Exploring the diversity of human-associated microbes and antimicrobial resistance in three species of Australian pinniped pups

A thesis submitted in fulfilment of the requirements for the degree of
Doctor of Philosophy
by

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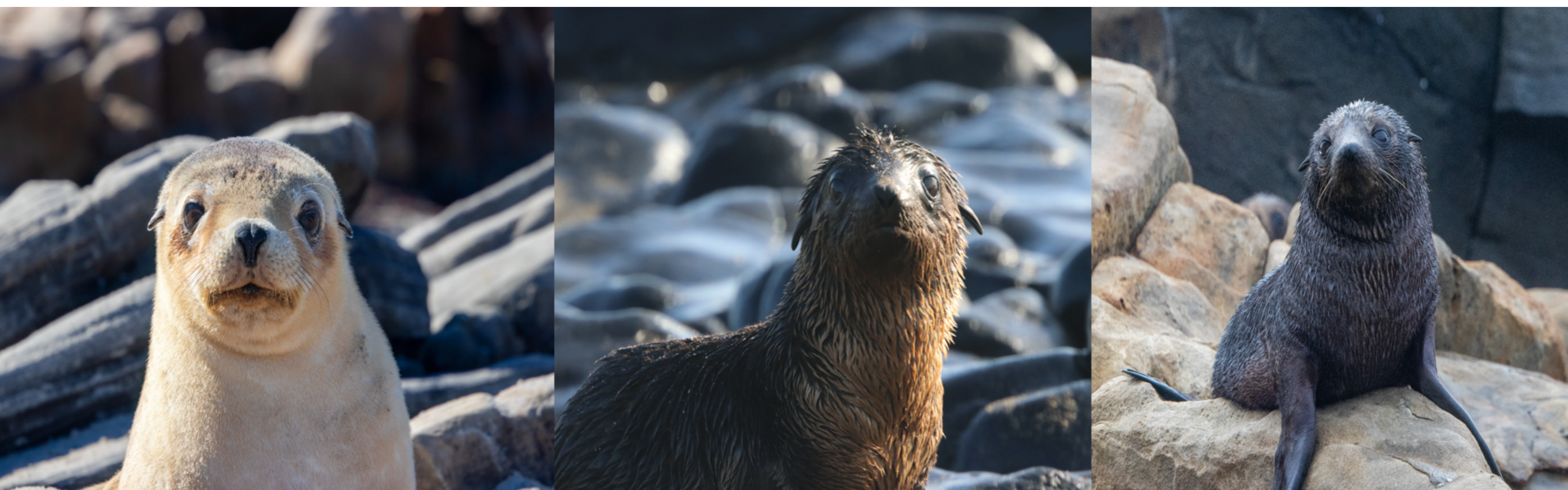

Statement of originality

To the best of my knowledge, the content presented in this thesis is my own work. All information derived from both published and unpublished sources has been acknowledged and referenced within this thesis. I declare that this thesis has not been submitted for any degree or other purpose at this or any other institution.

Mariel Fulham

29th August 2022

Summary of the thesis

The marine environment is being increasingly impacted and degraded by anthropogenic processes and pollution, threatening the ecosystems that marine mammals rely on for survival. Preliminary investigations identified the presence of class 1 integrons, genetic determinants of antimicrobial resistance, and human-associated phylotypes of *Escherichia coli* in free-ranging Australian sea lion (*Neophoca cinerea*) pups. However, no previous investigations have been conducted in sympatric pinniped species, the Australian fur seal (*Arctocephalus pusillus doriferus*) and the long-nosed fur seal (*Arctocephalus forsteri*). These findings were the impetus for this thesis to investigate the presence and diversity of *Escherichia coli* and class 1 integrons in three species of free-ranging pinniped pups that inhabit Australian waters. Understanding the presence of human-associated bacteria and antimicrobial resistance genes, both of which can be used as indicators of anthropogenic microbial pollution, is paramount for assessing the impact of anthropogenic processes on free-ranging pinniped populations.

Chapter 1 of this thesis outlines the context of this study, describing the life histories and key threats faced by *A. p. doriferus*, *A. forsteri,* and *N. cinerea* populations in Australia. The concept of anthropogenic microbial pollution is explored through the introduction of antimicrobial resistance and *E. coli*, and the importance of the gut microbiome discussed. The aims and objectives of this thesis are also outlined.

The carriage of antimicrobial resistant bacteria and resistance genes in free-ranging Australian pinniped pups is described in **Chapter 2**. Antibiotic resistance genes encoding resistance to four classes of antibiotics were detected in faecal DNA and *E. coli* isolated from *A. p. doriferus* and *N. cinerea* pups at six breeding colonies over multiple breeding seasons from 2016-2019. This chapter identified the presence of antimicrobial resistant bacteria in free-ranging pinniped pups in Australia, suggesting that these species are exposed to anthropogenic microbial pollution.

Chapters 3 and 4 explore the distribution and diversity of *E. coli* in free-ranging Australian pinniped pups. The presence and diversity of *E. coli* in *A. p. doriferus*, *A. forsteri* and *N. cinerea* pups from eight breeding colonies was characterised in **Chapter 3**, finding a dominance of the human-associated *E. coli* B2 phylotype across all species. There was no significant difference in the distribution of human-associated *E. coli* phylotypes across the

eight breeding colonies, indicating that pinniped colonies along the southern Australian coast are vulnerable to anthropogenic pollution. **Chapter 4** further explored *E. coli* in pinniped pups, characterising multiple *E. coli* isolates from each pup to determine whether a single *E. coli* isolate is representative of intra-individual phylotype diversity. The phylotype diversity was not significantly different within pups from each species, indicating that analysing a single *E. coli* isolate could be used as a fast and simple technique to assess the basic characteristics of *E. coli* in free-ranging pinniped pups.

The gut microbiota of *N. cinerea* pups and the impact of topical ivermectin treatment is addressed in **Chapter 5**, answering a key knowledge gap that needs to be considered when assessing human intervention as a management strategy for this endangered species. The gut microbiota of untreated (control) and treated *N. cinerea* pups was characterised, finding that topical ivermectin treatment and the removal of hookworm infection did not have any significant impact on the composition of the gut microbial community. The results from this study indicate that topical ivermectin treatment could be a safe and effective method to aid in the conservation of endangered *N. cinerea* pups.

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Author Contributions

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Mariel Fulham was the primary author of this chapter and contributed to all aspects of this publication including the design and conceptualization of the study, sample collection, methodology, analysis (laboratory, sequence and statistical), acquisition of funding and writing of the manuscript. Fiona McDougall contributed to methodology, sequence analysis and annotation. Rebecca R. McIntosh contributed to acquisition of funding and sample collection. Michelle Power contributed to conceptualization and design of the study, supervision and methodology. Rachael Gray contributed to study design and conceptualization, funding acquisition, sample collection, methodology and supervision. All authors reviewed and contributed to the editing of the final manuscript.

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In all publications mentioned above, the corresponding author (Dr Rachael Gray) has provided permission to include published material.

Mariel Fulham

26.08.2022

Attesting Author Contribution Statement

As the primary supervisor for the candidature upon which this thesis is based, as well as the corresponding author for all publications included, I can confirm that the authorship attribution statements above are correct.

Rachael Gray

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Peer-reviewed publications arising from candidature

- **Fulham, M,** Power, M, Gray, R. 2020. Diversity and distribution of *Escherichia coli* in three species of free-ranging Australian pinniped pups. *Frontiers in Marine Science*. 7:571171. https://doi.org/10.3389/fmars.2020.571171
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Additional Scientific Contributions

Co-supervisor of Animal and Veterinary Bioscience Honours Student, Bridget Webster

"Investigating the phylotype diversity of *Escherichia coli* in three species of Australian pinniped pups"

January – November 2019

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Thesis Abstract

Contamination of coastal marine environments with human-associated microbes and antibiotic resistance genes is continually increasing, impacting marine ecosystems and the wildlife species therein.

Australian fur seals (*Arctocephalus pusillus doriferus*), long-nosed fur seals (*Arctocephalus forsteri*) and Australian sea lions (*Neophoca cinerea*) inhabit numerous coastal and offshore sites along the Australian coastline. The overarching aim of this thesis was to investigate the presence of human-associated *Escherichia coli*, an indicator of faecal contamination, and class 1 integrons, genetic determinants of antimicrobial resistance, in neonatal pinniped pups. Faecal samples were collected from *A. p. doriferus*, *A. forsteri* and *N. cinerea* pups from eight breeding colonies between 2016-2021. *Escherichia coli* was cultured from faecal swabs and DNA was subsequently extracted from *E. coli* isolates and a subset of faecal swabs.

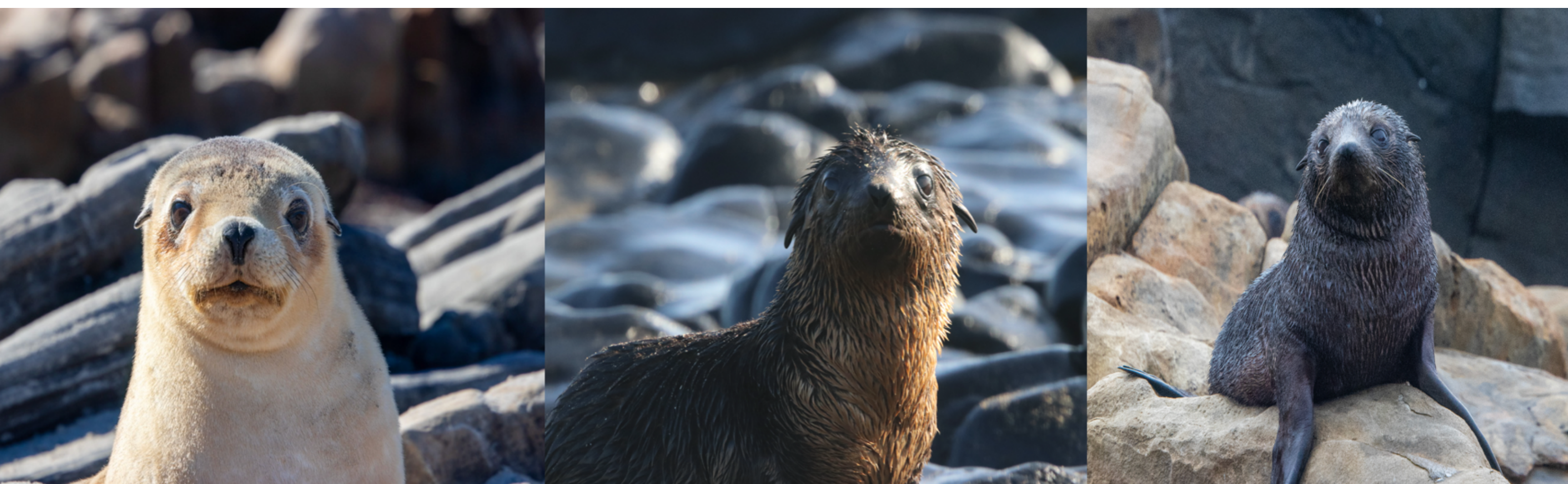
Phylotype diversity was not significantly different across species or breeding colonies and the human-associated *E. coli* B2 phylotype was the most prevalent phylotype across all species. Class 1 integrons were isolated from *E. coli* (*n*=36, 4.52%) and faecal DNA (*n*=15, 4.85%) in *A. p. doriferus* (*n*=40) and *N. cinerea* (*n*=11) pups but were absent in *A. forsteri* pups.

Finally, the faecal microbiota of endangered *N. cinerea* pups was characterised to investigate the impact of anthelmintic treatment to eliminate hookworm infection on the composition of the gut microbiota. Topical treatment did not significantly change the microbial composition, suggesting that it is a safe and minimally invasive management strategy to aid in the conservation of endangered *N. cinerea* pups.

The high prevalence of human-associated *E. coli* in addition to the detection of antimicrobial resistance genes highlights the imperative for ongoing monitoring and surveillance of microbes in marine sentinel species.

Chapter 1

Introduction, literature review, and aims of thesis



List of abbreviations used

3’-CS	3’ conserved segment
5’-CS	5’ conserved segment
AMR	antimicrobial resistance
ARB	antibiotic resistant bacteria
ARG	antibiotic resistance genes
GF	germ-free
HGT	horizontal gene transfer
intI	integron-integrase gene
intI1	class 1 integron-integrase gene
ISs	insertion sequences
MGE	mobile genetic element
MI	mobile integron
ORF	open reading frame
POPs	persistent organic pollutants
PFAS	per and polyfluoroalkyl substances
WWTP	wastewater treatment plant

1.1.Introduction

Marine ecosystems across the globe are being increasingly contaminated with pollutants from anthropogenic sources and contaminants have been found in even the most pristine environments, including Antarctica (Ansari and Matondkar, 2014; Beiras, 2018; Häder et al., 2020; Hernández et al., 2019, 2012; Power et al., 2016; Reisser et al., 2013). The majority of the Earth's surface is covered by marine environments (~71%), 7% of which are coastal areas that support a diverse array of organisms (Davidson et al., 2012; Häder et al., 2020). The increase in human populations near coastal ecosystems and constant growth in recent decades has resulted in rapidly increasing levels of anthropogenic pollution, pressure, and subsequent degradation of coastal environments (Pommepuy et al., 2006; Pompa et al., 2011). Coastal environments with cold currents and upwellings support a broad range of marine and terrestrial species (Davidson et al., 2012; Pompa et al., 2011), however, the species that inhabit coastal environments experience some of the highest levels of human impact, with those that live, forage and breed along coastlines at higher risk of exposure to threats and pollutants from anthropogenic sources (Davidson et al., 2012; P. J. H. Reijnders et al., 2009). Increasing anthropogenic activities are also contributing to unprecedented rates of extinction events of wildlife at both a population and species level (Pompa et al., 2011).

Marine environments are contaminated by a multitude of pollutants, including anthropogenic chemicals (heavy metals, organohalogen and organochlorine compounds e.g. persistent organic pollutants [POPs]) (Catania et al., 2020; Häder et al., 2020; Tchounwou et al., 2012), marine debris (Denuncio et al., 2017; Reisser et al., 2013), oil pollution from spills (Helm et al., 2014), and runoff from sewage and agriculture (Anastasi et al., 2012, 2010; Ansari and Matondkar, 2014; Beiras, 2018; Reisser et al., 2013). The presence of faecal coliform bacteria, such as *Escherichia coli*, is commonly used as an indicator of the contamination of

coastal marine environments with sewage and faecal bacteria from human sources (Ahmed et al., 2016; Beversdorf et al., 2007; Schaefer et al., 2011). While the presence of human-associated faecal coliforms is primarily monitored due to potential risks to human health, detection of these bacterium in wildlife species are increasingly being utilised as indicators of exposure to anthropogenic pollution (Ahmed et al., 2016; Schaefer et al., 2011). In addition to the presence of human-associated bacteria, there is increasing recognition of antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) as important emerging contaminants of marine ecosystems and indicators of exposure to anthropogenic pollution in wildlife species (Grilo et al., 2020).

The presence of ARB in coastal areas has increased in recent decades and is widespread, both in the environment and in wildlife species that inhabit these areas (Dolejska et al., 2007; M Fulham et al., 2018; Gillings et al., 2014; Lundbäck et al., 2020; Paulson et al., 2016). Investigations into the presence of ARB and ARGs in free-ranging wildlife have identified that animals living in closer proximity to human populations and activities are more likely to carry ARB and ARGs (Allen et al., 2010). Given the increasing human disturbance and pollution of coastal environments that many species rely on for survival, focussing on the impact of anthropogenic pollution on free-ranging wildlife species is paramount.

The increasing and constant pollution of coastal ecosystems has led to the consideration of wildlife species that inhabit these environments, mainly marine mammals, as sentinels for ecosystem health (Bossart, 2010; Furness et al., 2017; Hazen et al., 2019; Moore, 2008; Plaza-Rodríguez et al., 2021). Marine mammals are a diverse group of species belonging to three orders: Carnivora (sea otters, pinnipeds and polar bears), Cetacea (whales, dolphins and porpoises) and Sirenia (manatees and dugongs) (Fair and Becker, 2000). Marine mammals are generally long-lived, upper trophic level predators that are both physiologically

and anatomically adapted to aquatic environments and dependent upon healthy marine ecosystems for their survival (Moore, 2008). It is because of this dependency that marine mammals have been proposed as sentinels of ecosystem health (Bossart, 2010; Hazen et al., 2019; Moore, 2008), with studies to date focussing on the investigation of heavy metals, viruses and pathogenic bacteria (Bossart, 2010; Moore, 2008), largely with respect to potential crossover to humans and subsequent impact on human health. Many species of marine mammals have experienced severe population depletion through harvesting and provide some of the most well-known examples of population and species extinction through over-harvesting (Lancaster et al., 2010; Pompa et al., 2011).

Marine mammals continue to face a variety of threats, including interactions with fisheries (Avila et al., 2018; Page et al., 2004; Shaughnessy et al., 2003), entanglement in marine debris (Denuncio et al., 2017; Reisser et al., 2013), climate change (e.g. rising sea levels and temperatures) (Han et al., 2010; McLean et al., 2018; Schumann et al., 2013), habitat degradation, reduction in prey availability through overfishing (Bearzi et al., 2006; DeMaster et al., 2001; Kaschner et al., 2011; McLean et al., 2018; Myers and Worm, 2003), and exposure to a variety of pollutants, including noise, chemicals, oil, heavy metals, pesticides and sewage (di Cesare et al., 2020; Fair and Becker, 2000; Karkman et al., 2019; Marcovecchio et al., 1994). The diversity of life histories, breeding strategies, behaviour, geographic distribution, and anatomical and physiological adaptations of marine mammals results in differing degrees of exposure to anthropogenic pressures, activities, and contaminants between species (Ashley et al., 2020; Fair and Becker, 2000; Kovacs et al., 2012; Kretzmann et al., 2010; Kreuder et al., 2003; Schipper et al., 2008; Van Bressem et al., 2009). However, marine mammal species are still greatly impacted by anthropogenic pressures, with 10% of all marine mammals considered vulnerable, 11% endangered and 3% critically endangered (Pompa et al., 2011).

Pinnipeds, in particular, seem to face a higher level of risk, with one in three species considered threatened (Kovacs et al., 2012), likely as a result of their reliance on the marine environment for foraging and terrestrial environments for breeding and hauling out. Pinnipeds also have more restricted geographical ranges and generally only occur in highly productive areas (Kovacs et al., 2012) due to their need to forage and return to land every few days when raising young. Species inhabiting coastal environments experience higher levels of human impact (Davidson et al., 2012), and given that most pinniped species inhabit breeding colonies in coastal environments, more emphasis should be placed on monitoring the impact of human activities on these species.

An emerging field in the investigations into marine mammal health is characterisation of the gut microbiome. In recent years, an increase in the understanding of the role of the gut microbiome for host health have shown that a healthy microbiome is essential for absorption of nutrients and vitamins, regulating cognition and behaviour, development of the immune system, and providing protection against pathogens (Kreisinger et al., 2015; Lynch and Pedersen, 2016; Martin et al., 2019). Disturbances to the gut microbiota caused by parasitic infections (Brosschot and Reynolds, 2018; Cortés et al., 2019; Giacomin et al., 2015), antibiotics (Modi et al., 2014), and dietary changes (Albenberg and Wu, 2014; Conlon and Bird, 2014; Turnbaugh et al., 2009) can result in imbalances to the microbial community and has been associated with the development of disease (Lynch and Pedersen, 2016). The interactions between the host gut microbiota and parasites, in particular, has received considerable attention due to the potential impact that parasites can have on the microbiome composition and/or modulation of immune responses (Kreisinger et al., 2015), however, this data has primarily come from studies in humans and mice (Brosschot and Reynolds, 2018; Cortés et al., 2019; Kreisinger et al., 2015; Lee et al., 2014; Reynolds et al., 2015). The

importance of the microbiome for host health highlights the need for investigations into the gut microbiome of pinnipeds, to provide enhanced understanding of which factors contribute to alterations and potentially disease.

The remainder of this chapter will introduce three species of Australian pinnipeds, the Australian fur seal (*Arctocephalus pusillus doriferus*), the long-nosed fur seal (*Arctocephalus forsteri*) and the Australian sea lion (*Neophoca cinerea*) and outline the current threats and knowledge gaps for these species. The utility of using class 1 integrons and *Escherichia coli* as indictors of anthropogenic pollution will then be reviewed. The final component of this review will focus on the potential impact of antiparasitic treatment on the gut microbiome and implications this may have for conservation strategies, followed by the aims of the thesis.

1.2. Pinniped species

Pinnipeds belong to the Pinnipedia suborder which consists of three extant families, Otariidae (sea lions and fur seals), Phocidae (true seals) and Odobenidae (walrus). There are fifteen species of sea lions and fur seals (otariids), commonly referred to as 'eared seals', that have significant anatomical and physiological differences to the closely related phocids (Jefferson et al., 2015). Otariids have external pinnae and are agile on land due to their ability to rotate their hindflippers in a forward motion. In contrast, phocids or 'true seals' lack external pinnae and rely mainly on body movement in an undulating motion to facilitate movement on land. One characteristic that is shared by all pinnipeds, however, is their need to return to a terrestrial environment to breed and raise offspring.

Australian coastlines are home to three species of pinnipeds, Australian fur seals (*Arctocephalus pusillus doriferus*), long-nosed fur seals (*Arctocephalus forsteri*) and Australian sea lions (*Neophoca cinerea*). These species inhabit numerous offshore colonies along the Australian coast from Western Australian to Tasmania and frequently overlap across their

geographic ranges (Figure 1) (Kirkwood and Goldsworthy, 2013). All three species of pinnipeds were subjected to sealing practices during the 19th and 20th centuries, with populations of Australian pinnipeds being severely depleted by 1809 (Ling, 1999). While all three pinniped species were hunted, different numbers were harvested from each species with the majority of skins (up to 244,000) coming from *A. p. doriferus* in the Bass Strait, followed by *A. forsteri* and to a lesser extent, *N. cinerea* (Kirkwood and Goldsworthy, 2013; Ling, 2002, 1999). Since the cessation of sealing and protection of all seals in Australia under the National Parks and Wildlife Conservation Act (Shaughnessy, 1999), each species has differed greatly in their recovery, reflected in their current distribution and varied population trends, as outlined in sections 1.2.1-1.2.4 of this review.

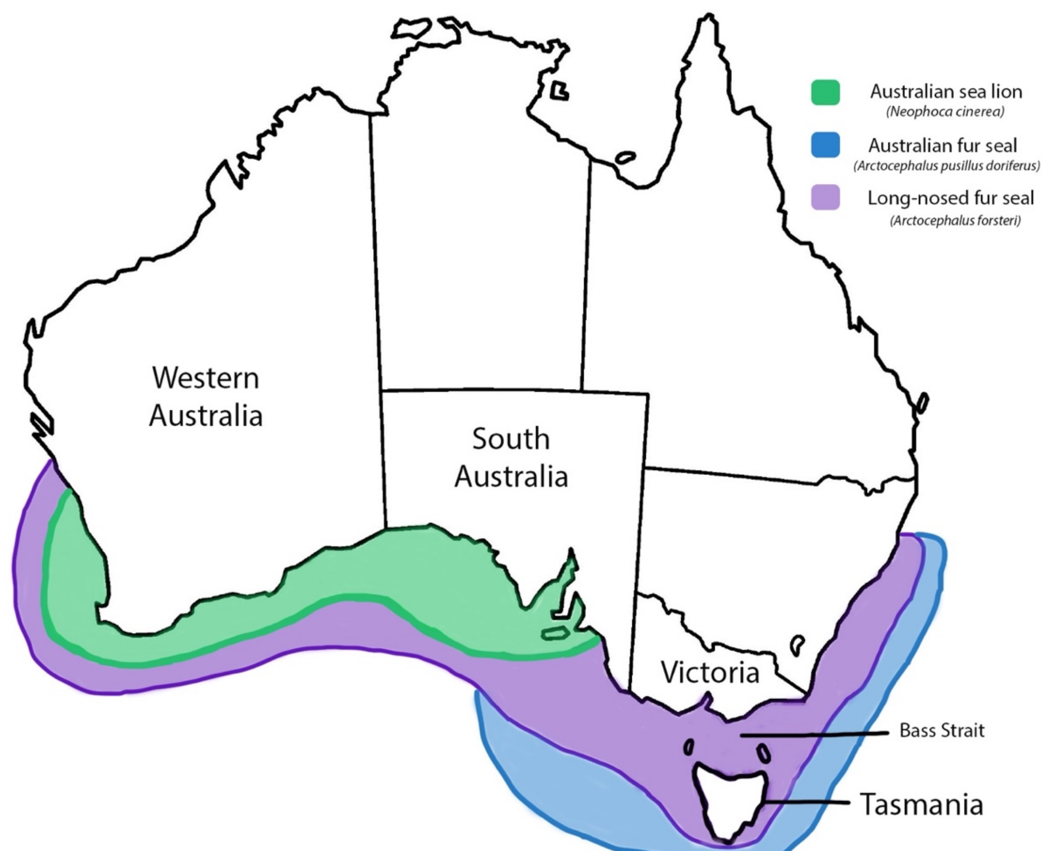


Figure 1. Map of the distribution of *A. p. doriferus, A. forsteri*, and *N. cinerea* along the Australian coastline, ranging from the Houtman Abrolhos Islands (Western Australia) through Bass Strait and Tasmania and along the eastern coast to Queensland.

1.2.1. Australian fur seals (*Arctocephalus pusillus doriferus*)

Arctocephalus pusillus doriferus breed mainly on small islands in the Bass Strait, between mainland Australia and Tasmania, across approximately 20 colonies (Kirkwood et al., 2010; McIntosh et al., 2018). Foraging predominantly over shelf waters of south-eastern Australia and within the Bass Strait, *A. p. doriferus* are a prominent marine mammal species (Kirkwood et al., 2010; Kirkwood and Arnould, 2012). The *A. p. doriferus* population in Australia was protected through the enforcement of the Environment Protection and Biodiversity Conservation Act 1999 (EPBC Act), as well as individual state legislation: National Parks and Wildlife Act 1972 (South Australia), Wildlife Act 1975 (Victoria), Threatened Species Protection Act 1995 and Nature Conservation Act 2002 (Tasmania) and the Threatened Species Protection Act 1995 (New South Wales). While the population has begun to recover since protection was enforced, the overall recovery of *A. p. doriferus* has been slower than that of other fur seal species. For example, the Cape fur seal (*Arctocephalus pusillus pusillus*) has increased from <100,000 individuals post-sealing to a current estimated population size of 1.5-2 million individuals (Arnould et al., 2003; Butterworth et al., 1995; Kirkman et al., 2007). In contrast, the most recent estimation of the *A. p. doriferus* population size suggests approximately 89,500 individuals with annual pup production at ~16,000 live pups (McIntosh et al., 2022), lower than the estimated pre-sealing numbers of up to 200,000 individuals and ~50,000 pups produced each season (Lancaster et al., 2010; Ling, 2002; Warneke and Shaughnessy, 1985).

A growth in population was observed from 1986-2002 and sustained at >5% per annum, followed by a stabilisation in pup production between 2002-2007, and a subsequent overall reduction in pup production was identified in the 2013-14 census (McIntosh et al., 2018). This decline continued in the most recent 2017-18 census with combined live pup

numbers reported at 16,903 in comparison to 17,503 reported in 2013 and 21,589 in 2007 (McIntosh et al., 2022). The decline in pup production is thought to be predominantly due to human activities including interactions with fisheries (McIntosh et al., 2015), climate change (for example rising sea levels, alterations to prey availability and food webs) (McLean et al., 2018), and anthropogenic pollutants, including POPs and per and polyfluoroalkyl substances (PFAS) (Taylor et al., 2021, 2018). The overall decline following a period of population increase and recovery highlights the need for ongoing monitoring to determine the factors that are limiting population recovery. Even with this decline, *A. p. doriferus* are listed as 'least concern' on the IUCN Red List (Hofmeyr, 2015).

Arctocephalus pusillus doriferus exhibit a high degree of sexual dimorphism: adult males are dark brown and weigh an average of 270kg and 2-2.3m standard length but can reach up to 360kg; adult females are light-dark brown with a lighter cream-coloured underside and weigh an average of 75kg and up to 110kg with standard length 1.4-1.7m (Figure 2) (Kirkwood and Goldsworthy, 2013). Pups are dark brown to black with a lighter brown underside and generally range between 5-12kg in weight and 60-80cm standard length at birth (Figure 2). Females reach sexual maturity earlier than males at 3-6 and 5 years of age, respectively, however males are unable to hold and establish mating territories until they are 8-13 years of age (Kirkwood and Goldsworthy, 2013). This species has a 12-month gestation period with a 3-month diapause; pupping occurs annually from early November until mid-December, usually reaching a peak in late November or early December and is synchronised across the species' range (Kirkwood et al., 2005; Kirkwood and Goldsworthy, 2013; Warneke and Shaughnessy, 1985). Weaning coincides with the breeding season with pups weaned when they are 10-12 months of age before the next breeding season begins.

Figure 2. Morphology of *A. p. doriferus* at differing life stages. (A) adult female (left) and male (right); (B) adult female with pup; (C) juvenile; (D) pup.

1.2.2. Long-nosed fur seals (*Arctocephalus forsteri*)

In Australia, *Arctocephalus forsteri* (now long-nosed fur seals, previously known as New Zealand fur seals) range from Western Australia to New South Wales, including Tasmania, breed predominantly in South Australia, and are also found in New Zealand and its subantarctic islands (Shaughnessy et al., 2015; Shaughnessy and Goldsworthy, 2020). Like *A. p. doriferus*, initial population recovery of *A. forsteri* was slow but has benefitted from protection under the EPBC Act 1999, as well as individual Acts in Australian states as mentioned in section 1.2.1, with the additional Wildlife Conservation Act 1950 in Western Australia. Long-term monitoring programs have shown continual population increase, establishment of new breeding colonies and expansion of the species' geographical range in

recent decades, with at least 40 breeding colonies now established (Shaughnessy et al., 2015; Shaughnessy and Goldsworthy, 2020). Of the three pinniped species that breed in South Australia, *A. p. doriferus, N. cinerea* and *A. forsteri*, *A. forsteri* is the most abundant (Shaughnessy and Goldsworthy, 2015). Some small declines in pup production have been observed, most likely as a result of decreases in prey availability arising from altered environmental conditions (Baylis et al., 2008; Shaughnessy and Goldsworthy, 2015), however, the general population trend is increasing, making it one of the few marine mammal species that have continued to increase in recent decades. A large proportion of the South Australian population is located on Kangaroo Island, where there has been an exponential increase in pup production (Shaughnessy et al., 2014). In the 2013-14 breeding season, approximately 20,000 pups were produced across 29 South Australian breeding colonies, giving an estimated population size of 97,200 in South Australia and a total Australian population of ~150,000 individuals (Kirkwood and Goldsworthy, 2013; Shaughnessy et al., 2015). The species is listed as 'least concern' on the IUCN Red List (Chilvers and Goldsworthy, 2015).

Arctocephalus forsteri are the smallest of the three pinniped species that inhabit Australian waters; males are larger than females weighing 120-180kg with standard lengths ranging from 150-250cm; females weigh 35-55kg, with standard lengths between 100-150cm (Figure 3) (Kirkwood and Goldsworthy, 2013). Pups are small at birth weighing between 4-6kg with standard lengths of 60-70cm. This species is visually similar to *A. p. doriferus* and often hard to distinguish based on colour alone, with males tending to be grey-dark brown, females grey-light brown with a lighter chest and throat and pups grey-black with light grey-brown undersides (Figure 3). *Arctocephalus forsteri* exhibit a typical otariid 12-month gestation and reproductive cycle; they breed and pup annually during the Austral summer with 90% of pups born between late November and early January. On Kangaroo Island, where a majority of the

South Australian pup production occurs, the peak of pupping generally occurs around the 25-26th December (Kirkwood and Goldsworthy, 2013). Both females and males are considered sexually mature at 4-5 years of age, however, like other otariids, males are generally not successful breeders or able to hold territories until they reach 9-12 years of age.

Figure 3. Morphology of *A. forsteri* at different life stages. (A) adult female (left) and male (right); (B) adult female with pup; (C) juvenile; (D) pup.

1.2.3. Australian sea lions (*Neophoca cinerea*)

Australian sea lions (*Neophoca cinerea*) are Australia's only endemic pinniped species and one of the rarest and most endangered pinniped species in the world, classified as endangered on the IUCN Red List (Goldsworthy et al., 2015) and recently up listed to endangered under Australian legislation through the EPBC Act (Committee, 2020). In addition, the *N. cinerea* population is protected under legislation in Western Australia and South Australia, as listed

in section 1.2.1 and 1.2.2. Their range extends from the Houtman Abrolhos Islands off the coast of Western Australia to the Pages Islands in South Australia with breeding occurring across approximately 80 colonies with the majority (50) located in South Australia (Kirkwood and Goldsworthy, 2013). The population is in a continual decline with an estimated population size of 10, 402 individuals and a species-wide pup abundance of 2739 (Goldsworthy et al., 2021), making it the rarest pinniped in Australia.

The species has an extended 17.5-month breeding cycle that is temporally asynchronous, with pupping occurring at each colony at different times, regardless of colony proximity (Gales et al., 1994; Higgins and Gass, 1993; Kirkwood and Goldsworthy, 2013; Shaughnessy et al., 2011). The majority of *N. cinerea* pups are born over a 4–5-month period although breeding seasons of 7-9 months have been observed, unlike most pinniped species where breeding seasons occur over 1-2 months (Goldsworthy et al., 2012; McIntosh et al., 2012). This species also has the longest gestation period of any pinniped at a total of 18 months consisting of a 4–5-month diapause followed by a post-implantation period of up to 14 months (Gales et al., 1997). Female *N. cinerea* show a high level of maternal investment; pups are not weaned until they reach 15-18 months of age (Higgins and Gass, 1993; McIntosh et al., 2012) and may continue to suckle until they are 3-4 years of age depending on whether their mothers have successfully mated and if subsequent pups survive. This atypical extended gestation and breeding cycle has been suggested as a contributing factor to the continual population decline of this species as it limits the number of individuals that can be produced over the sealions lifetime.

Like *A. p. doriferus* and *A. forsteri*, there is clear sexual dimorphism in *N. cinerea*. Males weigh between 180-250kg with a standard length of 1.85-2.5m and distinct dark brown colouring with a yellow-cream coloured ‘crown’ on the top of their heads; adult females

weigh 61-104kg with standard length ranging from 1.3m-1.85m and are light brown-grey with a cream-coloured underside (Figure 4) (Kirkwood and Goldsworthy, 2013). *Neophoca cinerea* pups weigh between 6-8kg, have standard lengths of 62-68cm, and are dark brown/black at birth becoming lighter brown in the early neonatal period (Figure 4). Females reach sexual maturity earlier than males, breeding from ~4.5 years of age, while males are considered sexually mature at approximately 5 years of age, but generally unable to successfully contend with other males and breed until 7.5-12 years of age (Kirkwood and Goldsworthy, 2013).

Figure 4. Morphology of *N. cinerea* at differing life stages. (A) adult male; (B) adult female with pup; (C) juvenile; (D) pup.

1.2.4. Current threats to species and knowledge gaps

These three Australian pinniped species share many threats due to their overlapping geographical ranges and the location of breeding colonies in coastal environments on

offshore islands. As previously mentioned, coastal environments experience high levels of human impact, and marine mammal species that breed, forage and inhabit colonies along the coast face continual threats (Davidson et al., 2012) including climate change (Han et al., 2010; McLean et al., 2018), increasing interactions with humans and anthropogenic activities (Curtis et al., 2021; DeMaster et al., 2001; Gales et al., 2003; Hamer et al., 2013; Hamer and Goldsworthy, 2006), exposure to anthropogenic pollutants (including toxicants) (Ashley et al., 2020; Davidson et al., 2012; Kovacs et al., 2012; Taylor et al., 2021, 2018) and disease (Marcus et al., 2015a; Shapiro et al., 2012; Stoddard et al., 2008, 2005). Although these threats may be shared, the ability of each species to respond to and overcome threats will differ greatly depending upon their life histories and current population predictions. *Neophoca cinerea*, for example, have a small population size such that the loss of even a few individuals could have an impact on the population (Goldsworthy et al., 2009a).

In recent decades, the most prominent threats to marine mammals have been accidental mortality as a result of interactions with humans and human activities (e.g. fisheries interactions) (Gales et al., 2003; McLeay et al., 2015; Shaughnessy et al., 2003), and pollution (Schipper et al., 2008). Indirect fisheries interactions are one of the main threats to pinnipeds, often causing injury and death as a result of entanglement in fishing gear and nets, competition for prey, and alterations in the ecosystem due to exploitation by fishing industries (Curtis et al., 2021; Kovacs et al., 2012; McIntosh et al., 2015; Page et al., 2004). All three species of pinnipeds in South Australian waters have been known to interact with a variety of fishing industries, with severity of these interactions depending on the pinniped species itself (Hamer et al., 2013; Hamer and Goldsworthy, 2006; Page et al., 2004; Shaughnessy et al., 2003).

In Australia, there is extensive overlap between the foraging ranges of *N. cinerea* and gill-net and lobster fisheries in South Australia (Goldsworthy et al., 2009b; Hamer et al., 2013). Interactions with gill-net fisheries have resulted in high levels of mortality due to bycatch, primarily affecting juveniles and adult females (Campbell et al., 2008; Hamer et al., 2013) and has been mitigated to some extent by the implementation of management strategies by the Australian Fisheries Management Authority (Page et al., 2004). The impact of these changes on the Australian sea lion population are yet to be fully understood and it will likely take more time to quantify.

Increasing population abundance has increased interactions between *A. forsteri* and gill-net operations in South Australia resulting in depredation of catch and damage to fishing gear (Goldsworthy et al., 2009b). However, this has not resulted in increased bycatch or entanglement, with no mortalities reported from 2010-2014 (McLeay et al., 2015), suggesting that the threat of fisheries may be reduced for this species (Shaughnessy et al., 2003, 2014).

Arctocephalus pusillus doriferus are also known to interact with fisheries and appear to have higher rates of entanglement in comparison with other Australian pinniped species (Shaughnessy et al., 2003). Interactions mainly occur with trawl fisheries which lead to bycatch mortality (Hamer and Goldsworthy, 2006). The inclusion of seal exclusion devices in trawl fisheries has resulted in a reduction in the number of seals being trapped and appears to have reduced the severity of these interactions for this species (Hamer and Goldsworthy, 2006).

Rising sea levels and temperatures, acidification, and increases in extreme weather conditions are all changes that are expected to impact the marine environment as a result of climate change (Poloczanska et al., 2007). In addition to the threat of increased sea surface temperatures, rising sea levels are likely to result in a reduction in breeding habitat, which

will have differing impacts on each pinniped species. For example, *A. p. doriferus* have limited genetic structuring with high levels of gene flow and colony mixing and it is likely that individuals could easily relocate to different breeding colonies if breeding habitat is reduced (Kirkwood et al., 2005; Lancaster et al., 2010). In comparison, male and female *N. cinerea* exhibit a high degree of philopatry, with females returning to their natal colony to breed and males only dispersing between closely located colonies (<110km) (Ahonen et al., 2016; R. A. Campbell et al., 2008; Lowther et al., 2012). Given that each colony breeds at a different time, it is unlikely that females would be able to move between colonies and successfully breed, and the ability for individuals to relocate and establish at a new location is unknown. Furthermore, the unique, temporally asynchronous reproductive cycle of *N. cinerea* makes population recovery more challenging as the extended breeding period limits the number of individuals that can be recruited into the breeding population (Gales et al., 1997; Higgins and Gass, 1993).

Pollutants in the marine environment are one of most well-known factors that impact the health of marine mammals (Fair and Becker, 2000; Kovacs et al., 2012). In this case, 'pollutants' refer to agricultural and industrial chemicals, sewage and wastewater runoff, toxic algal blooms, metals and oil pollution (Bossart, 2010; Fair and Becker, 2000; P. J. H. Reijnders et al., 2009). Organochlorine and organohalogen compounds are persistent and bioaccumulative such that they can persist in the environment for long periods of time without degrading resulting in bioaccumulation, especially in lipid-rich tissue such as blubber (Fair et al., 2010; Fair and Becker, 2000; P. J. H. Reijnders et al., 2009). Persistent organic pollutants (POPs) are a type of organohalogen that have been identified in a range of pinniped species and can cause a multitude of pathologies, reproductive impairment and can suppress the immune system, making individuals more vulnerable to other pathogens (Ashley et al.,

2020; Reijnders et al., 2009; Taylor et al., 2021). Per and polyfluoroalkyl substances (PFAS) including perflurooctanoic acid (PFOA) and perflurooctane sulfonate (PFOS) have also been found in Australian pinnipeds, with higher concentrations in pups and juveniles suggesting maternal transfer of these compounds (Taylor et al., 2021). These compounds were also present at higher levels than what has previously been reported in Northern and Southern Hemisphere pinnipeds (Taylor et al., 2021), representing a key threat to these populations, given the impact of these compounds on the health of marine mammals (P. J. H. Reijnders et al., 2009; Taylor et al., 2021).

Aquatic environments can act as reservoirs for bacteria and viruses and contribute to the dissemination of these biological pollutants across greater timescales than terrestrial environments (Marti et al., 2014). Wastewater from human origins can contain high numbers of viruses and potentially pathogenic and antimicrobial resistant bacteria (ARB) (Baquero et al., 2008). An increase in pinniped strandings in the Northern Hemisphere has been associated with a higher prevalence of pathogenic and antimicrobial resistant bacteria (Stoddard et al., 2008, 2005; Wallace et al., 2013). However, the presence and impact of ARB in pinnipeds remains poorly understood, representing both a threat and knowledge gap for Australian pinnipeds, and will be more thoroughly reviewed in sections 1.3 and 1.4.

Disease is a well-known contributor to mortality in most pinniped species. A number of diseases caused by viruses, protozoal and fungal pathogens have also been reported in a wide range of marine mammal hosts including Morbillivirus infection in cetaceans (Duignan et al., 1995; Lipscomb et al., 1994; Raga et al., 2008; Van Bressem et al., 2009), infection with *Toxoplasma gondii* in sea otters (*Enhydra lutris nereis*) (Kreuder et al., 2003), brucellosis in seals, porpoises and dolphins (Moreno et al., 2012; Nymo et al., 2011; Ross et al., 1996), and *Klebsiella pneumoniae* in New Zealand sea lions (*Phocarctos hookeri*) (Castinel et al., 2007a;

Michael et al., 2019). The presence and impact of pathogens is still largely unexplored in Australian pinnipeds despite the threat of continual growth and encroachment of human populations into coastal habitats that could increase the dissemination and potential exposure of pinnipeds to pathogens from anthropogenic sources (Davidson et al., 2012; Kovacs et al., 2012). The threat of novel pathogens from anthropogenic sources is likely to be important for *N. cinerea*, with recent genetic analysis by Bilgmann et al. (2021) determining that infectious disease poses a major threat to the future of the *N. cinerea* population. The presence of human-associated bacteria, such as *E. coli*, in Australian pinniped species has the potential to serve as an indicator of exposure to anthropogenic pollution and represents a key knowledge gap.

One of the perhaps more well-understood contributors to disease in pinniped species is infection with hookworm (*Uncinaria* sp.), which has been recorded in almost all otariid species. The contribution of hookworm to disease and mortality differs between otariid species and is also dependent upon the characteristics of the hookworm itself (Lyons et al., 2001; Marcus et al., 2014; Seguel et al., 2016). Disease caused by hookworm infection is an important contributor to pup mortality in Northern fur seals (*Callorhinus ursinus*) (Lyons et al., 2011, 2001), South American fur seals (*Arctocephalus australis*) (Seguel et al., 2016, 2011), California sea lions (*Zalophus californianus*) (Lyons et al., 2001) and *N. cinerea* (Marcus et al., 2014). Infection with the haematophagous hookworm, *Uncinaria sanguinis*, is endemic and present in 100% of neonatal *N. cinerea* pups at two of the most important colonies for this species, Seal Bay on Kangaroo Island and Dangerous Reef in the Spencer Gulf, both located in South Australia (Marcus et al., 2014). Pups are infected with *U. sanguinis* via the transmammary route immediately after birth and remain infected for 2-3 months (Marcus et al., 2014). Hookworms attach to the small intestine and feed on blood, causing anaemia and

hypoproteinaemia (Marcus et al., 2014). The pathologies caused by *U. sanguinis* infection are thought to contribute to up to 40% of pup mortality (Lindsay and Gray, unpublished) directly from disease, and indirectly by making pups lethargic and more vulnerable to conspecific trauma (Marcus et al., 2014). Hookworms (*Uncinaria* sp.) are also present in *A. p. doriferus* and *A. forsteri* pups and genetically cluster with *U. sanguinis*, however, hookworm infection occurs at a lower prevalence, lower infection intensities, and does not appear to be as pathogenic in these species (Ramos et al., 2013).

The high levels of pup mortality associated with *U. sanguinis* infection in *N. cinerea* pups have led to investigations into potential mitigation. The use of ivermectin, an anthelmintic treatment, has been tested on *N. cinerea* pups at Dangerous Reef over multiple breeding seasons, finding that it is a safe and highly effective treatment for *U. sanguinis* infection (Lindsay et al., 2021; Marcus et al., 2015b). Ivermectin treatment caused a significant reduction in hookworm burden with treated pups clearing the infection, resulting in improved pup growth and overall health, as seen through improved haematological parameters (Lindsay et al., 2021). However, one key knowledge gap is the impact of treatment on the gut microbiome, which is critical for host development and immunofunction, with changes to the microbiota in early life stages potentially causing long-term disruption, as described in human and murine models (Cortés et al., 2019; Hooper et al., 2012; Jenkins et al., 2018). Studies investigating alterations to the gut microbiota as a result of removal of parasites in humans show a variety of changes, mostly dependent upon the parasite species itself (Easton et al., 2019; Zaiss et al., 2015). When considering treatment as part of disease mitigation, it is critical to assess whether treatment alters the host gut microbiota given the potential consequences this can have for life-long immunity and host fitness.

1.3. Antimicrobial resistant bacteria and resistance genes in wildlife species

1.3.1. Overview of antimicrobial resistance

In the environment, bacteria are exposed to a variety of antimicrobials including antibiotics, heavy metals and organic solvents (Nguyen et al., 2019). Antibiotics target processes and structures within bacteria to either inhibit growth (bacteriostatic), directly kill bacteria (bactericidal), or utilise a combination of bacteriostatic and bactericidal mechanisms (Pal et al., 2017). Antibiotics are extensively used in human clinical settings to treat infections and are commonly overused and misused through incorrect administration and prescriptions (e.g., for viruses) (Chaw et al., 2018; Chokshi et al., 2019). Antibiotics have also been used extensively in animal and livestock operations (including aquaculture) to prevent and treat infections and are often administered in subtherapeutic doses for growth promotion (Ghosh and LaPara, 2007; Mann et al., 2021). However, in these operations, antibiotics are not fully absorbed by the host and large amounts are frequently excreted in urine and faeces (Byrne-Bailey et al., 2009; Ghosh and LaPara, 2007; Mann et al., 2021), which can be readily spread into the environment. The use of antibiotics in agricultural settings has increased selective pressures and concentrations of antibiotics in the environment, facilitating the spread and evolution of resistance in pathogenic, environmental, and opportunistic bacterial pathogens (Larsson, 2014; Peterson and Kaur, 2018). This has ultimately led to the widespread selection and dissemination of antimicrobial resistance (AMR), mainly through horizontal gene transfer (HGT) (von Wintersdorff et al., 2016).

The effectiveness of antibiotics to treat bacterial infections is continuously being reduced constituting one of the greatest modern threats to human health; it is estimated that if resistance to antibiotics continues to increase, there will be 10 million deaths annually attributable to infections with antibiotic resistant bacteria (ARB) by 2050 (Robinson et al.,

2016). However, AMR is no longer an issue just in human and agricultural sectors, with ARB increasingly detected in wildlife species (Vittecoq et al., 2016).

Resistance to antimicrobials can occur through mutations or through the acquisition of genes conferring resistance via HGT, with the latter identified as one of the most important factors leading to the rapid proliferation and spread of AMR (Stalder et al., 2012). Horizontal gene transfer involves the transfer of genetic material between bacteria. In the context of AMR, bacteria utilise HGT to acquire new genetic material from their environment and other bacteria when faced with selective pressures, such as the presence of antibiotics, facilitating the exchange of adaptive genes that rapidly increase fitness (Domingues et al., 2012; Lerminiaux and Cameron, 2018; Reygaert, 2018; Stalder et al., 2012). Furthermore, HGT can readily occur when resistance determinants and antibiotics are present in high bacterial densities which are commonly found in human and animal digestive tracts and in wastewater treatment plants (WWTPs) (Paulson et al., 2016). Horizontal gene transfer is mediated by mobile genetic elements (MGEs), including transposons, plasmids, insertion sequences (ISs), bacteriophages and integrons. Multiple mechanisms facilitate HGT; conjugation (bacterial plasmids and conjugative transposons), transformation (acquisition of free naked DNA in the environment) and transduction (bacteriophages) (Marti et al., 2014).

Increasing levels of ARB and antibiotic residues are being detected in diverse environments where their presence can apply selective pressures for bacterial species and lead to the proliferation of more ARB (Paulson et al., 2016; Stalder et al., 2012). A diverse array of antibiotics, ARB, and other pollutants are spread into the environment through stormwater and agricultural runoff, discharge from aquaculture, untreated and treated sewage (Marti et al., 2014). Wastewater treatment plants represent one of the main sources of antibiotics and ARB found in the environment (Baquero et al., 2008; Martinez, 2009), with

increased concentrations in areas that experience higher anthropogenic impact (Ahmed et al., 2016; Berendonk et al., 2015; Chen et al., 2019; Grilo et al., 2020; le Page et al., 2017). Water is also an ideal medium for the mixing of these pollutants and subsequent dissemination can occur across greater distances than is possible in the terrestrial environment.

1.3.2. Structure and function of integrons

Integrons are genetic elements that confer AMR primarily in Gram-negative bacteria and are increasingly being identified in Gram-positive bacteria (Domingues et al., 2012). Integrons have been integral to the rapid emergence and spread of AMR, in part due to their ability to capture and express a wide variety of antibiotic resistance genes (ARGs) (Gaze et al., 2011; Gillings, 2017). Integrons are localised on MGEs, including transposons and plasmids, which facilitate the transfer of multiple genes in a single event, assisting the rapid spread of ARGs (Lupo et al., 2012). Structurally, integrons have three main components: a 5' conserved segment (5'-CS) encoding the integron-integrase gene (*intI*) that encodes a tyrosine recombinase and is required for site-specific recombination; the recombination site (*attI*) that sits adjacent to and is recognised by *intI*; and the promotor (Pc) which is required for transcription and expression of gene cassettes carried by the integron (Boucher et al., 2007; Cambray et al., 2010; Domingues et al., 2012; Escudero et al., 2015; Gillings, 2014; Mazel, 2006). Integrons can carry a variable number of gene cassettes that form what is referred to as the gene cassette array (Hall and Collis, 1995).

Gene cassettes are circular, non-replicating DNA molecules that contain a cassette-associated recombination site (*attC*), which allows insertion into the cassette array at *attI* on the integron (Collis and Hall, 1992). The insertion of gene cassettes is mediated by *intI* and within the cassette array, the arrangement and carriage of genes can be modified by *intI*

activity (Boucher et al., 2007; Collis and Hall, 1992). Newly acquired gene cassettes are highly expressed by the promoter, but the regulation of the integrase gene is strictly controlled by the SOS response, a bacterial response to stressors that cause DNA damage (Baharoglu and Mazel, 2014; Guerin et al., 2009). Under normal conditions, *intI* maintains the gene cassettes in a stable array, however, when triggered by stressors, the SOS response is induced ensuring that existing cassettes are reordered or excised to allow capture and expression of cassettes that may be beneficial to survival (Guerin et al., 2009). One such stress is the presence of antibiotics, which can induce the SOS response and promote integrase expression (Guerin et al., 2009). Over 130 different gene cassettes have been identified, encoding resistance to virtually all classes of antibiotics that are currently used against Gram-negative bacteria (Cambray et al., 2010). In bacterium carrying integrons, having access to a broad pool of gene cassettes can contribute to genome plasticity and provide a greater ability to adapt to rapidly changing environmental conditions (Boucher et al., 2007; Escudero et al., 2015; Ghaly et al., 2021b). In environments where diverse gene cassettes are present, those conferring advantageous phenotypes will be retained and expressed (Gillings et al., 2009). Depending on environmental conditions, these cassettes can be expressed in many different orders, each providing a potential adaptive benefit (Hall, 2012).

There are five classes of mobile integrons (MIs) which are categorised based on differences in their *intI* sequences and classes 1, 2 and 3 are the most commonly detected (Labbate et al., 2009; Stalder et al., 2012). Of these three more common classes, class 1 integrons are the most prevalent, frequently identified in bacteria isolated from clinical settings, have been found in virtually all species of Gram-negative pathogens (Martinez-Freijo et al., 1998), and are considered clinically important, commonly referred to as ‘clinical class 1 integrons’ (Deng et al., 2015; Ghaly et al., 2021a; Zheng et al., 2020). Clinical class 1 integrons

are also commonly linked with genes that confer resistance to disinfectants, heavy metals, and sulfonamides (Gillings, 2018). In typical clinical class 1 integrons, downstream of the gene cassette is a 3' conserved segment (3'-CS) that usually contains *qacE*Δ and *sul1* genes, which encode resistance to quaternary ammonium compounds and sulfonamides, respectively (Deng et al. 2015), as well as an open reading frame (ORF) (Gillings et al., 2009). Carriage of the *qac* gene provides a selective advantage in clinical environments where bacteria are often exposed to disinfectants (Gillings et al., 2009). Class 1 integrons with atypical 3'-CS are increasingly being identified; instead of a *qacE*Δ gene, these atypical integrons have been modified by an IS, a small DNA segment that is able to randomly translocate itself and any associated resistance genes to new locations, facilitating the movement of clusters of resistance genes (Partridge et al., 2018). One particular IS, IS*26*, has been associated with class 1 integrons and has played an important role in the spread of resistance determinants among Gram-negative bacteria (Partridge et al., 2018; Varani et al., 2021).

