum *Proteobacteria* (42.9±19.2%) than any other colony, contributing greatly to dissimilarity from the typical wild profile (SIMPER contribution= 41.3%). In contrast *Firmicutes* (48.4±19.2%) represented a lower proportion of the gut microbial community in this colony (SIMPER contribution= 42.7%). Inter-individual variation was high in both the *Firmicutes* and *Proteobacteria* phylum groups (Table 13).

OTUs from two *Proteobacteria* families, Xanthomonadaceae (20±16.1%) and Moraxellaceae (18.1±14.8%) contributed to the observed increase in *Proteobacteria* and the average dissimilarity of gut microbiota of seals from Lilliput and the typical wild profile (SIMPER cumulative contribution= 26.1%).

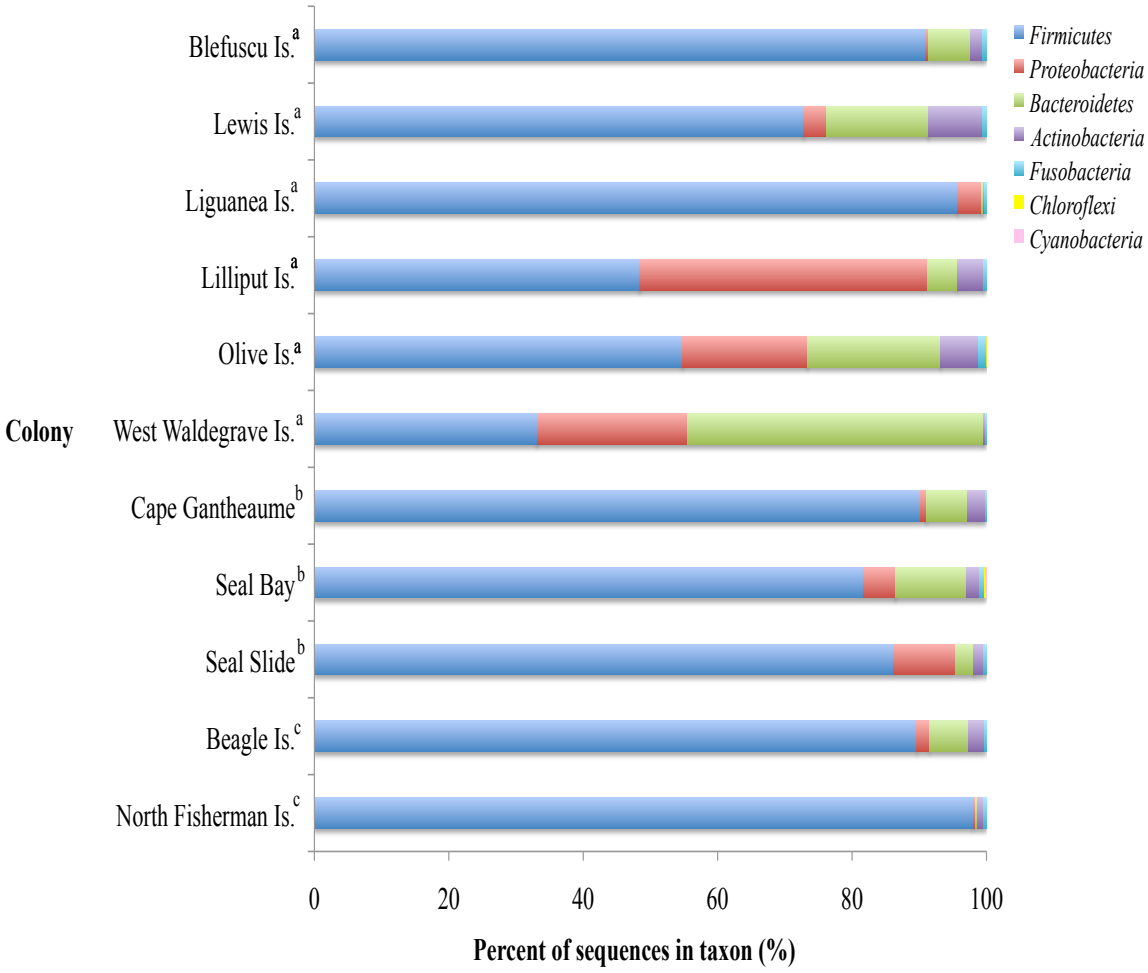

Figure 12. Relative abundance of bacterial phyla from geographically disparate sea lion populations.

The collective gut microbial communities of most wild colonies showed similar patterns of abundance at the phylum level. Notable differences in phyla distribution were observed in three colonies from South Australia: Lilliput, Olive and West Waldegrave Islands and one West Australian colony; North Fisherman Island. Superscript letters refer to sea lion colonies in; **(a)** South Australia, **(b)** Kangaroo Island and **(c)** Western Australia.

Table 13. The relative abundances of dominant bacterial phyla in the gut microbial communities of wild sea lion colonies with atypical microbial taxon abundance profiles.
Classification is based on 16S rRNA sequences from the hyper-variable V1-V3 region.