1.3.3. Class 1 integrons and usefulness as indicators of anthropogenic pollution

Areas with high levels of anthropogenic activity can become 'hot-spots' for the development of AMR through the exchange of genetic material as they create an environment with high bacterial densities, toxic materials, sub-inhibitory concentrations of antibiotics, heavy metals, and disinfectants (Pal et al., 2017; Peterson and Kaur, 2018). Such conditions can be found in sewage, WWTPs, hospital effluent, and agricultural runoff (von Wintersdorff et al., 2016). Wastewater treatment plants represent a significant interface between different environments by bringing together animal, human, and industrial wastes from diverse sources that contain bacteria (which carry MGEs and ARGs), antibiotics, and other pollutants (Baquero et al., 2008; Kümmerer, 2003; Marti et al., 2014). The presence of selective agents and resistance determinants facilitates the occurrence of HGT and can facilitate the

development and evolution of AMR as well as the dissemination of ARB and ARGs (Baquero et al., 2008; Marti et al., 2014). Furthermore, numerous studies have shown that treatment of wastewater in WWTPs it not always sufficient for the removal of antibiotics and ARB, with high levels of ARB detected in effluent and rivers downstream of WWTPs (Amos et al., 2014; Rosewarne et al., 2010; Wright et al., 2008) which eventually pollute the sea (Pommepuy et al., 2006). There is sufficient evidence to suggest that treated effluent from WWTPs is one of the most significant sources of ARB and ARGs in the environment (Karkman et al., 2019; Rizzo et al., 2013).

Increasing levels of MGEs, ARGs, ARB and antibiotics in the environment has facilitated the almost global presence of selective pressures, providing more opportunities for HGT to occur, and promoting the acquisition of ARGs by bacteria in all environments (Gillings et al., 2008; Stokes and Gillings, 2011). Class 1 integrons, for example, are now present in environments and ecosystems that are far removed from anthropogenic activities and associated selective pressures (Domingues et al., 2015; Stokes and Gillings, 2011). Class 1 integrons have been detected in over 100 bacterial species and are now widespread in humans and agricultural animals, with a single gram of faeces estimated to carry millions to billions of integron copies (Ghaly et al., 2021a; Zhu et al., 2017). It is estimated that class 1 integrons became integrated into human and animal gut microbiota prior to the 1940s (Gillings, 2018). Since colonisation, class 1 integrons have continued to increase in abundance with studies suggesting each gram of faeces from humans and animals could contain 10^8-10^{10} integron copies, with up to 10^{23} copies of class 1 integrons spread into the environment every day (Zhu et al., 2017). Given the high carriage of class 1 integrons in human faeces, it is unsurprising that waste from humans contain integrons and associated resistance genes, which are brought together in WWTPs (Schlüter et al., 2007; Zhang et al., 2009). Class 1

integrons have been identified at all stages of the WWTP process and are poorly removed during treatment processes, resulting in their release into the environment post-treatment (Baquero et al., 2008; LaPara et al., 2011; Stalder et al., 2014). Along with the release of class 1 integrons, effluents from WWTPs also commonly contain selective agents, meaning that the integron is likely able to persist in the environment for more extended periods of time, presenting opportunities for HGT to occur and further dissemination of ARGs into the environment (Gillings, 2018).

The class 1 integron-integrase gene, *intI1*, has been proposed as a useful indicator of antimicrobial pollution as it is highly conserved, abundant in humans, domestic animals and waste streams, and is rarely present in environments unaffected by anthropogenic activities (Gillings et al., 2014). The inefficient removal of class 1 integrons in WWTPs further supports the use of *intI1* as an indicator of anthropogenic pollution (LaPara et al., 2011; Rosewarne et al., 2010; Wright et al., 2008). Multiple studies have provided evidence to support the association between the *intI1* gene and anthropogenic pollution - areas exposed to higher levels of anthropogenic pollution, often downstream of sewage and industrial waste outputs, have significantly higher abundances of *intI1* in comparison to sites located further from outputs (Rosewarne et al., 2010; Stalder et al., 2014; Wright et al., 2008). Class 1 integrons and other MGEs are continuously released into marine environments via polluted rivers and waterways (Ansari and Matondkar, 2014; Häder et al., 2020; Zhang et al., 2009) at such high levels that water, and aquatic environments have become vectors of pollutants and have significantly contributed to the dissemination of ARB and MGEs (Lupo et al., 2012; Stalder et al., 2012).

Along with the *intI1* gene, the presence of bacteria with modified integrons, such as those carrying atypical ISs, could be used to indicate the spread of bacteria from human and

domesticated animals. Integrons with the IS*26* modification have been identified in *E. coli* from human, bovine and porcine sources in Australia (Dawes et al., 2010; Reid et al., 2017). Reid et al. (2017) showed that these modified class 1 integrons are highly abundant in pigs, suggesting that pigs in Australia may be reservoirs of these variants. The presence of class 1 integrons with IS*26* in bacteria from environmental sources and wildlife hosts could indicate not only exposure to waste from human and livestock animals, but the pollution of the marine environment with faecal waste and potential pathogens from livestock.

1.3.4. Antimicrobial resistance in wildlife species

There is increasing interest in AMR in wildlife and the number of studies that investigate factors influencing the presence of ARB in wildlife is growing (Dolejska and Literak, 2019). Wildlife species often harbour ARB; the primary factor influencing the levels of ARB and ARGs in wildlife species is the relative level of exposure to anthropogenic pollution and activities, with higher abundances associated with higher levels of impact (Ahlstrom et al., 2018; Allen et al., 2011; Furness et al., 2017; Guenther et al., 2011; Radhouani et al., 2014, 2009; Ramey and Ahlstrom, 2020; Rolland et al., 1985; Weiss et al., 2022). The biology and ecology of wildlife species has also been found to influence the presence of ARB (Dolejska et al., 2007; Guenther et al., 2011; Ramey and Ahlstrom, 2020).

Foraging represents an opportunity for wildlife species to be exposed to varying levels of anthropogenic pollutants and ARB and is a contributor to the prevalence of AMR in wildlife (Ramey and Ahlstrom, 2020). Studies have shown that terrestrial wildlife species feeding on human refuse carried significantly higher levels of ARB compared to individuals inhabiting areas more remote from human activities (Rolland et al., 1985; Souza et al., 1999). Most studies investigating the relationship between foraging and AMR have primarily focused on wild birds given they are abundant and occur in diverse environments, frequently associate

with environments of high human impact, and commonly migrate, such that they have the capacity for long-range dispersal of ARB and ARGs (Dolejska and Literak, 2019; Guenther et al., 2011). Gulls (family *Laridae*) have been studied in diverse environments, with investigations finding that gulls nesting near waste or agricultural water harboured higher levels of ARB than gulls associated with unpolluted water (Ahlstrom et al., 2019, 2018; Bonnedahl et al., 2009; Dolejska et al., 2007), and proximity to human activity increased the prevalence of ARB (Allen et al., 2010; Sjölund et al., 2008).

The widespread presence of ARB and ARGs in marine ecosystems poses largely unknown threats to wildlife species that inhabit these environments. However, given the high levels of ARB and ARGs that are disseminated into aquatic and marine environments via effluent from human and animal waste, it is unsurprising that higher levels of ARB have been detected in water-associated species (Furness et al., 2017; Jobbins and Alexander, 2015). In African wildlife, a greater prevalence of multidrug-resistant bacteria was detected in species that lived and foraged in aquatic environments (Jobbins and Alexander, 2015). In small wild mammals, closer proximities to aquatic environments were associated with higher levels of AMR and it was hypothesised that this increase was due to contamination with WWTP effluent and agricultural runoff (Furness et al., 2017). The prevalence of AMR in commensal bacteria in wildlife has also been linked to the geographic distance of the host species from wastes that contain ARB and antibiotic residues from humans, with closer proximities associated with higher levels of AMR (Swift et al., 2019).

Pinnipeds inhabit and forage in coastal environments that usually experience high levels of human impact, increasing opportunities for exposure to anthropogenic pollution. Despite their association with aquatic environments, limited investigations into the presence of AMR in pinnipeds has been undertaken. A study in northern elephant seals (*Mirounga*

angustirostris) in California demonstrated that exposure to freshwater outflow and runoff was associated with higher levels of AMR *E. coli* and was identified as an important risk factor for acquisition of faecal-associated pathogens (Stoddard et al., 2008). Isolation of ARB has been demonstrated in stranded harp seals (*Pagophilus gorenlandicus*), grey seals (*Halichoerus grypus*) and harbor seals (*Phoca vitulina*) in the northwest Atlantic (Wallace et al., 2013) and the Salish sea (Norman et al., 2021), although the focus of these studies has been to determine the prevalence of phenotypic antibiotic resistance, with almost no literature investigating the abundance of MGEs, such as class 1 integrons, in these marine mammals.

The *intI1* gene is increasingly being used as an indicator of exposure to anthropogenic pollution in free-ranging wildlife species, with greater abundances of *intI1* detected in species in environments with higher levels of human impact (Skurnik et al., 2006). Class 1 integrons have been detected in a diverse array of wildlife species in the Southern Hemisphere, including southern elephant seals (*Mirounga leonina*) and Weddell seals (*Leptonychotes weddellii*) in Antarctica (Power et al., 2016), free-ranging and captive *N. cinerea* (Delport et al., 2015; M Fulham et al., 2018), little penguins (*Eudyptula minor*) (Lundbäck et al., 2020), grey-headed flying foxes (*Pteropus poliocephalus*) (McDougall et al., 2019) and brush-tailed rock-wallabies (*Petrogale penicillata*) (Power et al., 2013). Skurnik et al. (2006) detected *intI1* in *E. coli* from wildlife species, finding that increased abundance of *intI1* correlated with proximity to humans. The authors also suggested a gradient of resistance, with absence to high prevalence directly related to the level of exposure to human activities (Skurnik et al., 2006).

Differences in prevalence of ARB and ARG between captive and free-ranging pinnipeds provides further evidence to support the influence of human proximity on AMR carriage

(Delport et al., 2015; Stoddard et al., 2009). In captive and free-ranging adult *N. cinerea*, a low prevalence of class 1 integrons was detected in captive individuals and there was a comparative absence in free-ranging adults (Delport et al., 2015). Similarly, Stoddard et al. (2009) determined that northern elephant seals admitted to a rehabilitation centre rapidly developed resistance to antibiotics, regardless of whether individuals were treated or not, suggesting that the environment of the centre itself and interactions with humans facilitated the transfer of resistant bacteria.

There is sufficient evidence to support association between the presence of ARB and ARGs in wildlife species exposed to anthropogenic pollution, however this remains a knowledge gap for free-ranging Australian pinniped populations. Investigations are required to firstly determine the presence of ARB and ARGs in the three pinniped species that inhabit Australian waters and secondly, to characterise the resistance determinants that may be present. Understanding the prevalence and characteristics of ARB and ARGs will provide insights into the impact of anthropogenic pollution on these populations, which is critical for ongoing monitoring and management of these species and utilising pinnipeds as sentinels of marine ecosystem pollution.

1.4. *Escherichia coli*

1.4.1. Ecology and life history of *Escherichia coli*

Escherichia coli is a gram-negative, facultatively anaerobic bacteria that is both a widespread commensal in the gut of vertebrates, and a significant pathogen in humans and domestic animals (Tenaillon et al., 2010). Commensal *E. coli* are the predominant aerobic bacteria in the gastrointestinal tract in humans residing in the caecum and colon and providing benefits to the host by inducing colonisation resistance (i.e., preventing colonisation by other pathogenic bacteria) (Gordon, 2013; Savageau, 1983; Tenaillon et al., 2010). *Escherichia coli*

population structure is clonal with low levels of recombination, with at least eight distinct genetic groups or phylotypes; seven of these phylotypes, A, B1, B2, C, D, E, and F, belong to *E. coli sensu stricto*, which refers to classic *E. coli* strains (Clermont et al., 2013). The eighth consists of *Escherichia* cryptic clade I, with strains of *E. coli* that are phenotypically indistinguishable, yet genetically distinct from *E. coli sensu stricto* assigned to this phylotype (Clermont et al., 2013, 2000; Escobar-Páramo et al., 2006; Selander and Levin, 1980). Recent analysis of *E. coli* isolates through whole genome sequencing has also revealed additional phylotypes G and H (Denamur et al., 2021; Yu et al., 2021). These phylogenetic groups can be partitioned into two main clusters: B2, G, F and D, and A, B1, C and E (Denamur et al., 2021). Each phylotype has a unique life history, exist in varying ecological niches, and have varied pathogenicity (Gordon, 2010). *Escherichia coli* strains belonging to phylotypes A and B1 are generalists, occupying a diverse range of vertebrate hosts, whereas B2 and D strains are more frequently isolated from humans, mammals, and birds (Gordon, 2013; Gordon and Cowling, 2003).

Strains of *E.* coli have acquired virulence factors, facilitating the evolution of pathogenic *E. coli*, a major cause of both intestinal and extraintestinal disease in humans and many animal species (Croxen and Finlay, 2010; Kaper et al., 2004; Picard et al., 1999; Reid et al., 2000). The ability of *E. coli* strains to cause disease is associated with the genetic background of the isolate (Escobar-Páramo et al., 2004). Strains within the B1 phylotype are rarely identified as causative agents of disease in humans and have a reduced capacity to persist in the human intestinal environment compared to other phylotypes, specifically B2 and D (Nowrouzian et al., 2005). Strains belonging to the B2 phylotype are the most well adapted to humans and are clinically more important, with a vast majority of extraintestinal pathogenic *E. coli* (ExPEC) strains assigned to this phylotype (Johnson et al., 2001; Picard et

al., 1999). Even within commensal *E. coli* communities, strains belonging to the B2 phylotype appear to be more virulent than other phylotypes and carry a greater number of virulence factors that contribute to the pathogenicity of isolates in this genetic group (Duriez et al., 2001). The B2 phylotype can be further sub-divided into nine lineages (sub-types) that are associated with extra-intestinal infections (Clermont et al., 2014). All of the nine B2 sub-types (I, II, III, IV, V, VI, VII, IX and X) are associated with sequence type complexes (STc), which provide additional information about the potential pathogenicity of the bacterium (Clermont et al., 2014; Gordon et al., 2008). For example, sub-types I (STc131), II (STc73), VI (STc12), VII (STc14) and IX (STc95) are associated with extraintestinal disease in humans and avian colibacillosis in domestic and wild bird species (Clermont et al., 2014; Dheilly et al., 2011).

While *E. coli* is well adapted to the human intestinal tract, the ability of *E. coli* strains to persist within this environment varies and is again dependent upon the phylogenetic group of the strain (Johnson et al., 2001; Nowrouzian et al., 2005; Picard et al., 1999). Strains of *E. coli* within the intestine are considered 'transient' if they persist for days to weeks, while 'resident' strains are capable of persisting for weeks to months (Caugant et al., 1984; Johnson et al., 2003; Nowrouzian et al., 2005; Sears et al., 1950). Strains within each phylogenetic group possess different adhesins and virulence factors which influence their ability to successfully colonise the intestinal environment (Johnson et al., 2001). As a result, strains belonging to more than one phylotype can be present in an individual at a single point in time. Studies investigating the diversity of phylotypes within human hosts found that on average, 1.8 *E. coli* strains are present in each individual (Blyton et al., 2014). Furthermore, in individuals where the strains belonging to the B2 phylotype were present, the co-occurrence of strains belonging to other phylotypes was unlikely (Blyton et al., 2014; Nowrouzian et al.,

2005), suggesting that strains in the B2 group have evolved characteristics that allow them to persist and become dominant within the human intestinal environment.

There have been limited investigations into the diversity and structure of *E. coli* phylotypes within wildlife species. Escobar-Páramo et al. (2006) isolated multiple *E. coli* strains from a variety of wildlife species, finding that diet was a contributing factor to the diversity of *E. coli* phylotypes; carnivores, herbivores and omnivores carried, on average, 2.3, 2.2 and 1.7 strains, respectively. Characterisation of commensal *E. coli* isolates in mountain brushtail possums (*Trichosurus cunninghami*) found a similar level of diversity, with individuals carrying an average of 2.2 strains (Blyton et al., 2013).

1.4.2. *Escherichia coli* as an indicator of anthropogenic pollution

Given the association between *E. coli* and human and animal intestinal tracts, *E. coli* is commonly used worldwide as an indicator of faecal contamination of waterways (Ahmed et al., 2016; Beversdorf et al., 2007; Gordon, 2005; Gordon and Cowling, 2003; Power et al., 2005). The presence of faecal coliforms such as *E. coli* in water sources is usually monitored to determine the safety of recreational and drinking waters for human use (Ahmed et al., 2016; Beversdorf et al., 2007) due to the health risks posed by microbes associated with faecal pollution (Ahmed et al., 2016; Hartz et al., 2008).

The presence of microbes associated with faecal pollution in marine ecosystems is potentially more important than in terrestrial environments, given that the ocean has greater connectivity and microbes can be more easily disseminated over greater distances (Jönsson and Watson, 2016). Sewage effluent and land-based runoff (freshwater and agricultural) are some of the most important sources of faecal contamination to enter coastal marine environments (Jamieson et al., 2002), and growth of human populations has ultimately increased the risk of coastal wildlife species being exposed to microbes associated with faecal

pollution from human and domestic animal sources (Häder et al., 2020; Oates et al., 2012; Wear et al., 2021).

As outlined in section 1.4.1, the phylogenetic group of *E. coli* strains is non-random and phylotypes are associated with specific host species (Gordon and Cowling, 2003). The assignment of *E. coli* strains to phylotypes can therefore be used to provide additional information about the origin of the bacteria and indicate whether *E. coli* isolated from wildlife species is of human origin (Carlos et al., 2010; Gordon and Cowling, 2003). *Escherichia coli* phylotypes B2 and D, for example, are frequently identified in faecal samples collected from humans, and B2 phylotypes in particular are associated with human intestinal environments (Nowrouzian et al., 2006, 2005). Studies investigating the phylogroup membership of *E. coli* isolates from wastewater samples have also found that the majority of isolates belonged to phylotypes B2 and D (Anastasi et al., 2010; Koczura et al., 2012), providing further evidence to support the association between humans and the B2 phylotype. However, there have been very limited investigations into the presence and impact of microbes associated with faecal pollution on free-ranging wildlife species, especially in coastal marine areas.

1.4.3. *Escherichia coli* in wildlife species

Escherichia coli has been used as a model to examine the presence of human-associated faecal microorganisms and the transmission of bacteria between humans and wildlife species (Goldberg et al., 2007; Pesapane et al., 2013; Rwego et al., 2008b; Skurnik et al., 2006). While ubiquitous in humans, the presence and distribution of *E. coli* in wildlife species has been linked to interactions with humans (Gordon and Cowling, 2003; Pesapane et al., 2013), and also varies depending on gut morphology, host characteristics and diet, as well as the characteristics of the *E. coli* strain itself (Gordon, 2013; Gordon and Cowling, 2003).

In terrestrial environments, transmission of human and animal gastrointestinal bacteria can occur when hosts are in close proximity to one another (Goldberg et al., 2007). *Escherichia coli* isolated from mountain gorilla (*Gorilla beringei beringei*) and chimpanzee (*Pan troglodytes*) populations in Uganda that frequently overlapped and interacted with humans and livestock were genetically similar to *E. coli* isolates from human and domestic animal hosts (Goldberg et al., 2007; Rwego et al., 2008b). These findings indicated the genetics of *E. coli* isolated from wildlife species can be used as an indicator of exposure to bacteria originating in humans (Goldberg et al., 2007; Rwego et al., 2008b).

Perhaps the most extensive study examining the presence of *E. coli* in wildlife was conducted by Gordon and Cowling (2003), where the presence of *E. coli* in over 350 vertebrate species in Australia was investigated. This study showed that birds living in areas close to human habitation were more likely to carry *E. coli* than those in remote areas, suggesting that interaction and cohabitation with humans can influence the presence of *E. coli* (Gordon and Cowling, 2003). A similar trend was also observed in lizards and frogs where association with humans influenced the prevalence of *E. coli* in these hosts (Gordon and Cowling, 2003).

The presence of *E. coli* has been investigated in Southern Hemisphere pinniped species including *M. leonina*, *L. weddellii*, *A. gazella*, (Mora et al., 2018; Power et al., 2016), and *N. cinerea* (Delport et al., 2015; Fulham et al., 2018). The prevalence of *E. coli* differed between captive (84%) and free-ranging (7.7%) adult *N. cinerea*, suggesting that human association influences the presence of *E. coli* in this species (Delport et al., 2015). In free-ranging *N. cinerea* pups, the prevalence of *E. coli* was 85% (Fulham et al., 2018), similar to what was observed in captive adults (Delport et al., 2015). In both *N. cinerea* adults and pups, isolates were most frequently assigned to the B2 phylotype, with 67% and 45% of isolates identified as B2 phylogroup members, respectively (Delport et al., 2015; Fulham et al., 2018).

Both Power et al. (2016) and Mora et al. (2018) observed a similar dominance of the B2 phylotype in *E. coli* isolated from Antarctic pinnipeds, with 44.5% and 52.2% of isolates assigned to the B2 phylotype, respectively. The high carriage of both *E. coli* and *E. coli* strains belonging to the B2 phylotype in pinnipeds in the Southern Hemisphere suggests that these populations are vulnerable to exposure to microbes associated with faecal pollution from human sources. While samples have been collected from *N. cinerea* pups to determine the presence of *E. coli*, there has been no investigations into the prevalence and distribution of *E. coli* in pups of sympatric pinniped species, *A. p. doriferus* and *A. forsteri*.

1.5. The gut microbiota and potential importance for wildlife species

1.5.1. Overview of gut microbiota, functions and factors shaping composition

The human gut consists of over 100 trillion microorganisms which have been collectively termed the 'microbiota' and the genes they encode the 'microbiome' (Clemente et al., 2012; Thursby and Juge, 2017; Ursell et al., 2012). The gut microbiome contains over 5 million genes, 150x larger than the human genome, and gene products of the microbiome provide a diverse array of biochemical and metabolic activities (Consortium, 2012; Sommer and Bäckhed, 2013). Mammals have co-evolved with their intestinal microbiota over millions of years to establish a mutualistic relationship where commensal bacteria are critical for host health: they contribute to the digestion of food to provide nutrients (Hooper et al., 2002), synthesise vitamins (Yatsunenko et al., 2012), protect against colonisation by pathogens (Bäumler and Sperandio, 2016) and contribute to the development, homeostasis and regulation of immune functions (Brestoff and Artis, 2013; Cebra, 1999).

In the past, understanding the composition of the gut microbiota has been limited to culture-based studies, however, an increase in culture-independent (metagenomic) sequencing technologies has expanded the knowledge base, allowing the identification and

quantification of organisms that cannot be cultured (Lozupone et al., 2012). All domains of life are represented within the gut microbiota including Archaea, Eukarya, Viruses and Bacteria (Eckburg et al., 2005; Hill and Artis, 2010; Whitman et al., 1998), where bacteria are the most abundant. The microbiota of healthy mammals is dominated by three main bacterial phyla: *Firmicutes, Bacteroidetes* and *Actinobacteria* (Bäckhed et al., 2005), which are essential for the breakdown of complex polysaccharides and production of essential vitamins, for example, vitamin K, B group vitamins, folates, riboflavin and thiamine (Hill, 1997), minerals, and short chain fatty acids (Brestoff and Artis, 2013; Ley et al., 2008b). Furthermore, communication, or cross-talk, between the host and its microbial community can occur when the products derived from the gut microbiota are absorbed by the host, and the host in turn produces metabolites that are secreted into the gut and utilised by microbes (Rowland et al., 2018).

Sequencing of human intestinal microbiota led to the proposal of 'enterotypes', distinct community composition types considered to encompass the 'core microbiota' (Costea et al., 2018). However, the composition of the gut microbial community, including proportions and presence of certain bacterial species, can change throughout the lifetime of the host, has been found to vary between individuals, and is influenced by age, genetics, diet, and the host's external environment (Lozupone et al., 2012; Yatsunenko et al., 2012). In the early life stages of mammals, two major shifts in the composition of the gut microbiota have been observed; an initial colonisation after birth, and a second colonisation during the transition from milk to solid food diets during infancy (Odamaki et al., 2016; Palmer et al., 2007). In humans, the composition of the intestinal microbiota is highly dependent on early life stages (Fallani et al., 2010; J.E. Koenig et al., 2011), the mode of delivery (vaginal or caesarean), diet (breast-fed vs. formula-fed) and introduction of solid foods (Fanaro et al.,

2003). The composition of the gut microbiota undergoes marked fluctuations during infancy where it is more variable and less stable (J.E. Koenig et al., 2011; Palmer et al., 2007; Rodríguez et al., 2015), until a relatively stable structure is reached between 2-4 years of age that largely resembles the gut microbiota of an adult (Bäckhed et al., 2015; Nagpal et al., 2018; Odamaki et al., 2016). Furthermore, the development of the gut microbiota in the first three years of life has the greatest overall impact on the microbial community throughout the lifetime of the host (Tanaka and Nakayama, 2017).

Host diet profoundly affects the composition of the gut microbiota (Kau et al., 2011). While the composition of the gut microbial community is more stable in adults, it is still susceptible to change and readily shifts in response to dietary changes (Thursby and Juge, 2017; Turnbaugh et al., 2006). Dietary intake generally varies on a day-to-day basis, representing short-term shifts that the microbial community must adapt to in order to utilise varying nutrient levels (Albenberg and Wu, 2014), providing a continual source of change that shapes the microbial ecology (Xu and Knight, 2015). Long-term diet, however, is the primary driver of gut microbial composition (Leeming et al., 2019).

The contribution of host genetics to the gut microbiota composition is an area of contention. Some studies have suggested that genetics is one of the factors that influences the establishment of the gut microbiota and shapes its composition (Clemente et al., 2012), with the abundance of specific bacterial phyla being influenced by the genetics of the host (Goodrich et al., 2014). However, more recent studies do not support this association (Rothschild et al., 2018; Xie et al., 2016). A study conducted by Xie et al. (2016) compared the gut microbiome of twins and found a greater impact of environment on gut microbiota composition, with the microbiota gradually becoming genetically dissimilar as individuals spent time living apart. Similarly, Rothschild et al. (2018) found no significant association

between host genetics and microbiome composition, instead concluding that environmental factors have a more significant role in shaping the gut microbiota.

1.5.2. Role of the gut microbiota in host health and development

Mammals have undergone millions of years of coevolution with their gut microbiota in order to contain microbes within the intestinal environment and provide protection against pathogens while simultaneously preserving the symbiotic relationship (Hooper et al., 2012). Both the innate and adaptive immune systems have also evolved to require microbial interaction for development and functioning (Chow et al., 2010).

The intestinal microbiota plays a key role in the maturation of the immune system and the function of the intestinal barrier that keeps microbes limited to the gut environment (Hooper and Gordon, 2001; Smith et al., 2007). Colonisation of the intestinal environment with microbes has a profound impact on the development and formation of gut-associated lymphoid tissues and immune system development (Hooper et al., 2012). The importance of the gut microbiota for the development of host tissues and the immune system has mainly been realised through studies in germ-free (GF) mice, which lack all microorganisms and are kept in sterile environments (Bauer et al., 1963; Macpherson and Harris, 2004). Comparative studies between GF mice and mice with 'normal' gut colonisation have revealed that GF animals have impaired intestinal morphology, architecture, immune responses to pathogens, are more susceptible to infection, have reduced vascularity and digestive enzyme activity, and smaller Peyer's patches (Hooper and Gordon, 2001; Shanahan, 2002; Smith et al., 2007; Thursby and Juge, 2017).

Aside from the critical contribution of the gut microbiota to immune system development and response to pathogens, the commensal microbes can also provide protection against pathogens, known as colonisation resistance (CR), which can occur through

multiple mechanisms (Stecher and Hardt, 2011). Firstly, the physical presence of commensal microorganisms limits the ability of pathogens to colonise by competing for attachment sites and nutrients (Bäumler and Sperandio, 2016; Stecher and Hardt, 2011). In addition, the microbiota can produce antimicrobial compounds to prevent the overgrowth of potential pathogens that already exist within the microbial community (Kamada et al., 2013), and signals derived from commensal bacteria are also critical for the regulation of host-defence mechanisms. These signals can enhance expression of host defence genes in the gastrointestinal tract, improve immunity against bacterial and protozoan pathogens, and prime the immune system to respond more rapidly to potential pathogens (Abt and Artis, 2013). Perturbations or disruptions to the microbial community can result in the loss of commensal bacterial members, which ultimately improves the ability of pathogenic bacteria to infect the host (Abt and Artis, 2013).

A stable gut microbiota and maintenance of the symbiotic relationship is critical to overall health and development of the host. Whether the microbiota changes as a result of disturbances depends on the resilience of the microbial community. In this context, resilience refers to the amount of stress that can be tolerated before a shift towards a different equilibrium state occurs (Lozupone et al., 2012). Disruptions to gut microbiota can result in dysbiosis, a shift away from a stable composition (Carding et al., 2015; Littman and Pamer, 2011). Dysbiosis can be caused by alterations to diet and exposure to drugs (e.g. antibiotics), toxins, or pathogens (e.g. parasitic worms) (Carding et al., 2015). The development of numerous gastrointestinal, neurological, respiratory, metabolic, and cardiovascular diseases have been associated with dysbiosis (Honda and Littman, 2016; Lynch and Pedersen, 2016). Dysbiosis can also disrupt regular immune function, further contributing to development of inflammatory disease in the intestinal tract (Round and Mazmanian, 2009).

1.5.3. Impact of parasitic infection on the gut microbiota

Colonisation of the intestinal environment by parasites threatens the homeostasis of the host-microbiota relationship (Kreisinger et al., 2015; Walk et al., 2010; Zaiss et al., 2015). Parasitic infection can physically change the gut ecosystem via direct damage to the epithelial lining, alteration in mucus production and composition, and increased rates of epithelial cell turnover (Cliffe et al., 2005; Leung et al., 2018), impacting the ability of commensal microbes to persist in the gut environment. Disruptions to the gastrointestinal mucosa can profoundly alter the host's interaction with the gut microbiota and can facilitate the movement of microbes out of the intestinal environment and result in disease (Leung et al., 2018; Natividad and Verdu, 2013).

While parasitic infection can cause physical alterations of the intestinal environment, the impact of the infection on the gut microbiota and on host health appears to be dependent upon the parasite itself (Lee et al., 2014; Ramanan et al., 2016). However, even when looking at single parasitic species, changes in the gut microbiota that result from infection are rarely consistent (Cortés et al., 2019). In controlled laboratory experiments, a clear relationship has been demonstrated between infection of mice with the parasitic roundworm *Heligmosomoides polygyrus* and shifts in the composition of the gut microbiota (Walk et al., 2010; Weinstock and Elliott, 2013). In comparison, another study found no clear relationship between hookworm infection and microbiota composition or diversity (Cantacessi et al., 2014).

In addition to physical changes, intestinal parasites can alter the microbial community composition and immune responses. The parasites themselves can promote their own survival by modulating the innate immune response, that in turn can alter the hosts response to pathogenic bacteria (Brosschot and Reynolds, 2018; Ezenwa and Jolles, 2015). Multiple

species of helminths (e.g. roundworms and tapeworms) have also been found to induce change in the composition and abundance of nutrients and metabolites, which directly affects the growth of commensal and pathogenic microbes within the intestinal environment (Vogt et al., 2015). Infection with parasites is common in wildlife species, with many hosts infected with multiple parasitic species at any point in time. As has been observed in human hosts, the impact of parasitic infection on the gut microbiota differs across host and parasite species. In wild rodents, infection with helminths was associated with alterations in the composition of the microbial community but without altering overall diversity (Kreisinger et al. 2015). Similar infection-associated changes were observed in African buffalo (*Syncerus caffer*) but were highly dependent on the duration of infection and whether the host was infected with numerous parasitic species (Sabey et al., 2021).

Given that helminth infection can alter many physical and biological components of the gut microbiota, it could be expected that the treatment of helminth infection and subsequent clearance of these parasites can alter the intestinal microbial community. The results from studies investigating the impact of helminth treatment on the gut microbiota are mixed. In humans treated with ivermectin, a compositional change in the faecal microbiota was observed six months post treatment, which became more similar to uninfected control subjects (Jenkins et al., 2018). A study conducted by Houlden et al. (2015) found that following clearance of parasites, microbiota changes reverted to ‘normal’ or ‘pre-infection’, indicating that infection is needed to sustain changes to the microbiota. However, other studies have found no changes to the microbiota associated with anthelmintic treatment (Cooper et al., 2013; Ramanan et al., 2016; Rosa et al., 2018). The impact of parasitic treatment has also been considered in some wildlife species and is largely host, and potentially treatment, specific. For example, treatment of African buffalo had no overall impact on the community

structure of the intestinal microbiota (Sabey et al., 2021), while anthelminthic treatment of Asian elephants (*Elephas maximus*) resulted in significant variation in gut microbial diversity, but whether this change was due to the removal of the parasite or from the treatment itself was unclear (Moustafa et al., 2021). In Amur tigers (*Panthera tigris altaica*), anthelminthic treatment significantly altered gut microbiota composition as well as the metabolic phenotype of faecal samples, indicating that anthelminthic treatment of this species altered metabolic homeostasis (He et al., 2018). Given that changes in gut microbial composition can alter interactions between microbes, affecting energy production and therefore host nutrition, it is vital to understand the impact of parasitic treatment on the intestinal microbiota of wildlife species.

1.5.4. Current knowledge of the gut microbiota in wildlife species

Understanding the importance of the gut microbiota for host health in humans and domestic animal species has resulted in an increasing number of studies investigating the host-microbe relationship in wildlife species. Similar to finding in humans, external rather than host factors contribute more to the microbial composition of the gut in wildlife species (Amato, 2013; Maurice et al., 2015) with dietary variation one of the most important factors shaping the microbiota (Amato, 2013; Maurice et al., 2015; Sabey et al., 2021; Smith et al., 2013). Furthermore, shifts in gut microbial community due to dietary and environmental changes are likely amplified in wildlife species as they are subject to greater variations in food availability due to climatic changes across habitats and seasons, as well as anthropogenic habitat disturbances (Amato, 2013). Perhaps one of the most convincing findings to support the importance of external factors is that captivity altered the gut microbiota of numerous wildlife species (Clayton et al., 2016; McKenzie et al., 2017). Differences in bacterial richness

have been observed between captive individuals and their wild counterparts, with captivity significantly reducing bacterial diversity (McKenzie et al., 2017).

The composition of the intestinal microbiota has been characterised in numerous terrestrial wildlife species including *S. caffer* (Sabey et al., 2021), *E. maximus* (Moustafa et al., 2021), brown bears (*Ursus arctos*) (Sommer et al., 2016), Southeast African cheetahs (*Acinonyx jubatus jubatus*) (Wasimuddin et al., 2017), *P. t. altaica* (He et al., 2018), and primates (Clayton et al., 2016). The majority of studies that have investigated wildlife gut microbiota are limited to describing the bacterial phyla that are present, with few considering host traits (for example, host age and sex) (Ezenwa and Jolles, 2015; Moustafa et al., 2021; Sabey et al., 2021). However, knowledge of whether the microbiota influences the health of wild animals is limited.

The gut microbiota composition has been investigated in a surprising number of pinniped species, including hooded seals (*Cystophora cristata*) (Acquarone et al., 2020; Glad et al., 2010), Pacific harbor seals (*Phoca vitulina richardii*) (Pacheco-Sandoval et al., 2019), *M. leonina* and *M. angustirostris* (Nelson et al., 2013; Stoffel et al., 2020), leopard seals (*Hydrurga leptonyx*) (Nelson et al., 2013), Juan Fernández fur seals (*Arctocephalus philippii*) (Toro-Valdivieso et al., 2021), spotted seals (*Phoca largha*) (Tian et al., 2020), *Z. californianus* (Bik et al., 2016), *A. australis* and Subantarctic fur seals (*Arctocephalus tropicalis*) (Medeiros et al., 2016), *A. p. doriferus* (Smith et al., 2013), and adult *N. cinerea* (Delport et al., 2016). The gut microbiota of pinnipeds appears to be dominated by bacteria belonging to the *Firmicutes, Bacteroidetes, Actinobacteria* and *Proteobacteria* phyla, with the relative abundance of each phylum differing across species (Delport et al., 2016; Medeiros et al., 2016; Nelson et al., 2013; Smith et al., 2013; Stoffel et al., 2020; Toro-Valdivieso et al., 2021). Most of these studies have investigated the gut microbiota composition in adult pinnipeds, with

limited knowledge regarding differences in microbial composition in pups. The variation in gut microbiota composition due to age has been investigated in *A. p. doriferus* (Smith et al., 2013), *M. leonina* (Nelson et al., 2013) and captive *P. largha* (Tian et al., 2020) where changes in the relative abundance of bacterial phyla occurred as individuals aged, and significant shifts coincided with transitions in age classes (i.e., between pups, juvenile and adult life stages). Studies of gut microbial community structure in *N. cinerea* have been limited to captive and free-ranging adults (Delport et al., 2016), and have so far not considered the composition of the gut microbiota in free-ranging pups.

1.6. Aims of the thesis

The studies conducted as part of this thesis aim to investigate the presence of antimicrobial resistant bacteria and diversity of *Escherichia coli* in three species of Australian pinniped pups to determine the potential exposure to anthropogenic pollution, which will be presented in three research chapters. The fourth research chapter will investigate the impact of topical ivermectin treatment on the gut microbiota of *N. cinerea* pups to determine the safety and efficacy of this treatment. The results from the four research chapters presented will be explored in the general discussion of this thesis.

This thesis aims to:

1. Determine the presence of bacteria carrying class 1 integrons in Australian pinniped pups (Chapter 2)
2. Characterise the types of resistance genes carried by bacteria in Australian pinniped pups (Chapter 2)
3. Define the presence and diversity of *E. coli* in pinniped pups (Chapter 3)
4. Investigate whether there is a difference in *E. coli* phylotype diversity across species (Chapter 3)

5. Test whether pinniped pups carry *E. coli* isolates belonging to more than one phylotype (Chapter 4)

6. Characterise the diversity of multiple *E. coli* isolates within individual pups, and determine whether any differences are species dependent (Chapter 4)

7. Characterise and compare the microbiome of anthelminthic treated and non-treated Australian sea lion pups (Chapter 5)

8. Test whether topical ivermectin anthelminthic treatment alters the composition of the gut microbiota in Australian sea lion pups (Chapter 5)

1.7. References

Abt, M. C., and Artis, D. (2013). The dynamic influence of commensal bacteria on the immune response to pathogens. *Curr Opin Microbiol* 16, 4–9. doi: 10.1016/j.mib.2012.12.002.

Acquarone, M., Salgado-Flores, A., and Sundset, M. A. (2020). The bacterial microbiome in the small intestine of hooded seals (*Cystophora cristata*). *Microorganisms* 8, 1664. doi: 10.3390/microorganisms8111664.

Ahlstrom, C. A., Bonnedahl, J., Woksepp, H., Hernandez, J., Olsen, B., and Ramey, A. M. (2018). Acquisition and dissemination of cephalosporin-resistant *E. coli* in migratory birds sampled at an Alaska landfill as inferred through genomic analysis. *Sci Rep* 8, 7361. doi: 10.1038/s41598-018-25474-w.

Ahlstrom, C. A., Ramey, A. M., Woksepp, H., and Bonnedahl, J. (2019). Repeated detection of carbapenemase-producing *Escherichia coli* in Gulls inhabiting Alaska. *Antimicrob Agents Chemother* 63, e00758-19. doi: 10.1128/AAC.00758-19.

Ahmed, W., Sidhu, J. P. S., Smith, K., Beale, D. J., Gyawali, P., and Toze, S. (2016). Distributions of fecal markers in wastewater from different climatic zones for human fecal pollution tracking in Australian surface waters. *Appl Environ Microbiol* 82, 1316–1323. doi: 10.1128/AEM.03765-15.

Ahonen, H., Lowther, A. D., Harcourt, R. G., Goldsworthy, S. D., Charrier, I., and Stow, A. J. (2016). The limits of dispersal: fine scale spatial genetic structure in Australian sea lions. *Front Mar Sci* 3. doi: 10.3389/fmars.2016.00065.

Albenberg, L. G., and Wu, G. D. (2014). Diet and the intestinal microbiome: associations, functions, and implications for health and disease. *Gastroenterology* 146, 1564–1572. doi: 10.1053/j.gastro.2014.01.058.

Allen, H. K., Donato, J., Wang, H. H., Cloud-Hansen, K. A., Davies, J., and Handelsman, J. (2010). Call of the wild: antibiotic resistance genes in natural environments. *Nat Rev Microbiol* 8, 251. doi: 10.1038/nrmicro2312.

Allen, S. E., Boerlin, P., Janecko, N., Lumsden, J. S., Barker, I. K., Pearl, D. L., et al. (2011). Antimicrobial resistance in generic *Escherichia coli* isolates from wild small mammals living in swine farm, residential, landfill, and natural environments in southern Ontario, Canada. *Appl Environ Microbiol* 77, 882–888. doi: 10.1128/AEM.01111-10.

Amato, K. R. (2013). Co-evolution in context: The importance of studying gut microbiomes in wild animals. *Microbiome Science and Medicine* 1, 10–29. doi: 10.2478/micsm-2013-0002.

Amos, G. C. A., Hawkey, P. M., Gaze, W. H., and Wellington, E. M. (2014). Waste water effluent contributes to the dissemination of CTX-M-15 in the natural environment. *J Antimicrob Chemother* 69, 1785–1791. doi: 10.1093/jac/dku079.

Anastasi, E. M., Matthews, B., Gundogdu, A., Vollmerhausen, T. L., Ramos, N. L., Stratton, H., et al. (2010). Prevalence and persistence of *Escherichia coli* strains with uropathogenic virulence characteristics in sewage treatment plants. *Appl Environ Microbiol* 76, 5882–5886. doi: 10.1128/AEM.00141-10.

Anastasi, E. M., Matthews, B., Stratton, H. M., and Katouli, M. (2012). Pathogenic *Escherichia coli* found in sewage treatment plants and environmental waters. *Appl Environ Microbiol* 78, 5536–5541. doi: 10.1128/AEM.00657-12.

Ansari, Z. A., and Matondkar, S. G. P. (2014). Anthropogenic activities including pollution and contamination of coastal marine environment. *J Ecophysiol Occup Health* 14, 71–78. doi: 10.15512/joeoh/2014/v14i1-2/50743.

Arnould, J. P. Y., Boyd, I. L., and Warneke, R. M. (2003). Historical dynamics of the Australian fur seal population: evidence of regulation by man? *Can J Zool* 81, 1428–1436. doi: 10.1139/z03-134.

Ashley, E. A., Olson, J. K., Raverty, S., Wilkinson, K., and Gaydos, J. K. (2020). Trace element concentrations in livers of pacific harbor seals (*Phoca vitulina richardii*) from San Juan County, Washington, USA. *J Wildl Dis* 56, 429–436. doi: 10.7589/2019-04-087.

Avila, I. C., Kaschner, K., and Dormann, C. F. (2018). Current global risks to marine mammals: Taking stock of the threats. *Biol Conserv* 221, 44–58. doi: 10.1016/j.biocon.2018.02.021.

Bäckhed, F., Ley, R. E., Sonnenburg, J. L., Peterson, D. A., and Gordon, J. I. (2005). Host-bacterial mutualism in the human intestine. *Science* 307, 1915–1920. doi: 10.1126/science.1104816.

Bäckhed, F., Roswall, J., Peng, Y., Feng, Q., Jia, H., Kovatcheva-Datchary, P., et al. (2015). Dynamics and stabilization of the human gut microbiome during the first year of life. *Cell Host Microbe* 17, 690–703. doi: 10.1016/j.chom.2015.04.004.

Baharoglu, Z., and Mazel, D. (2014). SOS, the formidable strategy of bacteria against aggressions. *FEMS Microbiol Rev* 38, 1126–1145. doi: 10.1111/1574-6976.12077.

Baquero, F., Martínez, J.-L., and Cantón, R. (2008). Antibiotics and antibiotic resistance in water environments. *Curr Opin Biotechnol* 19, 260–265. doi: 10.1016/j.copbio.2008.05.006.

Bauer, H., Horowitz, R. E., Levenson, S. M., and Popper, H. (1963). The response of the lymphatic tissue to the microbial flora. Studies on germfree mice. *Am J Pathol* 42, 471–483.

Bäumler, A. J., and Sperandio, V. (2016). Interactions between the microbiota and pathogenic bacteria in the gut. *Nature* 535, 85–93. doi: 10.1038/nature18849.

Baylis, A. M. M., Page, B., and Goldsworthy, S. D. (2008). Effect of seasonal changes in upwelling activity on the foraging locations of a wide-ranging central-place forager, the New Zealand fur seal. *Can J Zool* 86, 774–789. doi: 10.1139/Z08-055.

Bearzi, G., Politi, E., Agazzi, S., and Azzellino, A. (2006). Prey depletion caused by overfishing and the decline of marine megafauna in eastern Ionian Sea coastal waters (central Mediterranean). *Biol Conserv* 127, 373–382. doi: 10.1016/j.biocon.2005.08.017.

Beiras, R. (2018). *Marine pollution: sources, fate and effects of pollutants in coastal ecosystems*. Amsterdam, Netherlands: Elsevier doi: 10.1016/C2017-0-00260-4.

Berendonk, T. U., Manaia, C. M., Merlin, C., Fatta-Kassinos, D., Cytryn, E., Walsh, F., et al. (2015). Tackling antibiotic resistance: the environmental framework. *Nat Rev Microbiol* 13. doi: 10.1038/nrmicro3439.

Beversdorf, L. J., Bornstein-Forst, S. M., and McLellan, S. L. (2007). The potential for beach sand to serve as a reservoir for *Escherichia coli* and the physical influences on cell die-off. *J Appl Microbiol* 102, 1372–1381. doi: 10.1111/j.1365-2672.2006.03177.x.

Bik, E. M., Costello, E. K., Switzer, A. D., Callahan, B. J., Holmes, S. P., Wells, R. S., et al. (2016). Marine mammals harbor unique microbiotas shaped by and yet distinct from the sea. *Nat Commun* 7, 10516. doi: 10.1038/ncomms10516.

Bilgmann, K., Armansin, N., Ferchaud, A. L., Normandeau, E., Bernatchez, L., Harcourt, R., et al. (2021). Low effective population size in the genetically bottlenecked Australian sea lion is insufficient to maintain genetic variation. *Anim Conserv* 24, 847–861. doi: 10.1111/acv.12688.

Blyton, M. D. J., Banks, S. C., Peakall, R., and Gordon, D. M. (2013). High temporal variability in commensal *Escherichia coli* strain communities of a herbivorous marsupial. *Environ Microbiol* 15, 2162–2172. doi: 10.1111/1462-2920.12088.

Blyton, M. D. J., Cornall, S. J., Kennedy, K., Colligon, P., and Gordon, D. M. (2014). Sex-dependent competitive dominance of phylogenetic group B2 *Escherichia coli* strains within human hosts. *Environ Microbiol Rep* 6, 605–610. doi: 10.1111/1758-2229.12168.

Bonnedahl, J., Drobni, M., Gauthier-Clerc, M., Hernandez, J., Granholm, S., Kayser, Y., et al. (2009). Dissemination of *Escherichia coli* with CTX-M Type ESBL between humans and yellow-legged gulls in the South of France. *PLOS ONE* 4, e5958. doi: 10.1371/journal.pone.0005958.

Bossart, G. D. (2010). Marine mammals as sentinel species for oceans and human health. *Vet Pathol* 48, 676–690. doi: 10.1177/0300985810388525.

Boucher, Y., Labbate, M., Koenig, J. E., and Stokes, H. W. (2007). Integrons: mobilizable platforms that promote genetic diversity in bacteria. *Trends Microbiol* 15, 301–309. doi: 10.1016/j.tim.2007.05.004.

Brestoff, J. R., and Artis, D. (2013). Commensal bacteria at the interface of host metabolism and the immune system. *Nat Immunol* 14, 676–684. doi: 10.1038/ni.2640.

Brosschot, T. P., and Reynolds, L. A. (2018). The impact of a helminth-modified microbiome on host immunity. *Mucosal Immunol* 11, 1039–1046. doi: 10.1038/s41385-018-0008-5.

Butterworth, D. S., Punt, A. E., Oosthuizen, W. H., and Wickens, P. A. (1995). The effects of future consumption by the Cape fur seal on catches and catch rates of the Cape hakes. 3. Modelling the dynamics of the Cape fur seal *Arctocephalus pusillus pusillus*. *S Afr Mar Sci* 16, 161–183. doi: 10.2989/025776195784156511.

Byrne-Bailey, K. G., Gaze, W. H., Kay, P., Boxall, A. B. A., Hawkey, P. M., and Wellington, E. M. H. (2009). Prevalence of sulfonamide resistance genes in bacterial isolates from manured agricultural soils and pig slurry in the United Kingdom. *Antimicrob Agents Chemother* 53, 696–702. doi: 10.1128/AAC.00652-07.

Cambray, G., Guerout, A.-M., and Mazel, D. (2010). Integrons. *Annu Rev Genet* 44, 141–166. doi: 10.1146/annurev-genet-102209-163504.

Campbell, R. A., Gales, N. J., Lento, G. M., and Baker, C. S. (2008a). Islands in the sea: extreme female natal site fidelity in the Australian sea lion, *Neophoca cinerea*. *Biol Lett* 4, 139–142. doi: 10.1016/j.anbehav.2011.12.015.

Campbell, R., Holley, D., and Christianopolous, D. (2008b). Mitigation of incidental mortality of Australian sea lions in the west coast rock lobster fishery. *Endanger Species Res* 5, 345–358. doi: 10.3354/esr00155.

Cantacessi, C., Giacomin, P., Croese, J., Zakrzewski, M., Sotillo, J., McCann, L., et al. (2014). Impact of experimental hookworm infection on the human gut microbiota. *J Infect Dis* 210, 1431–1434. doi: 10.1093/infdis/jiu256.

Carding, S., Verbeke, K., Vipond, D. T., Corfe, B. M., and Owen, L. J. (2015). Dysbiosis of the gut microbiota in disease. *Microb Ecol Health Dis* 26, 26191. doi: 10.3402/mehd.v26.26191.

Carlos, C., Pires, M. M., Stoppe, N. C., Hachich, E. M., Sato, M. I. Z., Gomes, T. A. T., et al. (2010). *Escherichia coli* phylogenetic group determination and its application in the identification of

the major animal source of fecal contamination. *BMC Microbiol* 10, 161. doi: 10.1186/1471-2180-10-161.

Castinel, A., Duignan, P. J., Pomroy, W. E., López-Villalobos, N., Gibbs, N. J., Chilvers, B. L., et al. (2007). Neonatal mortality in New Zealand sea lions (*Phocarctos hookeri*) at Sandy Bay, Enderby Island, Auckland Islands from 1998 to 2005. *J Wildl Dis* 43, 461–474. doi: 10.7589/0090-3558-43.3.461.

Catania, V., Cascio Diliberto, C., Cigna, V., and Quatrini, P. (2020). Microbes and persistent organic pollutants in the marine environment. *Water Air Soil Pollut* 231, 354. doi: 10.1007/s11270-020-04712-w.

Caugant, D. A., Levin, B. R., and Selander, R. K. (1984). Distribution of multilocus genotypes of *Escherichia coli* within and between host families. *J Hyg (Lond)* 92, 377–384. doi: 10.1017/s0022172400064597.

Cebra, J. J. (1999). Influences of microbiota on intestinal immune system development. *Am J Clin Nutr* 69, 1046–1051. doi: 10.1093/ajcn/69.5.1046s.

Chaw, P. S., Höpner, J., and Mikolajczyk, R. (2018). The knowledge, attitude and practice of health practitioners towards antibiotic prescribing and resistance in developing countries—A systematic review. *J Clin Pharm Ther* 43, 606–613. doi: 10.1111/jcpt.12730.

Chen, H., Jing, L., Yao, Z., Meng, F., and Teng, Y. (2019). Prevalence, source and risk of antibiotic resistance genes in the sediments of Lake Tai (China) deciphered by metagenomic assembly: a comparison with other global lakes. *Environ Int* 127, 267–275. doi: 10.1016/j.envint.2019.03.048.

Chilvers, B. L., and Goldsworthy, S. D. (2015). *Arctocephalus forsteri*. *IUCN Red List of Threatened Species 2015: e.T41664A45230026*. doi: 10.2305/IUCN.UK.2015-2.RLTS.T41664A45230026.en.

Chokshi, A., Cennimo, D., Hrong, H., and Sifri, Z. (2019). Global contributors to antibiotic resistance. *J Glob Infect Dis* 11, 36–42. doi: 10.4103/jgid.jgid_110_18.

Chow, J., Lee, S. M., Shen, Y., Khosravi, A., and Mazmanian, S. K. (2010). Host-bacterial symbiosis in health and disease. *Adv Immunol* 107, 243–274. doi: 10.1016/B978-0-12-381300-8.00008-3.

Clayton, J. B., Vangay, P., Huang, H., Ward, T., Hillmann, B. M., Al-Ghalith, G. A., et al. (2016). Captivity humanizes the primate microbiome. *Proc Natl Acad Sci U S A* 113, 10376–10381. doi: 10.1073/pnas.1521835113.

Clemente, J. C., Ursell, L. K., Parfrey, L. W., and Knight, R. (2012). The impact of the gut microbiota on human health: an integrative view. *Cell* 148, 1258–1270. doi: 10.1016/j.cell.2012.01.035.

Clermont, O., Bonacorsi, S., and Bingen, E. (2000). Rapid and simple determination of the *Escherichia coli* phylogenetic group. *Appl Environ Microbiol* 66, 4555–4558. doi: 10.1128/AEM.66.10.4555-4558.2000.

Clermont, O., Christenson, J. K., Daubié, A.-S., Gordon, D. M., and Denamur, E. (2014). Development of an allele-specific PCR for *Escherichia coli* B2 sub-typing, a rapid and easy to perform substitute of multilocus sequence typing. *J Microbiol Methods* 101, 24–27. doi: 10.1016/j.mimet.2014.03.008.

Clermont, O., Christenson, J. K., Denamur, E., and Gordon, D. M. (2013). The Clermont *Escherichia coli* phylo-typing method revisited: improvement of specificity and detection of new phylo-groups. *Environ Microbiol Rep* 5, 58–65. doi: 10.1111/1758-2229.12019.

Cliffe, L., Humphreys, N., Lane, T., Potten, C., Booth, C., and Grencis, R. (2005). Accelerated intestinal epithelial cell turnover: a new mechanism of parasite expulsion. *Science* 308, 1463–1465. doi: 10.1126/science.1108661.

Collis, C. M., and Hall, R. M. (1992). Site-specific deletion and rearrangement of integron insert genes catalyzed by the integron DNA integrase. *J Bacteriol* 174, 1574–1585. doi: 10.1128/jb.174.5.1574-1585.1992.

Committee, T. S. S. (2020). Conservation advice. *Neophoca cinerea*, Australian sea lion. *Department of Agriculture, Water and the Environment, Canberra*.

Conlon, M. A., and Bird, A. R. (2014). The impact of diet and lifestyle on gut microbiota and human health. *Nutrients* 7, 17–44. doi: 10.3390/nu7010017.

Consortium, H. M. P. (2012). Structure, function and diversity of the healthy human microbiome. *Nature* 486, 207–214. doi: 10.1038/nature11234.

Cooper, P., Walker, A. W., Reyes, J., Chico, M., Salter, S. J., Vaca, M., et al. (2013). Patent human infections with the whipworm, *Trichuris trichiura*, are not associated with alterations in the faecal microbiota. *PLOS ONE* 8, e76573. doi: 10.1371/journal.pone.0076573.

Cortés, A., Peachey, L. E., Jenkins, T. P., Scotti, R., and Cantacessi, C. (2019). Helminths and microbes within the vertebrate gut – not all studies are created equal. *Parasitology* 146, 1371–1378. doi: 10.1017/S003118201900088X.

Costea, P. I., Hildebrand, F., Arumugam, M., Bäckhed, F., Blaser, M. J., Bushman, F. D., et al. (2018). Enterotypes in the landscape of gut microbial community composition. *Nat Microbiol* 3, 8–16. doi: 10.1038/s41564-017-0072-8.

Croxen, M. A., and Finlay, B. B. (2010). Molecular mechanisms of *Escherichia coli* pathogenicity. *Nat Rev Microbiol* 8, 26–38. doi: 10.1038/NRMICRO2265.

Curtis, S., Elwen, S. H., Dreyer, N., and Gridley, T. (2021). Entanglement of Cape fur seals (*Arctocephalus pusillus pusillus*) at colonies in central Namibia. *Mar Pollut Bull* 171, 112759. doi: 10.1016/j.marpolbul.2021.112759.

Davidson, A. D., Boyer, A. G., Kim, H., Pompa-Mansilla, S., Hamilton, M. J., Costa, D. P., et al. (2012). Drivers and hotspots of extinction risk in marine mammals. *Proc Natl Acad Sci U S A* 109, 3395–3400. doi: 10.1073/pnas.1121469109.

Dawes, F. E., Kuzevski, A., Bettelheim, K. A., Hornitzky, M. A., Djordjevic, S. P., and Walker, M. J. (2010). Distribution of class 1 integrons with IS*26*-mediated deletions in their 3'-conserved segments in *Escherichia coli* of human and animal origin. *PLOS ONE* 5, e12754–e12754. doi: 10.1371/journal.pone.0012754.

Delport, T. C., Harcourt, R. G., Beaumont, L. J., Webster, K. N., and Power, M. L. (2015). Molecular detection of antibiotic-resistance determinants in *Escherichia coli* isolated from the endangered Australian sea lion (*Neophoca cinerea*). *J Wildl Dis* 51, 555–563. doi: 10.7589/2014-08-200.

Delport, T. C., Power, M. L., Harcourt, R. G., Webster, K. N., and Tetu, S. G. (2016). Colony location and captivity influence the gut microbial community composition of the Australian sea lion (*Neophoca cinerea*). *Appl Environ Microbiol* 82, 3440–3449. doi: 10.1128/AEM.00192-16.

DeMaster, D. P., Fowler, C. W., Perry, S. L., and Richlen, M. F. (2001). Predation and competition: the impact of fisheries on marine-mammal populations over the next one hundred years. *J Mammal* 82, 641–651. doi: 10.1644/1545-1542(2001)082<0641:PACTIO>2.0.CO;2.

Denamur, E., Clermont, O., Bonacorsi, S., and Gordon, D. (2021). The population genetics of pathogenic *Escherichia coli*. *Nat Rev Microbiol* 19, 37–54. doi: 10.1038/s41579-020-0416-x.

Deng, Y., Bao, X., Ji, L., Chen, L., Liu, J., Miao, J., et al. (2015). Resistance integrons: class 1, 2 and 3 integrons. *Ann Clin Microbiol Antimicrob* 14, 45. doi: 10.1186/s12941-015-0100-6.

Denuncio, P., Mandiola, M. A., Pérez Salles, S. B., Machado, R., Ott, P. H., de Oliveira, L. R., et al. (2017). Marine debris ingestion by the South American fur seal from the Southwest Atlantic Ocean. *Mar Pollut Bull* 122, 420–425. doi: 10.1016/j.marpolbul.2017.07.013.

Dheilly, A., Bouder, A., le Devendec, L., Hellard, G., and Kempf, I. (2011). Clinical and microbial efficacy of antimicrobial treatments of experimental avian colibacillosis. *Vet Microbiol* 149, 422–429. doi: 10.1016/j.vetmic.2010.11.033.

di Cesare, A., Pjevac, P., Eckert, E., Curkov, N., Miko Šparica, M., Corno, G., et al. (2020). The role of metal contamination in shaping microbial communities in heavily polluted marine sediments. *Environ Pollut* 265, 114823. doi: 10.1016/j.envpol.2020.114823.

Dolejska, M., Cizek, A., and Literak, I. (2007). High prevalence of antimicrobial-resistant genes and integrons in *Escherichia coli* isolates from Black-headed Gulls in the Czech Republic. *J Appl Microbiol* 103, 11–19. doi: 10.1111/j.1365-2672.2006.03241.x.

Dolejska, M., and Literak, I. (2019). Wildlife is overlooked in the epidemiology of medically important antibiotic-resistant bacteria. *Antimicrob Agents Chemother* 8, e01167-19. doi: 10.1128/AAC.01167-19.

Domingues, S., da Silva, G. J., and Nielsen, K. M. (2015). Global dissemination patterns of common gene cassette arrays in class 1 integrons. *Microbiology (U.K.)* 161, 1313–1337. doi: 10.1099/MIC.0.000099.

Domingues, S., Silva, G. J. da, and Nielsen, K. M. (2012). Integrons: vehicles and pathways for horizontal dissemination in bacteria. *Mob Genet Elements* 2, 211. doi: 10.4161/MGE.22967.

Duignan, P. J., House, C., Geraci, J. R., Early, G., Copland, H. G., Walsh, M. T., et al. (1995). Morbillivirus infection in two species of pilot whale (*Globicephala* sp.) from the western Atlantic. *Mar Mamm Sci* 11, 150–162. doi: 10.1111/j.1748-7692.1995.tb00514.x.

Duriez, P., Clermont, O., Bonacorsi, S., Bingen, E., Chaventré, A., Elion, J., et al. (2001). Commensal *Escherichia coli* isolates are phylogenetically distributed among geographically distinct human populations. *Microbiology* 147, 1671–1676. doi: 10.1099/00221287-147-6-1671.

Easton, A. v., Quiñones, M., Vujkovic-Cvijin, I., Oliveira, R. G., Kepha, S., Odiere, M. R., et al. (2019). The impact of anthelmintic treatment on human gut microbiota based on cross-sectional and pre-and postdeworming comparisons in Western Kenya. *mBio* 10, 1–14. doi: 10.1128/MBIO.00519-19.

Eckburg, P. B., Bik, E. M., Bernstein, C. N., Purdom, E., Dethlefsen, L., Sargent, M., et al. (2005). Diversity of the human intestinal microbial flora. *Science* 308, 1635–1638. doi: 10.1126/science.1110591.

Escobar-Páramo, P., Clermont, O., Blanc-Potard, A.-B., Bui, H., le Bouguénec, C., and Denamur, E. (2004). A specific genetic background Is required for acquisition and expression of virulence factors in *Escherichia coli*. *Mol Biol Evol* 21, 1085–1094. doi: 10.1093/molbev/msh118.

Escobar-Páramo, P., Le Menac'h, A., Le Gall, T., Amorin, C., Gouriou, S., Picard, B., et al. (2006). Identification of forces shaping the commensal *Escherichia coli* genetic structure by comparing animal and human isolates. *Environ Microbiol* 8, 1975–1984. doi: 10.1111/j.1462-2920.2006.01077.x.

Escudero, J., Loot, C., Nivina, A., and Mazel, D. (2015). The integron: adaptation on demand. *Microbiol Spectr* 3, 139–161. doi: 10.1128/microbiolspec.MDNA3-0019-2014.

Ezenwa, V. O., and Jolles, A. E. (2015). Opposite effects of anthelmintic treatment on microbial infection at individual versus population scales. *Science* 347, 175–177. doi: 10.1126/science.1261714.

Fair, P. A., Adams, J., Mitchum, G., Hulsey, T. C., Reif, J. S., Houde, M., et al. (2010). Contaminant blubber burdens in Atlantic bottlenose dolphins (*Tursiops truncatus*) from two southeastern US estuarine areas: concentrations and patterns of PCBs, pesticides, PBDEs, PFCs, and PAHs. *Sci Total Environ* 408, 1577–1597. doi: 10.1016/j.scitotenv.2009.12.021.

Fair, P. A., and Becker, P. R. (2000). Review of stress in marine mammals. *J Aquat Ecosyst Stress Recovery* 7, 335–354. doi: 10.1023/A:1009968113079.

Fallani, M., Young, D., Scott, J., Norin, E., Amarri, S., Adam, R., et al. (2010). Intestinal microbiota of 6-week-old infants across Europe: geographic influence beyond delivery mode, breast-feeding, and antibiotics. *J Pediatr Gastroenterol Nutr* 51. doi: 10.1097/MPG.0b013e3181d1b11e.

Fanaro, S., Chierici, R., Guerrini, P., and Vigi, V. (2003). Intestinal microflora in early infancy: composition and development. *Acta Paediatr* 92, 48055. doi: 10.1111/j.1651-2227.2003.tb00646.x.

Fulham, M., Power, M., and Gray, R. (2018). Comparative ecology of *Escherichia coli* in endangered Australian sea lion (*Neophoca cinerea*) pups. *Infect Genet Evol* 62, 262–269. doi: 10.1016/j.meegid.2018.05.002.

Furness, L. E., Campbell, A., Zhang, L., Gaze, W. H., and McDonald, R. A. (2017). Wild small mammals as sentinels for the environmental transmission of antimicrobial resistance. *Environ Res* 154, 28–34. doi: 10.1016/j.envres.2016.12.014.

Gales, N. J., Shaughnessy, P. D., and Dennis, T. E. (1994). Distribution, abundance and breeding cycle of the Australian sea lion *Neophoca cinerea* (Mammalia: Pinnipedia). *J Zool* 234, 353–370. doi: 10.1111/j.1469-7998.1994.tb04853.x.

Gales, N. J., Williamson, P., Higgins, L. V., Blackberry, M. A., and James, I. (1997). Evidence for a prolonged post implantation period in the Australian sea lion (*Neophoca cinerea*). *J Reprod Fertil* 111, 159–163. doi: 10.1530/jrf.0.1110159.

Gales, Nick., Hindell, M., and Kirkwood, Roger. (2003). *Marine mammals: fisheries, tourism and management issues*. Collingwood, Vic: CSIRO Publishing.

Gaze, W. H., Zhang, L., Abdouslam, N. A., Hawkey, P. M., Calvo-Bado, L., Royle, J., et al. (2011). Impacts of anthropogenic activity on the ecology of class 1 integrons and integron-associated genes in the environment. *ISME J* 5, 1253–1261. doi: 10.1038/ismej.2011.15.

Ghaly, T. M., Gillings, M. R., Penesyan, A., Qi, Q., Rajabal, V., and Tetu, S. G. (2021a). The natural history of integrons. *Microorganisms* 9. doi: 10.3390/microorganisms9112212.

Ghaly, T. M., Tetu, S. G., and Gillings, M. R. (2021b). Predicting the taxonomic and environmental sources of integron gene cassettes using structural and sequence homology of attC sites. *Commun Biol* 4, 946. doi: 10.1038/s42003-021-02489-0.

Ghosh, S., and LaPara, T. M. (2007). The effects of subtherapeutic antibiotic use in farm animals on the proliferation and persistence of antibiotic resistance among soil bacteria. *ISME J* 1, 191–203. doi: 10.1038/ismej.2007.31.

Giacomin, P., Croese, J., Krause, L., Loukas, A., and Cantacessi, C. (2015). Suppression of inflammation by helminths: a role for the gut microbiota? *Philos Trans R Soc Lond B Biol Sci* 370. doi: 10.1098/RSTB.2014.0296.

Gillings, M., Boucher, Y., Labbate, M., Holmes, A., Krishnan, S., Holley, M., et al. (2008). The evolution of class 1 integrons and the rise of antibiotic resistance. *J Bacteriol* 190, 5095–5100. doi: 10.1128/JB.00152-08.

Gillings, M. R. (2014). Integrons: past, present, and future. *Microbiol Mol Biol Rev* 78, 257–277. doi: 10.1128/MMBR.00056-13.

Gillings, M. R. (2017). Class 1 integrons as invasive species. *Curr Opin Microbiol* 38, 10–15. doi: 10.1016/j.mib.2017.03.002.

Gillings, M. R. (2018). DNA as a Pollutant: the clinical class 1 integron. *Curr Pollut Rep* 4, 49–55. doi: 10.1007/s40726-018-0076-x.

Gillings, M. R., Gaze, W. H., Pruden, A., Smalla, K., Tiedje, J. M., and Zhu, Y.-G. (2014). Using the class 1 integron-integrase gene as a proxy for anthropogenic pollution. *ISME J* 9. doi: 10.1038/ismej.2014.226.

Gillings, M. R., Xuejun, D., Hardwick, S. A., Holley, M. P., and Stokes, H. W. (2009). Gene cassettes encoding resistance to quaternary ammonium compounds: a role in the origin of clinical class 1 integrons? *ISME J* 3, 209–215. doi: 10.1038/ismej.2008.98.

Glad, T., Kristiansen, V. F., Nielsen, K. M., Brusetti, L., Wright, A. G., and Sundset, M. A. (2010). Ecological characterisation of the colonic microbiota in Arctic and sub-Arctic seals. *Microb Ecol* 60, 320–330. doi: 10.1007/s00248-010-9690-x.

Goldberg, T. L., Gillespie, T. R., Rwego, I. B., Wheeler, E., Estoff, E. L., and Chapman, C. A. (2007). Patterns of gastrointestinal bacterial exchange between chimpanzees and humans involved in research and tourism in western Uganda. *Biol Conserv* 135, 511–517. doi: 10.1016/j.biocon.2006.10.048.

Goldsworthy, S. D., Gales, N. J., and Chilvers, B. L. (2015). *Neophoca cinerea*. *IUCN Red List of Threatened Species 2015: e.T14549A45228341*.

Goldsworthy, S. D., McKenzie, J., Shaughnessy, P. D., McIntosh, R. R., Page, B., and Campbell, R. (2009a). An update of the report: understanding the impediments to the growth of Australian sea lion populations. *SARDI Publication Number F2008/00847-1 SARDI Research Report Series No. 297*.

Goldsworthy, S. D., Page, B., Lowther, A. D., and Shaughnessy, P. D. (2012). Maintaining the monitoring of pup production at key Australian sea lion colonies in South Australia (2010/11). *SARDI Publication Number F2010/000665-2. SARDI Research Report Series No. 601 (Adelaide, Australia)*.

Goldsworthy, S. D., Page, B., Shaughnessy, P. D., Hamer, D. J., Peters, K. D., McIntosh, R. R., et al. (2009b). Innovative solutions for aquaculture planning and management: addressing seal interactions in the finfish aquaculture industry. FRDC Project Number: 2004/201. SARDI Aquatic Sciences.

Goldsworthy, S. D., Shaughnessy, P. D., Mackay, A. I., Bailleul, F., Holman, D., Lowther, A. D., et al. (2021). Assessment of the status and trends in abundance of a coastal pinniped, the Australian sea lion *Neophoca cinerea*. *Endanger Species Res* 44, 421–437. doi: 10.3354/ESR01118.

Goodrich, J. K., Waters, J. L., Poole, A. C., Sutter, J. L., Koren, O., Blekhman, R., et al. (2014). Human genetics shape the gut microbiome. *Cell* 159, 789–799. doi: 10.1016/j.cell.2014.09.053.

Gordon, D. M. (2005). The influence of ecological factors on the distribution and the genetic structure of *Escherichia coli*. *EcoSal Plus* 1. doi: 10.1128/ecosalplus.6.4.1.

Gordon, D. M. (2010). Strain typing and the ecological structure of *Escherichia coli*. *J AOAC Int* 93, 974–984. doi: 10.1093/jaoac/93.3.974.

Gordon, D. M. (2013). "Chapter 1 - The ecology of *Escherichia coli*," in *Escherichia coli (Second Edition)*, ed. M. S. Donnenberg (Boston: Academic Press), 3–20. doi: 10.1016/B978-0-12-397048-0.00001-2.

Gordon, D. M., Clermont, O., Tolley, H., and Denamur, E. (2008). Assigning *Escherichia coli* strains to phylogenetic groups: multi-locus sequence typing versus the PCR triplex method. *Environ Microbiol* 10, 2484–2496. doi: 10.1111/j.1462-2920.2008.01669.x.

Gordon, D. M., and Cowling, A. (2003). The distribution and genetic structure of *Escherichia coli* in Australian vertebrates: host and geographic effects. *Microbiology* 149, 3575–3586. doi: 10.1099/mic.0.26486-0.

Grilo, M. L., Sousa-Santos, C., Robalo, J., and Oliveira, M. (2020). The potential of *Aeromonas* spp. from wildlife as antimicrobial resistance indicators in aquatic environments. *Ecol Indic* 115. doi: 10.1016/j.ecolind.2020.106396.

Guenther, S., Ewers, C., and Wieler, L. H. (2011). Extended-spectrum beta-lactamases producing *E. coli* in wildlife, yet another form of environmental pollution? *Front Microbiol* 2, 246. doi: 10.3389/fmicb.2011.00246.

Guerin, É., Cambray, G., Sanchez-Alberola, N., Campoy, S., Erill, I., Re, S. da, et al. (2009). The SOS response controls integron recombination. *Science* 324, 1034. doi: 10.1126/science.1172914.

Häder, D.-P., Banaszak, A. T., Villafañe, V. E., Narvarte, M. A., González, R. A., and Helbling, E. W. (2020). Anthropogenic pollution of aquatic ecosystems: emerging problems with global implications. *Sci Total Environ* 713, 136586. doi: 10.1016/j.scitotenv.2020.136586.

Hall, R. M. (2012). Integrons and gene cassettes: hotspots of diversity in bacterial genomes. *Ann N. Y. Acad Sci* 1267, 71–78. doi: 10.1111/j.1749-6632.2012.06588.x.

Hall, R. M., and Collis, C. M. (1995). Mobile gene cassettes and integrons: capture and spread of genes by site-specific recombination. *Mol Microbiol* 15, 593–600. doi: 10.1111/j.1365-2958.1995.tb02368.x.

Hamer, D. J., and Goldsworthy, S. D. (2006). Seal-fishery operational interactions: identifying the environmental and operational aspects of a trawl fishery that contribute to by-catch and mortality of Australian fur seals (*Arctocephalus pusillus doriferus*). *Biol Conserv* 130, 517–529. doi: 10.1016/j.biocon.2006.01.014.

Hamer, D. J., Goldsworthy, S. D., Costa, D. P., Folwer, S., Page, M., and Sumner, M. D. (2013). The endangered Australian sea lion extensively overlaps with and regularly becomes by-catch in demersal shark gill-nets in South Australian shelf waters. *Biol Conserv* 157, 386–400. doi: 10.1016/j.biocon.2012.07.010.

Han, W., Meehl, G. A., Rajagopalan, B., Fasullo, J. T., Hu, A., Lin, J., et al. (2010). Patterns of Indian Ocean sea-level change in a warming climate. *Nat Geosci* 3, 546–550. doi: 10.1038/ngeo901.

Hartz, A., Cuvelier, M., Nowosielski, K., Bonilla, T. D., Green, M., Esiobu, N., et al. (2008). Survival potential of *Escherichia coli* and Enterococci in subtropical beach sand: implications for water quality managers. *J Environ Qual* 37, 898. doi: 10.2134/jeq2007.0312.

Hazen, E. L., Abrahms, B., Brodie, S., Carroll, G., Jacox, M. G., Savoca, M. S., et al. (2019). Marine top predators as climate and ecosystem sentinels. *Front Ecol Environ* 17, 565–574. doi: 10.1002/FEE.2125.

He, F., Zhai, J., Zhang, L., Liu, D., Ma, Y., Rong, K., et al. (2018). Variations in gut microbiota and fecal metabolic phenotype associated with Fenbendazole and Ivermectin tablets by 16S rRNA gene sequencing and LC/MS-based metabolomics in Amur tiger. *Biochem Biophys Res Commun* 499, 447–453. doi: 10.1016/j.bbrc.2018.03.158.

Helm, R. C., Costa, D. P., DeBruyn, T. D., O'Shea, T. J., Wells, R. S., and Williams, T. M. (2014). "Overview of effects of oil spills on marine mammals," in *Handbook of Oil Spill Science and Technology*, ed. M. Fingas.

Hernández, F., Calısto-Ulloa, N., Gómez-Fuentes, C., Gómez, M., Ferrer, J., González-Rocha, G., et al. (2019). Occurrence of antibiotics and bacterial resistance in wastewater and sea water from the Antarctic. *J Hazard Mater* 363, 447–456. doi: 10.1016/j.jhazmat.2018.07.027.

Hernández, J., Stedt, J., Bonnedahl, J., Molin, Y., Drobni, M., Calisto-Ulloa, N., et al. (2012). Human-associated extended-spectrum β-lactamase in the Antarctic. *Appl Environ Microbiol* 78, 2056–2058. doi: 10.1128/AEM.07320-11.

Higgins, L. V., and Gass, L. (1993). Birth to weaning: parturition, duration of lactation, and attendance cycles of Australian sea lions (*Neophoca cinerea*). *Can J Zool* 71, 2047–2055. doi: 10.1139/z93-290.

Hill, D. A., and Artis, D. (2010). Intestinal bacteria and the regulation of immune cell homeostasis. *Annu Rev Immunol* 28, 623–667. doi: 10.1146/annurev-immunol-030409-101330.

Hill, M. J. (1997). Intestinal flora and endogenous vitamin synthesis. *Eur J Cancer Prev* 6, S43–S45. doi: 10.1097/00008469-199703001-00009.

Hofmeyr, G. J. G. (2015). *Arctocephalus pusillus*. *IUCN Red List of Threatened Species 2015: e.T2060A45224212*. doi: 10.2305/IUCN.UK.2015-4.RLTS.T2060A45224212.en.

Honda, K., and Littman, D. R. (2016). The microbiota in adaptive immune homeostasis and disease. *Nature* 535, 75–84. doi: 10.1038/nature18848.

Hooper, L. V., and Gordon, J. I. (2001). Commensal host-bacterial relationships in the gut. *Science* 292, 1115–1118. doi: 10.1126/science.1058709.

Hooper, L. V., Littman, D. R., and Macpherson, A. J. (2012). Interactions between the microbiota and the immune system. *Science* 336, 1268–1273. doi: 10.1126/science.1223490.

Hooper, L. V., Midtvedt, T., and Gordon, J. I. (2002). How host-microbial interactions shape the nutrient environment of the mammalian intestine. *Annu Rev Nutr* 22, 283–307. doi: 10.1146/annurev.nutr.22.011602.092259.

Houlden, A., Hayes, K. S., Bancroft, A. J., Worthington, J. J., Wang, P., Grencis, R. K., et al. (2015). Chronic *Trichuris muris* infection in C57BL/6 mice causes significant changes in host microbiota and metabolome: effects reversed by pathogen clearance. *PLOS ONE* 10, e0125945. doi: 10.1371/journal.pone.0125945.

Jamieson, R. C., Gordon, R. J., Sharples, K. E., Stratton, G. W., and Madani, A. (2002). Movement and persistence of fecal bacteria in agricultural soils and subsurface drainage water: A review. *Can Biosyst Eng* 44, 1.1-1.9.

Jefferson, T. A., Webber, M. A., and Pitman, R. L. (2015). “3 - Taxonomic groupings above the species level,” in *Marine Mammals of the World (Second Edition)*, eds. T. A. Jefferson, M. A. Webber, and R. L. Pitman (San Diego: Academic Press), 17–23. doi: 10.1016/B978-0-12-409542-7.50003-2.

Jenkins, T. P., Formenti, F., Castro, C., Piubelli, C., Perandin, F., Buonfrate, D., et al. (2018). A comprehensive analysis of the faecal microbiome and metabolome of *Strongyloides stercoralis* infected volunteers from a non-endemic area. *Sci Rep* 8, 15651. doi: 10.1038/s41598-018-33937-3.

Jobbins, S. E., and Alexander, K. A. (2015). From whence they came - Antibiotic-resistant *Escherichia coli* in African wildlife. *J Wildl Dis* 51, 811–820. doi: 10.7589/2014-11-257.

Johnson, J. R., Delavari, P., Kuskowski, M., and Stell, A. L. (2001). Phylogenetic distribution of extraintestinal virulence-associated traits in *Escherichia coli*. *J Infect Dis* 183, 78–88. doi: 10.1086/317656.

Johnson, J. R., Gajewski, A., Lesse, A. J., and Russo, T. A. (2003). Extraintestinal pathogenic *Escherichia coli* as a cause of invasive nonurinary infections. *J Clin Microbiol* 41, 5798–5802. doi: 10.1128/JCM.41.12.5798-5802.2003.

Jönsson, B. F., and Watson, J. R. (2016). The timescales of global surface-ocean connectivity. *Nat Commun* 7, 11239. doi: 10.1038/ncomms11239.

Kamada, N., Chen, G. Y., Inohara, N., and Núñez, G. (2013). Control of pathogens and pathobionts by the gut microbiota. *Nat Immunol* 14, 685–690. doi: 10.1038/ni.2608.

Kaper, J. B., Nataro, J. P., and Mobley, H. L. T. (2004). Pathogenic *Escherichia coli*. *Nat Rev Microbiol* 2, 123–140. doi: 10.1038/nrmicro818.

Karkman, A., Pärnänen, K., and Larsson, D. G. J. (2019). Fecal pollution can explain antibiotic resistance gene abundances in anthropogenically impacted environments. *Nat Commun* 10, 80. doi: 10.1038/s41467-018-07992-3.

Kaschner, K., Tittensor, D. P., Ready, J., Gerrodette, T., and Worm, B. (2011). Current and future patterns of global marine mammal biodiversity. *PLOS ONE* 6, e19653. doi: 10.1371/journal.pone.0019653.

Kau, A. L., Ahern, P. P., Griffin, N. W., Goodman, A. L., and Gordon, J. I. (2011). Human nutrition, the gut microbiome and the immune system. *Nature* 474, 327–336. doi: 10.1038/nature10213.

Kirkman, S., Oosthuizen, W., Meÿer, M., Kotze, P., Roux, J.-P., and Underhill, L. (2007). Making sense of censuses and dealing with missing data: trends in pup counts of Cape fur seal *Arctocephalus pusillus pusillus* for the period 1972–2004. *Afr J Mar Sci* 29, 161–176. doi: 10.2989/AJMS.2007.29.2.2.185.

Kirkwood, R., and Arnould, J. P. Y. (2012). Foraging trip strategies and habitat use during late pup rearing by lactating Australian fur seals. *Aust J Zool* 59, 216–226. doi: 10.1071/ZO11080.

Kirkwood, R., Gales, R., Terauds, A., Arnould, J. P. Y., Pemberton, D., Shaughnessy, P. D., et al. (2005). Pup production and population trends of the Australian fur seal (*Arctocephalus pusillus doriferus*). *Mar Mamm Sci* 21, 260–282. doi: 10.1111/j.1748-7692.2005.tb01227.x.

Kirkwood, R., Pemberton, D., Gales, R., Hoskins, A. J., Mitchell, T., Shaughnessy, P. D., et al. (2010). Continued population recovery by Australian fur seals. *Mar Freshw Res* 61, 695–701. doi: 10.1071/MF09213.

Kirkwood, Roger., and Goldsworthy, Simon. (2013). *Fur Seals and Sea Lions*. Collingwood, VIC: CSIRO Publishing.

Koczura, R., Mokracka, J., Jabłońska, L., Gozdecka, E., Kubek, M., and Kaznowski, A. (2012). Antimicrobial resistance of integron-harboring *Escherichia coli* isolates from clinical samples, wastewater treatment plant and river water. *Sci Total Environ* 414, 680–685. doi: 10.1016/j.scitotenv.2011.10.036.

Koenig, J. E., Spor, A., Scalfone, N., Fricker, A. D., Stombaugh, J., Knight, R., et al. (2011). Succession of microbial consortia in the developing infant gut microbiome. *Proc Natl Acad Sci U S A* 108, 4578–4585. doi: 10.1073/pnas.1000081107.

Kovacs, K. M., Aguilar, A., Aurioles, D., Burkanov, V., Campagna, C., Gales, N., et al. (2012). Global threats to pinnipeds. *Mar Mamm Sci* 28, 414–436. doi: 10.1111/j.1748-7692.2011.00479.x.

Kreisinger, J., Bastien, G., Hauffe, H. C., Marchesi, J., and Perkins, S. E. (2015). Interactions between multiple helminths and the gut microbiota in wild rodents. *Philos Trans R Soc Lond B Biol Sci* 370. doi: 10.1098/RSTB.2014.0295.

Kretzmann, M., Rohrbach, L., Durham, K., Digiovanni, R., and Sañudo-Wilhelmy, S. (2010). Trace metal burdens in stranded seals from Long Island, New York: potential evidence for species differences in foraging. *Aquat Mamm* 36, 178–187. doi: 10.1578/AM.36.2.2010.178.

Kreuder, C., Miller, M. A., Jessup, D. A., Lowenstine, L. J., Harris, M. D., Ames, J. A., et al. (2003). Patterns of mortality in southern sea otters (*Enhydra lutris nereis*) from 1998-2001. *J Wildl Dis* 39, 495–509. doi: 10.7589/0090-3558-39.3.495.

Kümmerer, K. (2003). Significance of antibiotics in the environment. *J Antimicrob Chemother* 52, 5–7. doi: 10.1093/jac/dkg293.

Labbate, M., Case, R. J., and Stokes, H. W. (2009). The integron/gene cassette system: an active player in bacterial adaptation. *Methods Mol Biol*, 103–125. doi: 10.1007/978-1-60327-853-9_6.

Lancaster, M. L., Arnould, J. P. Y., and Kirkwood, R. (2010). Genetic status of an endemic marine mammal, the Australian fur seal, following historical harvesting. *Anim Conserv* 13, 247–255. doi: 10.1111/j.1469-1795.2009.00325.x.

LaPara, T. M., Burch, T. R., McNamara, P. J., Tan, D. T., Yan, M., and Eichmiller, J. J. (2011). Tertiary-treated municipal wastewater is a significant point source of antibiotic resistance genes into Duluth-Superior harbor. *Environ Sci Technol* 45, 9543–9549. doi: 10.1021/es202775r.

Larsson, D. G. J. (2014). Pollution from drug manufacturing: review and perspectives. *Philos Trans R Soc Lond B Biol Sci* 369. doi: 10.1098/RSTB.2013.0571.

le Page, G., Gunnarsson, L., Snape, J., and Tyler, C. R. (2017). Integrating human and environmental health in antibiotic risk assessment: a critical analysis of protection goals, species sensitivity and antimicrobial resistance. *Environ Int* 109, 155–169. doi: 10.1016/j.envint.2017.09.013.

Lee, S. C., Tang, M. S., Lim, Y. A. L., Choy, S. H., Kurtz, Z. D., Cox, L. M., et al. (2014). Helminth colonization is associated with increased diversity of the gut microbiota. *PLoS Negl Trop Dis* 8, e2880. doi: 10.1371/journal.pntd.0009325.

Leeming, E. R., Johnson, A. J., Spector, T. D., and le Roy, C. I. (2019). Effect of diet on the gut microbiota: Rethinking intervention duration. *Nutrients* 11, 2862. doi: 10.3390/nu11122862.

Lerminiaux, N. A., and Cameron, A. D. S. (2018). Horizontal transfer of antibiotic resistance genes in clinical environments. *Can J Microbiol* 65, 34–44. doi: 10.1139/cjm-2018-0275.

Leung, J. M., Graham, A. L., and Knowles, S. C. L. (2018). Parasite-microbiota interactions with the vertebrate gut: Synthesis through an ecological lens. *Front Microbiol* 9. doi: 10.3389/fmicb.2018.00.

Ley, R. E., Lozupone, C. A., Hamady, M., Knight, R., and Gordon, J. I. (2008). Worlds within worlds: evolution of the vertebrate gut microbiota. *Nat Rev Microbiol* 6, 776–788. doi: 10.1038/nrmicro1978.

Lindsay, S. A., Caraguel, C. G. B., and Gray, R. (2021). Topical ivermectin is a highly effective seal 'spot-on': A randomised trial of hookworm and lice treatment in the endangered Australian sea lion (*Neophoca cinerea*). *Int J Parasitol Parasites Wildl* 16, 275–284. doi: 10.1016/j.ijppaw.2021.11.002.

Ling, J. K. (1999). Exploitation of fur seals and sea lions from Australian, New Zealand and adjacent subantarctic islands during the eighteenth, nineteenth and twentieth centuries. *Aust Zool* 31, 323–350. doi: 10.7882/AZ.1999.036.

Ling, J. K. (2002). Impact of colonial sealing on seal stocks around Australia, New Zealand and subantarctic islands between 150 And 170 degrees east. *Aust Mammal* 24, 117–126. doi: 10.1071/AM02117.

Lipscomb, T. P., Schulman, F. Y., Moffett, D., and Kennedy, S. (1994). Morbilliviral disease in Atlantic bottlenose dolphins (*Tursiops truncatus*) from the 1987-1988 epizootic. *J Wildl Dis* 30, 567–571. doi: 10.7589/0090-3558-30.4.567.

Littman, D. R., and Pamer, E. G. (2011). Role of the commensal microbiota in normal and pathogenic host immune responses. *Cell Host Microbe* 10, 311–323. doi: 10.1016/j.chom.2011.10.004.

Lowther, A. D., Harcourt, R. G., Goldsworthy, S. D., and Stow, A. (2012). Population structure of adult female Australian sea lions is driven by fine-scale foraging site fidelity. *Anim Behav* 83, 691–701. doi: 10.1016/j.anbehav.2011.12.015.

Lozupone, C. A., Stombaugh, J. I., Gordon, J. I., Jansson, J. K., and Knight, R. (2012). Diversity, stability and resilience of the human gut microbiota. *Nature* 489, 220–230. doi: 10.1038/nature11550.

Lundbäck, I. C., McDougall, F. K., Dann, P., Slip, D. J., Gray, R., and Power, M. L. (2020). Into the sea: Antimicrobial resistance determinants in the microbiota of little penguins (*Eudyptula minor*). *Infect Genet Evol*, 104697. doi: 10.1016/j.meegid.2020.104697.

Lupo, A., Coyne, S., and Berendonk, T. U. (2012). Origin and evolution of antibiotic resistance: the common mechanisms of emergence and spread in water bodies. *Front Microbiol* 3, 18. doi: 10.3389/fmicb.2012.00018.

Lynch, S. v, and Pedersen, O. (2016). The human intestinal microbiome in health and disease. *N Engl J Med* 375, 2369–2379. doi: 10.1056/NEJMra1600266.

Lyons, E. T., DeLong, R. L., Nadler, S. A., Laake, J. L., Orr, A. J., DeLong, B. L., et al. (2011). Investigations of peritoneal and intestinal infections of adult hookworms (*Uncinaria* spp.) in northern fur seal (*Callorhinus ursinus*) and California sea lion (*Zalophus californianus*) pups on San Miguel Island, California (2003). *Parasitol Res* 109, 581–589. doi: 10.1007/s00436-011-2289-4.

Lyons, E. T., Melin, S. R., DeLong, R. L., Orr, A. J., Gulland, F. M., and Tolliver, S. C. (2001). Current prevalence of adult *Uncinaria* spp. in northern fur seal (*Callorhinus ursinus*) and California sea lion (*Zalophus californianus*) pups on San Miguel Island, California, with notes on the biology of these hookworms. *Vet Parasitol* 97, 309–318. doi: 10.1016/S0304-4017(01)00418-6.

Macpherson, A. J., and Harris, N. L. (2004). Interactions between commensal intestinal bacteria and the immune system. *Nat Rev Immunol* 4, 478–485. doi: 10.1038/nri1373.

Mann, A., Nehra, K., Rana, J. S., and Dahiya, T. (2021). Antibiotic resistance in agriculture: perspectives on upcoming strategies to overcome upsurge in resistance. *Curr Res Microb Sci* 2, 100030. doi: 10.1016/j.crmicr.2021.100030.

Marcovecchio, J. E., Gerpe, M. S., Bastida, R. O., Rodríguez, D. H., and Morón, S. G. (1994). Environmental contamination and marine mammals in coastal waters from Argentina: an overview. *Sci Total Environ* 154, 141–151. doi: 10.1016/0048-9697(94)90084-1.

Marcus, A. D., Higgins, D. P., and Gray, R. (2014). Epidemiology of hookworm (*Uncinaria sanguinis*) infection in free-ranging Australian sea lion (*Neophoca cinerea*) pups. *Parasitol Res* 113, 3341–3353. doi: 10.1007/s00436-014-3997-3.

Marcus, A. D., Higgins, D. P., and Gray, R. (2015a). Health assessment of free-ranging endangered Australian sea lion (*Neophoca cinerea*) pups: effect of haematophagous parasites on haematological parameters. *Comp Biochem Physiol A Mol Integr Physiol* 184, 132–143. doi: 10.1016/j.cbpa.2015.02.017.

Marcus, A. D., Higgins, D. P., and Gray, R. (2015b). Ivermectin treatment of free-ranging endangered Australian sea lion (*Neophoca cinerea*) pups: effect on hookworm and lice infection status, haematological parameters, growth, and survival. *Parasitol Res* 114, 2743–2755. doi: 10.1007/s00436-015-4481-4.

Marti, E., Variatza, E., and Balcazar, J. L. (2014). The role of aquatic ecosystems as reservoirs of antibiotic resistance. *Trends Microbiol* 22, 36–41. doi: 10.1016/j.tim.2013.11.001.

Martin, I., Kaisar, M. M. M., Wiria, A. E., Hamid, F., Djuardi, Y., Sartono, E., et al. (2019). The effect of gut microbiome composition on human immune responses: an exploration of interference by helminth infections. *Front Genet* 10, 1028. doi: 10.3389/fgene.2019.01028.

Martinez, J. L. (2009). Environmental pollution by antibiotics and by antibiotic resistance determinants. *Environ Pollut* 157, 2893–2902. doi: 10.1016/j.envpol.2009.05.051.

Martinez-Freijo, P., Fluit, A. C., Schmitz, F. J., Grek, V. S., Verhoef, J., and Jones, M. E. (1998). Class I integrons in Gram-negative isolates from different European hospitals and association with decreased susceptibility to multiple antibiotic compounds. *J Antimicrob Chemother* 42, 689–696. doi: 10.1093/jac/42.6.689.

Maurice, C. F., CL Knowles, S., Ladau, J., Pollard, K. S., Fenton, A., Pedersen, A. B., et al. (2015). Marked seasonal variation in the wild mouse gut microbiota. *ISME J* 9, 2423–2434. doi: 10.1038/ismej.2015.53.

Mazel, D. (2006). Integrons: agents of bacterial evolution. *Nat Rev Microbiol* 4, 608–620. doi: 10.1038/nrmicro1462.

McDougall, F., Boardman, W., Gillings, M., and Power, M. (2019). Bats as reservoirs of antibiotic resistance determinants: a survey of class 1 integrons in Grey-headed Flying Foxes (*Pteropus poliocephalus*). *Infect Genet Evol* 70, 107–113. doi: 10.1016/j.meegid.2019.02.022.

McIntosh, R. R., Goldsworthy, S. D., Shaughnessy, P. D., Kennedy, C. W., and Burch, P. (2012). Estimating pup production in a mammal with an extended and aseasonal breeding season, the Australian sea lion (*Neophoca cinerea*). *Wildl Res* 39, 137–148. doi: 10.1071/WR10222.

McIntosh, R. R., Kirkman, S. P., Thalmann, S., Sutherland, D. R., Mitchell, A., Arnould, J. P. Y., et al. (2018). Understanding meta-population trends of the Australian fur seal, with insights for adaptive monitoring. *PLOS ONE* 13, e0200253. doi: 10.1371/journal.pone.0200253.

McIntosh, R. R., Kirkwood, R., Sutherland, D. R., and Dann, P. (2015). Drivers and annual estimates of marine wildlife entanglement rates: a long-term case study with Australian fur seals. *Mar Pollut Bull* 101, 716–725. doi: 10.1016/j.marpolbul.2015.10.007.

McIntosh, R. R., Sorrell, K. J., Thalmann, S., Mitchell, A., Gray, R., Schinagl, H., et al. (2022). Sustained reduction in numbers of Australian fur seal pups: Implications for future population monitoring. *PLOS ONE* 17, e0265610. doi: 10.1371/journal.pone.0265610.

McKenzie, V. J., Song, S. J., Delsuc, F., Prest, T. L., Oliverio, A. M., Korpita, T. M., et al. (2017). The effects of captivity on the mammalian gut microbiome. *Integr Comp Biol* 57, 690–704. doi: 10.1093/icb/icx090.

McLean, L. J., George, S., Ierodiaconou, D., Kirkwood, R. J., and Arnould, J. P. Y. (2018). Impact of rising sea levels on Australian fur seals. *PeerJ* 6, e5786–e5786. doi: 10.7717/peerj.5786.

McLeay, L., Tsolos, A., and Boyle, M. K. (2015). Operational interactions with threatened, endangered or protected species in South Australian managed fisheries data summary: 2007/08-2013/14. *SARDI Publication No. F2009/000544-5 SARDI Research Report Series No. 851*.

Medeiros, A. W., Giongo, A., Valdez, F. P., Blaese de Amorin, D., Tavares, M., d'Azevedo, P. A., et al. (2016). Characterization of the faecal bacterial community of wild young South American (*Arctocephalus australis*) and Subantarctic fur seals (*Arctocephalus tropicalis*). *FEMS Microbiol Ecol* 92. doi: 10.1093/femsec/fiw029.

Michael, S. A., Hayman, D. T. S., Gray, R., Zhang, J., Rogers, L., and Roe, W. D. (2019). Pup mortality in New Zealand sea lions (*Phocarctos hookeri*) at Enderby Island, Auckland Islands, 2013-18. *PLOS ONE* 14, e0225461. doi: 10.1371/journal.pone.0225461.

Modi, S. R., Collins, J. J., and Relman, D. A. (2014). Antibiotics and the gut microbiota. *J Clin Invest* 124, 4212–4218. doi: 10.1172/JCI72333.

Moore, S. E. (2008). Marine mammals as ecosystem sentinels. *J Mammal* 89, 534–540. doi: 10.1644/07-MAMM-S-312R1.1.

Mora, A., García-Peña, F., Alonso, M., Pedraza-Diaz, S., Ortega-Mora, L., Garcia-Parraga, D., et al. (2018). Impact of human-associated *Escherichia coli* clonal groups in Antarctic pinnipeds: presence of ST73, ST95, ST141 and ST131. *Sci Rep* 8, 4678. doi: 10.1038/s41598-018-22943-0.

Moreno, E., Guzmán-Verri, C., Gonzalez-Barrios, R., Hernandez, G., Morales, J., Barquero-Calvo, E., et al. (2012). *Brucella ceti* and brucellosis in Cetaceans. *Front Cell Infect Microbiol* 2. doi: 10.3389/fcimb.2012.00003.

Moustafa, M. A. M., Chel, H. M., Thu, M. J., Bawm, S., Htun, L. L., Win, M. M., et al. (2021). Anthropogenic interferences lead to gut microbiome dysbiosis in Asian elephants and may alter adaptation processes to surrounding environments. *Sci Rep* 11, 741. doi: 10.1038/s41598-020-80537-1.

Myers, R. A., and Worm, B. (2003). Rapid worldwide depletion of predatory fish communities. *Nature* 423, 280–283. doi: 10.1038/nature01610.

Nagpal, R., Mainali, R., Ahmadi, S., Wang, S., Singh, R., Kavanagh, K., et al. (2018). Gut microbiome and aging: physiological and mechanistic insights. *J Nutr Health Aging* 4, 267–285. doi: 10.3233/NHA-170030.

Natividad, J. M. M., and Verdu, E. F. (2013). Modulation of intestinal barrier by intestinal microbiota: pathological and therapeutic implications. *Pharmacol Res* 69, 42–51. doi: 10.1016/j.phrs.2012.10.007.

Nelson, T. M., Rogers, T. L., Carlini, A. R., and Brown, M. v (2013). Diet and phylogeny shape the gut microbiota of Antarctic seals: a comparison of wild and captive animals. *Environ Microbiol* 15, 1132–1145. doi: 10.1111/1462-2920.12022.

Nguyen, C. C., Hugie, C. N., Kile, M. L., and Navab-Daneshmand, T. (2019). Association between heavy metals and antibiotic-resistant human pathogens in environmental reservoirs: A review. *Front Environ Sci Eng* 13, 46. doi: 10.1007/s11783-019-1129-0.

Norman, S. A., Lambourn, D. M., Huggins, J. L., Gaydos, J. K., Dubpernell, S., Berta, S., et al. (2021). Antibiotic resistance of bacteria in two marine mammal species, harbor seals and harbor porpoises, living in an urban marine ecosystem, the Salish Sea, Washington State, USA. *Oceans* 2. doi: 10.3390/oceans2010006.

Nowrouzian, F. L., Adlerberth, I., and Wold, A. E. (2006). Enhanced persistence in the colonic microbiota of *Escherichia coli* strains belonging to phylogenetic group B2: role of virulence factors and adherence to colonic cells. *Microbes Infect* 8, 834–840. doi: 10.1016/j.micinf.2005.10.011.

Nowrouzian, F. L., Wold, A. E., and Adlerberth, I. (2005). *Escherichia coli* strains belonging to phylogenetic group B2 have superior capacity to persist in the intestinal microflora of infants. *J Infect Dis* 191, 1078–1083. doi: 10.1086/427996.

Nymo, I. H., Tryland, M., and Godfroid, J. (2011). A review of *Brucella* infection in marine mammals, with special emphasis on *Brucella pinnipedialis* in the hooded seal (*Cystophora cristata*). *Vet Res* 42, 93. doi: 10.1186/1297-9716-42-93.

Oates, S. C., Miller, M. A., Byrne, B. A., Chouicha, N., Hardin, D., Jessup, D., et al. (2012). Epidemiology and potential land-sea transfer of enteric bacteria from terrestrial to marine species in the Monterey Bay region of California. *J Wildl Dis* 48, 654–668. doi: 10.7589/0090-3558-48.3.654.

Odamaki, T., Kato, K., Sugahara, H., Hashikura, N., Takahashi, S., Xiao, J., et al. (2016). Age-related changes in gut microbiota composition from newborn to centenarian: a cross-sectional study. *BMC Microbiol* 16, 90. doi: 10.1186/s12866-016-0708-5.

Pacheco-Sandoval, A., Schramm, Y., Heckel, G., Brassea-Pérez, E., Martínez-Porchas, M., and Lago-Lestón, A. (2019). The Pacific harbor seal gut microbiota in Mexico: Its relationship with diet and functional inferences. *PLOS ONE* 14, e0221770. doi: 10.1371/journal.pone.0221770.

Page, B., McKenzie, J., McIntosh, R., Baylis, A., Morrissey, A., Calvert, N., et al. (2004). Entanglement of Australian sea lions and New Zealand fur seals in lost fishing gear and other marine debris before and after Government and industry attempts to reduce the problem. *Mar Pollut Bull* 49, 33–42. doi: 10.1016/j.marpolbul.2004.01.006.

Pal, C., Asiani, K., Arya, S., Rensing, C., Stekel, D. J., Larsson, D. G. J., et al. (2017). “Metal resistance and its association with antibiotic resistance,” in *Advanced Microbial Physiology*, ed. R. K. B. T.-A. in M. P. Poole (Academic Press), 261–313. doi: 10.1016/bs.ampbs.2017.02.001.

Palmer, C., Bik, E. M., Digiulio, D. B., Relman, D. A., and Brown, P. O. (2007). Development of the human infant intestinal microbiota. *PLoS Biol* 5, 177. doi: 10.1371/journal.pbio.

Partridge, S. R., Kwong, S. M., Firth, N., and Jensen, S. O. (2018). Mobile genetic elements associated with antimicrobial resistance. *Clin Microbiol Rev* 31, e00088-17. doi: 10.1128/CMR.00088-17.

Paulson, J. R., Mahmoud, I. Y., Al-Musharafi, S. K., and Al-Bahry, S. N. (2016). Antibiotic resistant bacteria in the environment as bio-indicators of pollution. *Open Biotechnol J* 10, 342. doi: 10.2174/1874070701610010342.

Pesapane, R., Ponder, M., and Alexander, K. A. (2013). Tracking pathogen transmission at the human–wildlife interface: banded mongoose and *Escherichia coli*. *Ecohealth* 10, 115–128. doi: 10.1007/s10393-013-0838-2.

Peterson, E., and Kaur, P. (2018). Antibiotic resistance mechanisms in bacteria: relationships between resistance determinants of antibiotic producers, environmental bacteria, and clinical pathogens. *Front Microbiol* 9, 2928. doi: 10.3389/fmicb.2018.02928.

Picard, B., Garcia, J. S., Gouriou, S., Duriez, P., Brahimi, N., Bingen, E., et al. (1999). The link between phylogeny and virulence in *Escherichia coli* extraintestinal infection. *Infect Immun* 67, 546. doi: 10.1128/iai.67.2.546-553.1999.

Plaza-Rodríguez, C., Alt, K., Grobbel, M., Hammerl, J. A., Irrgang, A., Szabo, I., et al. (2021). Wildlife as sentinels of antimicrobial resistance in Germany? *Front Vet Sci* 7. doi: 10.3389/fvets.2020.627821.

Poloczanska, E. S., Babcock, R. C., Butler, A., Hobday, A. J., Hoegh-Guldberg, O., Kunz, T. J., et al. (2007). “Climate change and Australian marine life,” in *Oceanography and Marine Biology: An Annual Review*, eds. R. N. Gibson, R. J. A. Atkinson, and J. D. M. Gordon, 407–478.

Pommepuy, M., Hervio-Heath, D., Caprais, M. P., Gourmelon, M., le Saux, J. C., and le Guyader, F. (2006). “Fecal contamination in coastal areas: an engineering approach,” in *Oceans and Health: Pathogens in The Marine Environment*, eds. B. S. and R. R. Colwell (Boston, MA: Springer), 331–359. doi: 10.1007/0-387-23709-7_14.

Pompa, S., Ehrlich, P. R., and Ceballos, G. (2011). Global distribution and conservation of marine mammals. *Proc Natl Acad Sci U S A* 108, 13600 –13605. doi: 10.1073/pnas.1101525108.

Power, M. L., Emery, S., and Gillings, M. R. (2013). Into the wild: dissemination of antibiotic resistance determinants via a species recovery program. *PLOS ONE* 8, e63017. doi: 10.1371/journal.pone.0063017.

Power, M. L., Littlefield-Wyer, J., Gordon, D. M., Veal, D. A., and Slade, M. B. (2005). Phenotypic and genotypic characterization of encapsulated *Escherichia coli* isolated from blooms in two Australian lakes. *Environ Microbiol* 7, 631–640. doi: 10.1111/j.1462-2920.2005.00729.x.

Power, M. L., Samuel, A., Smith, J. J., Stark, J. S., Gillings, M. R., and Gordon, D. M. (2016). *Escherichia coli* out in the cold: Dissemination of human-derived bacteria into the Antarctic microbiome. *Environ Pollut* 215, 58–65. doi: 10.1016/j.envpol.2016.04.013.

Radhouani, H., Poeta, P., Igrejas, G., Goncalves, A., Vinué, L., and Torres, C. (2009). Antimicrobial resistance and phylogenetic groups in isolates of *Escherichia coli* from seagulls at the Berlengas nature reserve. *Vet Rec* 165, 138. doi: 10.1136/vr.165.5.138.

Radhouani, H., Silva, N., Poeta, P., Torres, C., Correia, S., and Igrejas, G. (2014). Potential impact of antimicrobial resistance in wildlife, environment, and human health. *Front Microbiol* 5. doi: 10.3389/fmicb.2014.00023.