	Mean relative abundance of phyla (%) (SD) [Minimum-Maximum]						
	Firmicutes	*Proteobacteria*	*Bacteroidetes*	*Actinobacteria*	*Fusobacteria*	*Chloroflexi*	*Cyanobacteria*
Sea lion colony							
South Australia							
Lilliput Is.	48.4 (38.5) [11.5-82.6]	42.9 (39.7) [4.33-79.4]	4.47 (6.24) [0.07-13.5]	3.81 (4.30) [0.05-8.63]	0.473 (0.606) [0.04-1.33]	-	-
Olive Is.	54.8 (40.2) [11.7-91.4]	18.5 (14.7) [2.13-30.6]	19.8 (25.7) [4.03-49.5]	5.71 (8.66) [0.42-15.7]	1.08 (0.862) [0.1-1.71]	0.04 (0.0693) [0-0.12]	-
West Waldegrave Is.	33.2 (11.1) [25.4-41.0]	22.4 (24.0) [5.37-39.4]	44.0 (13.3) [34.6-53.4]	0.35 (0.424) [0.05-0.65]	0.075 (0.0495) [0.04-0.11]	-	-
Western Australia							
North Fisherman Is.	98.1 (0.771) [97.3-98.8]	0.323 (0.176) [0.18-0.52]	0.26 (0.128) [0.12-0.37]	0.92 (1.03) [0.28-2.11]	0.423 (0.200) [0.22-0.62]	-	-

There was a high degree of inter-individual variation in family abundances contributing to the dissimilarity of gut microbiota (SIMPER overall average dissimilarity= 76.4) (Table 14A). Collectively, microbiota of sea lions from Lilliput Island were comprised of a greater number of genera (n= 72) than most other wild colonies (mean= 55±4.01).

In sea lions from Olive Island, gut microbial community profiles showed a higher proportion of taxa from *Bacteroidetes* (19.8±14.9) and *Proteobacteria* phyla (18.5±8.51) than was seen in the typical wild profile (SIMPER contribution= 23.7 and 22.8% respectively) (Table 14B). While *Firmicutes* (54.8±23.2%) formed a smaller contribution to microbial community composition, low relative abundance drove the observed dissimilarity between groups (SIMPER contribution= 44.9%) (Table 14B). Inter-individual variation of abundance was highest in the *Firmicutes* phylum in individual sea lion gut microbiota (Table 13). Decreased characteristic OTUs from *Firmicutes* family Clostridiaceae (11.9±6.71%) and increased Ruminococcaceae composition (34.5±18.3) contributed most to the dissimilarity between sea lions from Olive Island and the typical wild profile (SIMPER contribution= 20.8 and 19.9% respectively). The *Bacteroidetes* family Bacteroidaceae (12.1±8.07%) was more abundant in Olive Island sea lion gut microbiota and contributed to the observed dissimilarity between groups (SIMPER contribution= 8.07%). Inter-individual variation of gut microbiota was high across all familial taxa and contributed to the overall dissimilarity between groups (SIMPER overall average dissimilarity= 69.6). Microbiota of sea lions from Olive Island had relatively greater numbers of microbial genera (n= 72) than most other wild colonies (mean= 55±4.01).

On West Waldegrave Island the *Bacteroidetes* phylum (44.0±9.43%) formed a greater contribution to sea lion gut microbial composition than other wild colonies and the

typical wild profile (SIMPER contribution= 33.4%) (Table 14C). While the phylum *Proteobacteria* (22.4±17.0%) also represented a greater composition of gut microbial communities, decreased abundance of *Firmicutes* (33.2±7.84%) contributed most to the dissimilarity between groups (SIMPER contribution= 20.1 and 43.5% respectively) (Table 14C). Inter-individual variation at the phylum level of individual sea lion gut microbiota was relatively high in the *Proteobacteria* group (Table 13). Three *Bacteroidete*s families, Porphyromonadaceae (17.0±16.9%), Bacteroidaceae (14.1±13.6%) and Rikenellaceae (12.9±18.8%) were more abundant in gut microbiota of West Waldegrave sea lions and contributed greatly to the dissimilarity between groups (SIMPER cumulative contribution= 28.0%). Inter-individual variation was high contributing to the dissimilarity of West Waldegrave sea lions at the family level (SIMPER overall average dissimilarity= 78.7). However, interestingly fewer genera (n= 40) were observed in microbiota of West Waldegrave Island sea lions than any other colony (mean= 55±4.01).

In sea lions from North Fisherman Island, characteristic OTUs from the phylum *Firmicutes* (98.1±0.445%) contributed more to gut microbiota than was seen in the typical wild profile (SIMPER contribution= 48.8%) (Table 14D). *Proteobacteria* (0.323±0.102%) and *Bacteroidetes* (0.26±0.0737%) phyla represented a smaller contribution to gut microbiota than other colonies (SIMPER cumulative contribution= 44.0%) (Table 14D). Inter-individual variation of phylum group abundance between individual sea lion gut microbiota was low (Table 13). *Firmicutes* families Clostridaceae (58.6±28.0%) and Carnobacteriaceae (29.2±29.2%) were more abundant in gut microbiota of North Fisherman sea lions than the typical wild profile and contributed most to the average dissimilarity between groups (SIMPER cumulative contribution= 58.8%).