Raga, J., Banyard, A., Domingo, M., Corteyn, M., van Bressem, M., Fernández, M., et al. (2008). Dolphin morbillivirus epizootic resurgence, Mediterranean Sea. *Emerg Infect Dis* 14, 471–473. doi: 10.3201/eid1403.071230.

Ramanan, D., Bowcutt, R., Lee, S. C., Tang, M. S., Kurtz, Z. D., Ding, Y., et al. (2016). Helminth infection promotes colonization resistance via type 2 immunity. *Science* 352, 608–612. doi: 10.1126/science.aaf3229.

Ramey, A. M., and Ahlstrom, C. A. (2020). Antibiotic resistant bacteria in wildlife: perspectives on trends, acquisition and dissemination, data gaps, and future directions. *J Wildl Dis* 56, 1–15. doi: 10.7589/2019-04-099.

Ramos, P., Lynch, M., Hu, M., Arnould, J. P. Y., Norman, R., and Beveridge, I. (2013). Morphometric and molecular characterization of the species of Uncinaria Frölich, 1789 (Nematoda) parasitic in the Australian fur seal *Arctocephalus pusillus doriferus* (Schreber), with notes on hookworms in three other pinniped hosts. *Syst Parasitol* 85, 65–78. doi: 10.1007/s11230-013-9407-x.

Reid, C., Wyrsch, E., Zingali, T., Liu, M., Darling, A., Chapman, T., et al. (2017). Porcine commensal *Escherichia coli*: a reservoir for class 1 integrons associated with IS*26*. *BioRxiv* 3, e000143. doi: 10.1099/mgen.0.000143.

Reid, S. D., Herbelin, C. J., Bumbaugh, A. C., Selander, R. K., and Whittam, T. S. (2000). Parallel evolution of virulence in pathogenic *Escherichia coli*. *Nature* 406, 64–67. doi: 10.1038/35017546.

Reijnders, P. J. H., Aguilar, A., and Borrell, A. (2009). “Pollution and marine mammals,” in *Encyclopedia of Marine Mammals*, eds. W. F. Perrin, B. Würsig, and J. G. M. B. T.-E. of M. M. (Second E. Thewissen, London: Academic Press), 890–898. doi: 10.1016/B978-0-12-373553-9.00205-4.

Reisser, J., Shaw, J., Wilcox, C., Hardesty, B. D., Proietti, M., Thums, M., et al. (2013). Marine plastic pollution in waters around Australia: characteristics, concentrations, and pathways. *PLOS ONE* 8, e80466. doi: 10.1371/journal.pone.0080466.

Reygaert, W. C. (2018). An overview of the antimicrobial resistance mechanisms of bacteria. *AIMS Microbiol* 4, 482–501. doi: 10.3934/microbiol.2018.3.482.

Reynolds, L. A., Finlay, B. B., and Maizels, R. M. (2015). Cohabitation in the intestine: interactions among helminth parasites, bacterial microbiota, and host immunity. *J Immunol* 195, 4059–4066. doi: 10.4049/jimmunol.1501432.

Rizzo, L., Manaia, C., Merlin, C., Schwartz, T., Dagot, C., Ploy, M. C., et al. (2013). Urban wastewater treatment plants as hotspots for antibiotic resistant bacteria and genes spread into the environment: a review. *Sci Total Environ* 447, 345–360. doi: 10.1016/j.scitotenv.2013.01.032.

Robinson, T. P., Bu, D. P., Carrique-Mas, J., Fèvre, E. M., Gilbert, M., Grace, D., et al. (2016). Antibiotic resistance is the quintessential One Health issue. *Trans R Soc Trop Med Hyg* 110, 377–380. doi: 10.1093/trstmh/trw048.

Rodríguez, J. M., Murphy, K., Stanton, C., Ross, R. P., Kober, O. I., Juge, N., et al. (2015). The composition of the gut microbiota throughout life, with an emphasis on early life. *Microb Ecol Health Dis* 26, 26050. doi: 10.3402/mehd.v26.26050.

Rolland, R. M., Hausfater, G., Marshall, B., and Levy, S. B. (1985). Antibiotic-resistant bacteria in wild primates: increased prevalence in baboons feeding on human refuse. *Appl Environ Microbiol* 49, 791. doi: 10.1128/aem.49.4.791-794.1985.

Rosa, B. A., Supali, T., Gankpala, L., Djuardi, Y., Sartono, E., Zhou, Y., et al. (2018). Differential human gut microbiome assemblages during soil-transmitted helminth infections in Indonesia and Liberia. *Microbiome* 6, 33. doi: 10.1186/s40168-018-0416-5.

Rosewarne, C. P., Pettigrove, V., Stokes, H. W., and Parsons, Y. M. (2010). Class 1 integrons in benthic bacterial communities: abundance, association with Tn402-like transposition modules and evidence for coselection with heavy-metal resistance. *FEMS Microbiol Ecol* 72, 35–46. doi: 10.1111/j.1574-6941.2009.00823.x.

Ross, H., Jahans, K., MacMillan, A., Reid, R., Thompson, P., and Foster, G. (1996). *Brucella* species infection in North Sea seal and cetacean populations. *Vet Rec* 138, 647–648. doi: 10.1136/vr.138.26.647.

Rothschild, D., Weissbrod, O., Barkan, E., Kurilshikov, A., Korem, T., Zeevi, D., et al. (2018). Environment dominates over host genetics in shaping human gut microbiota. *Nature* 555, 210–215. doi: 10.1038/nature25973.

Round, J. L., and Mazmanian, S. K. (2009). The gut microbiota shapes intestinal immune responses during health and disease. *Nat Rev Immunol* 9, 313–323. doi: 10.1038/nri2515.

Rowland, I., Gibson, G., Heinken, A., Scott, K., Swann, J., Thiele, I., et al. (2018). Gut microbiota functions: metabolism of nutrients and other food components. *Eur J Nutr* 57, 1–24. doi: 10.1007/s00394-017-1445-8.

Rwego, I. B., Isabirye-Basuta, G., Gillespie, T. R., and Goldberg, T. L. (2008). Gastrointestinal bacterial transmission among humans, mountain gorillas, and livestock in Bwindi Impenetrable National Park, Uganda. *Conserv Biol* 22, 1600–1607. doi: 10.1111/j.1523-1739.2008.01018.x.

Sabey, K. A., Song, S. J., Jolles, A., Knight, R., and Ezenwa, V. O. (2021). Coinfection and infection duration shape how pathogens affect the African buffalo gut microbiota. *ISME J* 15, 1359–1371. doi: 10.1038/s41396-020-00855-0.

Savageau, M. A. (1983). *Escherichia coli* habitats, cell types, and molecular mechanisms of gene control. *Am Nat* 122, 732–744.

Schaefer, A. M., Bossart, G. D., Mazzoil, M., Fair, P. A., and Reif, J. S. (2011). Risk factors for colonization of *E. coli* in Atlantic Bottlenose Dolphins (*Tursiops truncatus*) in the Indian River Lagoon, Florida. *J Environ Public Health* 2011, 597073. doi: 10.1155/2011/597073.

Schipper, J., Chanson, J. S., Chiozza, F., Cox, N. A., Hoffmann, M., Katariya, V., et al. (2008). The status of the world's land and marine mammals: diversity, threat, and knowledge. *Science* 322, 225–230. doi: 10.1126/science.1165115.

Schlüter, A., Szczepanowski, R., Pühler, A., and Top, E. M. (2007). Genomics of IncP-1 antibiotic resistance plasmids isolated from wastewater treatment plants provides evidence for a widely accessible drug resistance gene pool. *FEMS Microbiol Rev* 31, 449–477. doi: 10.1111/j.1574-6976.2007.00074.x.

Schumann, N., Gales, N. J., Harcourt, R. G., and Arnould, J. P. Y. (2013). Impacts of climate change on Australian marine mammals. *Aust J Zool* 61, 146–159. doi: 10.1071/ZO12131.

Sears, H. J., Brownlee, I., and Uchiyama, J. K. (1950). Persistence of individual strains of *Escherichia coli* in the intestinal tract of man. *J Bacteriol* 59, 293–301. doi: 10.1128/jb.59.2.293-301.1950.

Seguel, M., Muñoz, F., Navarrete, M. J., Paredes, E., Howerth, E., and Gottdenker, N. (2016). Hookworm infection in South American fur seal (*Arctocephalus australis*) pups: pathology and factors associated with host tissue damage and mortality. *Vet Pathol* 54, 288–297. doi: 10.1177/0300985816677151.

Seguel, M., Paredes, E., Pavés, H., Molina, R., Henríquez, F., de Groote, F., et al. (2011). Pathological findings in South American fur seal pups (*Arctocephalus australis gracilis*) found dead at Guafo Island, Chile. *J Comp Pathol* 145, 308–317. doi: 10.1016/j.jcpa.2011.01.006.

Selander, R. K., and Levin, B. R. (1980). Genetic diversity and structure in *Escherichia coli* populations. *Science* 210, 545–547. doi: 10.1126/science.6999623.

Shanahan, F. (2002). The host–microbe interface within the gut. *Best Pract Res Clin Gastroenterol* 16, 915–931. doi: 10.1053/bega.2002.0342.

Shapiro, K., Miller, M., and Mazet, J. (2012). Temporal association between land-based runoff events and California sea otter (*Enhydra lutris nereis*) protozoal mortalities. *J Wildl Dis* 48, 393–404. doi: 10.7589/0090-3558-48.2.394.

Shaughnessy, P. D. (1999). *The action plan for Australian seals*. Canberra: Environment Australia, Biodiversity Group, Threatened Species and Communities Section.

Shaughnessy, P. D., and Goldsworthy, S. D. (2015). Increasing abundance of pups of the long-nosed fur seal (*Arctocephalus forsteri*) on Kangaroo Island, South Australia, over 26 breeding seasons to 2013–14. *Wildl Res* 42, 619–632. doi: 10.1071/WR14209.

Shaughnessy, P. D., and Goldsworthy, S. D. (2020). Dispersion of long-nosed fur seals (*Arctocephalus forsteri*) determined by tagging. *Aust J Zool* 67, 173–179. doi: 10.1071/ZO20032.

Shaughnessy, P. D., Goldsworthy, S. D., Hamer, D. J., Page, B., and McIntosh, R. R. (2011). Australian sea lions *Neophoca cinerea* at colonies in South Australia: distribution and abundance, 2004 to 2008. *Endanger Species Res* 13, 87–98. doi: 10.3354/esr00317.

Shaughnessy, P. D., Goldsworthy, S. D., and Mackay, A. I. (2014). Status and trends in abundance of New Zealand fur seal populations in South Australia. *SARDI Publication Number F2014/000338-1. SARDI Research Report Series No. 781 (Adelaide, South Australia)*.

Shaughnessy, P. D., Goldsworthy, S. D., and Mackay, A. I. (2015). The long-nosed fur seal (*Arctocephalus forsteri*) in South Australia in 2013–14: abundance, status and trends. *Aust J Zool* 63, 101. doi: 10.1071/ZO14103.

Shaughnessy, P., Kirkwood, R., Cawthorn, M., Kemper, C., and Pemberton, D. (2003). "Pinnipeds, cetaceans and fisheries in Australia: a review of operational interactions," in *Marine mammals: fisheries, tourism and management issues*, eds. N. J. Gales, M. A. Hindell, and R. Kirkwood (Melbourne: CSIRO Publishing), 136–152.

Sjölund, M., Bonnedahl, J., Hernandez, J., Bengtsson, S., Cederbrant, G., Pinhassi, J., et al. (2008). Dissemination of multidrug-resistant bacteria into the Arctic. *Emerg Infect Dis* 14, 70–72. doi: 10.3201/eid1401.070704.

Skurnik, D., Ruimy, R., Andremont, A., Amorin, C., Rouquet, P., Picard, B., et al. (2006). Effect of human vicinity on antimicrobial resistance and integrons in animal faecal *Escherichia coli*. *J Antimicrob Chemother* 57, 1215–1219. doi: 10.1093/jac/dkl122.

Smith, K., McCoy, K. D., and Macpherson, A. J. (2007). Use of axenic animals in studying the adaptation of mammals to their commensal intestinal microbiota. *Semin Immunol* 19, 59–69. doi: 10.1016/j.smim.2006.10.002.

Smith, S. C., Chalker, A., Dewar, M. L., and Arnould, J. P. Y. (2013). Age-related differences revealed in Australian fur seal *Arctocephalus pusillus doriferus* gut microbiota. *FEMS Microbiol Ecol* 86, 246–255. doi: 10.1111/1574-6941.12157.

Sommer, F., and Bäckhed, F. (2013). The gut microbiota — masters of host development and physiology. *Nat Rev Microbiol* 11, 227–238. doi: 10.1038/nrmicro2974.

Sommer, F., Ståhlman, M., Ilkayeva, O., Arnemo, J. M., Kindberg, J., Josefsson, J., et al. (2016). The gut microbiota modulates energy metabolism in the hibernating brown bear *Ursus arctos*. *Cell Rep* 14, 1655–1661. doi: 10.1016/j.celrep.2016.01.026.

Souza, V., Rocha, M., Valera, A., and Eguiarte, L. E. (1999). Genetic structure of natural populations of *Escherichia coli* in wild hosts on different continents. *Appl Environ Microbiol* 65, 3373–3385. doi: 10.1128/AEM.65.8.3373-3385.1999.

Stalder, T., Barraud, O., Casellas, M., Dagot, C., and Ploy, M.-C. (2012). Integron involvement in environmental spread of antibiotic resistance. *Front Microbiol* 3. doi: 10.3389/fmicb.2012.00119.

Stalder, T., Barraud, O., Jové, T., Casellas, M., Gaschet, M., Dagot, C., et al. (2014). Quantitative and qualitative impact of hospital effluent on dissemination of the integron pool. *ISME J* 8, 768–777. doi: 10.1038/ismej.2013.189.

Stecher, B., and Hardt, W.-D. (2011). Mechanisms controlling pathogen colonization of the gut. *Curr Opin Microbiol* 14, 82–91. doi: 10.1016/j.mib.2010.10.003.

Stoddard, R. A., Atwill, E. R., Conrad, P. A., Byrne, B. A., Jang, S., Lawrence, J., et al. (2009). The effect of rehabilitation of northern elephant seals (*Mirounga angustirostris*) on antimicrobial resistance of commensal *Escherichia coli*. *Vet Microbiol* 133, 264–271. doi: 10.1016/j.vetmic.2008.07.022.

Stoddard, R. A., Atwill, E. R., Gulland, F. M. D., Miller, M. A., Dabritz, H. A., Paradies, D. M., et al. (2008). Risk factors for infection with pathogenic and antimicrobial-resistant fecal bacteria in northern elephant seals in California. *Public Health Rep* 123, 360–370. doi: 10.1177/003335490812300316.

Stoddard, R. A., Gulland, M. D. F., Atwill, E. R., Lawrence, J., Jang, S., and Conrad, P. A. (2005). *Salmonella* and *Campylobacter* spp. in northern elephant seals, California. *Emerg Infect Dis* 11, 1967–1969. doi: 10.3201/eid1112.050752.

Stoffel, M. A., Acevedo-Whitehouse, K., Morales-Durán, N., Grosser, S., Chakarov, N., Krüger, O., et al. (2020). Early sexual dimorphism in the developing gut microbiome of northern elephant seals. *Mol Ecol* 29, 2109–2122. doi: 10.1111/mec.15385.

Stokes, H. W., and Gillings, M. R. (2011). Gene flow, mobile genetic elements and the recruitment of antibiotic resistance genes into Gram-negative pathogens. *FEMS Microbiol Rev* 35, 790–819. doi: 10.1111/j.1574-6976.2011.00273.x.

Swift, B. M. C., Bennett, M., Waller, K., Dodd, C., Murray, A., Gomes, R. L., et al. (2019). Anthropogenic environmental drivers of antimicrobial resistance in wildlife. *Sci Total Env* 649, 12–20. doi: 10.1016/j.scitotenv.2018.08.180.

Tanaka, M., and Nakayama, J. (2017). Development of the gut microbiota in infancy and its impact on health in later life. *Allergol Int* 66, 515–522. doi: 10.1016/j.alit.2017.07.010.

Taylor, S., Lynch, M., Terkildsen, M., Stevenson, G., Yates, A., Piro, N., et al. (2018). Utility of fur as a biomarker for persistent organic pollutants in Australian fur seals (*Arctocephalus pusillus doriferus*). *Sci Total Environ* 610–611, 1310–1320. doi: 10.1016/j.scitotenv.2017.08.204.

Taylor, S., Terkildsen, M., Stevenson, G., de Araujo, J., Yu, C., Yates, A., et al. (2021). Per and polyfluoroalkyl substances (PFAS) at high concentrations in neonatal Australian pinnipeds. *Sci Total Environ* 786, 147446. doi: 10.1016/j.scitotenv.2021.147446.

Tchounwou, P. B., Yedjou, C. G., Patlolla, A. K., and Sutton, D. J. (2012). Heavy metal toxicity and the environment. *Experientia Suppl* 101, 133–164. doi: 10.1007/978-3-7643-8340-4_6.

Tenaillon, O., Skurnik, D., Picard, B., and Denamur, E. (2010). The population genetics of commensal *Escherichia coli*. *Nat Rev Microbiol* 8, 207–217. doi: 10.1038/nrmicro2298.

Thursby, E., and Juge, N. (2017). Introduction to the human gut microbiota. *Biochem J* 474, 1823–1836. doi: 10.1042/BCJ20160510.

Tian, J., Du, J., Han, J., Song, X., and Lu, Z. (2020). Age-related differences in gut microbial community composition of captive spotted seals (*Phoca largha*). *Mar Mamm Sci* 36, 1231–1240. doi: 10.1111/mms.12728.

Toro-Valdivieso, C., Toro, F., Stubbs, S., Castro-Nallar, E., and Blacklaws, B. (2021). Patterns of the fecal microbiota in the Juan Fernández fur seal (*Arctocephalus philippii*). *Microbiology Open* 10, e1215–e1215. doi: 10.1002/mbo3.1215.

Turnbaugh, P. J., Ley, R. E., Mahowald, M. A., Magrini, V., Mardis, E. R., and Gordon, J. I. (2006). An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature* 444, 1027–1031. doi: 10.1038/nature05414.

Turnbaugh, P. J., Ridaura, V. K., Faith, J. J., Rey, F. E., Knight, R., and Gordon, J. I. (2009). The effect of diet on the human gut microbiome: a metagenomic analysis in humanized gnotobiotic mice. *Sci Transl Med* 1, 6ra14-6ra14. doi: 10.1126/scitranslmed.3000322.

Ursell, L. K., Metcalf, J. L., Parfrey, L. W., and Knight, R. (2012). Defining the human microbiome. *Nutr Rev* 70 Suppl 1, S38–S44. doi: 10.1111/j.1753-4887.2012.00493.x.

Van Bressem, M., Raga, J., Di Guardo, G., Jepson, P., Duignan, P., Siebert, U., et al. (2009). Emerging infectious diseases in cetaceans worldwide and the possible role of environmental stressors. *Dis Aquat Organ* 86, 143–157. doi: 10.3354/dao02101.

Varani, A., He, S., Siguier, P., Ross, K., and Chandler, M. (2021). The IS*6* family, a clinically important group of insertion sequences including IS*26*. *Mob DNA* 12, 11. doi: 10.1186/s13100-021-00239-x.

Vittecoq, M., Godreuil, S., Prugnolle, F., Durand, P., Brazier, L., Renaud, N., et al. (2016). Antimicrobial resistance in wildlife. *J Appl Ecol* 53, 519–529. doi: 10.1111/1365-2664.12596.

Vogt, S. L., Peña-Díaz, J., and Finlay, B. B. (2015). Chemical communication in the gut: Effects of microbiota-generated metabolites on gastrointestinal bacterial pathogens. *Anaerobe* 34, 106–115. doi: 10.1016/j.anaerobe.2015.05.002.

von Wintersdorff, C. J. H., Penders, J., van Niekerk, J. M., Mills, N. D., Majumder, S., van Alphen, L. B., et al. (2016). Dissemination of antimicrobial resistance in microbial ecosystems through horizontal gene transfer. *Front Microbiol* 7. doi: 10.3389/fmicb.2016.00173.

Walk, S. T., Blum, A. M., Ewing, S. A.-S., Weinstock, J. v, and Young, V. B. (2010). Alteration of the murine gut microbiota during infection with the parasitic helminth *Heligmosomoides polygyrus*. *Inflamm Bowel Dis* 16, 1841–1849. doi: 10.1002/ibd.21299.

Wallace, C., Yund, P., Ford, T., Matassa, K., and Bass, A. (2013). Increase in antimicrobial resistance in bacteria isolated from stranded marine mammals of the northwest Atlantic. *Ecohealth* 10, 201–210. doi: 10.1007/s10393-013-0842-6.

Warneke, R. M., and Shaughnessy, P. D. (1985). "*Arctocephalus pusillus*, the South African and Australian fur seal: taxonomy, evolution, biogeography, and life history," in *Studies of sea mammals in south latitudes*, eds. J. K. Ling and M. M. Bryden (Adelaide: South Australian Museum), 153–177.

Wasimuddin, Menke, S., Melzheimer, J., Thalwitzer, S., Heinrich, S., Wachter, B., et al. (2017). Gut microbiomes of free-ranging and captive Namibian cheetahs: Diversity, putative functions and occurrence of potential pathogens. *Mol Ecol* 26, 5515–5527. doi: 10.1111/mec.14278.

Wear, S. L., Acuña, V., McDonald, R., and Font, C. (2021). Sewage pollution, declining ecosystem health, and cross-sector collaboration. *Biol Conserv* 255, 109010. doi: 10.1016/j.biocon.2021.109010.

Weinstock, J. v, and Elliott, D. E. (2013). Translatability of helminth therapy in inflammatory bowel diseases. *Int J Parasitol* 43, 245–251. doi: 10.1016/j.ijpara.2012.10.016.

Weiss, D., Wallace, R. M., Rwego, I. B., Gillespie, T. R., Chapman, C. A., Singer, R. S., et al. (2022). Antibiotic-resistant *Escherichia coli* and class 1 integrons in humans, domestic animals, and wild primates in rural Uganda. *Appl Environ Microbiol* 84, e01632-18. doi: 10.1128/AEM.01632-18.

Whitman, W. B., Coleman, D. C., and Wiebe, W. J. (1998). Prokaryotes: the unseen majority. *Proc Natl Acad Sci U S A* 95, 6578–6583. doi: 10.1073/pnas.95.12.6578.

Wright, M. S., Baker-Austin, C., Lindell, A. H., Stepanauskas, R., Stokes, H. W., and Mcarthur, J. V. (2008). Influence of industrial contamination on mobile genetic elements: class 1 integron

abundance and gene cassette structure in aquatic bacterial communities. *ISME J* 2, 417. doi: 10.1038/ismej.2008.8.

Xie, H., Guo, R., Zhong, H., Feng, Q., Lan, Z., Qin, B., et al. (2016). Shotgun metagenomics of 250 adult twins reveals genetic and environmental impacts on the gut microbiome. *Cell Syst* 3, 572-584.e3. doi: 10.1016/j.cels.2016.10.004.

Xu, Z., and Knight, R. (2015). Dietary effects on human gut microbiome diversity. *Br J Nutr* 113 Suppl, S1–S5. doi: 10.1017/S0007114514004127.

Yatsunenko, T., Rey, F. E., Manary, M. J., Trehan, I., Dominguez-Bello, M. G., Contreras, M., et al. (2012). Human gut microbiome viewed across age and geography. *Nature* 486, 222–227. doi: 10.1038/nature11053.

Yu, D., Banting, G., and Neumann, N. F. (2021). A review of the taxonomy, genetics, and biology of the genus *Escherichia* and the type species *Escherichia coli*. *Can J Microbiol* 67, 553–571. doi: 10.1139/cjm-2020-0508.

Zaiss, M. M., Rapin, A., Lebon, L., Dubey, L. K., Mosconi, I., Sarter, K., et al. (2015). The intestinal microbiota contributes to the ability of helminths to modulate allergic inflammation. *Immunity* 43, 998–1010. doi: 10.1016/j.immuni.2015.09.012.

Zhang, X., Zhang, T., and Fang, H. H. P. (2009). Antibiotic resistance genes in water environment. *Appl Microbiol Biotechnol* 82, 397–414. doi: 10.1007/s00253-008-1829-z.

Zheng, W., Huyan, J., Tian, Z., Zhang, Y., and Wen, X. (2020). Clinical class 1 integron-integrase gene – a promising indicator to monitor the abundance and elimination of antibiotic resistance genes in an urban wastewater treatment plant. *Environ Int* 135, 105372. doi: 10.1016/j.envint.2019.105372.

Zhu, Y.-G., Gillings, M., Simonet, P., Stekel, D., Banwart, S., and Penuelas, J. (2017). Microbial mass movements. *Science* 357, 1099–1100. doi: 10.1126/science.aao3007.

Chapter 2.

Carriage of antibiotic resistant bacteria in endangered and declining Australian pinniped pups

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RESEARCH ARTICLE

Carriage of antibiotic resistant bacteria in endangered and declining Australian pinniped pups

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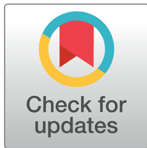

Abstract

The rapid emergence of antimicrobial resistance (AMR) is a major concern for wildlife and ecosystem health globally. Genetic determinants of AMR have become indicators of anthropogenic pollution due to their greater association with humans and rarer presence in environments less affected by humans. The objective of this study was to determine the distribution and frequency of the class 1 integron, a genetic determinant of AMR, in both the faecal microbiome and in *Escherichia coli* isolated from neonates of three pinniped species. Australian sea lion (*Neophoca cinerea*), Australian fur seal (*Arctocephalus pusillus doriferus*) and long-nosed fur seal (*Arctocephalus forsteri*) pups from eight breeding colonies along the Southern Australian coast were sampled between 2016–2019. DNA from faecal samples (n = 309) and from *E. coli* (n = 795) isolated from 884 faecal samples were analysed for class 1 integrons using PCRs targeting the conserved integrase gene (*intI*) and the gene cassette array. Class 1 integrons were detected in *A. p. doriferus* and *N. cinerea* pups sampled at seven of the eight breeding colonies investigated in 4.85% of faecal samples (n = 15) and 4.52% of *E. coli* isolates (n = 36). Integrons were not detected in any *A. forsteri* samples. DNA sequencing of the class 1 integron gene cassette array identified diverse genes conferring resistance to four antibiotic classes. The relationship between class 1 integron carriage and the concentration of five trace elements and heavy metals was also investigated, finding no significant association. The results of this study add to the growing evidence of the extent to which antimicrobial resistant bacteria are polluting the marine environment. As AMR determinants are frequently associated with bacterial pathogens, their occurrence suggests that these pinniped species are vulnerable to potential health risks. The implications for individual and population health as a consequence of AMR carriage is a critical component of ongoing health investigations.

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Introduction

Aquatic ecosystems are being increasingly identified as a sink for antimicrobial resistance (AMR) [1,2]. Aquatic systems provide a transport medium for the global dissemination of

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antibiotic resistant bacteria (ARB) and associated antibiotic resistance genes (ARGs) [1–3]. The combination of ARB with antibiotic residues and other pollutants in aquatic environments also promote the proliferation and establishment of resistant bacterial communities [2,4].

The widespread dissemination of AMR can partly be attributed to horizontal gene transfer (HGT) which allows the transfer of ARGs and associated genetic machinery between diverse bacterial species, facilitating the acquisition of novel traits from the environment and other bacteria [5,6]. In Gram-negative bacteria in particular, the rapid evolution of resistance has been linked to HGT and mobile genetic elements [7–9]. Class 1 integrons, for example, are mainly found in Gram-negative bacteria [10] and are able to capture and subsequently express a multitude of ARGs [11,12], which can be transferred between bacteria via their association with transposons and plasmids. In the human context, the clinical class 1 integron is considered to be of high importance for AMR dissemination [13,14]. The class 1 integron contains a conserved 5' segment which encodes the integrase gene (*intI1*) and a varying number of gene cassettes that together form a gene cassette array [15]. The conserved *intI1* is a useful genetic indicator of antimicrobial pollution as it is universally present, occurs in high abundance in humans and domestic animals, is highly abundant in waste streams and is rarely present in environments less affected by humans [16,17]. The recombination of gene cassettes is mediated by *intI1*, allowing the class 1 integron to capture, remove and express a variety of gene cassettes [15]. Variations of the class 1 integron are now emerging, with insertion sequences in the 3' segment, such as IS*26*, assisting in the dissemination of resistance genes in Gram-negative bacteria. These insertion sequences are associated with numerous genes that confer resistance to multiple antibiotic classes, and are able to promote and subsequently express these associated resistance genes [18,19].

Agricultural runoff, in addition to mining, municipal wastewater, and industrial and pharmaceutical waste are point sources of heavy metal pollutants frequently found in natural environments [20]. Heavy metals are considered to be co-selective agents of AMR [20]. Aquatic environments polluted by heavy metals have been associated with a greater incidence of class 1 integrons compared to non-polluted sites [21,22], through mechanisms of cross- and co-resistance [23]. The presence of heavy metals and antibiotic residues also common in the environment have the potential to exert a selective pressure that promotes the emergence and persistence of AMR in the environment [24–26]. As heavy metals can bioaccumulate and persist in the environment, such selective pressures are applied for extended periods of time, facilitating the development resistance traits in microbial communities [27]. However, there has been little investigation into whether there is increased acquisition of antibiotic resistant bacteria in humans and non-human animals in environments that have greater exposure to heavy metals.

Concentrations of essential trace elements and heavy metals including zinc (Zn), arsenic (As), selenium (Se), mercury (Hg) and lead (Pb) are of particular interest for wildlife health. The presence of Pb, even at low concentrations, can be associated with disease [28]. In contrast, Zn and Se are essential trace elements, but these too can have toxic effects at high concentrations [29]. Heavy metals have previously been identified in free-ranging pinnipeds [30–32], however, there has been no consideration of potential co-selection of ARGs in wildlife species associated with heavy metal exposure. Given the role that heavy metals play in the environmental amplification of ARB, investigating the levels of heavy metals and class 1 integron frequency in wildlife species could provide valuable insights into the factors contributing to the abundance and acquisition of ARB in free-ranging wildlife.

A diverse range of antibiotic resistant bacterial species have been detected in marine mammals [33,34], which are large-bodied, long-lived upper trophic predators considered to have a

role as sentinels of marine health [35]. *Escherichia coli* is a Gram-negative bacterium that is commonly used as an indicator of anthropogenic pollution [24], and has been used for the investigation of class 1 integrons in many species [36–40]. Evidence suggests that class 1 integrons are more prevalent in *E. coli* isolates that are closely associated with anthropogenic pollution and human environments [7]. The presence of class 1 integrons in *E. coli* has been investigated in some species of free-ranging pinnipeds in the Southern Hemisphere. An absence of class 1 integrons was reported in *E. coli* isolated from free-ranging southern elephant seals (*Mirounga leonina*), Weddell seals (*Leptonychotes weddellii*) [41] and adult Australian sea lions (*Neophoca cinerea*) [40], although class 1 integrons were detected in *E. coli* from captive adult *N. cinerea* [40]. The presence of class 1 integrons in captive wildlife and comparative absence in free-ranging individuals suggest that environmental conditions and the intimate proximity to humans experienced in captivity can impact the acquisition of ARGs by wildlife species and is consistent for many wildlife species [37,38,40]. Consistent with the hypothesis that the presence of ARGs in wildlife is associated with proximity to humans, a class 1 integron was recently detected in *E. coli* from a single free-ranging *N. cinerea* pup at a colony with comparatively high anthropogenic influence compared to a more remote colony [39].

The occurrence of class 1 integrons and ARG carriage in two additional pinniped species inhabiting Australian waters, namely Australian fur seals (*Arctocephalus pusillus doriferus*) and long-nosed fur seals (*Arctocephalus forsteri*), has not been investigated. All three species, *N. cinerea*, *A. p. doriferus*, and *A. forsteri*, inhabit numerous offshore islands along the Australian coast from Western Australia to Tasmania [42], with the ranges of these pinniped species overlapping in South Australia. Colonies of each species experience differing levels of human interaction; those on islands remote to mainland Australia experience little to no contact with humans while others are popular, frequently visited tourist sites. The differing proximities of sympatric colonies to human habitation and exposure to anthropogenic impacts creates a naturally occurring gradient ideal for studying anthropogenic pollution in the marine environment.

The main objective of this study was to determine the prevalence of class 1 integrons and ARG carriage in both the faecal microbiota and *E. coli* isolates from pups of three pinniped species sampled at multiple breeding colonies throughout Southern Australia. Given the role heavy metals have as a co-selective agent for AMR, an additional aim was to determine whether there was a relationship between the concentration of trace elements and heavy metals (Zn, Se, As, Hg and Pb) and class 1 integron prevalence. It was hypothesised that class 1 integrons would be more abundant in pups at colonies in closer proximity to sources of anthropogenic pollution. This paper reports the presence of ARGs in *E. coli* isolates and faecal microbiota of *N. cinerea* and *A. p. doriferus* pups at multiple breeding colonies along the Australian coast. We explore the differences between species and colonies and discuss factors contributing to changes in class 1 integron prevalence across breeding seasons and colonies. We provide recommendations for future investigations to further understand the dissemination of AMR in free-ranging pinniped species.

Methods

Study sites and sample collection

Faecal swabs (n = 884) were collected from neonatal pups sampled at eight breeding colonies across multiple breeding seasons from 2016–2019 (Fig 1 and Table 1). Breeding seasons are annual for both *A. p. doriferus* and *A. forsteri* with pupping beginning in November, while *N. cinerea* breeding seasons occur every 18 months. *Arctocephalus pusillus doriferus* and *A.*

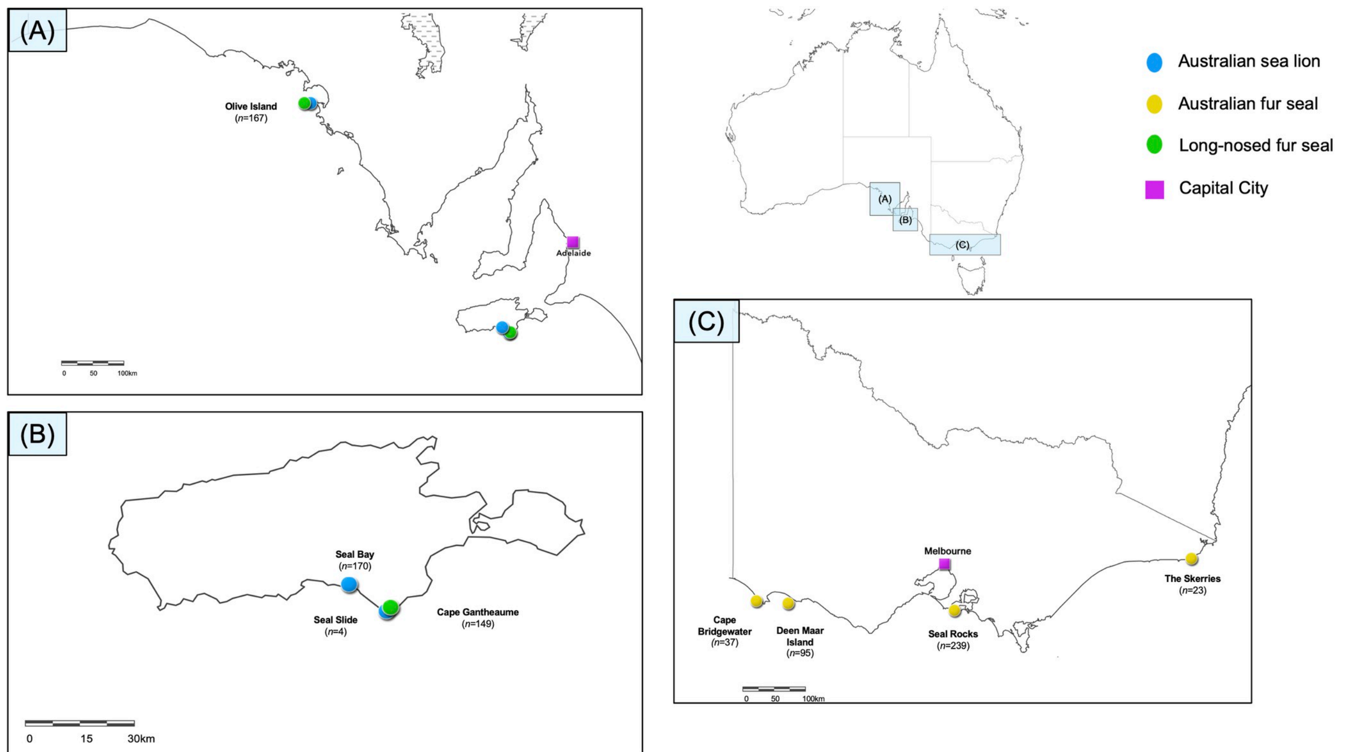

Fig 1. Map of the geographical locations of breeding colonies and pinniped species sampled. Breeding colonies in South Australia (A) include Olive Island, (B) Seal Bay, Seal Slide and Cape Gantheaume on Kangaroo Island, and (C) Cape Bridgewater, Deen Maar Island, Seal Rocks and The Skerries in Victoria. Total number of samples collected from each breeding colony across all breeding seasons is included for each colony. The closest capital city to breeding colonies in South Australia is Adelaide and capital city in Victoria is Melbourne. The map of breeding colony locations across South Australia and Victoria was developed using ArcGIS Online. Copyright © Mariel Fulham. All rights reserved.

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Table 1. Sample collection across breeding colonies and seasons.

Sampling site	Species	Year of breeding season and sample collection (*n* faecal samples collected)	Geographical coordinates
Seal Bay	*N. cinerea*	2016 (48) 2018 (59) 2019 (63)	35.95˚S, 137.32˚E
Seal Slide	*N. cinerea*	2018 (4)	36.03˚S, 137.29˚E
Olive Island	*N. cinerea* *A. forsteri*	2017 (89) 2019 (66) 2019 (12)	32.43˚S, 133.58˚E
Cape Gantheaume	*A. forsteri*	2016 (69) 2018 (80)	36.24˚S, 137.27˚E
Seal Rocks	*A. p. doriferus*	2017 (46) 2018 (99) 2019 (94)	38.31˚S, 145.5˚E
Deen Maar Island	*A. p. doriferus*	2017 (95)	38.24˚S, 142.0˚E
Cape Bridgewater	*A. p. doriferus*	2017 (37)	38.18˚S, 141.24˚E
The Skerries	*A. p. doriferus*	2017 (23)	37.45˚S, 149.31˚E

Location of sampling sites, species at each colony and number of faecal samples collected during each breeding season.

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forsteri pups were approximately 3–6 weeks of age and *N. cinerea* pups were 2–6 weeks of age at time of sampling. Samples were collected following methods described by Fulham et al. [39,43]. In brief, sterile swabs (Copan, Brescia, Italy) were inserted directly into the rectum of each pup and resulting samples were sub-sampled into sterile FecalSwab™ tubes (Copan, Brescia, Italy). FecalSwab samples were stored at 4˚C and cultured within 7–10 days of collection. Blood samples were collected from the brachial vein of pups as per methodology in Fulham et al. [39] and refrigerated at 4˚C prior to storage at -20˚C. Due to time and logistical constraints, blood collection was limited to pups sampled at Seal Bay, Olive Island, Cape Gantheaume, Seal Rocks (2018 and 2019) and Deen Maar Island. Sampling for *N. cinerea* and *A. forsteri* were approved by the Animal Ethics Committee at the University of Sydney (Protocol Nos. 2014/726 and 2017/1260); sampling methods for *A. p. doriferus* were approved by Phillip Island Nature Parks Animal Ethics Committee (Protocol No. 2.2016).

E. coli culture, isolation and DNA extraction

FecalSwab™ samples were cultured following the methodology described by Fulham et al. [39,43]. In summary, a selective media, Chromocult® coliform agar (Merck, Darmstadt, Germany), was used to isolate *E. coli*. After initial culture, *E. coli* colonies were sub-cultured and pure *E. coli* colonies were selected for DNA extraction based on colour and morphology. Pure *E. coli* colonies were inoculated into Luria-Bertani broth (5ml) and incubated at 37˚C for 24 hours in preparation for preservation and DNA extraction. DNA was extracted using a boil preparation method where the broth culture was centrifuged to pellet bacteria. Supernatant was decanted and bacterial pellet resuspended with sterile water (50μL). Samples were then heated for 5min at 95˚C followed by centrifugation and resulting bacterial lysates were stored at -30˚C.

Faecal DNA extraction and PCR competency

DNA was extracted from a subset of faecal samples (*n* = 309) as part of an exploratory analysis into the presence of *intI1* in pinniped microbiomes. Samples were randomly selected from each of the following colonies during each breeding season: Seal Bay 2016 (*n* = 48); Seal Rocks 2017 (*n* = 46), 2018 (*n* = 30), 2019 (*n* = 30); Deen Maar Island (*n* = 30); Cape Bridgewater (*n* = 30); the Skerries (*n* = 23); Cape Gantheaume 2018 (*n* = 30); Olive Island 2019 (*n* = 30 *N. cinerea*, *n* = 12 *A. forsteri*). Genomic DNA was extracted from FecalSwab™ sample media (200μL) using the ISOLATE Fecal DNA kit (Bioline, Sydney, Australia) as per manufacturer's instructions. PCR competency of DNA extracted from faecal samples and *E. coli* isolates was tested by a 16S PCR (Table 2) using methods described by Fulham et al. [39].

Table 2. PCR primers and target region.

Primer	Sequence 5'-3'	Target	Reference
f27	AGAGTTTGATCMTGGCTCAG	16S rRNA	[44]
r1492	TACGGYTACCTTGTTACGACTT	16S rRNA	[44]
HS463a	CTGGATTTCGATCACGGCACG	*intI1*	[45]
HS464	ACATGCGTGTAAATCATCGTCG	*intI1*	[45]
HS458	GTTTGATGTTATGGAGCAGCAACG	*attI1*	[46]
HS459	GCAAAAAGGCAGCAATTATGAGCC	*qacE1*	[46]
JL-D2	CGCATCACCTCAATACCTT	IS*26*	[18]

Primers used for amplification of 16S rRNA, class 1 integron components and sequencing.

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Screening for class 1 integrons

All faecal samples and *E. coli* isolates positive for 16S rDNA were further screened for the presence of the class 1 integron integrase gene (*intI1*) using HS463a and HS464 primers (Table 2) following the methods described by Waldron and Gillings [45].

Samples containing *intI1* were tested using additional PCRs to target the gene cassette array using HS458 and HS459 primers (Table 2) and PCR conditions described by Waldron and Gillings [45]. Any samples that did not produce a band for the HS458/HS459 PCR were analysed using a secondary primer set consisting of HS458 and JL-D2, which targets the IS*26* transposase, an alternate 3' terminus in integrons [18], using the conditions as described for HS458/HS459.

All PCRs included a positive control sample (integron positive *E. coli* KC2) and negative control (PCR-grade H_2O) and were resolved using gel electrophoresis (16S rRNA and HS463a/HS464 2% agarose w/v, HS458/459 and HS458/JL-D2 3% agarose w/v) with SYBR safe gel stain (Invitrogen, city, Australia). Electrophoresis was conducted at 100V for 30 min (16S) or 40 min (463/464; 458/459/JL-D2) in TBE (Tris, boric acid, ethylenediaminetetraacetic acid) and product size approximated using HyperLadderII 50bp DNA marker (Bioline, Sydney, Australia).

Cloning, sequencing and analysis

Using the MinElute PCR Purification Kit (Qiagen, Melbourne, Australia), amplicons from the two gene cassette array PCRs (HS458/459 and HS458/JL-D2) were purified following manufacturer's instructions. Amplicons containing only a single band were sequenced directly using the purified PCR product.

Amplicons containing multiple bands, indicating the presence of more than one gene cassette, were cloned using the TOPO TA cloning kit and transformed into One Shot ® DH5™-T1^R competent *E. coli* cells (Invitrogen, Carlsbad, CA, USA) as per manufacturer's protocol. Between six to twelve colonies of transformed *E. coli* were selected from each cloned sample and DNA from cell lysates were screened using HS458, HS459 and JL-D2 as described above. Amplicons of variable sizes were selected for sequencing.

Amplicons from HS463a/HS464 that did not amplify in HS458/HS459 or HS458/JL-D2 were purified and sequenced to confirm positive *intI* result.

Sequencing was performed at The Ramaciotti Centre for Genomics (University of New South Wales, Sydney, Australia) using Big Dye Terminator chemistry version 3.1 and ABI 3730/3730x1 Capillary Sequencers (Applied Biosystems, Foster City, CA, USA). Geneious Prime software (version 11.0.6; Biomatters Limited, Auckland, New Zealand) was employed to assemble and manually check sequences for quality. Assembled sequences were analysed for the presence of antibiotic resistance genes using Integrall (http:/integrall.bio.ua.pt). Class 1 integron gene cassette arrays were confirmed via detection of the 3' conserved region containing *qacE*. For arrays containing more than one gene cassette, the *attC* recombination site located between cassettes was identified using the highly conserved core sequence GTTRRRY and complementary inverse core sequence RYYAAC [47]. Representative sequences generated from this study have been lodged in GenBank under accession numbers OL960709-OL960714.

Trace element and heavy metal concentrations

The concentrations of Zn, As, Se, Hg, and Pb in whole blood of *A. p. doriferus* pups sampled at Seal Rocks in 2018 (n = 52) were provided by another study (Cobb-Clarke and Gray, personal communication) following previously described methods [31]. The data was derived from samples analysed using inductively coupled plasma-mass spectrometry (ICP-MS; Agilent

Technologies 7500 ce inductively coupled plasma mass spectroscopy, Santa Clara, CA). The median values and 95% confidence intervals (obtained from back transformed log data) for trace element and heavy metals in blood (μg/L) were Zn = 3.73 (95% CI 3.67–3.87), Se = 3.05 (95% CI 3.00–3.56), As = 0.06 (95% CI 0.05–0.07), Hg = 0.04 (95% CI 0.05–0.12) and Pb = 0.04 (95% CI 0.04–0.10).

Statistical analyses

All statistical analyses were conducted using RStudio software (V 1.2.5042, Boston, Massachusetts, USA). The Shapiro-Wilk's test was used to test for normality of data and any variables with significant (<0.05) and non-normal distribution were log transformed which normalised the data set and allowed for parametric statistical analysis. Significance was determined when $p < 0.05$.

The statistical analysis of class 1 integron distribution was conducted using Fisher's exact test to test for differences in class 1 integron occurrence between species. Pearson's chi-squared test was used to test for differences in class 1 integron occurrence within species across sampling sites and breeding seasons.

Welch's two sample t-test was used to test for significance in the relationship between integrons and trace element and heavy metal concentrations in *A. p. doriferus* pups ($n = 52$) sampled at Seal Rocks 2018. Of these 52 individuals, 14 were integron positive.

Results

Detection of class 1 integrons

Escherichia coli was isolated from a total of 795 faecal samples (89.9%) with PCR screening for *intI1* revealing 36 isolates (4.52%, $n = 795$) that tested positive based on the presence of the expected 473bp product. Seven of the positive *E. coli* isolates were from *N. cinerea* and 29 from *A. p. doriferus*. Screening of faecal DNA detected *intI1* in 15 faecal samples (4.85%, $n = 309$), with four from *N. cinerea* and 11 from *A. p. doriferus*. All faecal samples and *E. coli* isolates from *A. forsteri* were negative for *intI1* (Fig 2).

Class 1 integrons were detected at all *N. cinerea* and *A. p. doriferus* colonies sampled (Fig 2). There was a significant difference in the prevalence of class 1 integrons across *A. p. doriferus* colonies sampled ($\chi^2_{3, 40} = 58.8$, $p<0.001$). There was no statistically significant difference in prevalence across *N. cinerea* colonies ($\chi^2_{2, 11} = 2.90$, $p = 0.234$). The highest number of class 1 integrons ($n = 27$) was observed in *A. p. doriferus* pups at Seal Rocks in 2018. The analysis of prevalence within colonies across breeding seasons revealed a significant difference at Seal Rocks ($\chi^2_{2, 31} = 40.51$, $p<0.001$) and Seal Bay ($\chi^2_{2, 5} = 10$, $p<0.01$), but there was no significant difference at Olive Island ($\chi^2_{1, 5} = 1.8$, $p = 0.179$; Fig 2).

Gene cassette array diversity

DNA sequencing identified five different gene cassette arrays from the 51 positive samples. The majority of the samples ($n = 40$) contained gene cassette arrays void of ARGs. Of the samples containing integrons with ARGs, seven contained a single gene cassette and the remaining four arrays each had two gene cassettes (Fig 3).

Class 1 integrons identified in samples from *A. p. doriferus* were the most diverse, encoding seven different ARGs, while only two types of ARGs were detected in *N. cinerea* (Fig 3). The most common cassette array was *dfrA7* ($n = 4$), identified in *E. coli* isolate DNA from both *N. cinerea* and *A. p. doriferus* pups.

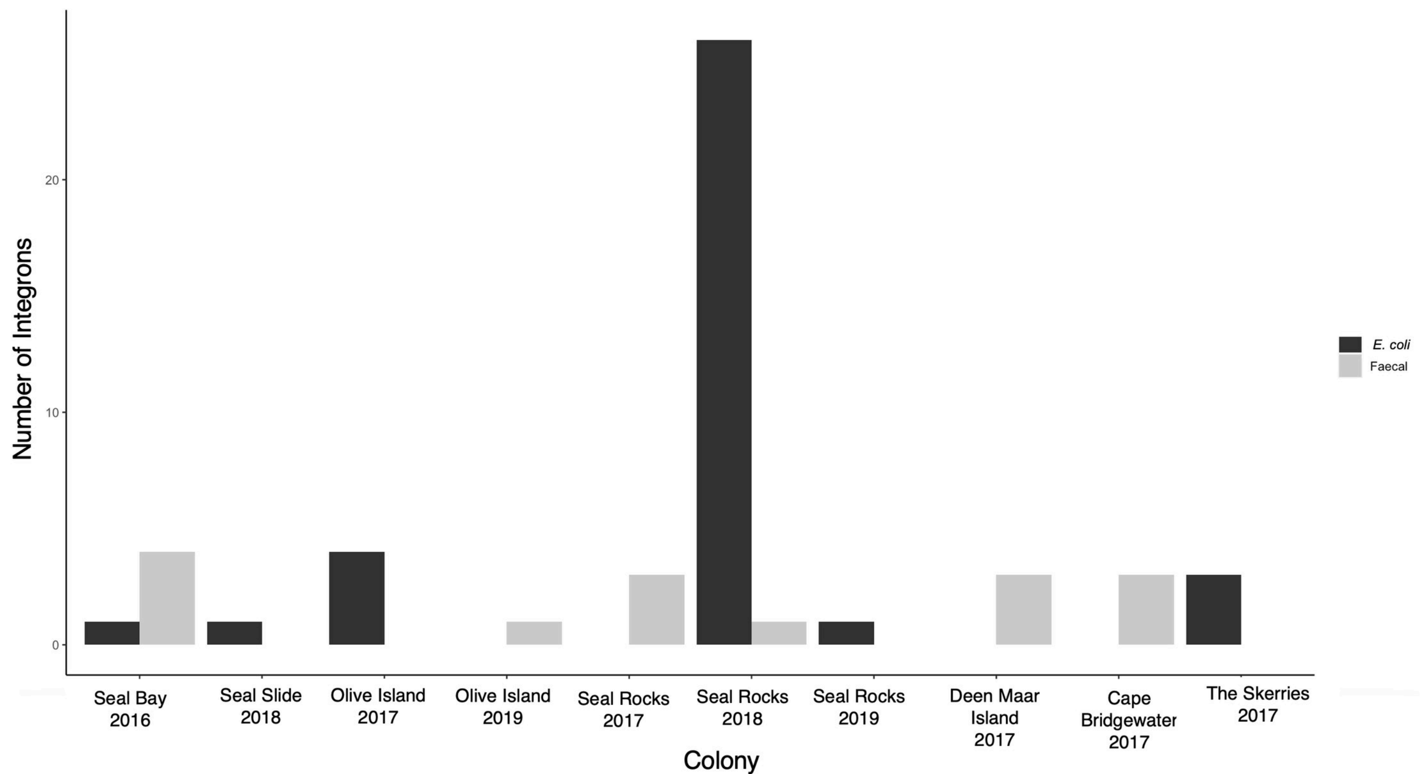

Fig 2. Graph of class 1 integrons detected in pinniped pups. Total number of class 1 integrons detected in faecal and *E. coli* isolate DNA in pups at each colony where integrons were detected during each breeding season sampled.

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The vast majority (49 of 51) of gene cassette arrays detected in this study contained the typical 3' conserved segment (*qacEΔ*), whereas, in the remaining two gene cassette arrays, *qacEΔ* was replaced with an IS*26* transposase (Fig 3).

Trace element and heavy metals and class 1 integron co-selection

There was no significant relationship between the concentrations of Zn (p = 0.905; 95% CI 3.67–3.87), Se (p = 0.507; 3.00–3.56), As (p = 0.446; 0.05–0.07), Hg (p = 0.335; 0.05–0.12) or Pb (p = 0.937; 0.04–0.10) in whole blood and integron carriage in *A*. *p*. *doriferus* (*n* = 52) sampled at Seal Rocks in 2018.

Discussion

This study identified class 1 integrons in both *E. coli* and faecal DNA from free-ranging *N. cinerea* and *A*. *p*. *doriferus* pups at seven breeding colonies in Southern Australia, representing the first time ARGs have been detected in *A*. *p*. *doriferus*, and in the faecal microbiota from *N. cinerea* pups. The occurrence of class 1 integrons in *A*. *p*. *doriferus* pups is of particular interest given the higher carriage of *intI1* in comparison with the other pinniped species studied.