Table 14. SIMPER analysis of phyla driving dissimilarity of gut microbial communities of sea lions from **(A)** Lilliput Island; **(B)** Olive Island; **(C)** West Waldegrave Island and **(D)** North Fisherman Island.

(A) **Taxon***	**Average dissimilarity**[a]	**Contribution**[b] **(%)**	**Wild Abundance**[c]	**Lilliput Island Abundance**[c]
Firmicutes	20.4	42.7	76.4	48.4
Proteobacteria	19.7	41.3	10.6	42.9
Bacteroidetes	5.08	10.6	9.53	4.47
Actinobacteria	2.26	4.75	2.95	3.81
Fusobacteria	0.264	0.554	0.505	0.473
Chloroflexi	0.00697	0.0146	0.0139	0
Cyanobacteria	0.00591	0.0124	0.0118	0
		Overall average dissimilarity: 47.7		

(B) **Taxon***	**Average dissimilarity**[a]	**Contribution**[b] **(%)**	**Wild Abundance**[c]	**Olive Island Abundance**[c]
Firmicutes	18.6	44.9	76.4	54.8
Bacteroidetes	9.82	23.7	9.53	19.8
Proteobacteria	9.44	22.8	10.6	18.5
Actinobacteria	3.14	7.57	2.95	5.71
Fusobacteria	0.430	1.04	0.505	1.08
Chloroflexi	0.0245	0.0592	0.0139	0.04
Cyanobacteria	0.00591	0.0142	0.0118	0
		Overall average dissimilarity: 41.5		

(C) **Taxon***	**Average dissimilarity**[a]	**Contribution**[b] **(%)**	**Wild Abundance**[c]	**West Waldegrave Abundance**[c]
Firmicutes	23.6	43.5	76.4	33.2
Bacteroidetes	18.1	33.4	9.53	44
Proteobacteria	10.9	20.12	10.6	22.4
Actinobacteria	1.4	2.58	2.95	0.35
Fusobacteria	0.221	0.407	0.505	0.075
Chloroflexi	0.00697	0.0129	0.0139	0
Cyanobacteria	0.00591	0.0109	0.0118	0

		Overall average dissimilarity: 54.3		
(D) Taxon*	**Average dissimilarity[a]**	**Contribution[b] (%)**	**Wild abundance[c]**	**North Fisherman Island Abundance[c]**
Firmicutes	10.9	44.8	76.4	98.1
Proteobacteria	5.13	23.0	10.6	0.323
Bacteroidetes	4.66	21.0	9.53	0.26
Actinobacteria	1.41	6.33	2.95	0.92
Fusobacteria	0.177	0.797	0.505	0.423
Chloroflexi	0.00697	0.0313	0.0139	0
Cyanobacteria	0.00591	0.0266	0.0118	0
		Overall average dissimilarity: 22.3		

* Phylum level.
[a] Bray-Curtis average dissimilarity between wild and captive groups.
[b] Contribution to dissimilarity between wild and captive groups.
[c] Mean abundance expressed as %.

There was a high degree of inter-individual variation in families contributing to the dissimilarity of gut microbiota (SIMPER overall average dissimilarity= 65.5). Samples from North Fisherman Island seals also contained relatively low numbers of microbial genera (n= 42) compared to most other wild colonies (mean= 55±4.01).

Discussion

Evidence of 'conserved' core gut microbiota in seal and sea lion species

In this study the gut microbiota of a large number of individuals representing both wild and captive Australian sea lion populations was found to be dominated by 5 bacterial

phyla; *Firmicutes, Proteobacteria, Bacteroidetes, Actinobacteria* and *Fusobacteria.* Our findings confirm preliminary observations describing the gut microbiota of a single sea lion determined from faeces collected from Seal Bay, Kangaroo Island Australia, in which *Firmicutes* contributed ~80% to the microbial community composition (Lavery et al., 2012). Similar gut microbiota composition and *Firmicutes* dominance has been observed in numerous pinniped species including Australian fur, leopard, southern elephant and Weddell seals suggesting a 'conserved' core gut microbial community in pinnipeds (Banks et al., 2014; Nelson et al., 2013; Smith et al., 2013).

Dietary behaviours drive gut microbial membership

Dietary resources have been shown to exert a strong influence on microbial community composition of both terrestrial and marine mammals (Dhanasiri et al., 2011; Ellison et al., 2014; Nelson et al., 2013). We hypothesize the diverse microbial communities observed in faecal samples of wild Australian sea lions may be contributed to by the broad diversity of prey consumed by sea lions. Australian sea lions are opportunistic foragers, feeding on a broad range of shallow-water benthic prey including teleost fish, cuttlefish, octopus, squid, rock lobster, rays, small sharks, penguins and small crustaceans (Gales and Cheal, 1992; McIntosh et al., 2007). In addition, engagement in behavioural practices such as the ingestion of pebbles or rocks and playing with seaweed, introduce diverse environmental microbes to gut microbial communities otherwise unexposed to in captivity (King, 1983). In captivity, sea lions are fed diversity-poor diets comprised of fresh or frozen fish of uniform size that have been processed in fish markets. For example, the diet of captive sea lions housed at Taronga Zoo, NSW, consists of small to medium size fish including; Australian herring (*Arripis georgianus*), red spot whiting (*Sillago flindersi*), Australian pilchards (*Sardinops sagax*), New Zealand arrow squid (*Nototodarus*

sloanii) and pacific saury (*Cololabis saira*), strongly contrasting with the highly variable diet of free-foraging sea lions (McIntosh et al., 2007). Recent studies observing the influence of diet on mammalian livestock and fish gut microbiota have shown that free-ranging animals manifest greater microbial diversity compared to those fed from artificial or concentrate sources (Dhanasiri et al., 2011; Ellison et al., 2014; Kohl and Dearing, 2014). While diet plays a substantial role in defining gut community membership and structure, the contribution of environmental microbes from ocean and coastal sources needs to be considered when assessing 'loss' of microbial richness in captive sea lion populations.

Natural habitats increase gut microbial community diversity

Environmental microbes common in natural host habitats are likely to have contributed to the observed diversity and richness of wild sea lion gut microbiota. Representatives from families Xanthomonadaceae, Rhodobacteraceae and Vibrionaceae, typically characterized as marine water, soil or plant based microbes, were commonly identified in faecal samples of wild sea lions but were observed less often or not at all in the samples from captive animals. Members of the Xanthomonadaceae were identified only in wild sea lions while Rhodobacteraceae and Vibrionaceae members were observed more often in samples from wild populations. Wild sea lions visit a variety of terrestrial habitats such as sandy beaches and rock outcroppings when breeding, raising young and resting (Walker and Ling, 1981). Additionally, sea lions seek shelter from sun and poor weather in coastal vegetation and sand dunes, providing opportunities for exposure to a variety of terrestrial microbes that may be absent from captive settings (Charrier et al., 2009). Recent observations of leopard and southern elephant seals similarly indicated increased gut bacterial richness in wild animals, which was attributed to contributions from

environmental microorganisms (Nelson et al., 2013). However, studies involving longer-term monitoring of faecal microbial communities would be useful in determining whether such environmental organisms are simply transiently passaged through the gut or are capable of longer-term establishment in the gastrointestinal tract.

Captivity influences gut microbial community composition

Captivity provides opportunities for the transmission and establishment of microbes from non-endemic sources in sea lion gut microbiota (Delport et al., 2015; Stoddard et al., 2009). In captivity marine mammals are exposed to a variety of non-endemic microbes via interactions with zookeepers, through animal interaction programs involving the general public, and through social interactions in holding pens with mammalian species not usually within their natural environment, thereby increasing the opportunity for microbial establishment from foreign sources (Delport et al., 2015). Characteristic OTUs from *Proteobacteria* families Enterobacteriaceae and Pseudoalteromonadacea were more abundant in captive sea lion gut microbiota, driving dissimilarity from wild animals. While many members of Enterobacteriaceae are harmless intestinal symbionts, this family also includes many well-known pathogenic species (Duignan et al., 2004). Our study confirms earlier observations of Enterobacteriaceae in Australian sea lion populations where enterobacteria were significantly higher in captive than wild sea lion faecal samples (Delport et al., 2015).

Members of the Pseudoalteromonadacea family were also more abundant in gut microbiota of captive sea lions. The *Pseudoalteromonas* genus contains a variety of marine species responsible for stabilizing biological molecules and preventing the settlement of fouling organisms (Holmström and Kjelleberg, 1999). Long-term observations of captive sea lion microbiota would better inform the impact of *Pseudoalteromonas* on the composition of

gut microbiota in captive sea lions. Future studies expanded to include information on specificities of diet, medicinal treatment history and co-habitation procedures, as well as enclosure sampling, would enable better understanding of microbial flow through the captive environment.

Localized foraging and behaviour influences the composition of gut microbiota

Colony dynamics, sea lion behaviour and foraging site fidelity are likely to play an important role in the composition of gut microbial communities in sea lions from geographically disparate colonies. OTU diversity was greatest in gut microbiota of sea lions from Olive and Lilliput Island, SA. Olive Island hosts a high-density sea lion colony that is sympatric with a small colony of New Zealand fur seals (*Arctocephalus forsteri*) also breeding on the island (Shaughnessy et al., 2005). Socialization and other interactions between these species is known to occur and could increase the potential for microbial transmission, thereby contributing to the richness of South Australian sea lion gut microbiota (Lombardo et al., 2008). Similar findings have been reported in studies observing southern elephant and leopard seal gut microbiota, where increased microbial richness in elephant seals was attributed to social nature of the species, often aggregating in great numbers ashore during breeding and molting periods compared with the solitary nature of leopard seals (Nelson et al., 2013).