There was similar class 1 integron abundance and gene cassette diversity across all four *A*. *p*. *doriferus* colonies sampled in 2017. These colonies differ in terms of size, topography, pup production and population density [48] and cover a wide geographical area. The similar *intI1* abundances indicates that these colonies are exposed to similar sources and levels of anthropogenic pollution, however, the number of *intI1* genes detected at Seal Rocks showed considerable change over sampling years (2017–2019), with a significant increase observed in 2018.

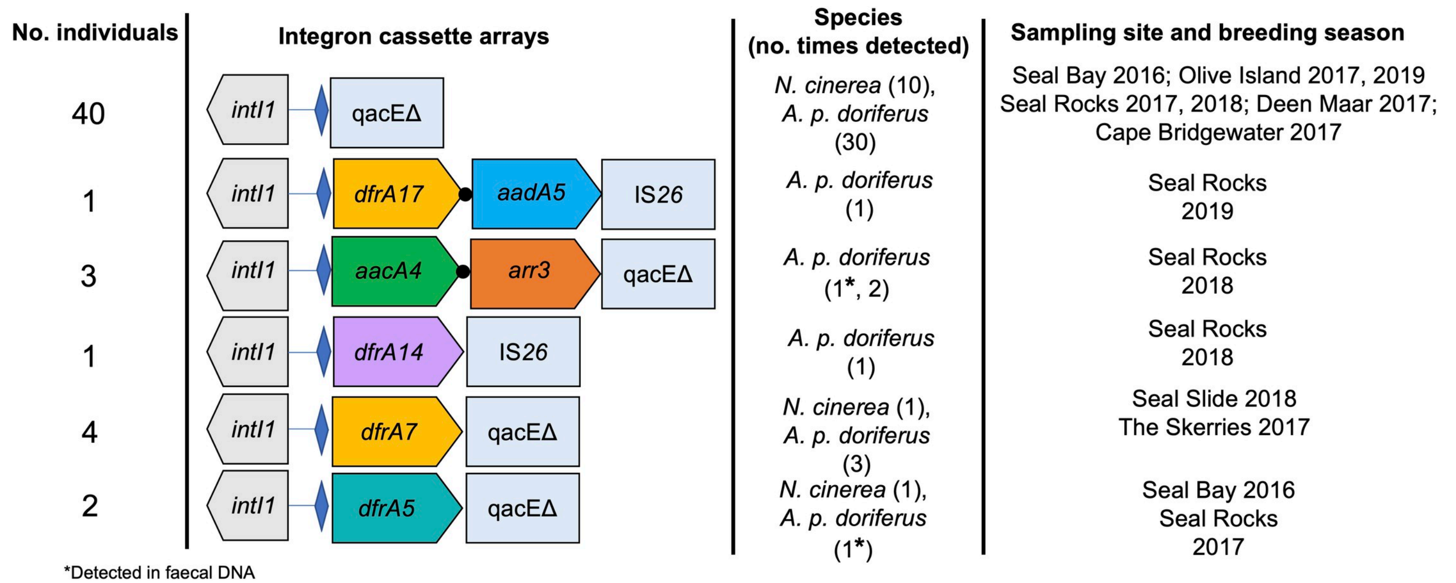

Fig 3. Schematic map of class 1 integron gene cassette arrays identified in *N. cinerea* and *A. p. doriferus* across all sampling sites and breeding seasons. Number of individuals with each array are listed on the left-hand side. Gene cassettes are represented as broad arrows. Blue diamonds represent the primary integron recombination site, *attI1*, where gene cassettes are inserted following acquisition; black circles represent gene cassette recombination site, *attC*. Gene symbols are as follows: *dfrA* genes encode dihydrofolate reductases that confer resistance to trimethoprim; *aacA* genes encode aminoglycoside (6') acetyltransferases (*aacA*) that confer resistance to aminoglycoside antibiotics; *aadA* genes encode aminoglycoside (3") adenylyltransferases that confer resistance to streptomycin and spectinomycin; *arr3* genes encode ADP-ribosyl transferases that confer resistance to rifampin; *qac* genes encode efflux pumps that confer resistance to quaternary ammonium compounds; *qacEΔ* and IS*26* represent the 3' terminus of some the gene cassette arrays depicted.

https://doi.org/10.1371/journal.pone.0258978.g003

This increase was not sustained over multiple breeding seasons and further investigation is needed to determine if this increase is due to a gradient of anthropogenic pollution or whether it is an aberrant finding at this colony. Seal Rocks was the only *A. p. doriferus* colony sampled over multiple breeding seasons limiting our ability to fully assess and compare trends in *intI1* abundance across other *A. p. doriferus* colonies over time.

The highest number of class 1 integrons was detected in *A. p. doriferus* pups at Seal Rocks. Class 1 integrons are generally more prevalent in locations closer to urbanised environments that are exposed to higher levels of anthropogenic pollution [16,49]. Seal Rocks is located 1.8km from Phillip Island and approximately 7km from the Mornington Peninsula, both of which are densely populated over summer months when pups are born and sampled. Seal Rocks is also located within 150km of Melbourne, Australia's second largest city (~5 million people), which could result in continuous exposure of pinnipeds to a higher number of anthropogenic sources of pollution compared to *A. p. doriferus* pups at the other colonies sampled (for example, Deen Maar Island and Cape Bridgewater are ~250km and 300km respectively from Melbourne), thereby facilitating the greater acquisition of class 1 integrons.

The abundance of *intI1* is presumed to change rapidly based on environmental factors [16]. Rapid changes in environments caused by extreme weather events can also introduce higher levels of runoff and industrial waste into the marine environment and distribute pollutants across wider geographical ranges [50]. Environmental conditions at pinniped breeding colonies were not considered as part of this study, however, given the differences in *intI1* abundance observed, the influence of stochastic events on the presence of class 1 integrons would need to be considered when attempting to understand the trends observed in *A. p. doriferus* pups.

In addition to the higher abundance of *intI1* genes detection in *A. p. doriferus* pups at Seal Rocks in 2018, was the presence of two IS*26* class 1 integron variants in *E. coli* isolates. There

are multiple reports of IS*26* class 1 integron variants in *E. coli* from animals in Australia, including cows and pigs [18,51,52] and Grey-headed flying foxes (*Pteropus poliocephalus*) [53], as well as in human clinical cases [54]. The presence of class 1 integrons with IS*26* variants in *E. coli* isolates from free-ranging *A. p. doriferus* pups provides further evidence to suggest that this colony is exposed to a marine environment that is contaminated by various sources of anthropogenic pollution.

The finding of the *arr3* gene cassette in multiple *A. p. doriferus* pups at Seal Rocks in 2018 was unexpected. This gene cassette, *arr3*, encodes ADP-ribosyl transferases which potentially confers resistance to rifampicin and has been identified in numerous Gram-negative pathogens (such as *Proteus spp*.) [55,56] and integrons in bacterial isolates from hospital patients [57]. The *aacA4-arr3-qacEΔ* cassette array detected in *A. p. doriferus* is not widely reported in the literature [57], however, the closest matches on GenBank were predominately detected in *Proteus* spp. and *Klebsiella* spp. (e.g. CP053614, LC549808) isolates from hospital patients and zoo and production animals in China. In this study, the array was detected in faecal DNA and the bacterial carrier remains unknown. As the *arr3* cassette is associated with human and domestic animal pathogens, the presence of this gene cassette in free-ranging pinnipeds provides further evidence to suggest that the Seal Rocks colony is exposed to anthropogenic microbial pollution.

In contrast to *A. p. doriferus* and *N. cinerea*, class 1 integrons were not detected in *A. forsteri* pups ($n = 150$) sampled over multiple breeding seasons. Over 5000 pups are born at the Cape Gantheaume colony each breeding season [58] and there is a much higher population density compared to Seal Slide, a small *N. cinerea* colony <10km away. Despite the similarity in location and higher population density between these two pinniped species a class 1 integron was detected in one of four *N. cinerea* sampled pups during the 2018 breeding season. The difference in *intI1* abundance between species is likely multifactorial and further investigation is necessary to better understand the factors that contribute to the acquisition of class 1 integrons in free-ranging pinniped species.

Heavy metals are environmental pollutants that can act as co-selecting agents for antibiotic resistance [20]. Heavy metals do not degrade and therefore persist in the environment for long periods of time, maintaining selective pressure for extended periods [22]. Furthermore, the abundance of class 1 integrons has been found to correlate with heavy metal concentrations, with previous studies focusing on the relationship between heavy metal pollutants and *intI1* abundance in environmental samples including water and soil [21,59]. It has been well established that concentrations of heavy metals are present in free-ranging marine mammals [31,32] and can bioaccumulate in upper trophic predators [30,60]. Despite the association between heavy metal concentrations and class 1 integron abundance in environmental samples, this relationship has not previously been investigated in free-ranging wildlife. A significant relationship between the concentrations of ARB carriage and two trace elements and three heavy metals was not seen, although the small sample size for this specific comparison could have limited this analysis. In addition, the current comparison was limited to concentrations of trace and heavy metals in whole blood which reflect recent exposure. The relationship between ARB carriage and heavy metal concentrations in other tissues (for example, liver) could be explored, given the bioaccumulation potential of this tissue when compared to whole blood [61].

Free-ranging wildlife are exposed to differing environmental conditions, selective pressures and exposure to ARB that likely drive the acquisition of ARGs [62]. Pinniped pups sampled as part of this study were less than two months of age and confined to the breeding colonies. As such, the only potential source of exposure to bacteria and ARGs for pups are those present in the breeding colony environment, which could be contaminated by wastewater run-off, faecal

contamination from other wildlife species (including sea birds) and juvenile and adult pinnipeds that inhabit these colonies [36,63]. Evidence suggests that the prevalence of ARB in free-ranging wildlife is influenced by exposure to anthropogenic pollution and environmental contamination [62], with the latter varying depending on habitat occupation and behaviours exhibited by wildlife species [62]. For example, foraging is a behaviour that can increase the likelihood of wildlife species being exposed to ARB [3]. The pinniped species studied herein have differing foraging strategies. While *N. cinerea* and *A. p. doriferus* are benthic foragers [64–67], *A. forsteri* are pelagic foragers [68,69]. In some aquatic species, differences in toxicant accumulation between benthic and pelagic feeders has been observed [61], thus differences in foraging behaviours could lead to exposure of higher levels of pollutants and increased ARB acquisition in benthic feeding pinniped species.

Another factor to consider is the proximity to wastewater treatment plants and the release of effluent. The environment created within wastewater treatment plants has been found to promote the proliferation of ARB through the exposure of bacteria to sub-inhibitory concentrations of antibiotics [70], disinfectants, and heavy metals [16]. Effluent released from wastewater treatment plants is being increasingly identified as an important source of ARB, with some studies finding higher frequencies of integron-positive *E. coli* downstream of such effluent [71]. Colonies located in closer proximity to wastewater treatment plants could therefore be exposed to higher levels of ARB, which could influence the acquisition of ARB and ARGs in free-ranging pinniped populations.

Interactions with other species in ecosystems is another aspect to consider when attempting to understand the transfer of AMR. The presence of other species at pinniped breeding colonies such as sea birds, known carriers of numerous ARGs [63], could also influence the carriage of AMR in free-ranging pinnipeds. Sampling both environmental substrates and other wildlife species present within study areas is required to gain a greater understanding of the source of AMR and the transfer of ARGs in free-ranging wildlife species.

Conclusion

This study detected bacteria carrying diverse gene cassettes encoding resistance to multiple classes of antibiotics in two species of free-ranging pinniped pups in Australia. The detection of class 1 integrons, mobile genetic elements that have been identified as useful indicators of antimicrobial pollution, suggests these populations are exposed to anthropogenic pollution. Furthermore, the detection of *E. coli* carrying IS*26* class 1 integron variants in free-ranging pinniped pups indicates that these isolates originated from domestic animals and/or humans.

Further investigation to better understand how antibiotic resistant bacteria are being acquired by free-ranging pinniped pups is critical and could be used as an additional mechanism to monitor anthropogenic pollution in marine ecosystems. Ongoing monitoring of antibiotic resistant bacteria in these species will also assist in understanding the role of increasing anthropogenic pollution on the long-term survival of these marine sentinel species.

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References

1. Baquero F, Martínez J-L, Cantón R. Antibiotics and antibiotic resistance in water environments. Curr Opin Biotechnol. 2008; 19(3):260–5. https://doi.org/10.1016/j.copbio.2008.05.006 PMID: 18534838
2. Berendonk TU, Manaia CM, Merlin C, Fatta-Kassinos D, Cytryn E, Walsh F, et al. Tackling antibiotic resistance: the environmental framework. Nat Rev Microbiol. 2015; 13(5). https://doi.org/10.1038/nrmicro3439 PMID: 25817583
3. Vittecoq M, Godreuil S, Prugnolle F, Durand P, Brazier L, Renaud N, et al. Antimicrobial resistance in wildlife. J Appl Ecol. 2016; 53(2):519–29.
4. Grilo ML, Sousa-Santos C, Robalo J, Oliveira M. The potential of *Aeromonas* spp. from wildlife as antimicrobial resistance indicators in aquatic environments. Ecol Indic. 2020; 115. https://doi.org/10.1016/j.ecolind.2020.106382 PMID: 34121931
5. Dunning Hotopp JC. Horizontal gene transfer between bacteria and animals. Trends Genet. 2011; 27(4):157–63. https://doi.org/10.1016/j.tig.2011.01.005 PMID: 21334091
6. Thomas CM, Nielsen KM. Mechanisms of, and barriers to, horizontal gene transfer between bacteria. Nat Rev Microbiol. 2005; 3(9):711. https://doi.org/10.1038/nrmicro1234 PMID: 16138099
7. Díaz-Mejía JJ, Amábile-Cuevas CF, Rosas I, Souza V. An analysis of the evolutionary relationships of integron integrases, with emphasis on the prevalence of class 1 integrons in *Escherichia coli* isolates from clinical and environmental origins. Microbiology. 2008; 154(1):94–102. https://doi.org/10.1099/mic.0.2007/008649-0 PMID: 18174129
8. Partridge SR, Kwong SM, Firth N, Jensen SO. Mobile Genetic Elements Associated with Antimicrobial Resistance. Clin Microbiol Rev. 2018; 31(4):e00088–17. https://doi.org/10.1128/CMR.00088-17 PMID: 30068738
9. Botelho J, Schulenburg H. The Role of Integrative and Conjugative Elements in Antibiotic Resistance Evolution. Trends Microbiol. 2021; 29(1):8–18. https://doi.org/10.1016/j.tim.2020.05.011 PMID: 32536522
10. Domingues S, Silva GJ da, Nielsen KM. Integrons: Vehicles and pathways for horizontal dissemination in bacteria. Mob Genet Elements. 2012; 2(5):211–23. https://doi.org/10.4161/mge.22967 PMID: 23550063
11. Gaze WH, Zhang L, Abdouslam NA, Hawkey PM, Calvo-Bado L, Royle J, et al. Impacts of anthropogenic activity on the ecology of class 1 integrons and integron-associated genes in the environment. ISME J. 2011; 5(8):1253–61. https://doi.org/10.1038/ismej.2011.15 PMID: 21368907

12. Gillings MR. Class 1 integrons as invasive species. Curr Opin Microbiol. 2017; 38:10–5. https://doi.org/10.1016/j.mib.2017.03.002 PMID: 28414952

13. Stokes HW, Gillings MR. Gene flow, mobile genetic elements and the recruitment of antibiotic resistance genes into Gram-negative pathogens. Vol. 35, FEMS Microbiology Reviews. Oxford, UK: Blackwell Publishing Ltd; 2011. p. 790–819.

14. Souque C, Escudero JA, Maclean RC. Integron activity accelerates the evolution of antibiotic resistance. Elife. 2021; 10:1–47. https://doi.org/10.7554/eLife.62474 PMID: 33634790

15. Hall RM, Collis CM. Mobile gene cassettes and integrons: capture and spread of genes by site-specific recombination. Mol Microbiol. 1995; 15(4):593–600. https://doi.org/10.1111/j.1365-2958.1995.tb02368.x PMID: 7783631

16. Gillings MR, Gaze WH, Pruden A, Smalla K, Tiedje JM, Zhu Y-G. Using the class 1 integron-integrase gene as a proxy for anthropogenic pollution. ISME J. 2014; 9(6). https://doi.org/10.1038/ismej.2014.226 PMID: 25500508

17. Ghaly TM, Gillings MR, Penesyan A, Qi Q, Rajabal V, Tetu SG. The Natural History of Integrons. 2021; 9(11):2212. https://doi.org/10.3390/microorganisms9112212 PMID: 34835338

18. Dawes FE, Kuzevski A, Bettelheim KA, Hornitzky MA, Djordjevic SP, Walker MJ. Distribution of class 1 integrons with IS*26*-mediated deletions in their 3'-conserved segments in *Escherichia coli* of human and animal origin. PLoS One. 2010; 5(9):e12754–e12754. https://doi.org/10.1371/journal.pone.0012754 PMID: 20856797

19. Harmer CJ, Moran RA, Hall RM. Movement of IS26-associated antibiotic resistance genes occurs via a translocatable unit that includes a single IS26 and preferentially inserts adjacent to another IS26. MBio. 2014; 5(5):e01801–14. https://doi.org/10.1128/mBio.01801-14 PMID: 25293759

20. Nguyen CC, Hugie CN, Kile ML, Navab-Daneshmand T. Association between heavy metals and antibiotic-resistant human pathogens in environmental reservoirs: A review. Front Environ Sci Eng. 2019; 13(3):46.

21. Wright MS, Baker-Austin C, Lindell AH, Stepanauskas R, Stokes HW, Mcarthur JV. Influence of industrial contamination on mobile genetic elements: class 1 integron abundance and gene cassette structure in aquatic bacterial communities. ISME J. 2008; 2(4):417. https://doi.org/10.1038/ismej.2008.8 PMID: 18273063

22. Alonso A, Sánchez P, Martínez JL. Environmental selection of antibiotic resistance genes. Environ Microbiol. 2001; 3(1):1–9. https://doi.org/10.1046/j.1462-2920.2001.00161.x PMID: 11225718

23. Yazdankhah S, Skjerve E, Wasteson Y. Antimicrobial resistance due to the content of potentially toxic metals in soil and fertilizing products. Microb Ecol Health Dis. 2018; 29(1):1548212–48.

24. Radhouani H, Silva N, Poeta P, Torres C, Correia S, Igrejas G. Potential impact of antimicrobial resistance in wildlife, environment, and human health. Front Microbiol. 2014; 5.

25. Goulas A, Livoreil B, Grall N, Benoit P, Couderc-Obert C, Dagot C, et al. What are the effective solutions to control the dissemination of antibiotic resistance in the environment? A systematic review protocol. Environ Evid. 2018; 7(1):1–9.

26. Alonso CA, Alcalá L, Simón C, Torres C. Novel sequence types of extended-spectrum and acquired AmpC beta-lactamase producing *Escherichia coli* and *Escherichia coli* clade V isolated from wild mammals. FEMS Microbiol Ecol. 2017; 93(8). https://doi.org/10.1093/femsec/fix097 PMID: 28873943

27. Ali H, Khan E, Ilahi I. Environmental chemistry and ecotoxicology of hazardous heavy metals: Environmental persistence, toxicity, and bioaccumulation. Yang Y, editor. J Chem. 2019; 2019:6730305.

28. Daum JR, Shepherd DM, Noelle RJ. Immunotoxicology of cadmium and mercury on B-lymphocytes—I. Effects on lymphocyte function. Int J Immunopharmacol. 1993; 15(3):383–94. https://doi.org/10.1016/0192-0561(93)90049-5 PMID: 7685007

29. Plum LM, Rink L, Haase H. The essential toxin: impact of zinc on human health. Int J Environ Res Public Health. 2010; 7(4):1342–65. https://doi.org/10.3390/ijerph7041342 PMID: 20617034

30. Kretzmann M, Rohrbach L, Durham K, Digiovanni R, Sañudo-Wilhelmy S. Trace metal burdens in stranded seals from Long Island, New York: Potential evidence for species differences in foraging. Aquat Mamm. 2010; 36(2):178–87.

31. Gray R, Canfield P, Rogers T. Trace element analysis in the serum and hair of Antarctic leopard seal, *Hydrurga leptonyx*, and Weddell seal, *Leptonychotes weddellii*. Sci Total Environ. 2008; 399(1):202–15.

32. Ashley EA, Olson JK, Raverty S, Wilkinson K, Gaydos JK. Trace element concentrations in livers of Pacific harbor seals (*Phoca vitulina richardii*) from San Juan County, Washington, USA. J Wildl Dis. 2020; 56(2):429–36. PMID: 31622186

33. Schaefer AM, Bossart GD, Mazzoil M, Fair PA, Reif JS. Risk factors for colonization of *E. coli* in Atlantic Bottlenose Dolphins (*Tursiops truncatus*) in the Indian River Lagoon, Florida. Schaefer AM, editor. J

Environ Public Health. 2011; 2011(2011):597073. https://doi.org/10.1155/2011/597073 PMID: 21977048

34. Wallace C, Yund P, Ford T, Matassa K, Bass A. Increase in antimicrobial resistance in bacteria isolated from stranded marine mammals of the northwest Atlantic. Ecohealth. 2013; 10(2):201–10. https://doi.org/10.1007/s10393-013-0842-6 PMID: 23636484
35. Bossart GD. Marine mammals as sentinel species for oceans and human health. Vet Pathol. 2010; 48 (3):676–90. https://doi.org/10.1177/0300985810388525 PMID: 21160025
36. Dolejska M, Cizek A, Literak I. High prevalence of antimicrobial-resistant genes and integrons in *Escherichia coli* isolates from Black-headed Gulls in the Czech Republic. J Appl Microbiol. 2007; 103 (1):11–9. https://doi.org/10.1111/j.1365-2672.2006.03241.x PMID: 17584448
37. Lundbäck IC, McDougall FK, Dann P, Slip DJ, Gray R, Power ML. Into the sea: Antimicrobial resistance determinants in the microbiota of little penguins (*Eudyptula minor*). Infect Genet Evol. 2020;104697. https://doi.org/10.1016/j.meegid.2020.104697 PMID: 33370595
38. McDougall F, Boardman W, Gillings M, Power M. Bats as reservoirs of antibiotic resistance determinants: A survey of class 1 integrons in Grey-headed Flying Foxes (*Pteropus poliocephalus*). Infect Genet Evol. 2019; 70:107–13. https://doi.org/10.1016/j.meegid.2019.02.022 PMID: 30798035
39. Fulham M, Power M, Gray R. Comparative ecology of Escherichia coli in endangered Australian sea lion (*Neophoca cinerea*) pups. Infect Genet Evol. 2018; 62:262–9. https://doi.org/10.1016/j.meegid.2018.05.002 PMID: 29730275
40. Delport TC, Harcourt RG, Beaumont LJ, Webster KN, Power ML. Molecular detection of antibiotic-resistance determinants in Escherichia coli isolated from the endangered Australian sea lion (*Neophoca cinerea*). J Wildl Dis. 2015; 51(3):555–63. https://doi.org/10.7589/2014-08-200 PMID: 25919463
41. Power ML, Samuel A, Smith JJ, Stark JS, Gillings MR, Gordon DM. *Escherichia coli* out in the cold: Dissemination of human-derived bacteria into the Antarctic microbiome. Environ Pollut. 2016; 215:58–65. https://doi.org/10.1016/j.envpol.2016.04.013 PMID: 27179324
42. Kirkwood R, Goldsworthy S. Fur Seals and Sea Lions. Collingwood, VIC: CSIRO PUBLISHING; 2013. (Australian Natural History Series).
43. Fulham M, Power M, Gray R. Diversity and distribution of *Escherichia coli* in three species of free-ranging Australian pinniped pups. Vol. 7, Frontiers in Marine Science. 2020. p. 755.
44. Lane D. Nucleic acid techniques in bacterial systematics. In: Stackebrandt E, editor. Nucleic Acid Techniques in Bacterial Systematics. Hoboken, N.J.: John Wiley and Sons; 1991.
45. Waldron LS, Gillings MR. Screening foodstuffs for class 1 integrons and gene cassettes. Waldron LS, editor. J Vis Exp. 2015; 2015(100):e52889–e52889. https://doi.org/10.3791/52889 PMID: 26132232
46. Holmes AJ, Gillings MR, Nield BS, Mabbutt BC, Nevalainen KMH, Stokes HW. The gene cassette metagenome is a basic resource for bacterial genome evolution. Environ Microbiol. 2003 May 1; 5 (5):383–94. https://doi.org/10.1046/j.1462-2920.2003.00429.x PMID: 12713464
47. Escudero J, Loot C, Nivina A, Mazel D. The integron: adaptation on demand. In: Sandmeyer S, editor. Mobile DNA III. Washinton, DC: ASM Press; 2015. p. 139–61.
48. McIntosh RR, Kirkman SP, Thalmann S, Sutherland DR, Mitchell A, Arnould JPY, et al. Understanding meta-population trends of the Australian fur seal, with insights for adaptive monitoring. PLoS One. 2018; 13(9):e0200253. https://doi.org/10.1371/journal.pone.0200253 PMID: 30183713
49. Koczura R, Mokracka J, Taraszewska A, Łopacinska N. Abundance of class 1 integron-integrase and sulfonamide resistance genes in river water and sediment is affected by anthropogenic pressure and environmental factors. Microb Ecol. 2016; 72(4):909–16. https://doi.org/10.1007/s00248-016-0843-4 PMID: 27599709
50. Yin J, Gentine P, Zhou S, Sullivan SC, Wang R, Zhang Y, et al. Large increase in global storm runoff extremes driven by climate and anthropogenic changes. Nat Commun. 2018; 9(1):4310–89. https://doi.org/10.1038/s41467-018-06236-8 PMID: 30333496
51. Reid C, Wyrsch E, Zingali T, Liu M, Darling A, Chapman T, et al. Porcine commensal Escherichia coli: A reservoir for class 1 integrons associated with IS26. BioRxiv. 2017; 3(12):e000143. https://doi.org/10.1099/mgen.0.000143 PMID: 29306352
52. Zingali T, Reid C, Chapman T, Gaio D, Liu M, Darling A, et al. Whole genome sequencing analysis of porcine faecal commensal *Escherichia coli* carrying class 1 integrons from sows and their offspring. Microorganisms. 2020; 8:843. https://doi.org/10.3390/microorganisms8060843 PMID: 32512857
53. McDougall FK, Boardman WSJ, Power ML. Characterization of beta-lactam-resistant *Escherichia coli* from Australian fruit bats indicates anthropogenic origins. Microb Genomics. 2021; 7(5):000571. https://doi.org/10.1099/mgen.0.000571 PMID: 33950805

54. Hastak P, Cummins ML, Gottlieb T, Cheong E, Merlino J, Myers GSA, et al. Genomic profiling of *Escherichia coli* isolates from bacteraemia patients: a 3-year cohort study of isolates collected at a Sydney teaching hospital. Microb Genomics. 2020; 6(5):e000371.

55. Baysarowich J, Koteva K, Hughes DW, Ejim L, Griffiths E, Zhang K, et al. Rifamycin antibiotic resistance by ADP-ribosylation: Structure and diversity of Arr. Proc Natl Acad Sci U S A. 2008; 105(12):4886–91. https://doi.org/10.1073/pnas.0711939105 PMID: 18349144

56. Bie L, Fang M, Li Z, Wang M, Xu H. Identification and characterization of new resistance-conferring SGI1s (Salmonella Genomic Island 1) in *Proteus mirabilis*. Vol. 9, Frontiers in Microbiology. 2018. p. 3172. https://doi.org/10.3389/fmicb.2018.03172 PMID: 30619228

57. Xia W, Xu T, Qin T, Li P, Liu Y, Kang H, et al. Characterization of integrons and novel cassette arrays in bacteria from clinical isloates in China, 2000–2014. J Biomed Res. 2016/05/10. 2016; 30(4):292–303. https://doi.org/10.7555/JBR.30.20150153 PMID: 27533938

58. Shaughnessy PD, Goldsworthy SD. Increasing abundance of pups of the long-nosed fur seal (*Arctocephalus forsteri*) on Kangaroo Island, South Australia, over 26 breeding seasons to 2013–14. Wildl Res. 2015; 42(8):619–32.

59. Dickinson AW, Power A, Hansen MG, Brandt KK, Piliposian G, Appleby P, et al. Heavy metal pollution and co-selection for antibiotic resistance: A microbial palaeontology approach. Environ Int. 2019; 132:105117. https://doi.org/10.1016/j.envint.2019.105117 PMID: 31473413

60. Wintle NJP, Duffield DA, Barros NB, Jones RD, Rice JM. Total mercury in stranded marine mammals from the Oregon and southern Washington coasts. Mar Mammal Sci. 2011; 27(4):E268–78.

61. Bustamante P, Bocher P, Chérel Y, Miramand P, Caurant F. Distribution of trace elements in the tissues of benthic and pelagic fish from the Kerguelen Islands. Sci Total Environ. 2003; 313(1–3):25–39. https://doi.org/10.1016/S0048-9697(03)00265-1 PMID: 12922058

62. Ramey AM, Ahlstrom CA. Antibiotic resistant bacteria in wildlife: perspectives on trends, acquisition and dissemination, data gaps, and future directions. J Wildl Dis. 2020; 56(1):1–15. PMID: 31567035

63. Dolejska M, Literak I. Wildlife is overlooked in the epidemiology of medically important antibiotic-resistant bacteria. Antimicrob Agents Chemother. 2019; 8(63):e01167–19. https://doi.org/10.1128/AAC.01167-19 PMID: 31209001

64. Arnould JPY, Kirkwood R. Habitat selection by female Australian fur seals (*Arctocephalus pusillus doriferus*). Aquat Conserv Mar Freshw Ecosyst. 2007 Dec 1; 17(S1):S53–67.

65. Arnould JPY, Hindell MA. Dive behaviour, foraging locations, and maternal-attendance patterns of Australian fur seals (*Arctocephalus pusillus doriferus*). Can J Zool. 2001; 79(1):35–48.

66. Peters KJ, Ophelkeller K, Bott NJ, Deagle BE, Jarman SN, Goldsworthy SD. Fine-scale diet of the Australian sea lion (*Neophoca cinerea*) using DNA-based analysis of faeces. Mar Ecol. 2015; 36(3):347–67.

67. Fowler SL, Costa DP, Arnould JPY. Ontogeny of movements and foraging ranges in the Australian sea lion. Mar Mammal Sci. 2007; 23(3):598–614.

68. Baylis AMM, Page B, Goldsworthy SD. Effect of seasonal changes in upwelling activity on the foraging locations of a wide-ranging central-place forager, the New Zealand fur seal. Can J Zool. 2008; 86 (8):774–89.

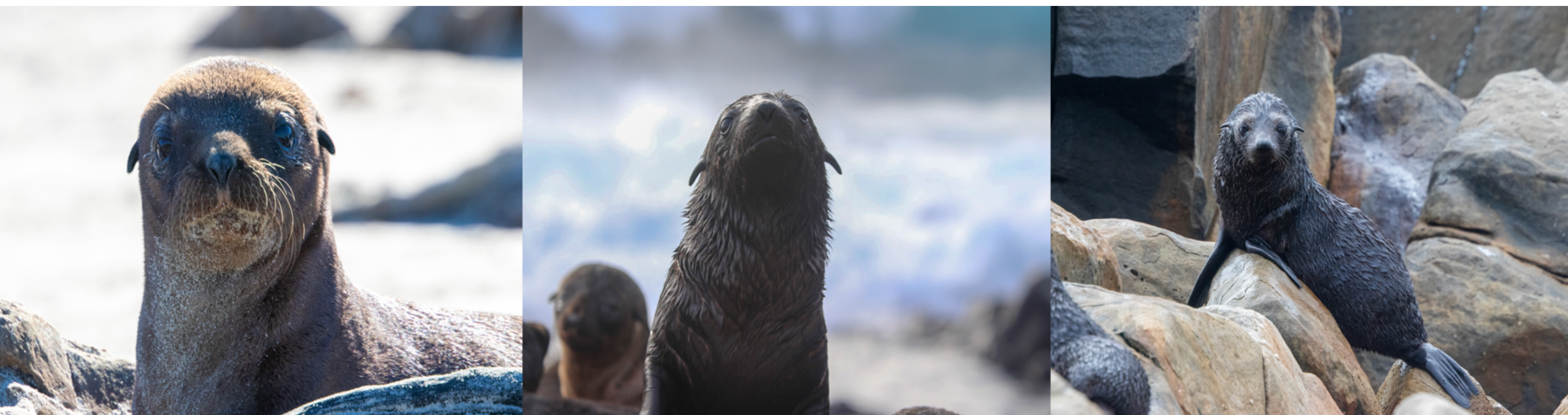
69. Page B, McKenzie J, Sumner MD, Coyne M, Goldsworthy SD. Spatial separation of foraging habitats among New Zealand fur seals. Mar Ecol Prog Ser. 2006; 323:263–79.

70. Rizzo L, Manaia C, Merlin C, Schwartz T, Dagot C, Ploy MC, et al. Urban wastewater treatment plants as hotspots for antibiotic resistant bacteria and genes spread into the environment: A review. Sci Total Environ. 2013; 447:345–60. https://doi.org/10.1016/j.scitotenv.2013.01.032 PMID: 23396083

71. Koczura R, Mokracka J, Jabłońska L, Gozdecka E, Kubek M, Kaznowski A. Antimicrobial resistance of integron-harboring *Escherichia coli* isolates from clinical samples, wastewater treatment plant and river water. Sci Total Environ. 2012; 414:680–5. https://doi.org/10.1016/j.scitotenv.2011.10.036 PMID: 22119028

Chapter 3.

Diversity and distribution of *Escherichia coli* in three species of free-ranging Australian pinniped pups



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Diversity and Distribution of *Escherichia coli* in Three Species of Free-Ranging Australian Pinniped Pups

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Anthropogenic activities and pollution are impacting marine environments globally. As a consequence, increasing numbers of human-associated phylotypes of *Escherichia coli*, an indicator of fecal contamination, have been found in both aquatic environments and marine mammals considered sentinels for marine health. The objective of this study was to determine the presence and diversity of *E. coli* in pups of three species of free-ranging pinnipeds in Australia. Fecal samples (n = 963) were collected between 2016 and 2019 from Australian sea lion (*Neophoca cinerea*), Australian fur seal (*Arctocephalus pusillus doriferus*) and long-nosed fur seal (*Arctocephalus forsteri*) pups from eight breeding colonies extending along the Southern Australian coast. *E. coli* were isolated from 842 (87.3%) samples and molecular screening was applied to assign isolates to *E. coli* phylotypes and sub-types. The human associated *E. coli* phylotype B2 was the most frequently isolated in all species at seven of the eight colonies, with 73.7% of all *E. coli* isolates belonging to this phylotype. Phylotype distribution did not differ significantly within or across species, breeding colonies or breeding seasons. Analysis of B2 isolates into sub-types showed a significant difference in sub-type distribution across breeding seasons at two colonies (Seal Rocks and Cape Gantheaume). The predominance of the B2 phylotype could indicate that all colonies are exposed to similar levels of anthropogenic pollution. This widespread occurrence of the human-associated *E. coli* phylotypes highlights the imperative for ongoing monitoring and surveillance of microbes in both the marine environment and sentinel species.

Keywords: *Escherichia coli*, pinnipeds, wildlife, anthropogenic pollution, human-associated bacteria

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INTRODUCTION

The contamination of the marine environment with atypical microorganisms as a result of anthropogenic pollution poses unknown risks to both marine wildlife and the wider ecosystem (Oates et al., 2012; Baily et al., 2015). Fecal coliforms such as *Escherichia coli* are used globally as indicators of anthropogenic fecal contamination and as a measure of pollution of waterways and coastal ecosystems (Beversdorf et al., 2007; Schaefer et al., 2011; Ahmed et al., 2016). *E. coli* is a commensal species colonizing the intestinal tract of birds and mammals (Gordon and Cowling, 2003; Guenther et al., 2011). However, some strains of *E. coli* have virulence traits that render

them opportunistic pathogens that can cause a range of diseases in multiple host species, including urinary tract infections, neonatal meningitis and septicemia (Russo and Johnson, 2000; Mora et al., 2009; Clermont et al., 2011). There are eight *E. coli* lineages (A, B1, B2, C, D, E, F, clade I), termed phylotypes, that differ in ecological niches, life histories, host affinities and pathogenic abilities; phylotypes B2 and D are commonly isolated from and associated with humans (Gordon and Cowling, 2003; Gordon et al., 2008). Many clinically relevant strains of *E. coli* that have the potential to cause extraintestinal disease in humans, domestic animals and birds belong to phylotype B2 (Johnson and Russo, 2002; Clermont et al., 2014).

Marine mammals are considered sentinels of ocean health with marine mammal health status providing insights into the health of the wider marine ecosystem (Bossart, 2010). *Escherichia coli* has been isolated from a number of aquatic mammal species including pinnipeds (Stoddard et al., 2008, 2009; Wallace et al., 2013; Delport et al., 2015; Power et al., 2016; Fulham et al., 2018; Mora et al., 2018), cetaceans (Schaefer et al., 2011; Melendez et al., 2019) and river otters (Oliveira et al., 2018). Despite this, little is known about the host and environmental factors that influence the diversity and prevalence of *E. coli* in marine mammals. Furthermore, the intestinal population of *E. coli* in mammals differs between individuals of the same species (Guenther et al., 2011). As a result, studies that focus on one species at a single time point are unlikely to accurately represent the diversity of *E. coli* within a species (Schierack et al., 2008, 2009; Leser and Mølbak, 2009).

In the Southern hemisphere, *E. coli* has been isolated from multiple pinniped species including southern elephant seals (*Mirounga leonina*), Weddell seals (*Leptonychotes weddellii*), Antarctic fur seals (*Arctocephalus gazella*) and Australian sea lions (*Neophoca cinerea*) (Hernandez et al., 2007; Delport et al., 2015; Power et al., 2016; Fulham et al., 2018; Mora et al., 2018). These studies have characterized *E. coli* isolated from free-ranging individuals with a majority of samples collected from adults and a predominance of phylotype B2 identified; the prevalence of the B2 phylotype ranging from 49% (Fulham et al., 2018) to 65% (Power et al., 2016). Comparisons of *E. coli* prevalence and phylotype distribution in captive and free-ranging adult *N. cinerea* (Delport et al., 2015) determined a higher prevalence in captive individuals; however, in both captive and free-ranging animals, the human-associated phylotype B2 was the most frequently identified (Delport et al., 2015). A higher *E. coli* prevalence and differing trends in phylotype distribution was described in free-ranging *N. cinerea* pups from two colonies in South Australia (Fulham et al., 2018). Of the two colonies, phylotype B2 was more prevalent in samples from the colony closer to anthropogenic sources of pollution than the colony located further from human habitation (Fulham et al., 2018). It is unknown whether host or environmental factors could be contributing to the differences in phylotype distribution observed in *E. coli* isolated from free-ranging *N. cinerea* pups. For this reason, further investigations are required to understand the distribution and diversity of *E. coli* in marine mammal species in Australian waters, including other pinniped species such as the Australian fur seal (*Arctocephalus pusillus doriferus*) and long-nosed fur seal (*Arctocephalus forsteri*) where the prevalence and diversity of *E. coli* has not been reported previously.

The three pinniped species, *N. cinerea, A. p. doriferus,* and *A. forsteri,* inhabit numerous offshore colonies along the Australian coast from Western Australia to Tasmania (Kirkwood and Goldsworthy, 2013). All three species were subjected to commercial sealing practices during the nineteenth and twentieth centuries which decimated their numbers (Ling, 1999). Population recovery of each species from historical harvesting has differed greatly, with numbers of *A. p. doriferus* and *A. forsteri* steadily increasing since the 1960s and 1970s (Shaughnessy et al., 2015; McIntosh et al., 2018). In contrast, *N. cinerea* have undergone continual decline; the species is listed as endangered on the IUCN Red list (Goldsworthy et al., 2015). The reasons underlying these differing rates of population recovery are likely complex and multi-factorial and include differing life histories, breeding strategies, geographical distribution, extent of fisheries interactions and disease (Goldsworthy et al., 2009; Shaughnessy et al., 2011; Marcus et al., 2015). The geographical range of these species frequently overlap, with some species breeding at the same sites. This geographical proximity provides an unparalleled opportunity for comparative investigations of *E. coli* to better understand the role of host and environmental factors on *E. coli* prevalence and diversity.

The objective of this study was to characterize the diversity of *E. coli* in three species of free-ranging pinniped pups in Southern Australia, establishing base line data for future comparative studies and to assess potential effects of anthropogenic impacts.

MATERIALS AND METHODS

Study Sites

Fecal swabs (n = 963) were collected from eight pinniped breeding colonies across multiple breeding seasons between 2016 and 2019 (**Figure 1** and **Table 1**). Of the 963 fecal swabs collected, a total of n = 401 fecal swabs were from *N. cinerea* pups, n = 400 from *A. p. doriferus* pups and n = 162 from *A. forsteri* pups. Samples collected from *N. cinerea* pups at Seal Bay in 2016 and Dangerous Reef 2017 were collected and analyzed as part of a previous study (Fulham et al., 2018). Samples were collected across two breeding seasons for all sites except *N. cinerea* at Dangerous Reef and *A. p. doriferus* at Deen Maar Island, Cape Bridgewater and The Skerries. The proximity to human settlements differs for each colony; Dangerous Reef and Olive Island are the most remote locations, located approximately 33 and 15 km offshore, respectively, while Seal Rocks and Deen Maar Island are located less than 10 km offshore (**Figure 1**).

Sample Collection

Pinniped pups, ranging in age from 3 to 6 weeks for *A. forsteri* and *A. p. doriferus* and 2 to 6 weeks for *N. cinerea,* were captured by hand as part of ongoing health investigations and restrained in canvas pup bags with breathing holes for the duration of capture and sample collection (Fulham et al., 2018). Pups were

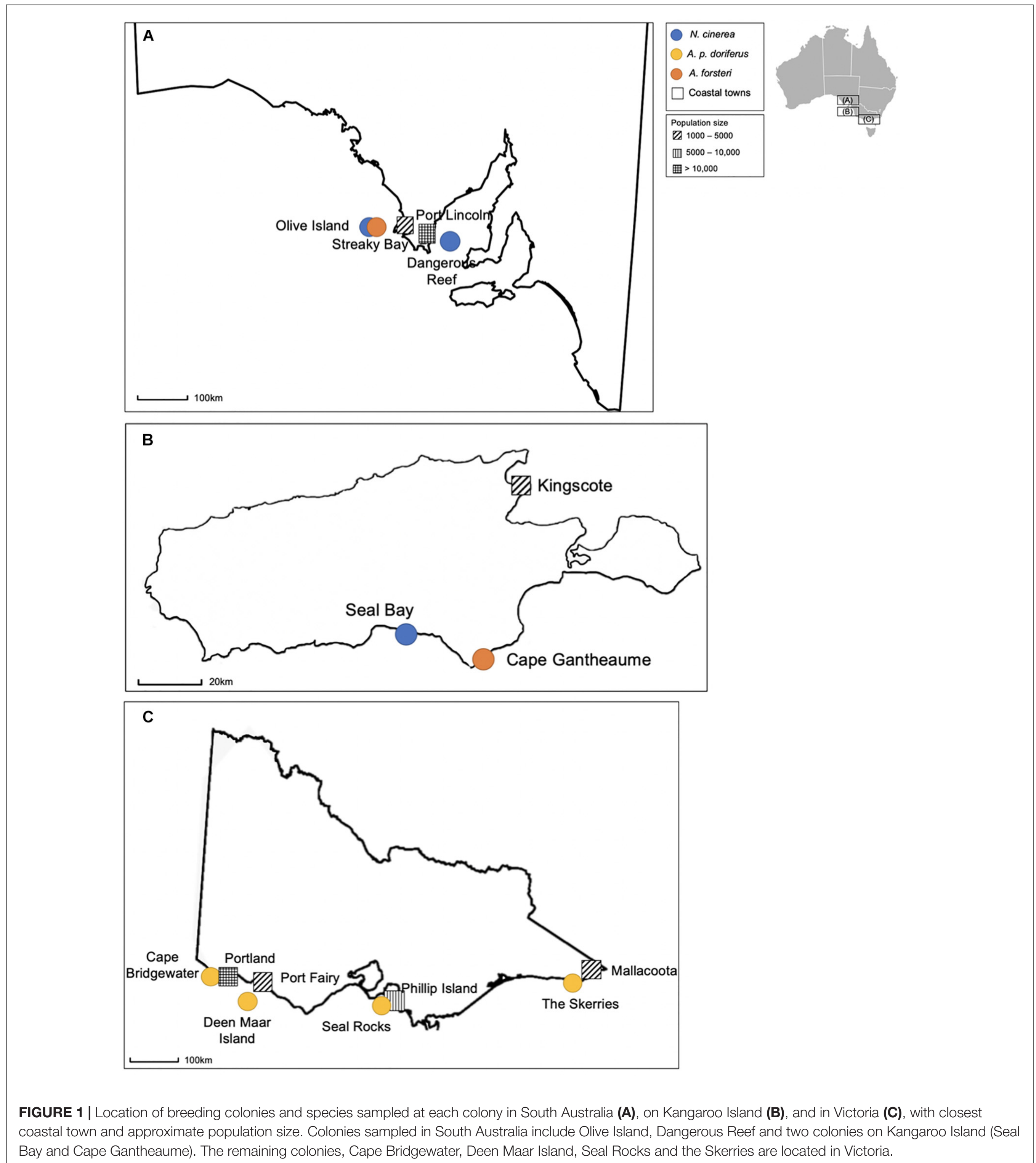

FIGURE 1 | Location of breeding colonies and species sampled at each colony in South Australia **(A)**, on Kangaroo Island **(B)**, and in Victoria **(C)**, with closest coastal town and approximate population size. Colonies sampled in South Australia include Olive Island, Dangerous Reef and two colonies on Kangaroo Island (Seal Bay and Cape Gantheaume). The remaining colonies, Cape Bridgewater, Deen Maar Island, Seal Rocks and the Skerries are located in Victoria.

sampled when mothers were absent. Fecal samples were collected by inserting a sterile swab (Copan, Brescia, Italy) covered by a lubricated sheath directly into the rectum or by swabbing a fecal sample passed by the pup during capture and restraint. Fecal swabs were then sub-sampled into Sterile FecalSwab™ (Copan, Brescia, Italy). All swabs were refrigerated at 4°C until culture, usually within 7 days of collection. All sampling methods for *N. cinerea* and *A. forsteri* were approved by the Animal Ethics Committee at the University of Sydney (Protocol Nos. 2014/726 and 2017/1260); sampling methods for *A. p. doriferus*

TABLE 1 | Geographical location for each breeding colony and total number of fecal samples collected each year at each breeding colony.

Sample site	State	Geographical coordinates	Species	Year collected	No. fecal samples collected
Seal Bay	SA	35.99°S, 137.32°E	*N. cinerea*	2016	48
				2018	72
				2019	63
Dangerous Reef	SA	34.82°S, 136.21°E	*N. cinerea*	2017	63
Olive Island	SA	32.43°S, 133.58°E	*N. cinerea*	2017	89
				2019	66
			A. forsteri	2019	12
Cape Gantheaume	SA	36.24°S, 137.27°E	*A. forsteri*	2016	69
				2018	81
Seal Rocks	VIC	38.31°S, 145.5°E	*A. p. doriferus*	2017	46
				2018	99
				2019	94
Deen Maar Island	VIC	38.24°S, 142.0°E	*A. p. doriferus*	2018	95
Cape Bridgewater	VIC	38.18°S, 141.24°E	*A. p. doriferus*	2018	43
The Skerries	VIC	37.45°S, 149.31°E	*A. p. doriferus*	2018	23

were approved by the Phillip Island Nature Park Animal Ethics Committee (Protocol No. 2.2016).

E. coli Culture, Isolation, and Preservation

Fecal swab media was inoculated onto Chromocult® coliform agar (Merck, Millipore, Australia) and cultured plates incubated at 37°C for 24 h. The *E. coli* colonies were identified by morphological features – being round, and dark blue-violet. Single colonies were selected and sub-cultured onto Chromocult® coliform agar plates to obtain pure cultures. To preserve cultures, pure *E. coli* isolates were grown in Luria Bertani (LB) broth (5 mL) at 37°C for 24 h and 500 μL of broth culture was combined with 500 μL 70% glycerol in 1.0 mL cryovials and samples were stored at −80°C.

Extraction of DNA From Preserved Bacteria

DNA was extracted using a boil preparation method. Preserved bacteria (40 μL) was inoculated into LB broth (150 μL) and incubated at 37°C for 24 h. Broth cultures were centrifuged for 5 min at 4000 rpm (Eppendorf 5810 R, rotor: A-4-62), the supernatant was removed and the bacterial pellet was re-suspended in molecular grade water (40 μL). Samples were then heated for 5 min at 95°C and centrifuged for 5 min at 4000 rpm (Eppendorf 5430 R, rotor: FA-45-24-11-HS). Lysates were stored in 1.5 mL microcentrifuge tubes at −30°C until PCR analysis.

Phylotyping of *E. coli* Isolates and Phylotype-Specific PCRs

To assign *E. coli* isolates to a phylotype, lysates were analyzed using a quadruplex PCR protocol following the methodology of Clermont et al. (2013). Isolates were assigned to a phylotype based on the presence or absence of four genes: *ChuA*, *yjaA*, *TspE4.C2,* and *arpA*. Isolates identified as A/C and D/E were further analyzed using two phylotype-specific PCRs. To assign isolates to phylotypes A or C, a singleplex PCR was conducted using the primers trpAgpC.1 and trpAgpC.2 with internal control primers trpBA.f and trpBA.r (Clermont et al., 2013). To assign isolates to phylotypes D or E, a singleplex PCR was conducted using primers ArpAgpE.f and ArpAgpE.r with internal control primers trpBA.f and trpBA.r (Clermont et al., 2013). All phylotyping PCRs were performed using GoTaq® Green Master Mix (Promega, Madison, United States) and included controls representing each phylotype (Power et al., 2016), and a negative control containing PCR water.

All reactions described above were resolved using gel electrophoresis (2% agarose w/v) conducted at 100 V for 30 min in TBE (Tris, boric acid, ethylenediaminetetraacetic acid) with SYBR safe gel stain (Invitrogen, Mulgrave, Australia). Product sizes were approximated against a HyperLadderII 50 bp DNA marker (Bioline, Sydney, Australia).

E. coli Phylotype B2 Sub-Typing

Isolates classified as B2 were further analyzed using two multiplex PCR panels to assign isolates to one of nine sub-groups using primers described by Clermont et al. (2014). Minor modifications were made to the PCR protocol by altering the annealing temperatures for both panels. Samples were assigned to sub-groups based on the presence of the following fragments: *putP*, *pabB*, *trpA*, *trpA*, *polB*, *dinB*, *icd*, *aes* (IX), and *aes* (X). The *chuA* gene was included as an internal control. Multiplex panel 1 tested for sub-groups II, III, VI, VII, and IX, and multiplex panel 2 tested for sub-groups I, VI, V, and X. The PCRs for panels 1 and 2 were performed using GoTaq® Green 2X (Promega, Madison, United States) with the addition of $MgCl_2$ to increase the concentration to 2.0 mM $MgCl_2$. The following PCR conditions were used: initial denaturation at 94°C for 4 min; 35 cycles at 94°C for 5 s, 59°C for 20 s (panel 1) or 58°C for 20 s (Panel 2), 72°C for 20 s, 72°C for 5 min and held at 4°C. Isolates were either assigned one of the nine sub-groups or considered unassigned. All reactions were resolved using gel electrophoresis using methods described in section "Phylotyping of *E. coli* Isolates and Phylotype-Specific PCRs".

TABLE 2 | *E. coli* isolates and prevalence of *E. coli* in pinniped pups at all eight colonies sampled for each breeding season.

Sample site	Species	Year collected	*E. coli* isolates (n fecal samples)	*E. coli* prevalence (%)	*E. coli* Phylotypes (n isolates assigned to phylotype)
Seal Bay	*N. cinerea*	2016	43 (48)	89.5[a]	B1 (16), B2 (24), D (3)
		2018	61 (72)	84.7[a]	B1 (17), B2 (37), D (1), F (4)
		2019	50 (63)	79.4	B1 (11), B2 (19), D (19), E (1)
Dangerous Reef	*N. cinerea*	2017	50 (63)	79.4	B1 (5), B2 (19), D (25), E (1)
Olive Island	*N. cinerea*	2017	81 (89)	91.0	B1 (2), B2 (66), D (1), F (12)
		2019	61 (66)	92.2	A (1), B1 (8), B2 (52), D (2), E (1)
	A. forsteri	2019	12 (12)	100	B2 (10), C (2)
Cape Gantheaume	*A. forsteri*	2016	52 (69)	75.3	B1 (9), B2 (36), D (1), F (6)
		2018	77 (81)	95.0	A 93), B1 (9), B2 (52), D (2), E (1), F (10)
Seal Rocks	*A. p. doriferus*	2017	41 (46)	89.1	A (1), B1 (9), B2 (27), D (1), F (3)
		2018	91 (99)	91.9	A (2), B1 (7), B2 (81), D (1)
		2019	76 (94)	80.8	A (1), B1 (4), B2 (67), D (3), F (1)
Deen Maar Island	*A. p. doriferus*	2018	86 (95)	90.5	A (2), B1 (1), B2 (75), D (6), F (2)
Cape Bridgewater	*A. p. doriferus*	2018	37 (43)	86.0	A (1), B1 (1), B2 (35)
The Skerries	*A. p. doriferus*	2018	23 (23)	100.0	B1 (2), B2 (21)

[a]*Previously published (Fulham et al., 2018).*

Statistical Analysis

RStudio (V 1.2.5042, Boston, MA, United States) software was utilized for all statistical analyses. A one-way ANOVA was used to compare *E. coli* prevalence across breeding seasons within each species. A generalized linear model was employed to analyze the relationship between the following factors: phylotype and sub-type prevalence, colony location, species, and breeding season. Normality of data was tested using the Shapiro-Wilk test. Factors were tested and included in the model based on AIC values. Statistical significance was determined when $p < 0.05$.