Lilliput Island is frequently visited by terrestrial bird species including the rock parrot (*Neophema petrophila*), ruddy turnstone (*Arenaria interpres*) and crested tern (*Sterna bergii*), increasing the potential for dissemination of terrestrial microbes to sea lion populations (Shaughnessy, 2007; Shaughnessy et al., 2008). Seabirds visiting terrestrial sources are exposed to a variety of microbes, atypical to the natural habitat of marine

mammals (Nelson et al., 2008). In addition to visitation from terrestrial species, sea lions from Lilliput Island often swim inshore close to the mainland (Lowther et al., 2012), increasing their exposure to microbes from wastewater run-off and terrestrial sources which may explain the elevated microbial diversity observed in faecal samples from this colony.

Interestingly, however, West Waldegrave Island, SA, which also experiences high visitation from terrestrial bird species and is frequently visited by New Zealand fur seals (Shaughnessy et al., 2005; Shaughnessy et al., 2007; Shaughnessy et al., 2008), showed the lowest overall faecal microbial diversity of any surveyed colony. One difference between this location and many other South Australian colonies is the absence of human visitation, reducing the likelihood of habitat disturbance. At this time it is unclear whether this contributes in some way to the lower observed OTU richness or whether this is linked to other factors, either environmental or perhaps relating to the age, sex, health or habits of the specimen donors at this location.

The gut microbial communities of sea lions from North Fisherman Island, WA, had substantially lower OTU richness than the majority of South Australian colonies. At this site sea lions demonstrate a strong tendency for limited dispersal from breeding colonies and foraging site fidelity (Campbell et al., 2008; Lowther et al., 2012; Lowther et al., 2013b). While determining diet variation of individual colonies is challenging due to the demersal nature of foraging, substantial regional differences in ocean trophic ecology are well documented (Lowther et al., 2013a). Recent studies observing sea lion foraging behaviours found significant differences in trophic diversity between South and Western Australian colonies, where western foraging sites had lower richness (Lowther et al., 2013a). Colony-centric foraging and limited dispersal suggests that habitat and the

availability of prey may contribute to decreased richness of microbes colonizing the gut of West Australian sea lions. Future investigations into dietary variation between South and Western Australia colonies may help further explain the observed differences in gut microbiota composition and diversity.

Conclusion

The gut microbial communities of Australian sea lions are impacted by diverse environmental and behavioural factors. As colonially breeding pinnipeds, sea lions are subject to high rates of microbial transfer between individuals. They are also likely to have exposure to a diverse array of microbes in their natural environments, from co-habiting species and anthropogenic sources. All of these factors can increase the risk of pathogen transfer and disease emergence in this endangered marine mammal (Cowan et al., 2011; Härkönen et al., 2006; Lynch et al., 2011). Understanding complex microbial interactions will inform current knowledge gaps in microbial movement and dissemination routes within high-density wildlife populations. The current study is the first step along this path. Future observations including monitoring of the biological environment would provide key information regarding potential dissemination routes of microbes to endangered species and assist in the development of long-term conservation management strategies.

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Chapter 5

Conclusions and future recommendations

Conclusions and future recommendations

Expansion of coastal settlements and human visitation to natural habitats of marine wildlife provide opportunities for the introduction of microorganisms from terrestrial sources (Baily et al., 2015; Bogomolni et al., 2008; Daszak et al., 2000). This is particularly evident in the Australian sea lion, where wild colonies within close proximity to human settlements, and those that experience near-constant human interaction, harbour parasitic protozoa and bacteria characteristic of terrestrial vertebrates (c.f. Feng and Xiao, 2011; c.f. Partridge et al., 2009), including humans (Delport et al., 2014; Delport et al., 2015). Using targeted screening for parasitic protozoa *Giardia* and *Cryptosporidium*, I identified human and terrestrial mammal genotypes of *G. duodenalis* in greater frequency in sea lions within close proximity to coastal settlements and captivity, than more isolated colonies. *Cryptosporidium* was not detected in the Australian sea lion (Chapter 2). Similarly, microbial analysis of faecal coliforms indicated that the presence of *E. coli* harbouring antibiotic resistance genes, common in clinical settings, was strongly influenced by exposure to captive environments (Chapter 3). Finally, comparative analysis of gut microbiota indicated a high level of dissimilarity of microbial community structure and membership between wild and captive animals (Chapter 4).

I provide the first evidence of microorganisms from terrestrial taxa establishing in Australian sea lions, and demonstrate the far-reaching effects of anthropogenic contamination of Australian marine ecosystems. While molecular typing of these microorganisms provides strong evidence of transmission from different terrestrial sources, the ability to draw inferences on precise sources of these organisms is hindered by limitations of current molecular methodologies.

Parasitic protozoa as an indicator of microorganism dissemination from anthropogenic and terrestrial sources

Molecular identification and characterization of the protozoan parasite, *G. duodenalis*, has proven to be a useful tool for preliminary identification of parasite dissemination from terrestrial taxa to marine mammals (Gaydos and Miller, 2008; Koehler et al., 2014; Lasek-Nesselquist et al., 2008). The specific aims of this investigation were to; first, determine the presence of terrestrial and human genotypes of *G. duodenalis* and *Cryptosporidium* in Australian sea lions, and secondly, determine if proximity to human settlements had an impact on occurrence (Chapter 2).

Giardia duodenalis occurrence was significantly higher in captive animals (36.8%) and wild sea lions within close proximity (23.8%) to human coastal settlements (<25km) than more isolated colonies (2.8%). Molecular typing identified *G. duodenalis* assemblages AI and B, genotypes common in human and terrestrial mammal hosts, in sea lions. *Cryptosporidium* was not detected. While identification of *G. duodenalis* sub-assemblage AI is a strong indicator of transmission from anthropogenic sources, absence of sub-type data from assemblage B isolates limited the ability to determine terrestrial origin.

Sub-typing of *G. duodenalis* is central to understanding the extent of parasite dissemination from terrestrial sources to marine mammals; however, the ability to draw such inferences is limited by the unreliability of current molecular protocols (Cacciò et al., 2002; Lalle et al., 2005; Lasek-Nesselquist et al., 2010; Read et al., 2004; Sulaiman et al., 2003). Nearly all 18S rRNA positive isolates failed to produce amplicons at loci used for routine typing of *Giardia*: β-giardin and *gdh* (Cacciò et al., 2002; Lalle et al., 2005; Read et al., 2004). In addition, attempts to optimize published *tpi* protocols (Sulaiman et al.,

2003), resulted in intermittent amplification of both human and sea lion controls. Furthermore, when applied to a single sample, genotyping to assemblage level was inconsistent across 18S rRNA and β-giardin loci. Intermittent amplification of the aforementioned loci has been documented in other marine vertebrates, including grey and Pacific harbor seals (Beck et al., 2011; Lasek-Nesselquist et al., 2010). Inconsistencies in sub-typing assemblage A and B isolates are commonly reported in both marine and terrestrial mammals including Pacific harbor seals, humans, captive non-human primates, cats and dogs (Cacciò et al., 2008; Geurden et al., 2009; Lasek-Nesselquist et al., 2010; Traub et al., 2004). Inconsistency of assemblage assignment is frequently reported in studies of *Giardia* in varied hosts (Cacciò and Sprong, 2010). A recent evaluation comparing *G. duodenalis* sequence data submitted to ZOOnotic Protozoa NETwork (ZOOPNET), a network primarily established to unify methodology for the detection of *Giardia* and *Cryptosporidium* and investigate the molecular epidemiology of protozoa infection, identified inconsistent typing between at least two of the aforementioned markers in approximately 15% of sequences (Cacciò and Sprong, 2010). The inconsistency of *G. duodenalis* genotyping in numerous marine and terrestrial mammal studies, combined with the findings from this investigation, emphasize the importance of (i) confirming genotyping data across multiple loci; (ii) improving current understanding of mixed infection with genotypes A and B and; (iii) applying new genetic markers when characterizing *G. duodenalis* sub-types in marine mammal investigations.

Sub-typing of *G. duodenalis* isolates is key to understanding terrestrial protozoa dissemination into the marine environment. In order to gain a more comprehensive understanding of the epidemiology of *Giardia* in marine wildlife, investigations should be expanded beyond PCR amplification of β-giardin, *gdh* and *tpi* loci, to additional genetic markers such as; elongation factor 1 alpha (*ef1α*), the *G. lamblia* open reading frame-C4

(GLORF-*C4*) and the second transcribed spacer (ITS-2) of nuclear ribosomal DNA (Hashimoto et al., 1994; Nash and Mowatt, 1992; Weiss et al., 1992). As these regions are less conserved than the 18S rRNA gene, the genetic variability is useful for both genotype and sub-type classification, and hence, can be used to make more accurate inferences on transmission routes of terrestrial genotypes to marine populations.

The effects of *G. duodenalis* establishment on marine mammal health are largely understudied. As human and terrestrial mammal genotypes of *Giardia* have been detected in numerous pinniped species, understanding the impact this has on animal health will be crucial to directing future conservation strategies targeted at managing potential *Giardia* infection in sensitive populations (eg. Appelbee et al., 2005; Benton et al., 2014; Gaydos and Miller, 2008; Lasek-Nesselquist et al., 2008).

Molecular typing of Escherichia coli and class I integrons isolated from faecal coliforms of Australian sea lions

While traditional culture-based screening and antimicrobial sensitivity tests enable determination of resistance to antimicrobials, the ability to identify unique genetic mechanisms facilitating resistance is not possible using such approaches (Stoddard et al., 2008; Stokes et al., 2001). The application of molecular tools bypasses the limitations of traditional culture-based techniques, enabling identification of resistance determinants, and allowing for source tracking and identification of possible transmission pathways (Gillings et al., 2014; Gillings et al., 2008; Glad et al., 2010). In this investigation, culture-based isolation of faecal coliforms was used to assess differences in *E. coli* presence between wild and captive sea lions, and molecular characterization used to phylotype *E. coli*

isolates and identify antibiotic resistance genes associated with clinical class I integrons (Chapter 3).