RESULTS

Prevalence of *E. coli* in Pinniped Pups

E. coli was detected in 842/963 (87.3%) fecal samples collected from *N. cinerea*, *A. p. doriferus,* and *A. forsteri* pups. The total prevalence of *E. coli* varied across pinniped species and sampling sites (**Table 2**). *E. coli* prevalence was highest in *A. p. doriferus* (88.7%), followed by *A. forsteri* (87.0%) and *N. cinerea* (86.2%). There was no significant difference in *E. coli* prevalence across sites ($p = 0.442$), species ($p = 0.564$) or breeding season ($p = 0.293$).

Distribution of *E. coli* Phylotypes in Pinniped Pups

Phylotyping of *E. coli* isolates showed only minor differences in distribution across the three species (**Figure 2**). There was no significant difference in *E. coli* phylotypes across species ($p = 0.055$), sampling sites ($p = 0.437$) or breeding season at colonies sampled over multiple breeding seasons; Seal Bay ($p = 0.272$), Olive Island ($p = 0.199$), Seal Rocks ($p = 0.880$), and Cape Gantheaume ($p = 0.076$) (**Figure 3**). The B2 phylotype was most frequently isolated from all samples with 73.7% of all *E. coli* isolates identified as B2, followed by B1, D, F, A, E, and C.

In *N. cinerea, A. forsteri,* and *A. p. doriferus* the most frequently identified phylotype was B2, with 62.5, 86.4, and 69.5% of isolates assigned to this phylotype, respectively (**Table 2** and **Figure 3**). There were no *E. coli* isolates from *A. p. doriferus* pups assigned to phylotype E. Isolates from *A. forsteri* pups were the most diverse with seven phylotypes identified and phylotype C was only found in *E. coli* isolates from *A. forsteri* pups at Olive Island.

Distribution of B2 Sub-Types in Pinniped Pups

Isolates from *N. cinerea*, *A. forsteri,* and *A. p. doriferus* pups that were assigned to the B2 phylotype were further analyzed and assigned to one of nine B2 sub-types. There was no significant difference in B2 sub-type distribution across sampling sites ($p = 0.768$) or species ($p = 0.121$) (**Figure 4**). At Seal Bay and Olive Island there was no significant difference in B2 sub-type distribution across breeding seasons ($p = 0.483$ and $p = 0.098$, respectively) (**Figure 5**). There was a significant difference in sub-type distribution across breeding seasons at Seal Rocks ($p = 0.046$) and Cape Gantheaume ($p < 0.001$). At Seal Rocks there was a decrease in the frequency of sub-types II, IV, VI, VII, IX, and X between seasons, while the frequency of sub-type III increased (**Figure 4**). The significant difference seen at Cape Gantheaume was due to an increase in sub-types II, V, VI, and IX and a decrease in the frequency of sub-types III, IV, and VII.

Sub-type distribution differed slightly between species with sub-type I only detected in a single *N. cinerea* pup sampled at Olive Island; sub-type X was only detected in one *A. p. doriferus* pup sampled at Seal Rocks. The most frequently isolated sub-type across all species was VI, followed by III, V, II, VII, IV, IX, I, and X (**Figure 4**). A total of 23.6% of B2 isolates could not be assigned (UA) to a sub-type.

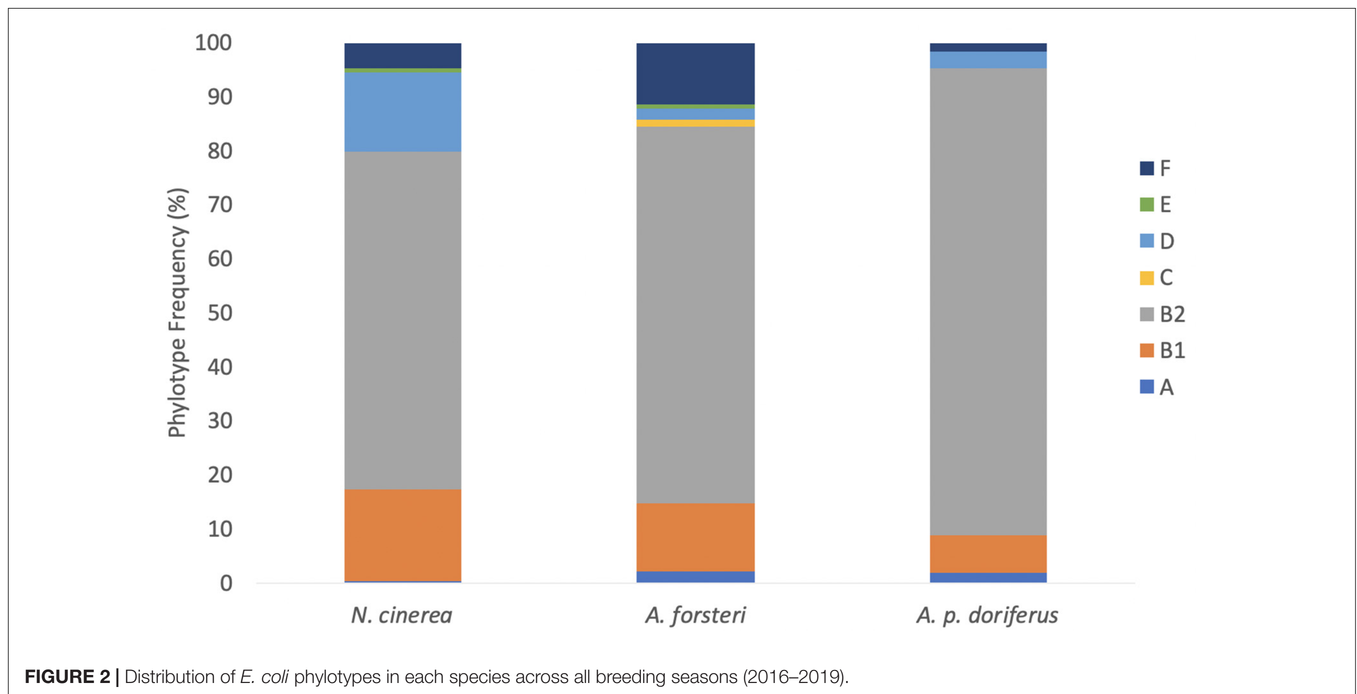

FIGURE 2 | Distribution of *E. coli* phylotypes in each species across all breeding seasons (2016–2019).

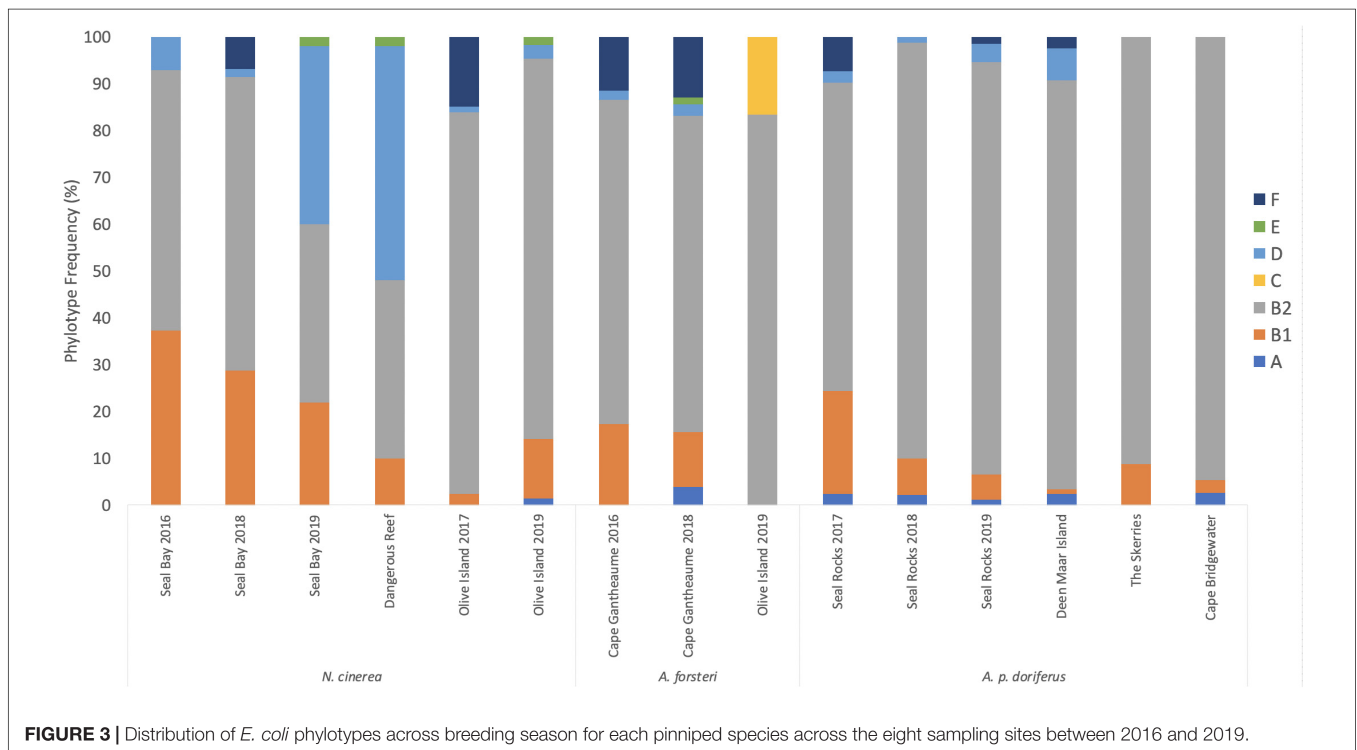

FIGURE 3 | Distribution of *E. coli* phylotypes across breeding season for each pinniped species across the eight sampling sites between 2016 and 2019.

DISCUSSION

This study reports the prevalence and diversity of *E. coli* isolated from free-ranging *N. cinerea*, *A. p. doriferus,* and *A. forsteri* pups at eight breeding colonies in Australia, finding no significant difference in *E. coli* prevalence or the distribution of phylotypes across species, colonies or breeding seasons.

This is the first investigation of the comparative presence and distribution of *E. coli* in free-ranging *A. p. doriferus* and *A. forsteri* pups. The prevalence of *E. coli* was similar across all three species and supports previous reports in Antarctic pinnipeds including Antarctic fur seals, Southern elephant seals and Weddell seals (Power et al., 2016; Mora et al., 2018), and captive adult *N. cinerea* (Delport et al., 2015).

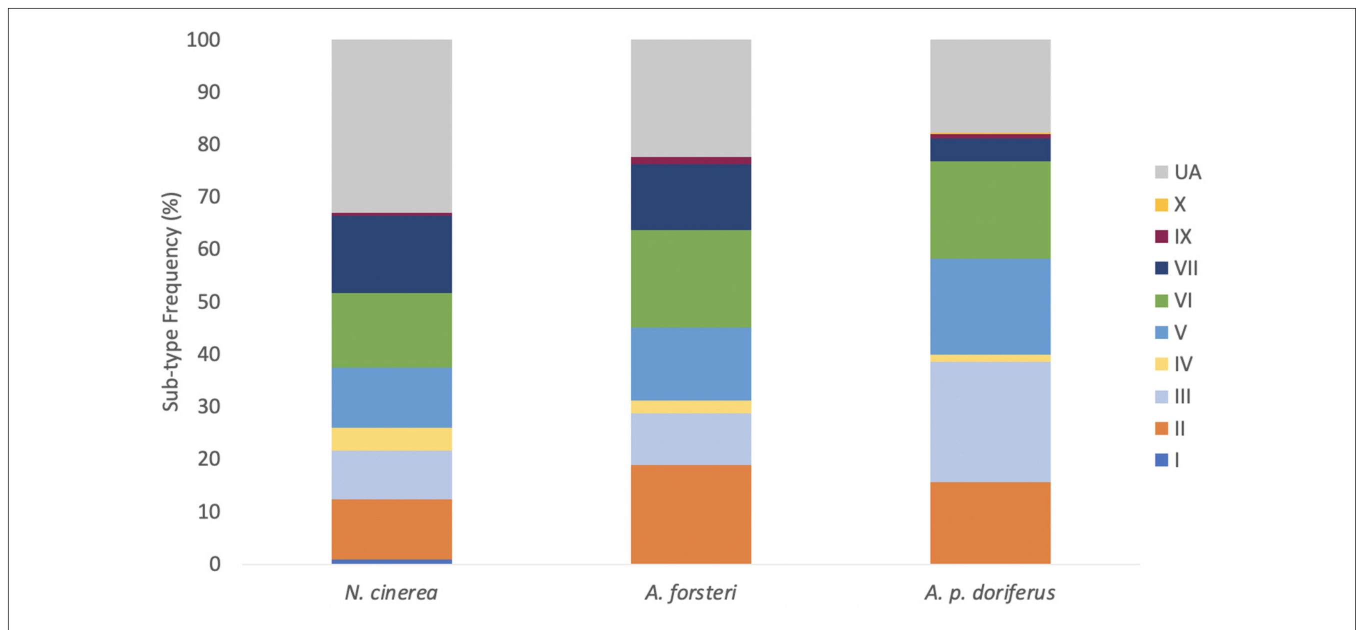

FIGURE 4 | Combined distribution of *E. coli* B2 sub-types for each species across breeding seasons sampled (2016–2019). Samples that could not be assigned to a sub-type were considered "unassigned" (UA). All nine sub-groups were identified in at least one of the three species.

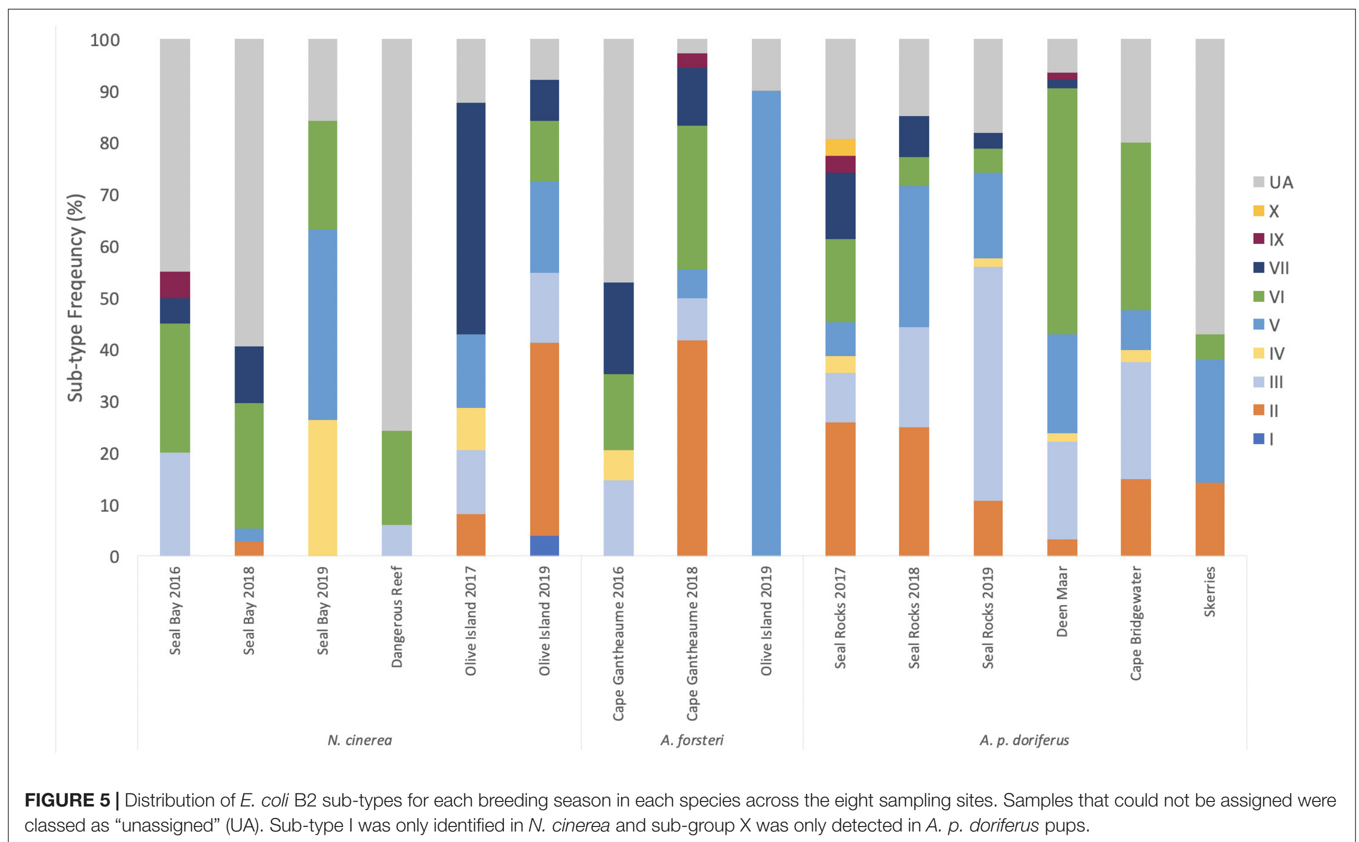

FIGURE 5 | Distribution of *E. coli* B2 sub-types for each breeding season in each species across the eight sampling sites. Samples that could not be assigned were classed as "unassigned" (UA). Sub-type I was only identified in *N. cinerea* and sub-group X was only detected in *A. p. doriferus* pups.

Previous studies have suggested that *E. coli* is uncommon in marine mammals (Johnson et al., 1998; Hernandez et al., 2007) with a higher prevalence associated with proximity to humans (Stoddard et al., 2008; Delport et al., 2015). In this study the differences in proximity to humans at each breeding colony and the high prevalence of *E. coli* observed in *N. cinerea*, *A. p. doriferus,* and *A. forsteri* pups could potentially indicate that proximity to humans

is not the only factor contributing to *E. coli* prevalence in marine mammals.

The B2 phylotype was most frequently identified from pups of all three species, with 73.7% of all *E. coli* isolates assigned to this phylotype. This is the highest frequency of the B2 phylotype identified in pinnipeds in Australia; in free-ranging *N. cinerea*, B2 made up 67% of all isolates in adults (Delport et al., 2015). It has been suggested that strains belonging to the B2 phylotype are well adapted to the intestinal environment of mammals (Gordon and Cowling, 2003; Nowrouzian et al., 2006), which could be a contributing factor to the high frequency identified in this study. The B2 phylotype was the most frequently isolated phylotype at seven of the eight breeding colonies during every breeding season sampled. It was previously hypothesized that the difference in phylotype distribution observed in free-ranging *N. cinerea* pups was due to colony location (Fulham et al., 2018), however, the results from this study indicate that geographical location and proximity to humans, is not a contributing factor to *E. coli* diversity in *N. cinerea*, *A. p. doriferus,* or *A. forsteri* pups.

The prevalence of *E. coli* in free-ranging pinniped pups is likely diet related; *E. coli* is a lactose fermenter and as the sampled pups were feeding solely on milk, high *E. coli* prevalence is not unexpected (Fulham et al., 2018). In adult *N. cinerea*, *E. coli* prevalence from free-ranging individuals was significantly lower (7.7%) compared to captive individuals (84%) suggesting that *E. coli* does not occur naturally in free-ranging adult *N. cinerea* (Delport et al., 2015). Marine wildlife species that forage in coastal or nearshore waters are at greater risk of exposure to pathogens in fecal bacteria as a result of anthropogenic pollution compared to species further removed from anthropogenic influence (Oates et al., 2012). In Antarctic pinnipeds, it was hypothesized that the presence of human-associated *E. coli* could be due to the geographic mobility of the animals sampled (Mora et al., 2018). Free-ranging *N. cinerea*, *A. p. doriferus,* and *A. forsteri* adults occupy large geographical ranges (Shaughnessy et al., 2011, 2015; McIntosh et al., 2018), encountering a variety of environments and environmental conditions that could influence their exposure to anthropogenic pollution. In other pinniped species, there is evidence of maternal transmission of gut microbes (Nelson et al., 2013b), however, it is unknown whether the acquisition of *E. coli* in pinniped pups in this study is environmental or through maternal transfer. Understanding the prevalence of *E. coli* and phylotype diversity in free-ranging adults could provide valuable insights into the factors that contribute to the acquisition of *E. coli* and the trends in *E. coli* prevalence and phylotype distribution seen in pups.

Studies investigating the transfer of *E. coli* between humans and wildlife species are limited to terrestrial environments. Generally, *E. coli* isolated from wildlife species that share habitats and have higher levels of interactions with humans and livestock were genetically similar to *E. coli* isolated from humans (Goldberg et al., 2007; Rwego et al., 2008a,b). This genetic similarity suggests *E. coli* transmission between these species in shared habitats is likely a result of indirect contact through contaminated environmental sources rather than direct contact (Goldberg et al., 2007). For example, *E. coli* isolated from the feces of banded mongoose (*Mungos mungo*) was genetically similar to *E. coli* isolated from human fecal waste in their environment, highlighting the importance of indirect routes of transmission (Pesapane et al., 2013). These results suggest that the acquisition of human-associated bacteria by wildlife species is linked to fecal contamination of the environment.

Coastal environments can be contaminated by fecal pollution through a number of different sources including sewage and storm water runoff from agricultural, urban and commercial land (Crain et al., 2009; Pandey et al., 2014). The population size and density of people in coastal towns will influence the amount of bacterial contamination of the environment from runoff, with higher density resulting in higher levels of fecal bacteria (Pandey et al., 2014). The population size of towns closest to the pinniped breeding colonies sampled in this study varied, however, the prevalence of *E. coli* did not differ across colonies or seasons, suggesting that the contribution of human population density to trends in *E. coli* prevalence observed requires further investigation. Previous studies have determined that *E. coli* isolates from wildlife species occupying habitats in close association with humans or that are exposed to fecal pollution are more likely to belong to phylotype B2 than those isolated from wildlife living in isolation from humans (Gordon and Cowling, 2003). *E. coli* isolates belonging to B2 and D phylotypes have been found in treated sewage, suggesting a greater ability to survive treatment processes (Anastasi et al., 2010). The survival of these phylotypes in wastewater coupled with increasing pollution of coastal ecosystems presents a higher risk of transfer of human-associated bacteria into the marine environment. The predominance of the B2 phylotype across the three pinniped species studied could therefore suggest that all species are exposed to similar levels of anthropogenic pollution. This could be important for pup health, given that strains belonging to this phylotype are associated with extraintestinal disease in humans (Dale and Woodford, 2015).

Ocean currents and tides are also potential factors contributing to the high prevalence of *E. coli* in pinnipeds. The main source of ocean pollution is of terrestrial origin (Robinson et al., 2017) and as a consequence, coastal areas in close proximity to populated areas are exposed to higher levels of anthropogenic pollution (Partelow et al., 2015). The marine environment, being connected over longer timescales (Jönsson and Watson, 2016) has greater connectivity compared to terrestrial environments, with fewer physical barriers between areas. Consequently, the pathways of water that flow along coastlines can influence the dispersal of anthropogenic pollutants. Wastewater effluent is a known source of human-associated *E. coli* phylotypes (de Stoppe et al., 2017) and *E. coli* that originate from wastewater have an enhanced capacity to survive in the marine environment, able to survive for several days outside of a host (Rozen and Belkin, 2001). In addition, *E. coli* can attach to particles in the water column, facilitating movement in the marine environment (Mallin et al., 2000). This enhanced survival and attachment to particles in the water column could result in prolonged environmental persistence of

E. coli, facilitating acquisition by marine wildlife. The similarity in *E. coli* distribution across species and breeding colonies in this study could be indicative of the high degree of connectivity of marine ecosystems (Crain et al., 2009; Robinson et al., 2017) or similar anthropogenic pollution across this large geographical range.

The identification of B2 sub-types I, II and IX are of particular interest given that clonal complexes that belong to these sub-types are commonly associated with disease in avian species and humans (Clermont et al., 2014; Riley, 2014). ST131 and ST95 strains belonging to sub-types I and IX, respectively, are associated with disease caused by extraintestinal pathogenic *E. coli* (ExPEC) (Dale and Woodford, 2015). These sub-types or strains have previously been identified in Antarctic pinnipeds (Power et al., 2016; Mora et al., 2018). Assignment of *E. coli* isolates to strains was not undertaken in this study, however, given the presence of B2 sub-types associated with pathogenic strains, further analysis of B2 isolates will assist our understanding of the potential risks posed to pup health by their presence.

It is also important to consider that *E. coli* is a very small component of the intestinal microbiota in pinnipeds (Nelson et al., 2013a,b; Delport et al., 2016). However, the ease with which *E. coli* can be cultured and characterized has resulted in *E. coli* being commonly used for monitoring fecal contamination of marine environments (Beversdorf et al., 2007). For this reason, it can be a useful indicator of the diversity of *E. coli* phylotypes present in marine mammals, and a relatively high prevalence may suggest greater exposure to anthropogenic pollution. Sampling of substrate and water surrounding breeding colonies and comparing those *E. coli* phylotypes with diversity seen in pinniped pups could provide useful insights into potential sources of environmental contamination. Investigating the presence of specific markers through microbial source tracking could also be utilized to determine the origin of *E. coli* found in both wildlife species and contaminated environments.

The presence of bacteria and protozoa that are associated with humans should be explored to further understand potential pathogen transmission from anthropogenic sources into the marine environment. *Toxoplasma gondii*, *Campylobacter* spp. and *Salmonella* spp. have been isolated from marine mammals inhabiting coastal environments, including gray seals (*Halichoerus grypus*), Californian sea otters (*Enhydra lutris nereis*) and northern elephant seals (Stoddard et al., 2005; Oates et al., 2012; Shapiro et al., 2012; Baily et al., 2015). *Campylobacter* spp. isolated from *H. grypus* were genetically similar to isolates commonly found in agricultural and human sources, demonstrating the dissemination of a human pathogen into the marine environment (Baily et al., 2015). Similar investigations in Australian pinniped species could enable the identification of the source and dissemination of anthropogenic microbial pollution into the Australian marine environment.

In this study, the predominance of the human associated B2 phylotype and similarity of *E. coli* prevalence and phylotype diversity seen across species, colonies and breeding seasons could indicate that all colonies are exposed to similar levels of anthropogenic pollution. This widespread occurrence of human associated phylotypes highlights the need for ongoing monitoring and surveillance of microbes in both the marine environment and sentinel species, particularly those with potential pathogenicity for marine mammals. Future investigations should focus on whether *E. coli* is an atypical bacterium in these pinniped species and determine its reliability as an indicator of marine pollution.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Ethics Committee at the University of Sydney and Phillip Island Nature Parks.

AUTHOR CONTRIBUTIONS

MF, RG, and MP contributed to conceptualization and design of the study. MF and RG collected samples from pinniped pups. MF completed laboratory analysis of samples and data analysis. MF drafted the manuscript. RG and MP participated in revising the manuscript. All authors contributed to the article and approved the submitted version.

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REFERENCES

Ahmed, W., Sidhu, J. P. S., Smith, K., Beale, D. J., Gyawali, P., and Toze, S. (2016). Distributions of fecal markers in wastewater from different climatic zones for human fecal pollution tracking in Australian surface waters. *Appl. Environ. Microbiol.* 82, 1316–1323. doi: 10.1128/AEM.03765-15

Anastasi, E. M., Matthews, B., Gundogdu, A., Vollmerhausen, T. L., Ramos, N. L., Stratton, H., et al. (2010). Prevalence and persistence of *Escherichia coli* strains with uropathogenic virulence characteristics in sewage treatment plants. *Appl. Environ. Microbiol.* 76, 5882–5886. doi: 10.1128/AEM.00141-10

Baily, J. L., Méric, G., Bayliss, S., Foster, G., Moss, S. E., Watson, E., et al. (2015). Evidence of land-sea transfer of the zoonotic pathogen *Campylobacter* to a wildlife marine sentinel species. *Mol. Ecol.* 24, 208–221. doi: 10.1111/mec.13001

Beversdorf, L. J., Bornstein-Forst, S. M., and McLellan, S. L. (2007). The potential for beach sand to serve as a reservoir for *Escherichia coli* and the physical influences on cell die-off. *J. Appl. Microbiol.* 102, 1372–1381. doi: 10.1111/j.1365-2672.2006.03177.x

Bossart, G. D. (2010). Marine mammals as sentinel species for oceans and human health. *Vet. Pathol.* 48, 676–690. doi: 10.1177/0300985810388525

Clermont, O., Christenson, J. K., Daubié, A.-S., Gordon, D. M., and Denamur, E. (2014). Development of an allele-specific PCR for *Escherichia coli* B2 subtyping, a rapid and easy to perform substitute of multilocus sequence typing. *J. Microbiol. Methods* 101, 24–27. doi: 10.1016/j.mimet.2014.03.008

Clermont, O., Christenson, J. K., Denamur, E., and Gordon, D. M. (2013). The Clermont *Escherichia coli* phylo-typing method revisited: improvement of specificity and detection of new phylo-groups. *Environ. Microbiol. Rep.* 5, 58–65. doi: 10.1111/1758-2229.12019

Clermont, O., Olier, M., Hoede, C., Diancourt, L., Brisse, S., Keroudean, M., et al. (2011). Animal and human pathogenic *Escherichia coli* strains share common genetic backgrounds. *Infect. Genet. Evol.* 11, 654–662. doi: 10.1016/j.meegid.2011.02.005

Crain, C. M., Halpern, B. S., Beck, M. W., and Kappel, C. V. (2009). Understanding and managing human threats to the coastal marine environment. *Ann. N. Y. Acad. Sci.* 1162, 39–62. doi: 10.1111/j.1749-6632.2009.04496.x

Dale, A. P., and Woodford, N. (2015). Extra-intestinal pathogenic *Escherichia coli* (ExPEC): disease, carriage and clones. *J. Infect.* 71, 615–626. doi: 10.1016/j.jinf.2015.09.009

de Stoppe, N. C., Silva, J. S., Carlos, C., Sato, M. I. Z., Saraiva, A. M., Ottoboni, L. M. M., et al. (2017). Worldwide phylogenetic group patterns of *Escherichia coli* from commensal human and wastewater treatment plant isolates. *Front. Microbiol.* 8:2512. doi: 10.3389/fmicb.2017.02512

Delport, T. C., Harcourt, R. G., Beaumont, L. J., Webster, K. N., and Power, M. L. (2015). Molecular detection of antibiotic-resistance determinants in *Escherichia coli* isolated from the endangered Australian sea lion (*Neophoca cinerea*). *J. Wildl. Dis.* 51, 555–563. doi: 10.7589/2014-08-200

Delport, T. C., Power, M. L., Harcourt, R. G., Webster, K. N., and Tetu, S. G. (2016). Colony location and captivity influence the gut microbial community composition of the Australian sea lion (*Neophoca cinerea*). *Appl. Environ. Microbiol.* 82, 3440–3449. doi: 10.1128/AEM.00192-16

Fulham, M., Power, M., and Gray, R. (2018). Comparative ecology of *Escherichia coli* in endangered Australian sea lion (*Neophoca cinerea*) pups. *Infect. Genet. Evol.* 62, 262–269. doi: 10.1016/j.meegid.2018.05.002

Goldberg, T. L., Gillespie, T. R., Rwego, I. B., Wheeler, E., Estoff, E. L., and Chapman, C. A. (2007). Patterns of gastrointestinal bacterial exchange between chimpanzees and humans involved in research and tourism in western Uganda. *Biol. Conserv.* 135, 511–517. doi: 10.1016/j.biocon.2006.10.048

Goldsworthy, S. D., Gales, N. J., and Chilvers, B. L. (2015). *Neophoca Cinerea. IUCN Red List Threat. Species 2015 e.T14549A45228341*. Gland: IUCN.

Goldsworthy, S. D., McKenzie, J., Shaughnessy, P. D., McIntosh, R. R., Page, B., and Campbell, R. (2009). *An Update of the Report: Understanding the Impediments to the Growth of Autralian Sea Lion Populations*. SARDI Publ. Number F2008/00847-1 SARDI Reearch Report Series. No. 297. Urrbrae, SA: South Australian Research and Development Institute

Gordon, D. M., Clermont, O., Tolley, H., and Denamur, E. (2008). Assigning *Escherichia coli* strains to phylogenetic groups: multi-locus sequence typing versus the PCR triplex method. *Environ. Microbiol.* 10, 2484–2496. doi: 10.1111/j.1462-2920.2008.01669.x

Gordon, D. M., and Cowling, A. (2003). The distribution and genetic structure of *Escherichia coli* in Australian vertebrates: host and geographic effects. *Microbiology* 149, 3575–3586. doi: 10.1099/mic.0.26486-0

Guenther, S., Ewers, C., and Wieler, L. H. (2011). Extended-spectrum Beta-Lactamases producing E. coli in wildlife, yet another form of environmental pollution? *Front. Microbiol.* 2:246. doi: 10.3389/fmicb.2011.00246

Hernandez, J., Prado, V., Torres, D., Waldenström, J., Haemig, P. D., and Olsen, B. (2007). Enteropathogenic *Escherichia coli* (EPEC) in Antarctic fur seals *Arctocephalus gazella*. *Polar Biol.* 30, 1227–1229. doi: 10.1007/s00300-007-0282-2

Johnson, J. R., and Russo, T. A. (2002). Extraintestinal pathogenic *Escherichia coli*: "The other bad E coli.". *J. Lab. Clin. Med.* 139, 155–162. doi: 10.1067/mlc.2002.121550

Johnson, S. P., Nolan, S., and Gulland, F. M. (1998). Antimicrobial susceptibility of bacteria isolated from pinnipeds stranded in central and northern California. *J. Zoo Wildl. Med.* 29, 288–294.

Jönsson, B. F., and Watson, J. R. (2016). The timescales of global surface-ocean connectivity. *Nat. Commun.* 7:11239. doi: 10.1038/ncomms11239

Kirkwood, R., and Goldsworthy, S. (2013). *Fur Seals and Sea Lions*. Collingwood, VIC: CSIRO PUBLISHING.

Leser, T. D., and Mølbak, L. (2009). Better living through microbial action: the benefits of the mammalian gastrointestinal microbiota on the host. *Environ. Microbiol.* 11, 2194–2206. doi: 10.1111/j.1462-2920.2009.01941.x

Ling, J. K. (1999). Exploitation of fur seals and sea lions from Australian, New Zealand and adjacent subantarctic islands during the eighteenth, nineteenth and twentieth centuries. *Aust. Zool.* 31, 323–350. doi: 10.7882/AZ.1999.036

Mallin, M. A., Williams, K. E., Esham, E. C., and Lowe, R. P. (2000). Effect of human development on bacteriological water quality in coastal watersheds. *Ecol. Appl.* 10, 1047–1056. doi: 10.1890/1051-07612000010

Marcus, A. D., Higgins, D. P., and Gray, R. (2015). Health assessment of free-ranging endangered Australian sea lion (*Neophoca cinerea*) pups: Effect of haematophagous parasites on haematological parameters. *Comp. Biochem. Physiol. Part A Mol. Integr. Physiol.* 184, 132–143. doi: 10.1016/j.cbpa.2015.02.017

McIntosh, R. R., Kirkman, S. P., Thalmann, S., Sutherland, D. R., Mitchell, A., Arnould, J. P. Y., et al. (2018). Understanding meta-population trends of the Australian fur seal, with insights for adaptive monitoring. *PLoS One* 13:e0200253. doi: 10.1371/journal.pone.0200253

Melendez, D., Roberts, M. C., Greninger, A. L., Weissman, S., No, D., Rabinowitz, P., et al. (2019). Whole-genome analysis of extraintestinal pathogenic *Escherichia coli* (ExPEC) MDR ST73 and ST127 isolated from endangered southern resident killer whales (*Orcinus orca*). *J. Antimicrob. Chemother.* 74:2176. doi: 10.1093/jac/dkz159

Mora, A., García-Peña, F., Alonso, M., Pedraza-Diaz, S., Ortega-Mora, L., Garcia-Parraga, D., et al. (2018). Impact of human-associated *Escherichia coli* clonal groups in Antarctic pinnipeds: presence of ST73, ST95, ST141 and ST131. *Sci. Rep.* 8:4678. doi: 10.1038/s41598-018-22943-0

Mora, A., López, C., Dabhi, G., Blanco, M., Blanco, J. E., Alonso, M. P., et al. (2009). Extraintestinal pathogenic *Escherichia coli* O1:K1:H7/NM from human and avian origin: detection of clonal groups B2 ST95 and D ST59 with different host distribution. *BMC Microbiol.* 9:132. doi: 10.1186/1471-2180-9-132

Nelson, T. M., Rogers, T. L., and Brown, M. V. (2013a). The gut bacterial community of mammals from marine and terrestrial habitats. *PLoS One* 8:e83655. doi: 10.1371/journal.pone.0083655

Nelson, T. M., Rogers, T. L., Carlini, A. R., and Brown, M. V. (2013b). Diet and phylogeny shape the gut microbiota of Antarctic seals: a comparison of wild and captive animals. *Environ. Microbiol.* 15, 1132–1145. doi: 10.1111/1462-2920.12022

Nowrouzian, F. L., Adlerberth, I., and Wold, A. E. (2006). Enhanced persistence in the colonic microbiota of *Escherichia coli* strains belonging to phylogenetic group B2: role of virulence factors and adherence to colonic cells. *Microbes Infect.* 8, 834–840. doi: 10.1016/j.micinf.2005.10.011

Oates, S. C., Miller, M. A., Byrne, B. A., Chouicha, N., Hardin, D., Jessup, D., et al. (2012). Epidemiology and potential land-sea transfer of enteric bacteria from terrestrial to marine species in the Monterey Bay region of California. *J. Wildl. Dis.* 48, 654–668. doi: 10.7589/0090-3558-48.3.654

Oliveira, M., Freire, D., and Pedroso, N. M. (2018). *Escherichia coli* is not a suitable fecal indicator to assess water fecal contamination by otters. *Braz. J. Biol.* 78, 155–159. doi: 10.1590/1519-6984.167279

Pandey, P. K., Kass, P. H., Soupir, M. L., Biswas, S., and Singh, V. P. (2014). Contamination of water resources by pathogenic bacteria. *AMB Express* 4:51. doi: 10.1186/s13568-014-0051-x

Partelow, S., von Wehrden, H., and Horn, O. (2015). Pollution exposure on marine protected areas: A global assessment. *Mar. Pollut. Bull.* 100, 352–358. doi: 10.1016/j.marpolbul.2015.08.026

Pesapane, R., Ponder, M., and Alexander, K. A. (2013). Tracking pathogen transmission at the human–wildlife interface: banded mongoose and *Escherichia coli*. *Ecohealth* 10, 115–128. doi: 10.1007/s10393-013-0838-2

Power, M. L., Samuel, A., Smith, J. J., Stark, J. S., Gillings, M. R., and Gordon, D. M. (2016). *Escherichia coli* out in the cold: dissemination of human-derived bacteria into the Antarctic microbiome. *Environ. Pollut.* 215, 58–65. doi: 10.1016/j.envpol.2016.04.013

Riley, L. W. (2014). Pandemic lineages of extraintestinal pathogenic *Escherichia coli*. *Clin. Microbiol. Infect* 20, 380–390. doi: 10.1111/1469-0691.12646

Robinson, J., New, A. L., Popova, E. E., Srokosz, M. A., and Yool, A. (2017). Far-field connectivity of the UK's four largest marine protected areas: Four of a kind? *Earth's Futur.* 5, 475–494. doi: 10.1002/2016EF000516

Rozen, Y., and Belkin, S. (2001). Survival of enteric bacteria in seawater. *FEMS Microbiol. Rev.* 25, 513–529. doi: 10.1111/j.1574-6976.2001.tb00589.x

Russo, T. A., and Johnson, J. R. (2000). Proposal for a new inclusive designation for extraintestinal pathogenic isolates of *Escherichia coli*: ExPEC. *J. Infect. Dis.* 181, 1753–1754. doi: 10.1086/315418

Rwego, I. B., Gillespie, T. R., Isabirye-Basuta, G., and Goldberg, T. L. (2008a). High rates of *Escherichia coli* transmission between livestock and humans in rural Uganda. *J. Clin. Microbiol.* 46, 3187–3191. doi: 10.1128/JCM.00285-08

Rwego, I. B., Isabirye-Basuta, G., Gillespie, T. R., and Goldberg, T. L. (2008b). Gastrointestinal bacterial transmission among humans, mountain gorillas, and livestock in Bwindi Impenetrable National Park. *Uganda. Conserv. Biol.* 22, 1600–1607. doi: 10.1111/j.1523-1739.2008.01018.x

Schaefer, A. M., Bossart, G. D., Mazzoil, M., Fair, P. A., and Reif, J. S. (2011). Risk factors for colonization of E. coli in atlantic bottlenose dolphins (*Tursiops truncatus*) in the Indian River Lagoon. *Florida. J. Environ. Public Health* 2011:597073. doi: 10.1155/2011/597073

Schierack, P., Romer, A., Jores, J., Kaspar, H., Guenther, S., Filter, M., et al. (2009). Isolation and characterization of intestinal *Escherichia coli* clones from wild boars in Germany. *Appl. Environ. Microbiol.* 75:695. doi: 10.1128/AEM.01650-08

Schierack, P., Walk, N., Ewers, C., Wilking, H., Steinrück, H., Filter, M., et al. (2008). ExPEC-typical virulence-associated genes correlate with successful colonization by intestinal *E.coli* in a small piglet group. *Environ. Microbiol.* 10, 1742–1751. doi: 10.1111/j.1462-2920.2008.01595.x

Shapiro, K., Miller, M., and Mazet, J. (2012). Temporal association between land-based runoff events and California sea otter (*Enhydra lutris* nereis) protozoal mortalities. *J. Wildl. Dis.* 48, 393–404. doi: 10.7589/0090-3558-48.2.394

Shaughnessy, P. D., Goldsworthy, S. D., Hamer, D. J., Page, B., and McIntosh, R. R. (2011). Australian sea lions *Neophoca cinerea* at colonies in South Australia: Distribution and abundance, 2004 to 2008. *Endanger. Species Res.* 13, 87–98. doi: 10.3354/esr00317

Shaughnessy, P. D., Goldsworthy, S. D., and Mackay, A. I. (2015). The long-nosed fur seal (Arctocephalus forsteri) in South Australia in 2013–14: abundance, status and trends. *Aust. J. Zool.* 63:101. doi: 10.1071/ZO14103

Stoddard, R. A., Atwill, E. R., Conrad, P. A., Byrne, B. A., Jang, S., Lawrence, J., et al. (2009). The effect of rehabilitation of northern elephant seals (*Mirounga angustirostris*) on antimicrobial resistance of commensal *Escherichia coli*. *Vet. Microbiol.* 133, 264–271. doi: 10.1016/j.vetmic.2008.07.022

Stoddard, R. A., Atwill, E. R., Gulland, F. M. D., Miller, M. A., Dabritz, H. A., Paradies, D. M., et al. (2008). Risk factors for infection with pathogenic and antimicrobial-resistant fecal bacteria in northern elephant seals in California. *Public Health Rep.* 123, 360–370. doi: 10.1177/003335490812300316

Stoddard, R. A., Gulland, M. D. F., Atwill, E. R., Lawrence, J., Jang, S., and Conrad, P. A. (2005). Salmonella and Campylobacter spp. in northern elephant seals. *California. Emerg. Infect. Dis.* 11, 1967–1969. doi: 10.3201/eid1112.050752

Wallace, C., Yund, P., Ford, T., Matassa, K., and Bass, A. (2013). Increase in antimicrobial resistance in bacteria isolated from stranded marine mammals of the northwest Atlantic. *Ecohealth* 10, 201–210. doi: 10.1007/s10393-013-0842-6

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Chapter 4.

Implications of *Escherichia coli* community diversity in free-ranging Australian pinniped pups

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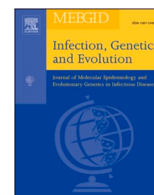

Implications of *Escherichia coli* community diversity in free-ranging Australian pinniped pups

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A B S T R A C T

Escherichia coli is a widely studied bacterium, commonly used as an indicator of faecal contamination. Investigations into the structure and diversity of *E. coli* in free-ranging wildlife species has been limited. The objective of this study was to characterise intra-individual and inter-species *E. coli* phylotype and B2 sub-type diversity in free-ranging Australian pinniped pups, to determine whether a single *E. coli* colony is representative of the phylotype and B2 sub-type diversity in these hosts. Faecal samples were collected from free-ranging Australian fur seal (*Arctocephalus pusillus doriferus*), Australian sea lion (*Neophoca cinerea*) and long-nosed fur seal (*Arctocephalus forsteri*) pups from three breeding colonies between 2018 and 2021. Faecal swabs from thirty randomly selected pups ($n = 10$ from each species) were cultured and ten *E. coli* colonies were selected from each culture based on morphology and separation between colonies on agar plates. Molecular screening techniques were utilised to assign isolates to phylotypes and B2 sub-types. There was no significant difference ($p > 0.05$) in either intra-individual or inter-species *E. coli* phylotype and B2 sub-type diversity. The B2 phylotype was the most dominant, with 78% of isolates ($n = 234$) assigned to this phylotype. Host factors (species, weight [kg] and standard length [cm]) did not significantly affect phylotype diversity.

The absence of intra-individual and inter-species differences in *E. coli* diversity at a phylotype level suggests that a single *E. coli* colony could be used as an indicator of overall diversity of *E. coli* at a phylotype level in *A. p. doriferus*, *N. cinerea* and *A. forsteri* pups. These findings can be used to simplify and improve the efficiency of sampling protocols for ongoing monitoring of human-associated *E. coli* phylotypes in free-ranging pinniped populations.

1. Introduction

Escherichia coli is a Gram-negative bacterium that is present universally in the intestinal tract of many vertebrate species that occupy a wide range of habitats (Gordon and Cowling, 2003). While *E. coli* commonly exists as a commensal in the intestinal tract of vertebrates (Tenaillon et al., 2010), the acquisition of virulence factors by various strains of *E. coli* has resulted in disease in humans and many other animal species (Croxen and Finlay, 2010; Kaper et al., 2004; Picard et al., 1999; Reid et al., 2000). Pathogenic strains of *E. coli* are primarily subdivided into intestinal pathogenic *E. coli* (InPEC) and extraintestinal pathogenic *E. coli* (ExPEC) (Denamur et al., 2021; Kaper et al., 2004). InPEC *E. coli* are one of the primary causes of diarrheal disease worldwide (Croxen and Finlay, 2010; Kaper et al., 2004), while ExPEC strains of *E. coli* are frequently implicated as the cause of urinary tract infections (uropathogenic *E. coli* [UPEC]) and meningitis in newborns (newborn meningitis *E. coli* [NMEC]), or avian pathogenic colibacillosis (APEC) (Denamur et al., 2021; Mora et al., 2013).

In-depth genetic analysis of *E. coli* multi-locus sequence typing (MLST) and whole genome sequencing (WGS) (Fratamico et al., 2016) has identified eight main *E. coli* phylotypes (Beghain et al., 2018; Clermont et al., 2013). Seven of these phylotypes (A, B1, B2, C, D, E, and F) belong to *E. coli sensu stricto*, referring to the classic strains of *E. coli* (Clermont et al., 2013), while the eighth is *Escherichia* cryptic clade I, which contains strains that are phenotypically indistinguishable, but genetically distinct, from *E. coli sensu stricto* (Clermont et al., 2013; Luo et al., 2011). The phylotypes of *E. coli sensu stricto* can be partitioned into two clusters: A, B1, C and E, and B2, F and D (Denamur et al., 2021); recent WGS analysis has identified phylotype G, an additional intermediate phylotype between B2 and F (Denamur et al., 2021; Yu et al.,

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2021). The distribution of *E. coli* in vertebrate hosts is not random and is dependent on host body mass, diet, age, the climate the host occupies and the characteristics of the *E. coli* itself (Alm et al., 2011; Blyton et al., 2014; Escobar-Páramo et al., 2006). Each phylotype has a differing life history, ecological niche, affinity for hosts and pathogenic features (Gordon et al., 2008; Gordon and Cowling, 2003). For example, phylotypes B2 and D are associated with humans and many strains belonging to the B2 phylotype can be opportunistic pathogens and are frequently implicated in extra-intestinal disease (Clermont et al., 2014; Johnson and Russo, 2002). Isolates assigned to the B2 phylotype can be further analysed using an allele-specific method which discriminates nine B2 sub-types (lineages) associated with extra-intestinal infections (Clermont et al., 2014). Each sub-type is associated with a sequence type complex (STc), which refers to the Achtman MLST scheme of seven genes (Clermont et al., 2014; Gordon et al., 2008; Jaureguy et al., 2008; Reid et al., 2000; Wirth et al., 2006). These B2 sub-types provide additional information about the potential pathogenicity of the bacteria, with *E. coli* isolates belonging to sub-types I (STc131), II (STc73), VI (STc12), VII (STc14) and IX (STc95) associated with disease caused by ExPEC in humans and avian species (Dale and Woodford, 2015; Denamur et al., 2021).

The persistence of commensal *E. coli* strains residing within the human intestinal tract differs. 'Resident' strains persist within a host for months to years while 'transient' strains undergo rapid turnover, generally only occurring for short periods (weeks) (Caugant et al., 1984; Johnson et al., 2003; Nowrouzian et al., 2005). The carriage of virulence-associated genes determines the ability of these phylotypes to persist in, and colonise, the intestinal tract (Blyton et al., 2014). Given that strains belonging to the B2 phylotype are frequently associated with disease, it is unsurprising that B2 isolates have been commonly identified as resident strains in the human intestine and have evolved traits that facilitate their persistence and survival in the intestinal tract (Nowrouzian et al., 2006; Touchon et al., 2020). On average, humans have been found to have 1.8 *E. coli* strains within individual gut microbiomes (Blyton et al., 2014). Individuals carrying B2 phylotypes are also more likely to have less diversity in their *E. coli* community structure (Blyton et al., 2014; Nowrouzian et al., 2006), with strains belonging to A and B1 rarely identified when B2 strains are present. Similar trends have been observed in wildlife species; Blyton et al. (2013) characterised the diversity of commensal *E. coli* within mountain brushtail possums (*Trichosurus cunninghami*), finding that on average, each individual carried 2.2 *E. coli* strains. There was also almost complete dominance of B2 phylotype commensal *E. coli* in possums, which is similar to what has been reported in humans, providing further evidence that *E. coli* belonging to the B2 phylotype could have specialised characteristics that support intestinal survival (Blyton et al., 2014).

Escherichia coli is one of the best-studied bacteria, is widely known, and has been identified as a useful indicator of both anthropogenic faecal pollution (Ahmed et al., 2016; Beversdorf et al., 2007; Gordon, 2005; Schaefer et al., 2011) and of the transmission of bacteria from human and domestic animal sources into the environment and wildlife species (Mercat et al., 2015; Pesapane et al., 2013). A relationship between *E. coli* prevalence and association with humans in wildlife species has previously been reported, with the prevalence of *E. coli* being highest in humans (100%), followed by human-associated (64%) and wild (45%) animals (Lescat et al., 2013). Furthermore, in one study, an increased frequency of contact between humans, livestock and wild primates in Uganda was associated with a higher prevalence of *E. coli* in wild primates (Rwego et al., 2008), suggesting that association with, or exposure to humans increases the prevalence of *E. coli* in domestic and wildlife species.

With the rapid emergence and dissemination of antibiotic resistant bacteria globally, there has been an increase in the number of studies investigating *E. coli* in wildlife species (Ahlstrom et al., 2019; Alonso et al., 2016; Dolejska et al., 2007; Lu et al., 2016; McDougall et al., 2021; Power et al., 2016; Radhouani et al., 2009). However, the primary interest in many studies is determining whether wild animals are reservoirs for antibiotic resistant *E. coli*, rather than the study of the native genetic structure of *E. coli* in these species and its modelling by anthropogenic impact (Bélanger et al., 2011; Mercat et al., 2015). Marine mammals are upper trophic predators and as such, are regarded as sentinels for ocean and marine ecosystem health (Bossart, 2010). The detection of *E. coli* as an indicator of exposure to anthropogenic bacterial pollution has been investigated in a number of marine mammals, including pinnipeds (Delport et al., 2015; Fulham et al., 2020, 2018; Power et al., 2016; Stoddard et al., 2009, 2008; Wallace et al., 2013), cetaceans (Melendez et al., 2019; Schaefer et al., 2011) and otters (Oliveira et al., 2018). A high prevalence of *E. coli* has previously been identified in three species of free-ranging Australian pinniped pups, namely Australian fur seal (*Arctocephalus pusillus doriferus*), Australian sea lion (*Neophoca cinerea*), and long-nosed fur seal (*Arctocephalus forsteri*) pups (Fulham et al., 2020, 2018). Samples were collected from eight breeding and *E. coli* was isolated from 87.3% samples (842/963), with 73.7% of all isolates belonging to the B2 phylotype (Fulham et al., 2020). In these free-ranging pinniped pups, the prevalence of *E. coli* was higher than what has previously been reported in animals associated with humans (Gordon and Cowling, 2003; Lescat et al., 2013), and together with the dominance of the B2 phylotype, it was suggested that free-ranging pinniped pups were being exposed to faecal pollution from anthropogenic sources (Fulham et al., 2020). However, this study was limited to analysis of a single *E. coli* isolate; the diversity and variation in the *E. coli* community structure in these free-ranging wildlife species remains unknown. Previous studies on *E. coli* in Australian wildlife have also utilised a single *E. coli* isolate (Delport et al., 2015; Fulham et al., 2020, 2018; Lundbäck et al., 2020; Power et al., 2016). Although low *E. coli* strain diversity has been observed in humans and possums (Blyton et al., 2014, 2013), to ensure the reliability of utilising a single isolate to investigate *E. coli* phylotype and sub-type diversity, a greater understanding of the diversity of *E. coli* phylotypes within individual pinniped pups (intra-individual diversity) is required. The ability to use a single *E. coli* isolate as an indicator of phylotype diversity, if confirmed to be a reliable measure of diversity, would provide enhanced efficiency and cost-effectiveness for ongoing monitoring of exposure to faecal pollution in free-ranging populations. *Arctocephalus pusillus doriferus, Neophoca cinerea* and *Arctocephaus forsteri* inhabit numerous offshore colonies along the east coast of Australia, ranging from Tasmania to Western Australia (Kirkwood and Goldsworthy, 2013). Breeding colonies of these three species are spread out along the coastline, encompassing a large geographical area. These colonies differ in their proximity to human populations and experience differing levels of exposure to anthropogenic activities and contaminants (Fulham et al., 2022; Taylor et al., 2021), providing a unique opportunity for comparative studies among free-ranging Australian pinniped populations.