Escherichia coli yields were significantly higher in faecal coliforms isolated from captive (84%) than wild animals (7.7%), suggesting that (i) *E. coli* may not be a dominant member of natural gut microbial communities and; (ii) *E. coli* presence is strongly influenced by anthropogenic influence and exposures in the captive environment. While *E. coli* is often absent in faecal coliforms recovered from wild pinnipeds (Johnson et al., 1998), it is a dominant member in gut microbial communities of humans and Australian terrestrial mammals (Gordon and Cowling, 2003), suggesting that increased presence in captive animals may be the result of dissemination of *E. coli* at the captive interface (Skurnik et al., 2006; Stoddard et al., 2009). Genetic characterisation of class I integrons in *E. coli* isolates from sea lions identified gene cassettes encoding resistance to streptomycin-spectinomycin and trimethoprim, antimicrobials common in human clinical settings (Partridge et al., 2009) in *E. coli* from captive animals only. The detection of *E. coli* harbouring resistance genes commonly identified in human clinical cases in captive sea lions may be the result of conditions experienced in captivity, and the dissemination of human-derived microbes at this interface (Sidjabat et al., 2006; Skurnik et al., 2006; Stoddard et al., 2008). Veterinary treatment with antimicrobials and antibiotic residue in enclosure water, may lead to selection for resistance genes in faecal coliforms of captive sea lions (Sidjabat et al., 2006; Stoddard et al., 2009; Skurnik et al., 2006). Screening enclosure water and animal housing may provide further insight into the role the captive environment plays in facilitating dissemination of antibiotic resistance to resident sea lions.

The distribution of *E. coli* phylotypes in marine mammals is largely unknown. To our knowledge, this is the first investigation of *E. coli* phylotype distribution in faecal

coliforms of a marine mammal. Of the four *E. coli* phylotypes (A, B1, B2, D), phylotype B2 is the most dominant phylotype group in both wild and captive Australian sea lions, representing greater than 50% of cultured isolates. While phylotype B2 is suggested to have the highest carriage of virulence factors and greatest resistance to antibiotics (Johnson et al., 2001), further molecular typing targeted at assessing virulence carriage of *E. coli* isolates from Australian sea lions is required to determine if this presents increased disease risks to wild sea lions (Skurnik et al., 2005). Diet is also suggested to contribute significantly to phylotype distribution in *E. coli* strains isolated from Australian mammalian taxa (Gordon and Cowling, 2003). Phylotype B2 dominance is common in *E. coli* strains isolated from humans and omnivorous Australian terrestrial mammals, while phylotype B1 is dominant in carnivorous species (Escobar-Páramo et al., 2006; Gordon and Cowling, 2003). Further typing of *E. coli* B2 isolates is required to determine if dominance in captive sea lion faecal coliforms is the result of artificial diet fed in captivity, colonization by human and terrestrial mammal *E. coli* strains, or the result of selective pressures of treatment with antibiotics.

Understanding microorganism flow through the captive environment is the first step in managing microbial transfer between animals and reducing potential emerging disease risks associated with increased interactions with humans. Molecular typing of *E. coli* B2 strains isolated from Australian sea lions would provide further insight into the extent of colonization by human strains in captive animals. The *E. coli* B2 phylotype can be further sub-typed into 10 distinctive groups that vary in their host preference. Hence, B2 sub-typing would allow for deeper analysis into anthropogenic impacts to captive animals (Clermont et al., 2014). Future investigations encompassing screening of animals housed together and environmental sampling of enclosures, coupled with information on animal medicinal treatment history and specificities of dietary components would further enhance

the understanding of microbial movement through captive environments.

Application of next-generation sequencing to assess anthropogenic influence on marine mammal gut microbial communities

Next-generation sequencing technologies offer a sophisticated approach to microbial ecology, allowing a broad and more accurate depiction of entire microbial communities, otherwise unidentified using culture-based techniques (Banks et al., 2014; Nelson et al., 2013; Smith et al., 2013). In this investigation, next-generation sequencing was used to; first, define the gut microbiome of the Australian sea lion, and secondly, compare microbial communities of wild and captive populations (Chapter 4).

The gut microbiome of the Australian sea lion was comprised of 5 dominant bacterial phyla; *Firmicutes, Proteobacteria, Bacteroidetes, Actinobacteria* and *Fusobacteria.* Dominance of the phylum *Firmicutes* and similar microbial phylum contributions, confirm earlier observations describing the gut microbiome of a single Australian sea lion (Lavery et al., 2012). Similar gut microbial composition has been identified in pinniped species including; the Australian fur, leopard, southern elephant and Weddell seals, suggesting a 'conserved' core gut microbiome in pinnipeds (Banks et al., 2014; Lavery et al., 2012; Nelson et al., 2013; Smith et al., 2013).

Next-generation sequencing of Australian sea lion gut microbial communities revealed a high level of dissimilarity in microbial membership (bacterial species present) and community structure (the relative abundance of bacterial species) between wild and captive sea lion gut microbiota. While the phylum *Firmicutes* was dominant in both wild and captive sea lions, *Proteobacteria* contributed more to the composition of captive

(29.3±11.5%) than wild animal gut communities (10.6±3.43%). Members of the Enterobacteriaceae family were more abundant in the collective captive microbiome. This finding confirms earlier culture-based observations of northern elephant seal and Australian sea lion faecal coliforms, where anthropogenic influence and exposures experienced in captivity had a significant effect on the abundance of enterobacteria (Chapter 3) (Stoddard et al., 2009). While many Enterobacteriaceae are harmless intestinal symbionts, the family also includes many well-known human and animal pathogenic species including; *Klebsiella pneumonia, Salmonella enterica* and *Pseudomonas aeruginosa* (Duignan et al., 2004; Fenwick et al., 2004). However, the absence of lower-level taxonomic classification prevented identification of enterobacteria species contributing to the observed higher frequency in captive animals, limiting inference on their potential pathogenicity and origin.

Most importantly, this study emphasizes the importance of culture-independent screening techniques in accurately assessing community structure of the gut microbiota. While *E. coli* was recovered from sea lions using culture-dependent screening (Chapter 3), the molecular data showed that the *Escherichia* genus contributed to no greater than 0.1% of the total gut microbial community. Understanding the ecology of sea lion gut microbiota and the impact of the external environment on microbial community dynamics are essential steps for defining microorganism movement and establishment in wild animals. Future applications of next generation sequencing technologies would aim to focus on lower taxonomic levels to identify the role socialization and environmental factors play in shaping marine mammal gut microbiota (Banks et al., 2014; Nelson et al., 2013; Smith et al., 2013).

Diet plays a substantial role in defining gut microbial membership and structure, however, due to the demersal nature of Australian sea lion foraging, prey specificities of individual colonies are largely unknown (Dhanasiri et al., 2011; Ellison et al., 2014; McIntosh et al., 2007). Given the demarcation in foraging ecotypes and population genetic structure found in Australian sea lions (Lowther et al., 2012, Lowther et al., 2013a), future studies should aim to quantify colony dietary niche of sea lions. Insights from such studies would enhance our understanding of bacterial diversity in microbial communities of sea lions from geographically disparate colonies. Long-term monitoring of faecal microbial communities, coupled with environmental sampling would facilitate a greater understanding of the role the biological environment plays in facilitating microbe transmission.

Conclusion

As coastal settlements expand, encroachment on natural habitats and increasing interactions between humans and marine wildlife populations poses significant risks for the introduction of non-endemic microorganisms to sensitive marine populations. As human and terrestrial genotypes of parasitic protozoa, and pathogenic microbes commonly identified in clinical cases are increasingly observed in pinniped populations, it is important that we bridge gaps in knowledge of microorganism dissemination to marine ecosystems (Bailey et al., 2015; Benton et al., 2014; Bossart, 2011). Microbial and protozoal monitoring protocols are non-invasive, and can be applied to advancing understanding of potential contamination sources of marine ecosystems, allowing for the development of effective conservation strategies aimed at reducing anthropogenic impacts to marine mammals.

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Appendices

Appendix A: *Giardia duodenalis* in Australian sea lions media report.

Friday, 17 October 2014

WA sea lions infected with high rate of Giardia

Written by Laura Glitsos (/topics/fisheries-a-water/itemlist/user/184-lauraglitsos) (/topics/fisheries-a-water/item/3131-wa-sea-lions-infected-with-high-rate-of-giardia?tmpl=component&print=1)

Little is known of the effect of Giardia infection on Australian sea lions. *Image: Nicolò Bonazzi (https://www.flickr.com/photos/8542653@N03/3446844581)*

A STARTLING proportion of West Australia's sea lion population is infected with the parasite *Giardia duodenalis*, research (http://www.sciencedirect.com/science/article/pii/S2213224414000285) has found.

Macquarie University's Tiffany Delport used genomic sequencing to test the faecal samples of wild and captive Australian sea lions (*Neophoca cinerea*), searching for the tell-tale DNA of parasites *Giardia* and *Cryptosporidium*.

Ms Delport found the rate of *Giardia* infection was highest in captive sea lions, closely followed by Australian sea lion colonies situated within 25km of human coastal settlements.

In WA, Beagle and North Fisherman Islands are home to two wild sea lion colonies, 17.7km and 21.6km from human settlement respectively.

Ms Delport says of the 30 faecal samples collected from these islands, 12 were positive for *Giardia*—an infection rate of about 40 per cent.

Parasite dispersed from human sources

Transmitted through the ingestion of contaminated food and water sources, *Giardia* can spread from human to mammal, and from land mammal to marine mammal, Ms Delport says.

"Taking steps to minimise environmental pollution with human and domestic animal faecal material infected with cysts may reduce the spread of *Giardia* to mammalian wildlife populations," she says.

"It is also important to consider that *Giardia* cysts are environmentally robust, capable of surviving extended periods of time outside of a host."

Ms Delport says in humans and domestic animals, *Giardia* can cause diarrhoea and loss of appetite.

"However, very little is known about the effects that *Giardia* has on many wildlife hosts, including marine mammals," she says.

"As *Giardia* is observed in many marine mammal species, including endangered animals such as the Australian sea lion, it is important that future investigations monitor the impact of infection on host health and identify potential pathways from humans and terrestrial animals to wildlife populations."

Ms Delport did not find evidence of *Cryptosporidium* cysts in wild or captive sea lion populations.

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