This study will characterise the intra-individual diversity of *E. coli* phylotypes and B2 sub-types in the pups of three free-ranging pinnipeds in Australia, *A. p. doriferus*, *N. cinerea*, and *A. forsteri*. In addition, intra-individual, intra-species (across pups of the same species) and inter-species (across species) variation in *E. coli* phylotypes and B2 sub-types will be explored to gain a better understanding of *E. coli* variation in wildlife species.

2. Methods

2.1. Study sites

Faecal swabs were collected from *A. p. doriferus* pups ($n = 99$) at Seal Rocks, Western Port, Victoria in December 2018, from *N. cinerea* pups ($n = 66$) at Olive Island, Eyre Peninsula, South Australia in January 2019, and from *A. forsteri* pups ($n = 48$) at Cape Linois, Kangaroo Island, South Australia in January 2021. From the total collection of faecal swabs, a subset of samples from each species (ten per species, $n = 30$ in total) were randomly selected for analysis by assigning a random number

(Microsoft Excel for Mac v16.61.1) to each pup identification. Samples from *A. p. doriferus* and *N. cinerea* pups were collected as part of a previous study (Fulham et al., 2020). The phylotypes of 20 isolates from *A. p. doriferus* and *N. cinerea* pups ($n = 10$ from each species), have been reported as part of this previous study (Fulham et al., 2020).

2.2. Sample collection

Pinniped pups were sampled when mothers were absent, following methods described by Fulham et al. (Fulham et al., 2022, 2020, 2018). In summary, a physical examination was performed where pup weight (kg), standard length (cm) and sex were determined. Faecal samples were collected by inserting a sterile swab (Copan, Brescia, Italy) into the rectum of each pup, followed by sub-sampling on to Sterile FecalSwab™ (Copan, Brescia, Italy). FecalSwabs were refrigerated at 4 °C and cultured within 7 days of collection.

All sample collection for *N. cinerea* and *A. forsteri* was approved by the Animal Ethics Committee at the University of Sydney (Protocol No. 2017/1260) and Phillip Island Nature Parks Animal Ethics Committee (Protocol No. 2.2016) for *A. p. doriferus* pups.

2.3. Escherichia coli culture, isolation, preservation and DNA extraction

Escherichia coli was cultured following methodology described by (Fulham et al., 2022, 2020, 2018). In brief, FecalSwabs were inoculated onto Chromocult® coliform agar (Merck, Darmstadt, Germany) plates and incubated at 37 °C for 24 h. Colonies of *E. coli* were identified based on morphological features including shape (round) and colour (dark blue-violet). Ten colonies from each sample ($n = 300$ colonies in total) that were physically isolated from each other on culture plates were selected and sub-cultured onto Chromocult® coliform agar plates to obtain pure cultures. Approximate size (mm) and colour of each colony was recorded. To preserve each bacterial isolate, one pure *E. coli* colony from each sub-cultured sampled was selected and inoculated into Luria Bertani (LB) broth (5 mL) and incubated at 37 °C for 24 h. Broth culture (500 μL) was combined with 70% glycerol (500 μL) in 1.0 mL cryovials and stored at −80 °C (Fulham et al., 2022, 2020).

A boil preparation method was utilised to extract DNA from each broth culture (Fulham et al., 2020). The broth culture remaining after preservation was centrifuged to pellet bacteria. After decanting supernatant, the bacterial pellet was resuspended with sterile PCR-grade H_2O (50 μL) and then heated for 5 min at 95 °C. Samples were then centrifuged and resulting bacterial lysates were stored at −30 °C.

2.4. Escherichia coli phylotyping and phylotype-specific PCRs

A quadruplex PCR protocol was utilised to assign bacterial lysates to *E. coli* phylotypes following the methodology described by Clermont et al. (2013). This method allows isolates to be assigned to a phylotype based on the presence or absence of four genes: *ChuA*, *yjaA*, *TspE4.C2*, and *arpA*. Singplex phylotype-specific PCRs were then utilised to further analyse isolates identified as A/C and D/E following methods described by Fulham et al. (2020) using primers designed by Clermont et al. (2013). Phylotyping PCRs, both singleplex and quadruplex were performed using GoTaq® Green 2× Master Mix (Promega, Madison, United States). Controls representing each phylotype and a negative control containing PCR water were included in each reaction.

2.5. Escherichia coli phylotype B2 sub-typing

Escherichia coli isolates identified as belonging to the B2 phylotype were further analysed and assigned to one of nine sub-groups using two multiplex PCRs and primers described by Clermont et al. (Clermont et al., 2014). The PCR protocols were modified as described by Fulham et al. (Fulham et al., 2020) and the internal control gene *chuA* was included in all panels. The first multiplex panel tested for sub-groups II, III, VI, VII, and IX, while the second panel tested for sub-groups I, VI, V, and X. Both multiplex PCR panels were performed using GoTaq® Green 2× Master Mix (Promega, Madison, United States).

Gel electrophoresis (2% agarose *w/v*) was utilised to resolve all phylotyping and B2 sub-typing reactions described above, conducted at 100 V for 30 min in TBE (Tris, boric acid, ethylenediaminetetraacetic acid) with SYBR safe gel stain (Invitrogen, Mulgrave, Australia). Product sizes were approximated against a HyperLadderII 50 bp DNA marker (Bioline, Sydney, Australia).

2.6. Statistical analysis

Statistical analyses were conducted in RStudio software (V 2021.09.1, Boston, Massachusetts, USA). The Shapiro-Wilk's test was employed to evaluate data distribution and one-way ANOVA was used to test for differences in intra-individual, intra-species and inter-species phylotype and B2 sub-type carriage. A generalised linear mixed model was utilised to test the significance of the relationship between the number of phylotypes and pup sex, weight, length, and species. Significance was determined when $p < 0.05$.

3. Results

3.1. Inter-species E. coli phylotype diversity

Escherichia coli was isolated from a total of 190/213 samples (89.2%), with the highest prevalence in *N. cinerea* pups at 92.4% (61/66), followed by *A. p. doriferus* pups at 91.9% (91/99) and *A. forsteri* at 84.4% (38/45). The prevalence of *E. coli* in *A. p. doriferus* and *N. cinerea* pups has previously been reported, however, the results from only one *E. coli* isolate from the randomly selected pups were included in the previous study (Fulham et al., 2020).

The majority of pups from all species carried isolates only belonging to one phylotype ($n = 23$, 76.7%), with two individuals carrying isolates belonging to two different phylotypes (6.7%), and five individuals carrying isolates belonging to three different phylotypes (16.7%).

The B2 phylotype was the most frequently identified, with 78.0% of isolates ($n = 234$) assigned to this phylotype across all three species, followed by B1 (13.3%, $n = 40$), D (8.3%, $n = 25$) and A (0.3%, $n = 1$) (Fig. 1). The inter-species frequency of phylotypes did not differ significantly ($p > 0.05$) for A, B2 or D, however there was a significant difference for the B1 phylotype ($p = 0.0162$) with higher carriage of this phylotype in *N. cinerea* pups ($n = 35$) and absence of the B1 phylotype in *A. p. doriferus* pups.

3.2. Inter-species diversity of E. coli phylotype B2 sub-types

A total of 234 *E. coli* B2 isolates were analysed and either assigned to one of the nine B2 sub-types or considered unassigned (UA). Eight of the nine B2 sub-types were identified in the study including sub-types I, II, III, IV, V, VI, VII and IX. Sub-type I was found only in *A. p. doriferus* pups ($n = 3$) while sub-type IX was only isolated from *A. forsteri* ($n = 2$) pups (Fig. 2). There was no significant difference ($p > 0.05$) in the distribution of sub-type I, II, III, IV, VI, VII, IX, or UA isolates across the three species (Fig. 2). There was, however, a significant difference in the distribution of sub-type V ($p = 0.031$), with a greater frequency of sub-type V in *A. p. doriferus* and *N. cinerea* pups, in comparison to *A. forsteri* pups.

The diversity of B2 sub-types in each species differed to the observed *E. coli* diversity, with *A. p. doriferus* pups carrying the highest number of B2 sub-types, with an average of 2.5 sub-types in each individual pup, followed by 1.8 in *N. cinerea* and 1.6 in *A. forsteri* pups (Fig. 3). There was no significant difference ($p > 0.05$) in the number of sub-types carried between the three species.

Frequency (%) of Phylotypes
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
A. p. doriferus
N. cinerea
A. forsteri
Phylotype
A
B1
B2
D

Fig. 1. Combined diversity and frequency (%) of phylotypes A, B1, B2 and D in the three pinniped species.

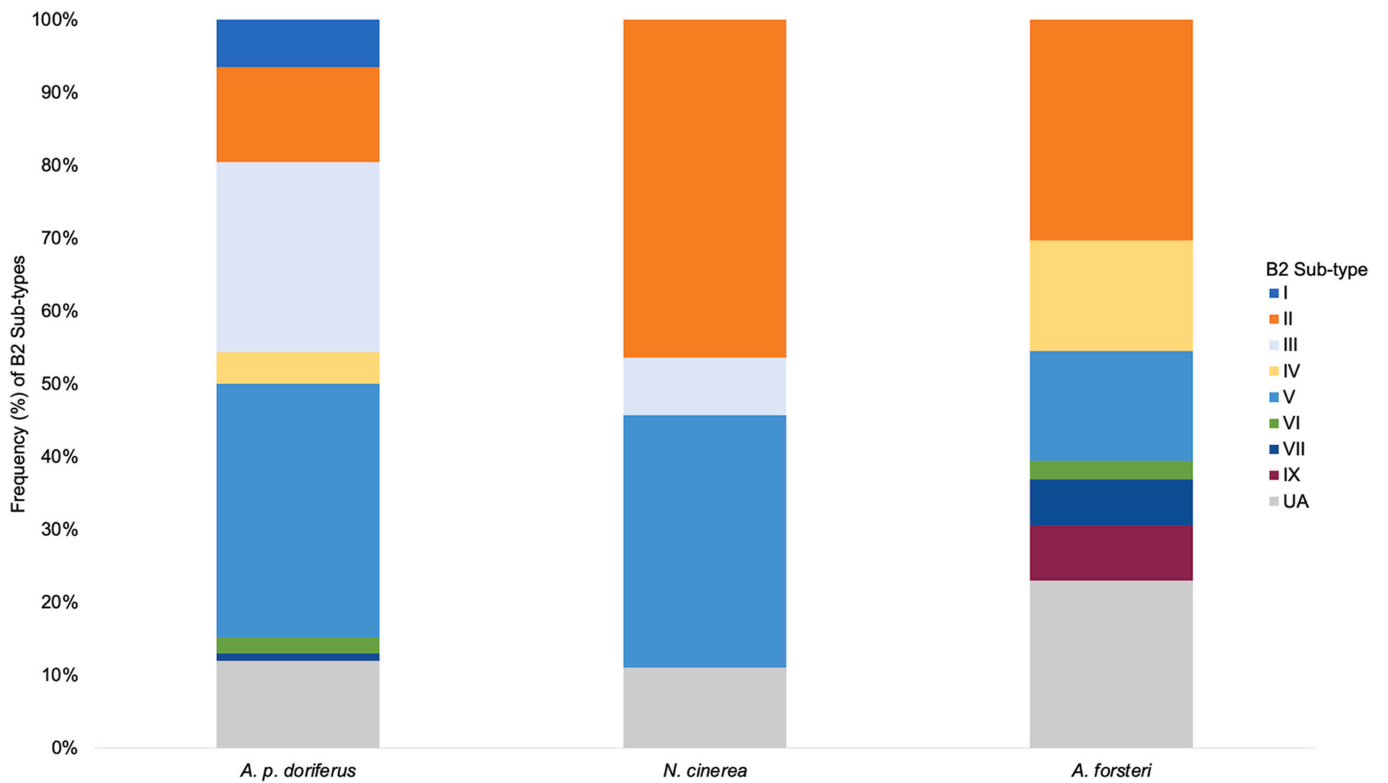

Fig. 2. Combined frequency of B2 sub-types I (STc131), II (STc73), III (STc127), IV (STc141), V (STc144), VI (STc14), VII (STc14) and IX (STc95) in all three species. Any isolates that could not be assigned were considered unassigned (UA).

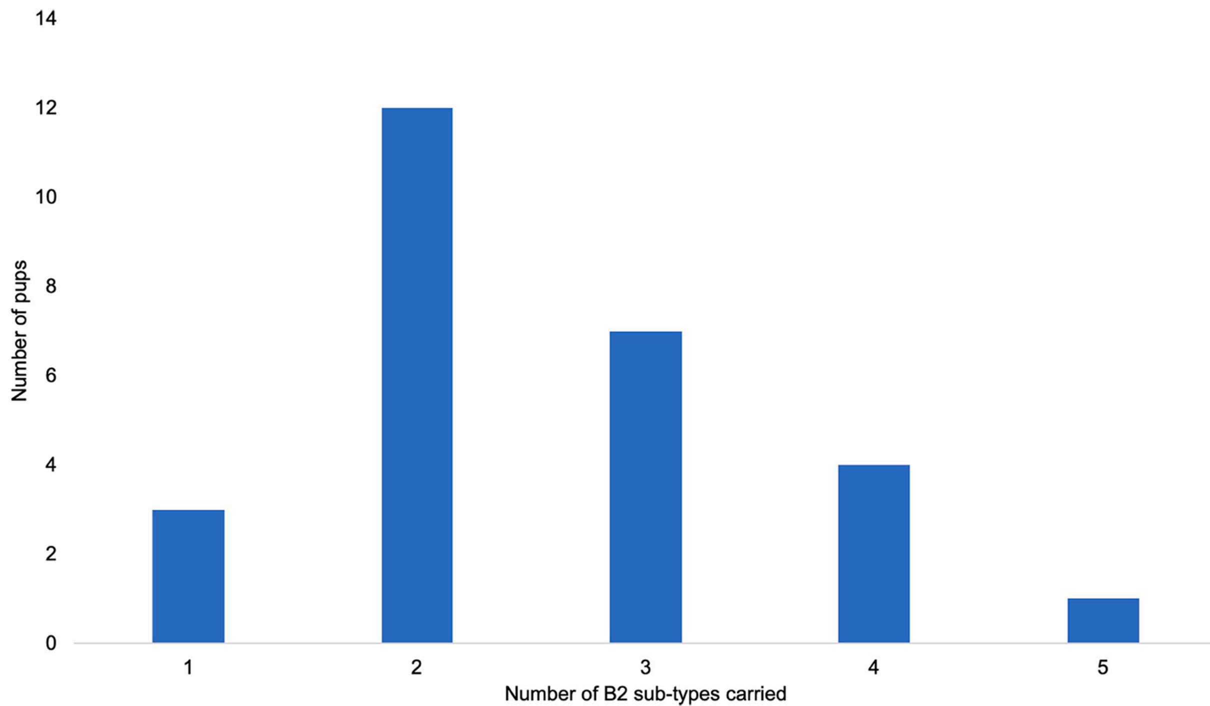

Fig. 3. Number of *E. coli* B2 sub-types carried by each pup combined across all three species.

3.3. Intra-individual *E. coli* phylotype and B2 sub-type diversity in *A. p. doriferus* pups

The most dominant phylotype present in *A. p. doriferus* pups was B2, with 92.0% of isolates ($n = 92$) assigned to this phylotype, followed by D (7.0%, $n = 7$) and A (1.0%, $n = 1$) (Fig. 4). Seven of the ten individuals only carried isolates belonging to the B2 phylotype, while two pups carried both B2 and D ($n = 9$ B2 and $n = 1$ D in both individuals), and

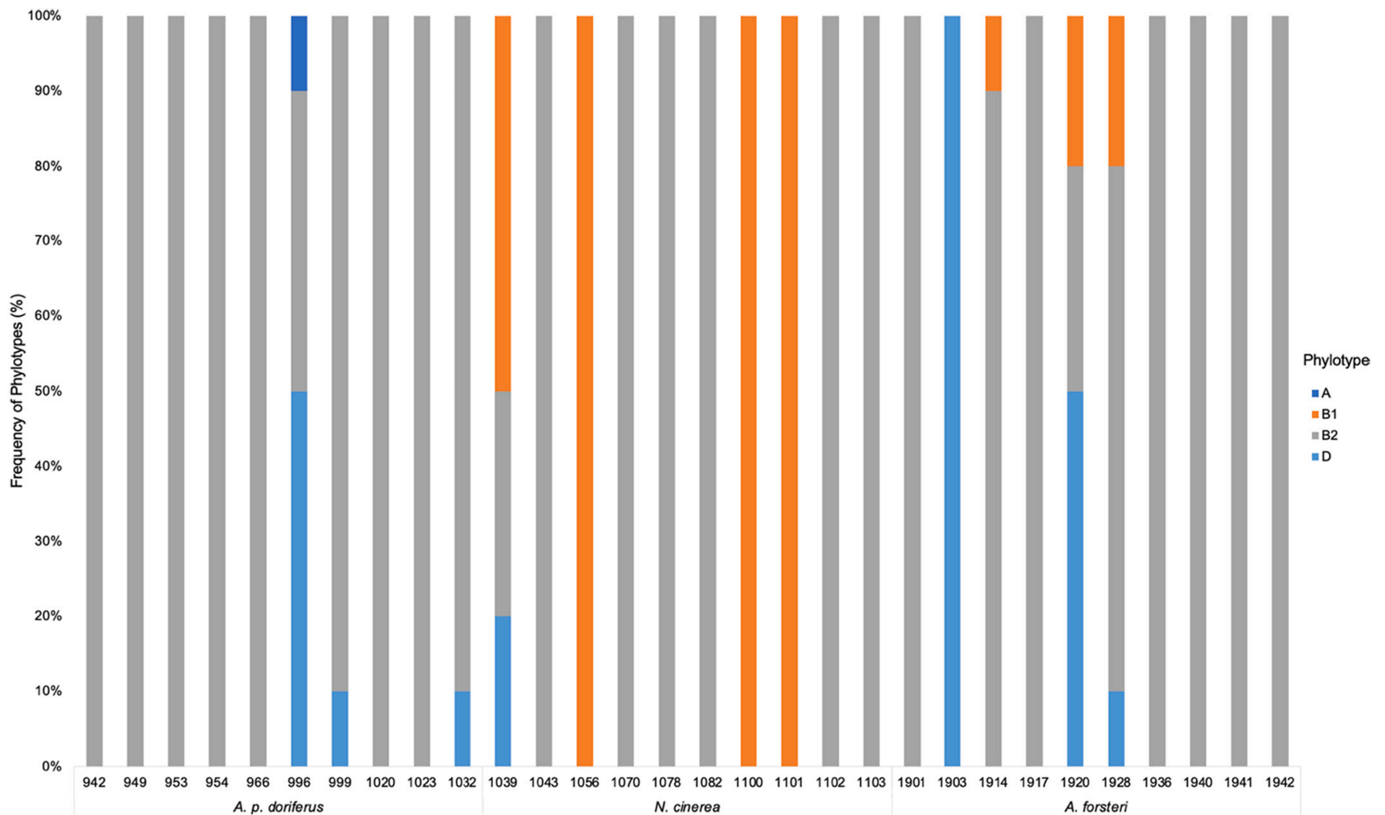

Fig. 4. Frequency (%) of each phylotype occurring within *A. p. doriferus*, *N cinerea* and *A. forsteri* pups, representing intra-individual and inter-species diversity. Numbers on the x-axis represent individual pup identifications from each species.

one pup carried isolates belonging to three phylotypes ($n = 1$ A, $n = 4$ B2 and $n = 5$ D).

Of the 92 isolates assigned to the B2 phylotype, 81 could be assigned to a B2 sub-type, with sub-types I, II, III, IV, V, VI and VII identified in isolates from ten pups. The most frequently identified sub-type was V ($n = 32$, 34.8%), followed by III ($n = 24$, 26.1%), II ($n = 12$, 13.0%), I ($n = 6$, 6.5%), IV ($n = 4$, 4.3%), VI ($n = 2$, 2.2%) and VII ($n = 1$, 1.1%), with 11 isolates considered UA (12.0%) (Fig. 5). There was no significant difference ($p > 0.05$) in the distribution of any of the B2 sub-types in *A. p. doriferus* pups.

3.4. Intra-individual E. coli phylotype and B2 sub-type diversity in N. cinerea pups

In *N. cinerea* pups, the B2 phylotype was the most frequently isolated, with 63.0% of isolates ($n = 63$) identified as B2, followed by B1 (35.0%, $n = 35$) and D (2.0%, $n = 2$) (Fig. 4). There was less diversity among individuals with nine pups carrying isolates of only one phylotype; six pups only carried B2 isolates, and three pups carried only B1 isolates. Only one *N. cinerea* pup carried three phylotypes ($n = 5$ B1, $n = 3$ B2 and $n = 2$ D) (Fig. 4).

Fewer B2 sub-types were identified in *N. cinerea* pups (Fig. 5). Of the 63 isolates assigned to the B2 phylotype, 56 could be assigned to B2 sub-types II, III or V and seven isolates considered unassigned. Unlike in *A. p. doriferus* pups, sub-type II was the most frequently identified ($n = 29$, 46.0%), followed by V ($n = 22$, 34.9%), and III ($n = 5$, 7.9%) (Fig. 5). There was no significant difference ($p > 0.05$) in the distribution of B2 sub-types in *N. cinerea* pups.

3.5. Intra-individual E. coli phylotype and B2 sub-type diversity in A. forsteri pups

The dominance of the B2 phylotype observed in *A. p. doriferus* and *N. cinerea* pups was similar in *A. forsteri* pups with 79% of all isolates assigned to this phylotype ($n = 79$), followed by D (16.0%, $n = 16$) and B1 (5.0%, $n = 5$) (Fig. 4). The diversity among individuals was similar to *N. cinerea* pups, with seven individuals carrying only one phylotype. Of these seven, six carried only B2 isolates; one carried only D isolates. The remaining three pups carried isolates belonging to multiple phylotypes, with one pup carrying two phylotypes ($n = 9$ B2, $n = 1$ B1) and two pups carried three phylotypes ($n = 2$ B1, $n = 3$ B2, $n = 5$ D and $n = 2$ B1, $n = 7$ B2 and $n = 1$ D).

A total of six B2 sub-types were identified in the 79 *E. coli* B2 isolates from *A. forsteri* pups. From the 79 B2 isolates, 61 could be assigned to one of the following sub-types, II, IV, V, VI, VII or IX with 18 isolates (22.8%) considered unassigned (Fig. 5). The majority of isolates were assigned to sub-type II ($n = 24$, 30.4%), followed by IV ($n = 12$, 15.2%) and V ($n = 12$, 15.2%), IX ($n = 6$, 7.6%), VII ($n = 5$, 6.3%) and VI ($n = 2$, 2.5%). Similar to what was found in the other pinniped species, there was no significant difference ($p > 0.05$) in the distribution of B2 sub-types in individual *A. forsteri* pups.

3.6. Number of phylotypes carried and host characteristics

There was a significant relationship between the number of phylotypes and sex of the host ($p = 0.0195$), while weight and standard length were not significant ($p > 0.05$). The significant relationship between number of phylotypes and sex is likely due to all *N. cinerea* pups sampled being male. A total of 19 pups were male and 11 were female, with an even division in *A. p. doriferus* pups (five female, five male) and more female *A. forsteri* pups (six female, four male). Interestingly, four females carried more than one phylotype while only two males carried more than one phylotype, but this difference in phylotype carriage was not significant ($p > 0.05$).

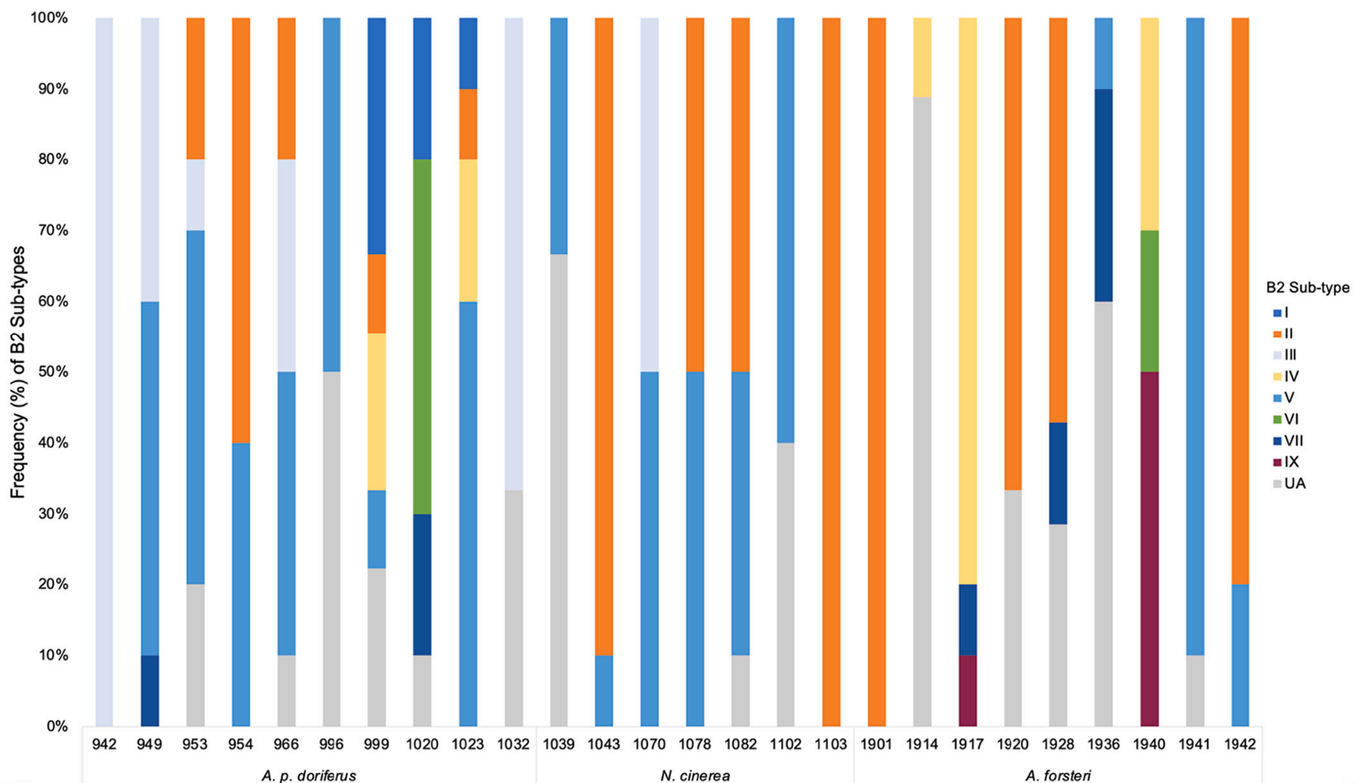

Fig. 5. Frequency (%) of B2 subtypes in individual *A. p. doriferus*, *N. cinerea,* and *A. forsteri*. Isolates were assigned to sub-types I (STc131), II (STc73), III (STc127), IV (STc141), V (STc144), VI (STc14), VII (STc14) or IX (STc95), or considered unassigned (UA). Numbers on the x-axis represent individual pup identifications from each species.

4. Discussion

Previous investigation of *E. coli* diversity in free-ranging Australian pinniped pups was based on analysis of a single *E. coli* isolate per host (Fulham et al., 2020). This study characterised the diversity and abundance of *E. coli* phylotypes and B2 sub-types in 300 *E. coli* isolates from *A. p. doriferus*, *N. cinerea* and *A. forsteri* pups to elucidate the utility of a single isolate for phylotype characterisation in these species. Low intra-individual *E. coli* phylotype diversity was observed, with only seven individuals across all three species carrying more than one phylotype. All of the four main *E. coli* phylotypes, A, B1, B2 and D (Clermont et al., 2013; Gordon and Cowling, 2003), were identified in *A. p. doriferus*. *N. cinerea*, and *A. forsteri* pups sampled in this study, with no significant differences in the diversity or abundance of *E. coli* phylotypes observed between the three species. Fulham et al. (2020) previously found a greater diversity of phylotypes (A, B1, B2, C, D, E and F) in pinniped pups of these same species, however, a greater number of individuals were sampled from a wider geographical range and over multiple breeding seasons. Furthermore, phylotypes C, E and F only represented 0.23%, 0.47% and 4.5% of the total isolates (Fulham et al., 2020), suggesting that these phylotypes are not common in free-ranging Australian pinniped pups.

It has previously been suggested that association with humans influences the presence and diversity of phylotypes in Australian pinnipeds (Delport et al., 2015), and it was hypothesised that pinniped pups in breeding colonies exposed to higher levels of anthropogenic and faecal pollution would have a higher prevalence of *E. coli* and the B2 phylotype. There was no significant difference in *E. coli* phylotype diversity between the breeding colonies, which is consistent with what has been observed in free-ranging Australian pinniped pups (Fulham et al., 2020), indicating that all colonies are exposed to some level of microbial pollution from anthropogenic sources.

Similar to the previous study in these species in which the B2 phylotype predominated (73.7%) (Fulham et al., 2020), a dominance of the B2 phylotype was also observed in the present study among repeat isolates from individuals. Only three individuals, all *N. cinerea* pups, did not carry isolates assigned to the B2 phylotype. In pups where the B2 phylotype was present, it was uncommon to observe any other phylotypes. Of the 30 individuals studied, $n = 19$ only carried the B2 phylotype, and a total of 234 isolates (78.0%) were assigned to this phylotype. This dominance of the B2 phylotype has also been observed in free-ranging and captive adult *N. cinerea* (Delport et al., 2015), Antarctic pinnipeds (Mora et al., 2018; Power et al., 2016), and mountain brushtail possums in Australia, where 86% of *E. coli* isolates from possums were assigned to the B2 group (Blyton et al., 2013). These findings are also consistent with previous observations that the B2 phylogenetic group has become specialised to the intestinal environment, when compared to other phylogenetic groups, carrying virulence genes that assist long-term survival in the intestinal tract (Blyton et al., 2013; Nowrouzian et al., 2006; Touchon et al., 2020). Furthermore, the results from this study support previous reports of other phylotypes being infrequently identified when *E. coli* strains belonging to the B2 phylotype predominate (Smati et al., 2013).

In this study, the carriage of the B2 phylotype was highest in *A. p. doriferus* pups ($n = 92$), followed by *A. forsteri* ($n = 79$) and *N. cinerea* ($n = 63$). Given the higher abundance of B2 isolates, it is not surprising that *A. p. doriferus* and *A. forsteri* pups carried a more diverse array of B2 sub-types, with seven and six sub-types identified in these species, respectively. A greater diversity of B2 sub-types was observed in comparison to *E. coli* phylotypes, with only three pups carrying a single B2 sub-type. Consequently, a single *E. coli* isolate may not be representative of B2 sub-type diversity in the host. Understanding the presence of strains belonging to the B2 phylotype is of importance, given that these strains are known to carry virulence traits and cause extraintestinal disease in humans, domestic animals and birds (Clermont et al., 2014; Johnson and Russo, 2002; Mora et al., 2013). A global survey of ExPEC *E. coli* conducted by Manges et al. (2019) identified the predominant ExPEC lineages, which include STc131, STc73 and STc95 (B2 sub-types I, II and IX, respectively). Sub-types I, II and IX were identified in free-ranging pinniped pups sampled as part of this study and have previously been reported in free-ranging Antarctic pinnipeds (Mora et al., 2018; Power et al., 2016), in adult *N. cinerea* (Delport et al., 2015) and in *A. p. doriferus, N. cinerea* and *A. forsteri* pups (Fulham et al., 2020, 2018). Importantly, STc131 has been found to be the predominant ExPEC lineage worldwide, colonising humans and human-associated animals, and appears to be one of the most competitive B2 strains (Nicolas-Chanoine et al., 2014), while STc95 and STc73 have been implicated as causes of ExPEC disease in humans and avian species (Clermont et al., 2014; Dheilly et al., 2011; Riley, 2014). Although strain-typing of *E. coli* isolates was not conducted in this study, the identification of B2 sub-types frequently implicated as ExPEC *E. coli* provides additional information about the potential pathogenicity and source of *E. coli* isolated from free-ranging Australian pinniped pups.

Previous studies investigating *E. coli* diversity in wildlife have found that a relationship exists between the relative abundance of *E. coli* phylotypes and host species, diet and body mass (Gordon and Cowling, 2003). Escobar-Páramo et al. (2006) determined that intra-individual *E. coli* strain diversity was influenced by diet, with carnivores having greater diversity (2.3 strains per individual) compared to herbivores (2.2 strains per individual) and omnivores (1.7 strains per individual). At the time of sampling, *A. p. doriferus*, *N. cinerea* and *A. forsteri* pups are not foraging independently, consume a milk-only diet and are confined to their breeding colonies. As a result, any impact of diet on pup phylotype diversity is likely influenced by maternal diet (Nelson et al., 2013).

In the present study, a significant relationship between phylotype and pup sex was observed, however, this is likely due to the sampling bias, despite random selection of individuals for study inclusion, toward only male *N. cinerea* pups. A significant interaction effect between host sex and the phylotype of the dominant *E. coli* strain has previously been reported in a human study (Blyton et al., 2014), with this study also finding that this interaction effect was greater in females, with the phylotype of the dominant strain having a greater influence on strain diversity in female subjects (Blyton et al., 2014). While our sample size is too low to fully understand the impact of pup sex on the carriage of phylotypes, pinniped pups carrying more than one phylotype were more likely to be female than male, despite having a greater number of samples from male pups. The difficulties of sampling free-ranging individuals is such that an equal inclusion of sexes is not always feasible; however future studies should aim to include a greater proportion of samples from female pups, to better understand the contribution of host sex to *E. coli* phylotype diversity in pinnipeds.

The low intra-individual *E. coli* phylotype diversity determined in this study suggests a single *E. coli* isolate can provide a baseline of the general abundance of *E. coli* phylotypes within individual *A. p. doriferus*, *N. cinerea* and *A. forsteri* pups when measured at the phylotype level, especially when the B2 phylotype is detected. However, the use of a single isolate does still have limitations: it is likely that not all diversity is captured, especially when considering phylotypes that are generally less prevalent (*e.g.*, phylotype F) (Touchon et al., 2020; Yu et al., 2021). However, phylotypes C, E and F appear to be rare in free-ranging pinniped populations in Australia, with the main *E. coli* phylotypes, A B1 B2 and D being the most prevalent (Delport et al., 2015; Fulham et al., 2020, 2018). Given that the B2 phylotype is associated with humans and ExPEC strains of *E. coli* (Dale and Woodford, 2015; Denamur et al., 2021), being able to monitor the presence of this phylotype is potentially the most important for free-ranging pinniped pup health. Importantly, in pups in which the B2 phylotype was identified, it was uncommon to identify another phylotype within those individuals. Even in pups that carried more than one phylotype, B2 remained the numerically dominant group, which is consistent with findings of previous studies (Blyton et al., 2013; Nowrouzian et al., 2006). In contrast,

greater intra-individual B2 sub-type diversity was seen, with only three pups carrying isolates belonging to a single B2 sub-type. It is therefore unlikely that identifying the B2 sub-type of a single isolate from an individual pinniped pup would be representative of the B2 sub-type diversity within that individual.

5. Conclusion

This study investigated the diversity of *E. coli* phylotypes and B2 subtypes within individuals belonging to the same species and between three species of free-ranging pinniped pups, finding limited differences. For investigations limited to phylotype classification of *E. coli* isolates, the characterisation of a single *E. coli* isolate can provide an understanding of the general abundance of phylotypes in free-ranging pinniped populations, as well as efficient and cost-effective screening tool for marine contamination, particularly when the B2 phylotype is detected. The diversity of *E. coli* phylotypes and B2 sub-types can provide a basic understanding of the genetic structure of *E. coli* within free-ranging pinniped pups. However, to gain a better understanding of the community structure of *E. coli* in these individuals, future studies should incorporate further analysis of isolates through strain-typing and whole genome sequencing, particularly given the high carriage of isolates belonging to the B2 phylotype in all species studied. Analysis of isolates to strain level would provide a greater understanding of the potential for this bacterium to cause disease in these pinniped hosts.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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References

Ahlstrom, C.A., Ramey, A.M., Woksepp, H., Bonnedahl, J., 2019. Repeated detection of carbapenemase-producing *Escherichia coli* in gulls inhabiting Alaska. Antimicrob. Agents Chemother. 63 https://doi.org/10.1128/AAC.00758-19.

Ahmed, W., Sidhu, J.P.S., Smith, K., Beale, D.J., Gyawali, P., Toze, S., 2016. Distributions of fecal markers in wastewater from different climatic zones for human fecal pollution tracking in Australian surface waters. Appl. Environ. Microbiol. 82, 1316–1323. https://doi.org/10.1128/AEM.03765-15.

Alm, E.W., Walk, S.T., Gordon, D.M., 2011. The niche of *Escherichia coli*. In: Walk, S.T., Feng, P.C.H. (Eds.), Population Genetics of Bacteria: A Tribute to S Whittam. ASM Press, Washington, DC, pp. 69–89. https://doi.org/10.1128/9781555817114.ch6.

Alonso, C.A., González-Barrio, D., Tenorio, C., Ruiz-Fons, F., Torres, C., 2016. Antimicrobial resistance in faecal *Escherichia coli* isolates from farmed red deer and wild small mammals. Detection of a multiresistant *E. coli* producing extended-spectrum beta-lactamase. Comp. Immunol. Microbiol. Infect. Dis. 45, 34–39. https://doi.org/10.1016/j.cimid.2016.02.003.

Beghain, J., Bridier-Nahmias, A., le Nagard, H., Denamur, E., Clermont, O., 2018. ClermonTyping: an easy-to-use and accurate in silico method for *Escherichia* genus strain phylotyping. Microb. Genom. 4, e000192 https://doi.org/10.1099/MGEN.0.000192.

Bélanger, L., Garenaux, A., Harel, J., Boulianne, M., Nadeau, E., Dozois, C.M., 2011. *Escherichia coli* from animal reservoirs as a potential source of human extraintestinal pathogenic *E. coli*. FEMS Immunol. Med. Microbiol. 62, 1–10. https://doi.org/10.1111/j.1574-695X.2011.00797.x.

Beversdorf, L.J., Bornstein-Forst, S.M., McLellan, S.L., 2007. The potential for beach sand to serve as a reservoir for *Escherichia coli* and the physical influences on cell die-off. J. Appl. Microbiol. 102, 1372–1381. https://doi.org/10.1111/j.1365-2672.2006.03177.x.

Blyton, M.D.J., Banks, S.C., Peakall, R., Gordon, D.M., 2013. High temporal variability in commensal *Escherichia coli* strain communities of a herbivorous marsupial. Environ. Microbiol. 15, 2162–2172. https://doi.org/10.1111/1462-2920.12088.

Blyton, M.D.J., Cornall, S.J., Kennedy, K., Colligon, P., Gordon, D.M., 2014. Sex-dependent competitive dominance of phylogenetic group B2 *Escherichia coli* strains within human hosts. Environ. Microbiol. Rep. 6, 605–610. https://doi.org/10.1111/1758-2229.12168.

Bossart, G.D., 2010. Marine mammals as sentinel species for oceans and human health. Vet. Pathol. 48, 676–690. https://doi.org/10.1177/0300985810388525.

Caugant, D.A., Levin, B.R., Selander, R.K., 1984. Distribution of multilocus genotypes of *Escherichia coli* within and between host families. J. Hyg. 92, 377–384. https://doi.org/10.1017/s0022172400064597.

Clermont, O., Christenson, J.K., Denamur, E., Gordon, D.M., 2013. The Clermont *Escherichia coli* phylo-typing method revisited: improvement of specificity and detection of new phylo-groups. Environ. Microbiol. Rep. 5, 58–65. https://doi.org/10.1111/1758-2229.12019.

Clermont, O., Christenson, J.K., Daubié, A.-S., Gordon, D.M., Denamur, E., 2014. Development of an allele-specific PCR for *Escherichia coli* B2 sub-typing, a rapid and easy to perform substitute of multilocus sequence typing. J. Microbiol. Methods 101, 24–27. https://doi.org/10.1016/j.mimet.2014.03.008.

Croxen, M.A., Finlay, B.B., 2010. Molecular mechanisms of *Escherichia coli* pathogenicity. Nat. Rev. Microbiol. 8, 26–38. https://doi.org/10.1038/NRMICRO2265.

Dale, A.P., Woodford, N., 2015. Extra-intestinal pathogenic *Escherichia coli* (ExPEC): disease, carriage and clones. J. Inf. Secur. 71, 615–626. https://doi.org/10.1016/j.jinf.2015.09.009.

Delport, T.C., Harcourt, R.G., Beaumont, L.J., Webster, K.N., Power, M.L., 2015. Molecular detection of antibiotic-resistance determinants in *Escherichia coli* isolated from the endangered Australian sea lion (*Neophoca cinerea*). J. Wildl. Dis. 51, 555–563. https://doi.org/10.7589/2014-08-200.

Denamur, E., Clermont, O., Bonacorsi, S., Gordon, D., 2021. The population genetics of pathogenic *Escherichia coli*. Nat. Rev. Microbiol. 19, 37–54. https://doi.org/10.1038/s41579-020-0416-x.

Dheilly, A., Bouder, A., le Devendec, L., Hellard, G., Kempf, I., 2011. Clinical and microbial efficacy of antimicrobial treatments of experimental avian colibacillosis. Vet. Microbiol. 149, 422–429. https://doi.org/10.1016/j.vetmic.2010.11.033.

Dolejska, M., Cizek, A., Literak, I., 2007. High prevalence of antimicrobial-resistant genes and integrons in *Escherichia coli* isolates from black-headed gulls in the Czech Republic. J. Appl. Microbiol. 103, 11–19. https://doi.org/10.1111/j.1365-2672.2006.03241.x.

Escobar-Páramo, P., Le Menac'h, A., Le Gall, T., Amorin, C., Gouriou, S., Picard, B., Skurnik, D., Denamur, E., 2006. Identification of forces shaping the commensal *Escherichia coli* genetic structure by comparing animal and human isolates. Environ. Microbiol. 8, 1975–1984. https://doi.org/10.1111/j.1462-2920.2006.01077.x.

Fratamico, P.M., DebRoy, C., Liu, Y., Needleman, D.S., Baranzoni, G.M., Feng, P., 2016. Advances in molecular serotyping and subtyping of *Escherichia coli*. Front. Microbiol. https://doi.org/10.3389/fmicb.2016.00644.

Fulham, M., Power, M., Gray, R., 2018. Comparative ecology of *Escherichia coli* in endangered Australian sea lion (*Neophoca cinerea*) pups. Infect. Genet. Evol. 62, 262–269. https://doi.org/10.1016/j.meegid.2018.05.002.

Fulham, M., Power, M., Gray, R., 2020. Diversity and distribution of *Escherichia coli* in three species of free-ranging Australian pinniped pups. Frontiers in marine. Science 7, 1–11 [571171]. https://doi.org/10.3389/fmars.2020.571171.

Fulham, M., McDougall, F., Power, M., McIntosh, R.R., Gray, R., 2022. Carriage of antibiotic resistant bacteria in endangered and declining Australian pinniped pups. PLoS One 17, e0258978.

Gordon, D.M., 2005. The influence of ecological factors on the distribution and the genetic structure of *Escherichia coli*. EcoSal Plus 1. https://doi.org/10.1128/ecosalplus.6.4.1.

Gordon, D.M., Cowling, A., 2003. The distribution and genetic structure of *Escherichia coli* in Australian vertebrates: host and geographic effects. Microbiology (NY) 149, 3575–3586. https://doi.org/10.1099/mic.0.26486-0.

Gordon, D.M., Clermont, O., Tolley, H., Denamur, E., 2008. Assigning *Escherichia coli* strains to phylogenetic groups: multi-locus sequence typing versus the PCR triplex method. Environ. Microbiol. 10, 2484–2496. https://doi.org/10.1111/j.1462-2920.2008.01669.x.

Jaureguy, F., Landraud, L., Passet, V., Diancourt, L., Frapy, E., Guigon, G., Carbonnelle, E., Lortholary, O., Clermont, O., Denamur, E., Nassif, X., Brisse, S., 2008. Phylogenetic and genomic diversity of human bacteremic *Escherichia coli* strains. BMC Genomics 9. https://doi.org/10.1186/1471-2164-9-560.

Johnson, J.R., Russo, T.A., 2002. Extraintestinal pathogenic *Escherichia coli*: "The other bad *E coli*". J. Lab. Clin. Med. 139, 155–162. https://doi.org/10.1067/mlc.2002.121550.

Johnson, J.R., Gajewski, A., Lesse, A.J., Russo, T.A., 2003. Extraintestinal pathogenic *Escherichia coli* as a cause of invasive nonurinary infections. J. Clin. Microbiol. 41, 5798–5802. https://doi.org/10.1128/JCM.41.12.5798-5802.2003.

Kaper, J.B., Nataro, J.P., Mobley, H.L.T., 2004. Pathogenic *Escherichia coli*. Nat. Rev. Microbiol. 2, 123–140. https://doi.org/10.1038/nrmicro818.

Kirkwood, Roger, Goldsworthy, Simon, 2013. Fur Seals and Sea Lions, Australian Natural History Series. CSIRO Publishing, Collingwood, VIC.

Lescat, M., Clermont, O., Woerther, P.L., Glodt, J., Dion, S., Skurnik, D., Djossou, F., Dupont, C., Perroz, G., Picard, B., Catzeflis, F., Andremont, A., Denamur, E., 2013. Commensal *Escherichia coli* strains in Guiana reveal a high genetic diversity with host-dependant population structure. Environ. Microbiol. Rep. 5, 49–57. https://doi.org/10.1111/j.1758-2229.2012.00374.x.

Lu, S., Jin, D., Wu, S., Yang, J., Lan, R., Bai, X., Liu, S., Meng, Q., Yuan, X., Zhou, J., Pu, J., Chen, Q., Dai, H., Hu, Y., Xiong, Y., Ye, C., Xu, J., 2016. Insights into the evolution of pathogenicity of *Escherichia coli* from genomic analysis of intestinal *E. coli* of *Marmota himalayana* in Qinghai-Tibet plateau of China. Emerg. Microb. Infect. 5, 122. https://doi.org/10.1038/emi.2016.122.

Lundbäck, I.C., McDougall, F.K., Dann, P., Slip, D.J., Gray, R., Power, M.L., 2020. Into the sea: antimicrobial resistance determinants in the microbiota of little penguins (*Eudyptula minor*). Infect. Genet. Evol. 104697 https://doi.org/10.1016/j.meegid.2020.104697.

Luo, C., Walk, S.T., Gordon, D.M., Feldgarden, M., Tiedje, J.M., Konstantinidis, K.T., 2011. Genome Sequencing of Environmental *Escherichia coli* Expands Understanding of the Ecology and Speciation of the Model Bacterial Species, p. 108. https://doi.org/10.1073/pnas.1015622108.

Manges, A.R., Geum, H.M., Guo, A., Edens, T.J., Fibke, C.D., Pitout, J.D.D., 2019. Global extraintestinal pathogenic *Escherichia coli* (ExPEC) lineages. Clin. Microbiol. Rev. 32 https://doi.org/10.1128/CMR.00135-18.

McDougall, F.K., Boardman, W.S.J., Power, M.L., 2021. Characterization of beta-lactam-resistant *Escherichia coli* from Australian fruit bats indicates anthropogenic origins. Microb. Genom. 7, 000571 https://doi.org/10.1099/mgen.0.000571.

Melendez, D., Roberts, M.C., Greninger, A.L., Weissman, S., No, D., Rabinowitz, P., Wasser, S., 2019. Whole-genome analysis of extraintestinal pathogenic *Escherichia coli* (ExPEC) MDR ST73 and ST127 isolated from endangered southern resident killer whales (*Orcinus orca*). J. Antimicrob. Chemother. 74, 2176. https://doi.org/10.1093/jac/dkz159.

Mercat, M., Clermont, O., Massot, M., Ruppe, E., de Garine-Wichatitsky, M., Miguel, E., Valls Fox, H., Cornelis, D., Andremont, A., Denamur, E., Caron, A., 2015. *Escherichia coli* population structure and antibiotic resistance at a buffalo/cattle interface in southern Africa. Appl. Environ. Microbiol. 82, 1459–1467.

Mora, A., Viso, S., López, C., Alonso, M.P., García-Garrote, F., Dabhi, G., Mamani, R., Herrera, A., Marzoa, J., Blanco, M., Blanco, J.E., Moulin-Schouleur, M., Schouler, C., Blanco, J., 2013. Poultry as reservoir for extraintestinal pathogenic *Escherichia coli* O45:K1:H7-B2-ST95 in humans. Vet. Microbiol. 167, 506–512. https://doi.org/10.1016/j.vetmic.2013.08.007.

Mora, A., García-Peña, F., Alonso, M., Pedraza-Diaz, S., Ortega-Mora, L., Garcia-Parraga, D., López, C., Viso, S., Dahbi, G., Marzoa, J., Sergeant, M., García, V., Blanco, J., 2018. Impact of human-associated *Escherichia coli* clonal groups in Antarctic pinnipeds: presence of ST73, ST95, ST141 and ST131. Sci. Rep. 8, 4678. https://doi.org/10.1038/s41598-018-22943-0.

Nelson, T.M., Rogers, T.L., Carlini, A.R., Brown, M.V., 2013. Diet and phylogeny shape the gut microbiota of Antarctic seals: a comparison of wild and captive animals. Environ. Microbiol. 15, 1132–1145. https://doi.org/10.1111/1462-2920.12022.

Nicolas-Chanoine, M.H., Bertrand, X., Madec, J.Y., 2014. *Escherichia coli* ST131, an intriguing clonal group. Clin. Microbiol. Rev. 27, 543. https://doi.org/10.1128/CMR.00125-13.

Nowrouzian, F.L., Wold, A.E., Adlerberth, I., 2005. *Escherichia coli* strains belonging to phylogenetic group B2 have superior capacity to persist in the intestinal microflora of infants. J. Infect. Dis. 191, 1078–1083. https://doi.org/10.1086/427996.

Nowrouzian, F.L., Adlerberth, I., Wold, A.E., 2006. Enhanced persistence in the colonic microbiota of *Escherichia coli* strains belonging to phylogenetic group B2: role of virulence factors and adherence to colonic cells. Microbes Infect. 8, 834–840. https://doi.org/10.1016/j.micinf.2005.10.011.

Oliveira, M., Freire, D., Pedroso, N.M., 2018. *Escherichia coli* is not a suitable fecal indicator to assess water fecal contamination by otters. Braz. J. Biol. 78, 155–159. https://doi.org/10.1590/1519-6984.167279.

Pesapane, R., Ponder, M., Alexander, K.A., 2013. Tracking pathogen transmission at the human–wildlife interface: banded mongoose and *Escherichia coli*. Ecohealth 10, 115–128. https://doi.org/10.1007/s10393-013-0838-2.

Picard, B., Garcia, J.S., Gouriou, S., Duriez, P., Brahimi, N., Bingen, E., Elion, J., Denamur, E., 1999. The link between phylogeny and virulence in *Escherichia coli* extraintestinal infection. Infect. Immun. 67, 546.

Power, M.L., Samuel, A., Smith, J.J., Stark, J.S., Gillings, M.R., Gordon, D.M., 2016. *Escherichia coli* out in the cold: dissemination of human-derived bacteria into the Antarctic microbiome. Environ. Pollut. 215, 58–65. https://doi.org/10.1016/j.envpol.2016.04.013.

Radhouani, H., Poeta, P., Igrejas, G., Goncalves, A., Vinué, L., Torres, C., 2009. Antimicrobial resistance and phylogenetic groups in isolates of *Escherichia coli* from seagulls at the Berlengas nature reserve. Veterin. Record 165, 138. https://doi.org/10.1136/vr.165.5.138.

Reid, S.D., Herbelin, C.J., Bumbaugh, A.C., Selander, R.K., Whittam, T.S., 2000. Parallel evolution of virulence in pathogenic *Escherichia coli*. Nature 406, 64–67. https://doi.org/10.1038/35017546.

Riley, L.W., 2014. Pandemic lineages of extraintestinal pathogenic *Escherichia coli*. Clin. Microbiol. Infect. 20, 380–390. https://doi.org/10.1111/1469-0691.12646.

Rwego, I.B., Isabirye-Basuta, G., Gillespie, T.R., Goldberg, T.L., 2008. Gastrointestinal bacterial transmission among humans, mountain gorillas, and livestock in Bwindi impenetrable National Park, Uganda. Conserv. Biol. 22, 1600–1607. https://doi.org/10.1111/j.1523-1739.2008.01018.x.

Schaefer, A.M., Bossart, G.D., Mazzoil, M., Fair, P.A., Reif, J.S., 2011. Risk factors for colonization of *E. coli* in Atlantic bottlenose dolphins (*Tursiops truncatus*) in the Indian River lagoon, Florida. J. Environ. Public Health, 597073. https://doi.org/10.1155/2011/597073.

Smati, M., Clermont, O., le Gal, F., Schichmanoff, O., Jauréguy, F., Eddi, A., Denamur, E., Picard, B., 2013. Real-time PCR for quantitative analysis of human commensal *Escherichia coli* populations reveals a high frequency of subdominant phylogroups. Appl. Environ. Microbiol. 79, 5005. https://doi.org/10.1128/AEM.01423-13.

Stoddard, R.A., Atwill, E.R., Gulland, F.M.D., Miller, M.A., Dabritz, H.A., Paradies, D.M., Worcester, K.R., Jang, S., Lawrence, J., Byrne, B.A., Conrad, P.A., 2008. Risk factors for infection with pathogenic and antimicrobial-resistant fecal bacteria in northern elephant seals in California. Public Health Rep. 123, 360–370. https://doi.org/10.1177/003335490812300316.

Stoddard, R.A., Atwill, E.R., Conrad, P.A., Byrne, B.A., Jang, S., Lawrence, J., McCowan, B., Gulland, F.M.D., 2009. The effect of rehabilitation of northern elephant seals (*Mirounga angustirostris*) on antimicrobial resistance of commensal *Escherichia coli*. Vet. Microbiol. 133, 264–271. https://doi.org/10.1016/j.vetmic.2008.07.022.

Taylor, S., Terkildsen, M., Stevenson, G., de Araujo, J., Yu, C., Yates, A., McIntosh, R.R., Gray, R., 2021. Per and polyfluoroalkyl substances (PFAS) at high concentrations in neonatal Australian pinnipeds. Sci. Total Environ. 786, 147446 https://doi.org/10.1016/j.scitotenv.2021.147446.

Tenaillon, O., Skurnik, D., Picard, B., Denamur, E., 2010. The population genetics of commensal *Escherichia coli*. Nat. Rev. Microbiol. 8, 207–217. https://doi.org/10.1038/nrmicro2298.

Touchon, M., Perrin, A., de Sousa, J.A.M., Vangchhia, B., Burn, S., O'Brien, C.L., Denamur, E., Gordon, D., Rocha, E.P., 2020. Phylogenetic background and habitat drive the genetic diversification of *Escherichia coli*. PLoS Genet. 16 https://doi.org/10.1371/journal.pgen.1008866 e1008866.

Wallace, C., Yund, P., Ford, T., Matassa, K., Bass, A., 2013. Increase in antimicrobial resistance in bacteria isolated from stranded marine mammals of the Northwest Atlantic. Ecohealth 10, 201–210. https://doi.org/10.1007/s10393-013-0842-6.

Wirth, T., Falush, D., Lan, R., Colles, F., Mensa, P., Wieler, L.H., Karch, H., Reeves, P.R., Maiden, M.C.J., Ochman, H., Achtman, M., 2006. Sex and virulence in *Escherichia coli*: an evolutionary perspective. Mol. Microbiol. 60, 1136–1151. https://doi.org/10.1111/j.1365-2958.2006.05172.x.

Yu, D., Banting, G., Neumann, N.F., 2021. A review of the taxonomy, genetics, and biology of the genus *Escherichia* and the type species *Escherichia coli*. Can. J. Microbiol. 67, 553–571. https://doi.org/10.1139/cjm-2020-0508.

Chapter 5.
Gut microbiota of endangered Australian sea lion pups is unchanged by topical ivermectin treatment for endemic hookworm infection

Australian sea lion mother and pup at Seal Bay, Kangaroo Island, April 2021.

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Gut microbiota of endangered Australian sea lion pups is unchanged by topical ivermectin treatment for endemic hookworm infection

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The gut microbiota is essential for the development and maintenance of the hosts' immune system. Disturbances to the gut microbiota in early life stages can result in long-lasting impacts on host health. This study aimed to determine if topical ivermectin treatment for endemic hookworm (*Uncinaria sanguinis*) infection in endangered Australian sea lion (*Neophoca cinerea*) pups resulted in gut microbial changes. The gut microbiota was characterised for untreated (control) (n=23) and treated (n=23) Australian sea lion pups sampled during the 2019 and 2020/21 breeding seasons at Seal Bay, Kangaroo Island. Samples were collected pre- and post-treatment on up to four occasions over a four-to-five-month period. The gut microbiota of untreated (control) and treated pups in both seasons was dominated by five bacterial phyla, *Fusobacteria*, *Firmicutes*, *Proteobacteria, Actinobacteria* and *Bacteroidetes*. A significant difference in alpha diversity between treatment groups was seen in pups sampled during the 2020/21 breeding season (p=0.008), with higher richness and diversity in treated pups. Modelling the impact of individual pup identification (ID), capture, pup weight (kg), standard length (cm), age and sex on beta diversity revealed that pup ID accounted for most of the variation (35% in 2019 and 42% in 2020/21), with pup ID, capture, and age being the only significant contributors to microbial variation (p<0.05). There were no statistically significant differences in the composition of the microbiota between treatment groups in both the 2019 and 2020/21 breeding seasons, indicating that topical ivermectin treatment did not alter the composition of the gut microbiota. To our knowledge, this is the first study to characterise the gut microbiota of free-ranging Australian pinniped pups, compare the composition across multiple time points, and to consider the impact of parasitic treatment on overall diversity and microbial composition of the gut microbiota. Importantly, the lack of compositional changes in the gut microbiota with treatment support the utility of topical ivermectin as a safe and minimally invasive management strategy to enhance pup survival in this endangered species.

KEYWORDS

microbiome, hookworm, ivermectin, pinniped, endangered

Introduction

The Australian sea lion is Australia's only endemic pinniped species and has a highly fragmented population, breeding across 80 colonies which extend from the Houtman Abrolhos in Western Australia to The Pages Islands in South Australia (Gales et al., 1994; Kirkwood and Goldsworthy, 2013). The Australian sea lion population has undergone continual decline since commercial harvesting in the 18th and 19th centuries (Ling, 1999). It is currently listed as endangered on both the IUCN Red List (Goldsworthy et al., 2015) and the Environmental Protection and Biodiversity Conservation (EPBC) ACT (Threatened Species Scientific Committee, 2020) with approximately 10,000 free-ranging individuals remaining (Goldsworthy et al., 2021), making it one of the rarest pinniped species in the world. The reasons for this decline are multifactorial, with fisheries interactions (Hamer et al., 2013), entanglement in marine debris (Page et al., 2004; Byard and Machado, 2019), anthropogenic pollution (antibiotic resistant and human-associated bacteria, persistent organic pollutants, and heavy metal contaminants; Fulham et al., 2020, 2022; Taylor et al., 2021, 2022), and disease (Marcus et al., 2014, 2015a) identified as contributing factors.

At two of the largest Australian sea lion breeding colonies, Seal Bay Conservation Park on Kangaroo Island and Dangerous Reef in the Spencer Gulf, high levels of pup mortality have been reported, with rates of up to 41.8 and 44.6%, respectively (Goldsworthy et al., 2007, 2019). Pup mortality within these colonies has been attributed to starvation, conspecific trauma, and stillbirths (McIntosh and Kennedy, 2013). Pups at both colonies are endemically infected with *Uncinaria sanguinis*, a haematophagous nematode (Marcus et al., 2014) that causes localised intestinal inflammation, anaemia and hypoproteinaemia (Marcus et al., 2015a). *Uncinaria sanguinis* infection is typically patent for 2–3 months, and after clearance of infection, pups do not become re-infected (Marcus et al., 2014). Given the continual population decline and high rates of pup mortality, recent conservation management for this species has included mitigation of pup mortality through hookworm treatment (Marcus et al., 2015b; Lindsay et al., 2021). Lindsay et al. (2021) determined that topical ivermectin treatment was 96.5% effective at eliminating hookworm with improved haematological parameters and increased bodyweight in treated compared to untreated (control) pups. Hookworm infection is also prevalent in other pinniped species, contributing to differing levels of clinical disease and mortality in northern fur seal (*Callorhinus ursinus*; DeLong et al., 2009; Lyons et al., 2011), California sea lion (*Zalophalus californianus*; Spraker et al., 2007), South American fur seal (*Arctocephalus australis*; Seguel et al., 2013, 2016) and New Zealand sea lion (*Phocarctos hookeri*; Castinel et al., 2007; Michael et al., 2019) pups. Ivermectin has been administered to New Zealand sea lion, northern fur seal, and South American fur seal pups to reduce hookworm-associated mortality, and in all studies treatment resulted in hookworm clearance and improved pup growth and survival (Castinel et al., 2007; DeLong et al., 2009; Seguel et al., 2016; Michael et al., 2021). However, previous studies have not considered the potential consequences of treatment intervention on the composition of the gut microbiota in pinniped pups.

The gut microbiota of numerous pinniped species has been characterised (Nelson et al., 2013; Smith et al., 2013; Bik et al., 2016; Pacheco-Sandoval et al., 2019; Stoffel et al., 2020; Tian et al., 2020; Toro-Valdivieso et al., 2021), including in adult Australian sea lions (Delport et al., 2016). In most of these pinniped species, the gut microbiota is usually dominated by the *Firmicutes, Bacteroidetes*, *Actinobacteria*, and *Proteobacteria* phyla, with the relative abundance differing between species and sampling site (Nelson et al., 2013; Bik et al., 2016; Delport et al., 2016; Pacheco-Sandoval et al., 2019). However, many of these studies have been limited to captive or adult pinnipeds, with the gut microbiota of pups only characterised in Juan Fernández fur seals (*Arctocephalus philippii*; Toro-Valdivieso et al., 2021), spotted seals (*Phoca largha*; Tian et al., 2020), Australian fur seals (*Arctocephalus pusillus doriferus*; Smith et al., 2013), and southern and northern elephant seals (*Mirounga leonina* and *Mirounga angustirostris*; Nelson et al., 2013; Stoffel et al., 2020).

The mammalian gastrointestinal tract is home to over 100 trillion microorganisms, collectively termed the microbiota (Clemente et al., 2012; Ursell et al., 2012; Thursby and Juge, 2017). Mammalian hosts and their microbiota have co-evolved over millions of years to establish a mutualistic relationship, and the importance of this relationship for host health has been established in recent decades (Hooper et al., 2012; Brestoff and Artis, 2013). Commensal bacteria contribute to digestion and nutrient provision (Hooper et al., 2002), synthesise essential vitamins and minerals (Yatsunenko et al., 2012), protect against pathogen colonisation through colonisation resistance (Bäumler and Sperandio, 2016) and are essential for the development and regulation of the immune system (Cebra, 1999; Brestoff and Artis, 2013).

The impact of intestinal parasites, such as helminths, on the composition of the gut microbiota remains relatively unknown, varying between host and parasite species (Walk et al., 2010; Rausch et al., 2013; Zaiss et al., 2015). Helminth infections in laboratory mice can result in substantial shifts in the gut microbiota composition (Li et al., 2012; Aivelo and Norberg, 2018; Peachey et al., 2018; Lee et al., 2020), which could be a consequence of multiple factors, including helminth secretory products (Zaiss et al., 2015). As intestinal helminths can both directly and indirectly interact with the gut microbiota, the removal of parasites *via* antiparasitic administration also has the potential to alter the composition of the microbial community (Giacomin et al., 2015; Reynolds et al., 2015). In humans and mice, the removal of intestinal helminths has varying impacts on the gut microbiota; outcomes of parasite treatment range from no associated change (Martin et al., 2018), minimal to moderate change in microbiota composition (Ramanan et al., 2016; Martin et al., 2018), to a shift in the composition of the microbiota to resemble individuals without helminth infection

(Jenkins et al., 2018). In wildlife species, treatment with antiparasiticides has been associated with alterations to the faecal gut microbiota and metabolic profile (He et al., 2018; Moustafa et al., 2021). Given the cross-talk between the gut microbiota and the immune system, it is likely that the modulation of the microbial community by intestinal helminths can have both direct and indirect impacts on the hosts' immune system and immune responses (Giacomin et al., 2015; Zaiss et al., 2015; Martin et al., 2019). Intestinal parasitism is prevalent in many free-ranging wildlife species (Spratt and Beveridge, 2019), for this reason, it is crucial to understand how parasitic infection (or removal thereof) influences gut microbial composition.

Just as microbes in the gut environment have evolved with their hosts, parasites can evolve with their hosts (Ebert and Fields, 2020), and it is likely *U. sanguinis* has evolved with its host species. Furthermore, interactions between the host, intestinal helminths and the gut microbiota are likely to be complex and multidirectional (Cortés et al., 2019). Whilst ivermectin treatment has been shown to improve Australian sea lion pup health parameters in the short term (Marcus et al., 2015b; Lindsay et al., 2021), the impact of removal of *U. sanguinis* on the gut microbial community, and potentially on pup health notwithstanding the improvements associated with mitigating hookworm disease, is unknown. The gut microbiota becomes established during early mammalian life stages, with disruptions or alterations potentially impacting the functional capacity of the microbiome, the development of the immune system (Johansson et al., 2012; Brestoff and Artis, 2013), altering the microbial composition, promoting colonisation of pathogenic bacteria, and most importantly, has the potential to influence lifelong host health and disease status (Guinane and Cotter, 2013; Schroeder and Bäckhed, 2016). Given the importance of the gut microbiota and its influence on development during early life stages, investigating whether treatment intervention and parasite elimination alters the gut microbiota of Australian sea lion pups is critical for understanding any potential consequences.

To establish the safety of a topical antiparasitic treatment as a potential management strategy to assist in the recovery of the Australian sea lion, the impact of treatment on the gut microbial community must be understood. To this end, this study aims to characterise and monitor the microbial composition of the gut microbiota of both untreated (control) and treated (and subsequently hookworm-free) Australian sea lion pups.

Materials and methods

Study site and sample collection

Faecal samples were collected from neonatal Australian sea lion pups at Seal Bay Conservation Park on Kangaroo Island, South Australia (35.99°S, 137.32°E). Pups were sampled during the 2019 winter ($n = 160$) and 2020/21 summer ($n = 184$) breeding seasons as part of an ivermectin treatment trial (Lindsay et al. unpublished).

In brief, pups were captured on up to four occasions, at approximately 4-week intervals. Pups were captured by hand and physically restrained in a ventilated canvas bag designed specifically for pinniped pups. During initial capture (capture 1 – the time point at which treatment intervention occurs but prior to impact of treatment), pups were assigned to an untreated (control) or treated (and subsequently hookworm-free, herein referred to as treated) group based on a randomised number chart, generated using Microsoft Excel. Morphometric data was collected from each pup during each capture event including bodyweight (kg), standard length (cm, measured from tip of the nose to tip of the tail), sex and body condition (poor, fair-thin, good, excellent). The initial capture also included a unique 'hair cut' on the dorsolumbar pelage and application of commercial hair dye to the hair cut (Schwarzkopf Nordic Blonde, Henkel Australia, Melbourne, Australia) to facilitate individual pup identification for recapture. Faecal swabs were collected *via* insertion of a sterile swab (Copan, Brescia, Italy) within a lubricated sheath into the rectum. Swabs were then subsampled into Sterile FecalSwab™ tubes (Copan, Brescia, Italy) and stored at 4°C for up to 2 months, followed by storage at −20°C for up to 2 months, and then at −80°C. Blood samples were collected from the brachial vein as previously described (Fulham et al., 2018, 2022). Up to 1 ml of blood was transferred into a tube containing ethylenediaminetetraacetic acid (EDTA; Sarstedt, Nümbrecht, Germany) for haematological analysis. Blood samples were stored at 4°C and processed within 10 h of collection. Sampling of Australian sea lion pups was approved by the Animal Ethics Committee at the University of Sydney (Protocol Number 2017/1260).

Of the total number of pups sampled in 2019 and 2020/21, a subset of pups ($n = 46$) that had been captured on four occasions were randomly selected from both the untreated (control) and treated groups by assigning a random number (Microsoft Excel for Mac v16.61.1) to each pup ID for inclusion in the microbial study.

Hookworm infection status

After collection, faecal swabs were kept at 4°C prior to storage at −20°C. Samples were processed within 14 days of collection. The hookworm status of each pup was determined following methods described by Lindsay et al. (2021). In summary, faecal material was transferred onto a clean glass slide and examined *via* light microscopy for the presence of *U. sanguinis* eggs. In samples where no eggs were detected, two additional smears were evaluated to confirm negative status. A grading system was used to grade hookworm burden in cases where samples were positive: grade 1 = 1–9 eggs, grade 2 = 10–19 eggs, grade 3 = 20–29 eggs, grade 4 ≥ 30 eggs (Lindsay, pers.

comm). Hookworm grade was determined using the mean value for the total number of smears performed for each individual.

Haematological analysis

Blood samples were processed following methodology described by Lindsay et al. (2021). In brief, the packed cell volume (PCV; L/L) was measured using the microhaematocrit method and total plasma protein (TPP; g/L) was estimated using a hand-held refractometer (Reichert TS Meter, Cambridge Instruments, Buffalo, United States). Blood smears were prepared in duplicate and fixed in 100% methanol (Chem-Supply Pty Ltd., Port Adelaide, South Australia) for 4 min. An aliquot (200 µl) of anticoagulated EDTA blood was transferred to a separate tube for preservation with an equal aliquot (200 µl) of Streck Cell preservative (Streck, Omaha, United States) and stored at 4°C prior to further analysis. Preserved samples were analysed on an automated haematology analyser (Sysmex XT-2000iV, Sysmex, Kobe, Japan) at the Veterinary Pathology Diagnostic Service, Sydney School of Veterinary Science, The University of Sydney, within 2–8 days of sample collection. From automated haematology analysis, the total erythrocyte count ($x10^{12}$/L), haemoglobin concentration (g/L), mean cell volume (fL), mean cell haemoglobin concentration (g/L), platelet count ($x10^{9}$/L) and total nucleated (leukocyte) cell count (TNCC, $\times10^{9}$/L) were determined. The differential leukocyte count was obtained by differentiating 100 leukocytes for every 10×10^{9}/L TNCC to determine the absolute neutrophil, lymphocyte, eosinophil and monocyte counts ($\times10^{9}$/L) by multiplying the percentage of each leukocyte by the TNCC. Nucleated red cell (nRBC) count was determined by counting the number of nucleated red blood cells per 100 leukocytes.

Age determination

As part of an ongoing monitoring program at Seal Bay Conservation Park, the breeding colony is frequently monitored during the breeding season, allowing birth dates to be recorded (Goldsworthy et al., 2014). Date of birth for pups was provided by the Department of Environment and Water (DEW), South Australia, and the South Australian Research and Development Institute (SARDI), as part of ongoing monitoring within each breeding season. For the 2020/21 breeding season, all pups included in the study had known dates of birth and age at each capture was calculated based on known birth date. For the 2019 breeding season, birth dates were only known for a subset of pups ($n=13$). For the remaining pups ($n=10$) sampled in 2019, a regression analysis was utilised to estimate pup age, following the approach of Bradshaw et al. (2000). The fitted age model for the 2019 data is:

$$\text{Age}\left[\text{days}\right]=\begin{pmatrix}-19.51+0.2825\times\left(\text{Length}\right)\\-0.0008976\times\left(\text{Sex}==\text{"Male"}\right)\\\times(\text{Length})+\left(5.3968\times\text{BCI}\right)\end{pmatrix}^{2}$$

where sex has the value of 1 for males and 0 for females, and BCI (body condition index) is calculated based on a regression of length on weight (Stokes et al., 2020).

DNA extraction and PCR amplification

From the randomised subset of pups, DNA was extracted from a total of 184 faecal swabs ($n=92$ from 2019 and $n=92$ from 2020/21). DNA was also extracted from two faecal samples collected from *N. cinerea* pups in 2019 to be used as control samples to account for variation between sequencing runs.

Faecal DNA was extracted from FecalSwab™ media (200 µl) using the ISOLATE II Fecal DNA Kit (Bioline, Sydney, Australia) following the manufacturer's protocol. Faecal DNA was tested for PCR competency by a 16S PCR using methods described by Fulham et al. (2020) using forward primer 27F and reverse primer 1492R (Lane, 1991). Amplicons were resolved using gel electrophoresis (2% agarose w/v) with SYBR safe gel stain (Invitrogen, Sydney, Australia) and conducted at 100 V for 30 min with product size approximated using HyperLadder II 50 bp DNA marker (Bioline, Sydney, Australia).

16S rRNA sequencing

Prior to sequencing, the concentration of DNA (ng/µl) and purity of nucleic acids in all faecal DNA was tested using a NanoDrop® ND-1000 UV–Vis Spectrophotometer (Thermo Fisher Scientific, Massachusetts, United States). DNA was submitted to the Ramaciotti Centre for Genomics (University of New South Wales, Sydney, Australia) for 16SV1-3 amplicon sequencing with an Illumina Miseq v3 2×300 bp sequencing kit using primers 27F and 519R, producing a ~ 530 bp fragment (Lane, 1991).

Analysis of sequences and taxonomic classification

Demultiplexed paired-end sequences were analysed using Quantitative Insights Into Microbial Ecology 2 (QIIME 2) version 2022.2 software (Bolyen et al., 2019). The DADA2 (Divisive Amplicon Denoising) plugin (Callahan et al., 2016) in QIIME 2 was used to trim and filter sequences for quality including the removal of primers, denoising and chimaera removal. Sequences were clustered into amplicon sequence variants (ASVs), also known as exact sequence variants, following denoising. Operational taxonomic units (OTUs) are also frequently used to

define the gut microbiota and clusters sequences either based on the observed sequences or using a reference database (Preheim et al., 2013). Recently, more focus has been on ASVs as they are more sensitive and specific than OTUs, can be reused across studies, and are reproducible for future data sets (Callahan et al., 2017).

Taxonomies were assigned to each ASV using a 16S rRNA V1-V3 classifier trained against the frequently updated SILVA database (release 138; Quast et al., 2013; Yilmaz et al., 2014; Glöckner et al., 2017) using the q2-feature-classifier plugin in QIIME 2 (Rognes et al., 2016). Low abundance ASVs that were not present in at least two samples with a total read count below 20 were filtered out and removed prior to further analysis.

Statistical analyses

All statistical analyses were conducted in either QIIME 2 or RStudio (v2022.2.3 + 492, Boston Massachusetts). Data was exported from QIIME 2 into R using the QIIME2R package (Hall and Beiko, 2018). Using both QIIME 2 and the vegan package (Oksanen et al., 2022), alpha diversity rarefaction curves were generated to filter samples based on sufficient sampling depth and data was rarefied with the threshold of 5,000 reads used as the cut-off for samples collected in both 2019 and 2020/21. Significance was determined when $p > 0.05$ for all statistical tests.

Mean pup age in untreated (control) and treated groups were calculated at each capture event for both breeding seasons. A paired sample t-test was used to determine whether there was a significant difference in pup age at each capture between treatment groups and breeding seasons.

For analysis of hookworm burden, the mean hookworm grade from samples collected from untreated (control) and treated pups during the initial capture was determined. A paired sample t-test was utilised to test for significant differences in hookworm grade between treatment groups at the time of first capture.

Alpha diversity analysis

The alpha (within-sample) diversity was estimated using the phyloseq package (McMurdie and Holmes, 2013). Richness, the total number of bacterial species present, and diversity, the amount of individual bacteria from each species identified, were measured using two metrics: Chao1, which estimates the richness in a sample through the estimation of the total number of species present (Chao, 1984); and the Shannon-Wiener index, which estimates diversity based on richness and abundance (Shannon, 1948). A Shapiro–Wilk test was used to test for data normality. Wilcoxon's Rank Sum Tests were employed to estimate the statistical differences in alpha diversity metrics due to treatment group (untreated [control] and treated) and breeding seasons (2019 and 2020/21). Statistical differences in alpha diversity pre-treatment (capture 1) and post-treatment (capture 2) for both treatment groups were also tested using Wilcoxon's Rank Sum Test.

To examine the correlations between host factors and alpha diversity, four linear mixed models (LMMs) were fitted using the lme4 package v1.1-25 (Bates et al., 2015). The first two models were conducted when analysing entire datasets within a season. The first model included treatment group, capture (1-4), pup sex, weight (kg), standard length (cm) and age as predictors and pup ID as a random effect, and the alpha diversity metric (Chao1 or Shannon-Wiener Index) as the response variable. In the second model, treatment group, hookworm status, age (months), TPP, TNCC, leukocyte counts (absolute neutrophil, absolute monocyte, absolute lymphocyte and absolute eosinophil; $\times 10^9$/L) were predictors, pup ID was a random effect, and the alpha diversity metric was the response variable. Models three and four tested the influence of host factors within treatment groups and were the same as models one and two, respectively, with treatment group excluded.

Beta diversity analysis

The variation between samples (beta diversity) was analysed using Bray–Curtis dissimilarity calculations using rarefied data based on the abundance of ASVs and using principal coordinate analysis (PCoA) plots. Using the adonis function in the vegan package (Oksanen et al., 2022), a permutational analysis of variance (PERMANOVA) was employed to test the statistical differences in ASV abundances due to treatment group, hookworm status, weight, standard length, sex, capture, and pup age, and computed with 999 permutations. Four PERMANOVA models were fitted; the first included pup ID, hookworm status and treatment group; the second model included pup ID, treatment group, capture, and age; the third model included treatment group, pup ID, weight, standard length and pup sex; and the final model included pup ID, treatment group, TPP, TNCC and leukocyte counts (neutrophil, lymphocyte, monocyte and eosinophil). The second model was compared *post hoc* to account for effects of repeated measures. The assumption of multivariate homogeneity was met across all models for PERMANOVA tests ($p > 0.05$).

Wilcoxon's Rank Sum Test was used to test for significant differences in the relative abundance of bacterial phyla and families between treatment groups and within and between breeding seasons. Finally, an analysis of composition of microbiomes (ANCOM) was used to test for differences in differentially abundant ASVs between treatment groups (Mandal et al., 2015), identifying any bacterial families that had the highest contribution to any dissimilarities observed in the gut microbial composition of untreated (control) and treated groups.

Results

Randomisation of the sampled pups resulted in the sequencing of samples from 12 untreated (control) and 11 treated pups from the 2019 breeding season, and 11 untreated (control) and 12

treated pups from the 2020/21 breeding season. Treatment with ivermectin resulted in 100% elimination of hookworm infection in the $n = 23$ Australian sea lion pups in the treated group (Lindsay et al. unpublished).

When comparing mean age of pups between untreated (control) and treated groups, there was no significant difference ($p > 0.05$) between groups at any of the capture events, in both the 2019 and 2020/21 cohorts (Table 1). There was, however, a significant difference in the age of treated pups at the third capture event between the 2019 and 2020/21 breeding seasons. There was no significant difference in the mean hookworm grade between untreated (control) and treated pups at first capture ($p > 0.05$) in both breeding seasons.

The hypervariable V1 to V3 region of the 16 s rRNA gene was sequenced in a total of 184 samples collected in 2019 and 2020/21, resulting in 12,078,034 and 8,439,797 sequences, respectively. After filtering steps and removal of low quality ($n = 32$) samples, 75 samples from 2019 (37 untreated [control], 38 treated) and 77 samples (38 untreated [control], 39 treated) from 2020/21 were imported into RStudio for analysis. The filtering of low abundance ASVs resulted in the clustering of 621 and 512 ASVs in the 2019 and 2020/21 datasets, respectively.

Gut microbiota composition of untreated (control) and treated Australian sea lion pups during the 2019 breeding season

Five bacterial phyla, *Fusobacteria*, *Firmicutes, Actinobacteria*, *Proteobacteria*, and *Bacteroidetes*, were present in untreated (control) and treated Australian sea lion pups (Figure 1). The majority of ASVs in both untreated (control) and treated pups were assigned to the *Fusobacteria* phylum (Table 2). In both groups, *Fusobacteria* had the highest average relative abundance (average relative abundance ± standard deviation = 55.4% ± 27.5 in untreated (control) and 61.3% ± 27.1 in treated), followed by *Firmicutes* (27.1% ± 12.9 and 20.5% ± 9.2), *Proteobacteria* (10.3% ± 4.3 and 9.2% ± 5.7), *Actinobacteria* (6.3% ± 3.3 and 6.5% ± 3.7) and *Bacteroidetes* (0.1% ± 0.1 in both treatment groups). There was no significant difference in the average relative abundance of each bacterial phyla pre- or post-treatment (capture 1-capture 2; $p > 0.05$) in both treatment groups and across captures 1 – 4 ($p > 0.05$). In both untreated (control) and treated pups, the relative abundance of *Fusobacteria* remained stable across all captures, whilst the abundance of *Firmicutes* decreased from capture 1 to capture 4 (Figure 1). The abundance of *Actinobacteria* increased after the second capture and then decreased in captures 3 and 4 in pups from both treatment groups, with the exception of one pup (pupID 19-141) in the untreated (control) group with a much greater abundance of *Actinobacteria* after capture 4 (Figure 1).

In both untreated (control) and treated pups, *Fusobacteriaceae* was the most dominant bacterial family. In untreated (control) pups, ASVs were assigned to five families from four bacterial phyla. The *Fusobacteriaceae* family occurred at the highest average relative abundance (54.1% ± 27.7), followed by families from the *Firmicutes* phyla, *Clostridiaceae* (17.8% ± 7.1) and *Ruminococcaceae* (7.4% ± 6.8), *Coriobacteriaceae* (4.4% ± 3.0) from the *Actinobacteria* phyla, and *Succinivibrionaceae* (2.7% ± 1.7) from

TABLE 1 Mean age (days) of pups in untreated (control) and treatment groups at each capture event during the 2019 and 2020/21 breeding seasons.

Capture event	Mean age (Days)	Standard deviation (Days)	Treatment group	Breeding season
1	16	7	Untreated (control)	2019
	17	7.5	Treated	2019
2	48	6	Untreated (control)	2019
	49	6	Treated	2019
3	86	11	Untreated (control)	2019
	78	6	Treated	2019
4	118	10	Untreated (control)	2019
	123	18	Treated	2019
1	13	6.5	Untreated (control)	2020/21
	13	4	Treated	2020/21
2	47	12.8	Untreated (control)	2020/21
	45	16.6	Treated	2020/21
3	84	13.9	Untreated (control)	2020/21
	75	16	Treated	2020/21
4	110	10	Untreated (control)	2020/21
	101	12	Treated	2020/21

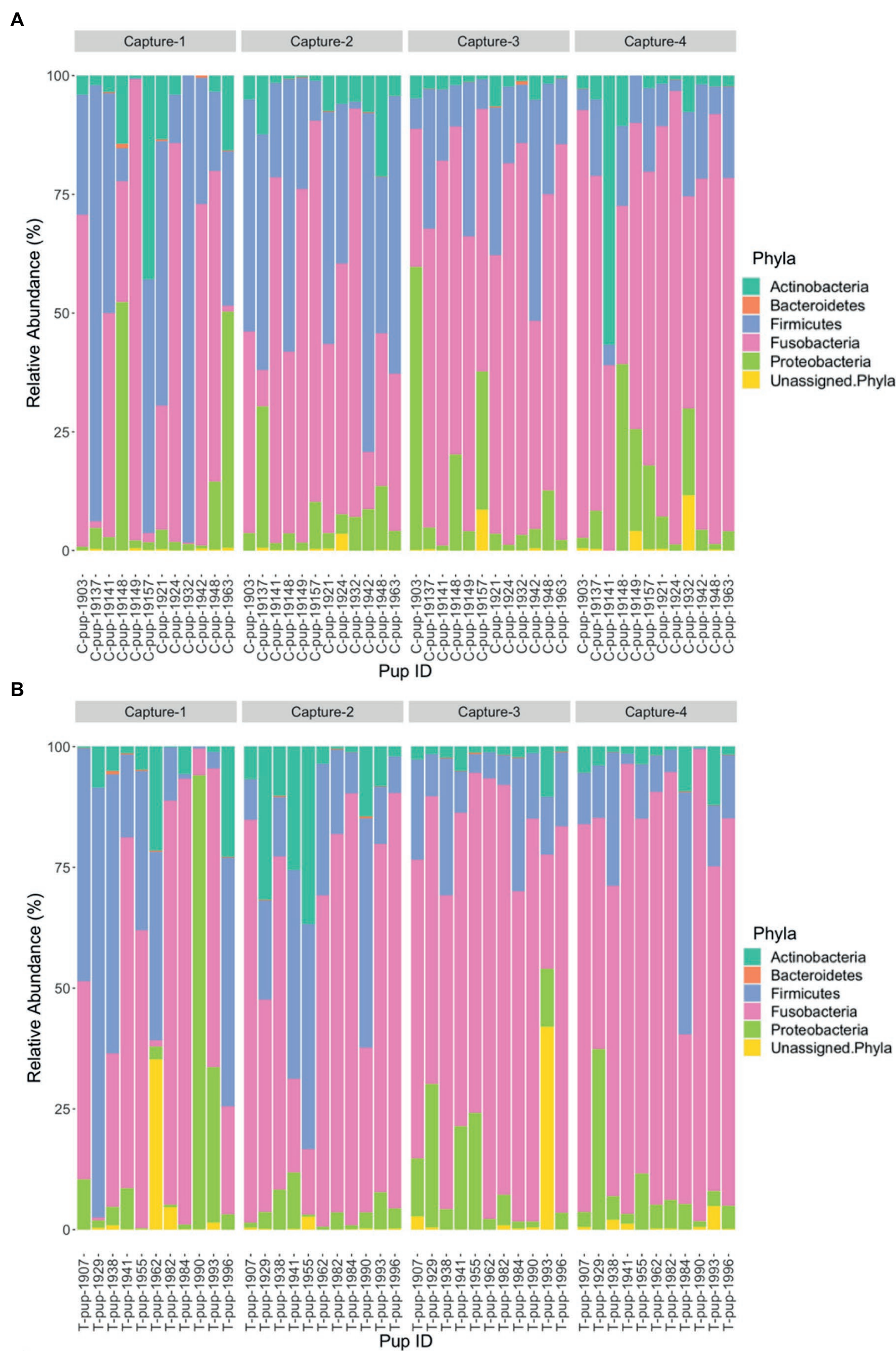

FIGURE 1
Relative abundance (%) of bacterial phyla in **(A)** untreated (control) and **(B)** treated Australian sea lion pups across four captures during the 2019 breeding season.

the *Proteobacteria* phyla. Seven bacterial families from four bacterial phyla were identified in treated pups, with *Fusobacteriaceae* being similarly dominant (55.2% ± 20.7). The *Clostridiaceae* family was also the second most abundant in treated pups (13.5% ± 9.4), followed by *Coriobacteriaceae* (5.7% ± 3.5), *Ruminococcaceae* (5.7% ± 4.1), *Succinivibrionaceae*

TABLE 2 Abundance of ASVs in untreated (control) and treated pups in captures 2–4, present in at least 90% of samples in each treatment group.

ASV	Phylum	Class	Family	Genus	Treatment group	Abundance (%)	Year
9ea04ed3882733d04922cb403e743965	Fusobacteria	Fusobacteriia	Fusobacteriaceae	Fusobacterium	Untreated (control)	6.1	2019
89f44d1e04224365282d4702046f1ce9	Fusobacteria	Fusobacteriia	Fusobacteriaceae	Fusobacterium	Untreated (control)	6	2019
147eb0d49c44569aa2f12c06fce289a0	Fusobacteria	Fusobacteriia	Fusobacteriaceae	Fusobacterium	Untreated (control)	4.7	2019
14ac82f71eee6441a2decab6f008b8e0	Actinobacteria	Coriobacteriia	Coriobacteriaceae	Collinsella	Untreated (control)	0.9	2019
147eb0d49c44569aa2f12c06fce289a0	Fusobacteria	Fusobacteriia	Fusobacteriaceae	Fusobacterium	Treated	8.8	2019
89f44d1e04224365282d4702046f1ce9	Fusobacteria	Fusobacteriia	Fusobacteriaceae	Fusobacterium	Treated	5.9	2019
9ea04ed3882733d04922cb403e743965	Fusobacteria	Fusobacteriia	Fusobacteriaceae	Fusobacterium	Treated	4.9	2019
89f44d1e04224365282d4702046f1ce9	Fusobacteria	Fusobacteriia	Fusobacteriaceae	Fusobacterium	Untreated (control)	3.5	2020/21
9ea04ed3882733d04922cb403e743965	Fusobacteria	Fusobacteriia	Fusobacteriaceae	Fusobacterium	Untreated (control)	3.1	2020/21
89f44d1e04224365282d4702046f1ce9	Fusobacteria	Fusobacteriia	Fusobacteriaceae	Fusobacterium	Treated	6.1	2020/21
9ea04ed3882733d04922cb403e743965	Fusobacteria	Fusobacteriia	Fusobacteriaceae	Fusobacterium	Treated	3.8	2020/21
d908d12d31a00d235b622945720d56bb	Firmicutes	Clostridia	Lachnospiraceae	Blautia	Treated	1	2020/21

The abundance of ASVs from first captures were excluded from both treatment groups to reflect any changes in abundance after treatment was administered.

(4.5% ± 2.3), *Rhodobacteraceae* (2.9% ± 1.1), a member of the *Proteobacteria* phyla, and *Leptotrichiaceae* (2.2% ± 0.5) a family belonging to *Fusobacteria* (Figure 2). There was no significant difference in the average relative abundance of each family between treatment groups ($p > 0.05$). The *Rhodobacteraceae*, *Leptotrichiaceae*, and *Succinivibrionaceae* families were only identified at an abundance ≥1% in ASVs in treated pups, with *Leptotrichiaceae* only present in treated pups after capture 4 (Figure 2).

Gut microbiota composition of untreated (control) and treated Australian sea lion pups during the 2020/21 breeding season

In Australian sea lion pups sampled during the 2020/21 breeding season, five bacterial phyla were identified. The gut microbiota of both untreated (control) and treated pups was dominated by *Fusobacteria*, which had the highest average relative abundance (38.6% ± 23.2 in untreated [control] and 42.7% ± 20.9 in treated pups), followed by *Firmicutes* (36.1% ± 20.4 and 36.3% ± 20.3), *Proteobacteria* (16.7% ± 8.3 and 14.7% ± 6.3), *Bacteroidetes* (5.0% ± 1.7 and 4.5% ± 1.2) and *Actinobacteria* (3.4% ± 1.2 and 1.1% ± 0.3). The majority of ASVs in both treatment groups were assigned to the phylum *Fusobacteria* (Table 2). A small percentage of ASVs could not be assigned to a bacterial phylum in both groups, 0.2% in untreated (control) and 0.7% in treated pups. The average relative abundance of each bacterial phyla did not change when compared pre- and post-treatment in the treated group ($p < 0.05$). There was no significant difference in the average relative abundance of each bacterial phyla between treatment groups and across captures 1 – 4 ($p > 0.05$). The relative abundance of *Fusobacteria* and *Firmicutes* decreased in both treatment groups across the four captures whilst *Proteobacteria* and *Actinobacteria* increased (Figure 3). The abundance of *Bacteroidetes* was greatest in samples from capture 4 in both treatment groups (Figure 3).

At the family level, seven bacterial families were present in untreated (control) pups and five families were identified in treated pups that had a total abundance ≥1%. In untreated (control) pups, the average relative abundance was highest for the *Fusobacteriaceae* family (39.4% ± 23.9), followed by families belonging to the *Firmicutes* phyla, *Lachnospiraceae* (19.3% ± 7.6), *Clostridiaceae* (8.9% ± 3.1) and *Ruminococcacae* (5.0% ± 2.2), *Halomonadaceae* (7.5% ± 1.5) and *Rhizobioaceae* (3.1% ± 0.9) from the *Proteobacteria* phyla, and *Flavobacteriaceae* (3.2% ± 1.0) from the *Bacteroidetes* phyla. The five families assigned to reads from treated pups were similarly abundant, with *Fusobacteriaceae* being the most dominant (42.4% ± 27.1), followed by *Lachnospiraceae* (18.9% ± 10.2), *Clostridiaceae* (10.3% ± 7.7), *Halomonadaceae* (6.0% ± 5.8) and *Ruminococcaceae* (6.0% ± 5.0). Whilst the abundance of *Fusobacteriaceae* decreased from capture 1 to capture 4 in both treatment groups, a more noticeable decrease in *Clostridiaceae* was apparent in treated pups after capture 1 (Figure 4). The *Flavobacteriaceae* and *Rhizobiaceae* families were only present in untreated (control) pups in captures 3 and 4 (Figure 4).

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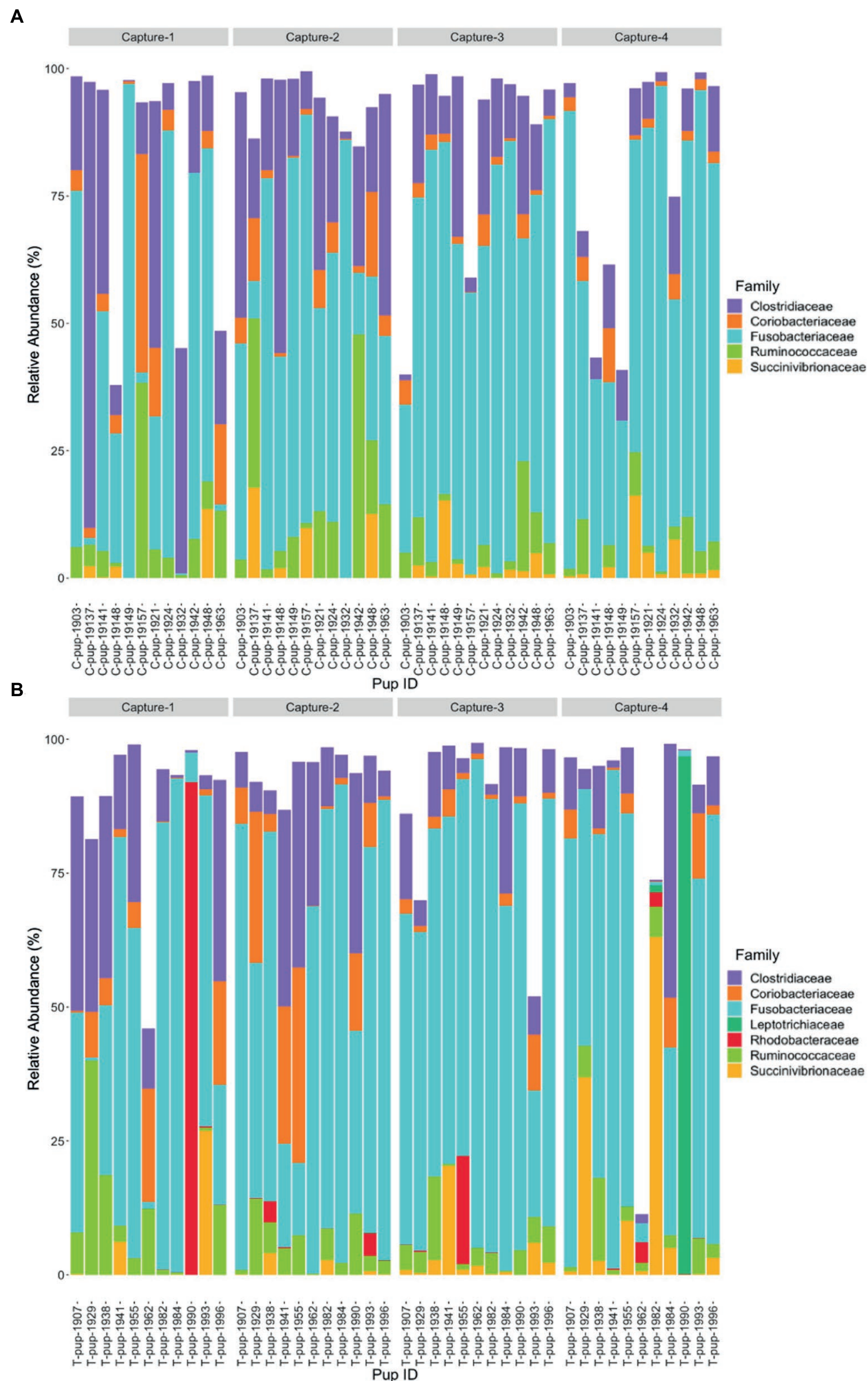

FIGURE 2
Relative abundance (%) of bacterial families in **(A)** untreated (control) and **(B)** treated Australian sea lion pups across all four captures during the 2019 breeding season. Only families that occurred at an abundance ≥1% in each treatment group are shown.

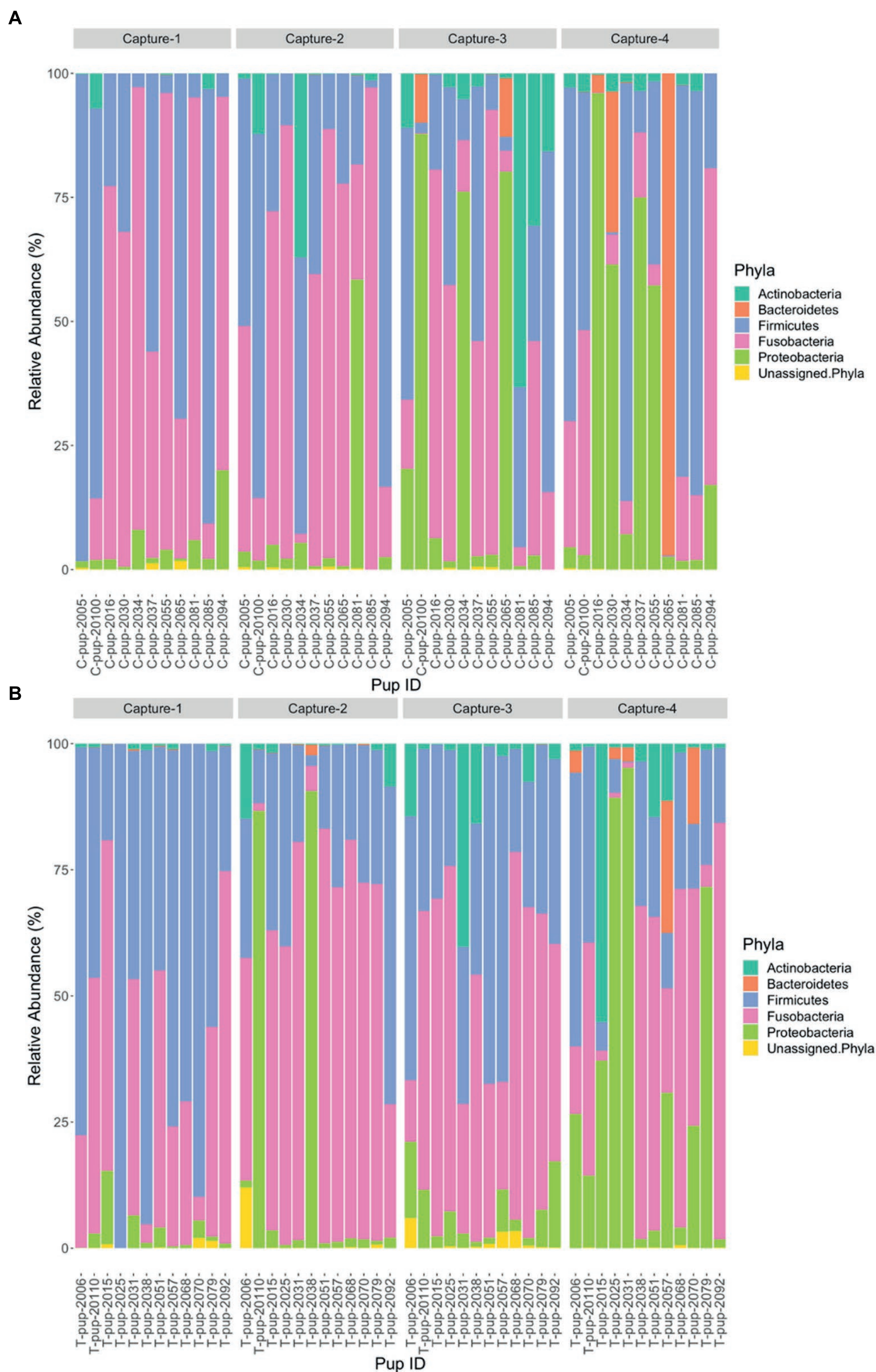

FIGURE 3
Relative abundance (%) of bacterial phyla in **(A)** untreated (control) and **(B)** treated Australian sea lion pups across the four captures during the 2020/21 breeding season.

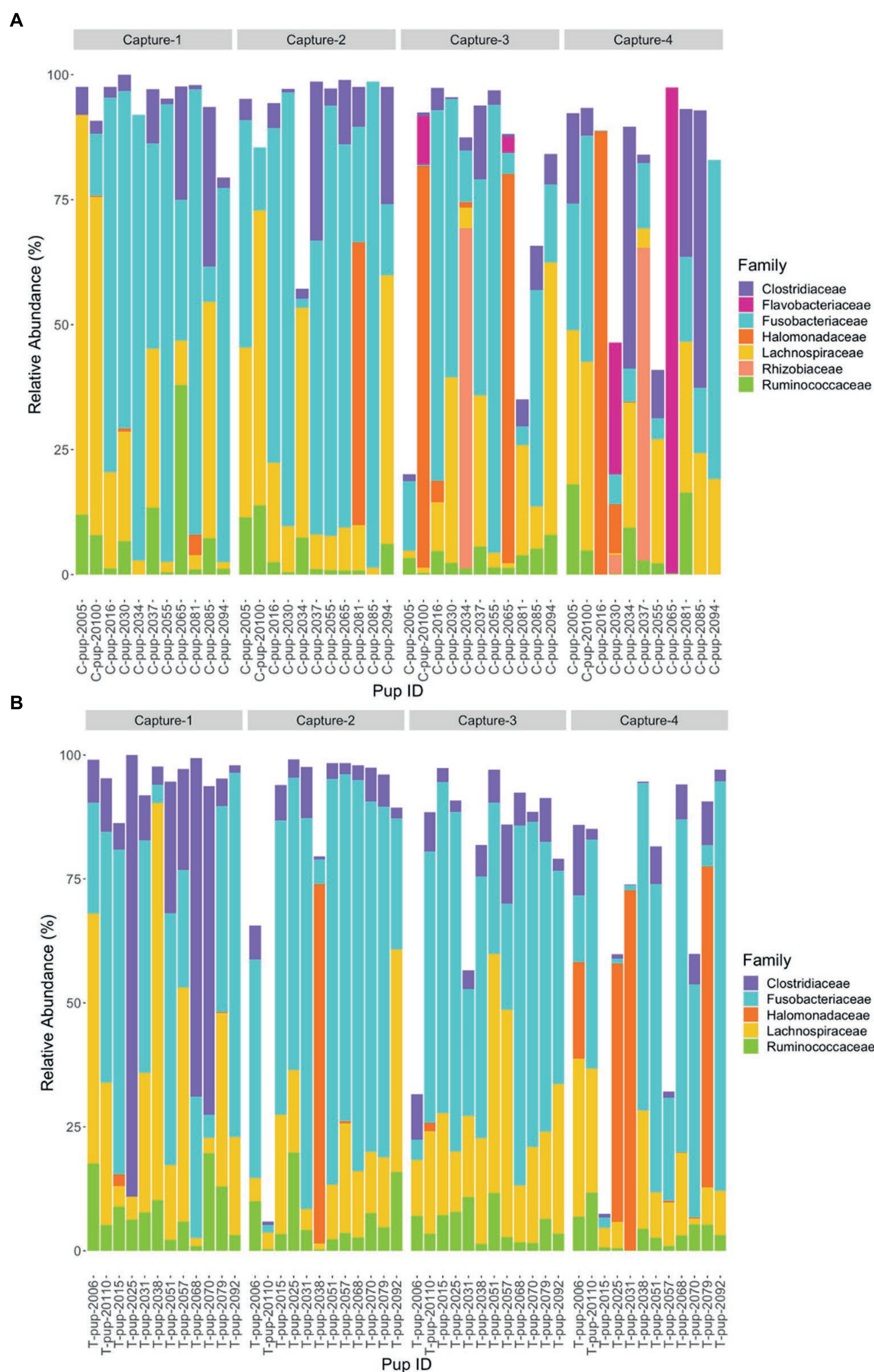

FIGURE 4
Relative abundance (%) of bacterial families in **(A)** untreated (control) and **(B)** treated Australian sea lion pups over four captures, sampled during the 2020/21 breeding season. Only families that occurred at an abundance ≥1% in each treatment group are shown.

Comparison of gut microbiota composition between breeding seasons

Five bacterial phyla were identified in both untreated (control) and treated pups in both breeding seasons. In both 2019 and 2020/21, the majority of ASV counts belonged to three phyla: *Fusobacteria* (53.5 and 39.0%), *Firmicutes* (22.1 and 33.8%) and *Proteobacteria* (9.6 and 14.4%). The *Actinobacteria* phyla was more dominant in 2019 (5.8 and 2.1%) whilst *Bacteroidetes* was more dominant in 2020/21 (0.1 and 4.4%). A small percent of ASVs, 1.6 and 0.43%, could not be assigned to a bacterial phylum in 2019 and 2020/21, respectively. There was a significant difference in the relative abundance of *Fusobacteria* ($p < 0.001$), *Firmicutes* ($p = 0.0021$), *Actinobacteria* ($p < 0.0001$) and ASVs that could not be assigned to a phylum ($p = 0.001$) between breeding seasons. The average relative abundance of *Fusobacteria* and *Actinobacteria* were higher in 2019 (58.6 ± 27.2 and 6.30 ± 3.43) compared to 2020/21 (41.4 ± 20.1 and 4.4 ± 2.3), whilst the opposite was observed for *Firmicutes* (23.8 ± 11.1 in 2019 and 33.8 ± 16.0 in 2020/21). There was no significant difference in the relative abundance of *Bacteroidetes* ($p = 0.11$) or *Proteobacteria* ($p = 0.29$) across the 2019 and 2020/21 breeding seasons.

When comparing the relative abundance of each phylum in untreated (control) pups across seasons, there was only a significant difference in *Fusobacteria* ($p = 0.021$) and *Actinobacteria* ($p = 0.0023$), with a greater relative abundance of both phyla in 2019. In treated pups, there was a significant difference between breeding seasons in the relative abundance of *Fusobacteria* ($p < 0.01$), *Firmicutes* ($p < 0.01$) and *Actinobacteria* ($p < 0.01$). The relative abundance of *Fusobacteria* and *Actinobacteria* was higher in treated pups in 2019, whilst *Firmicutes* occurred at a higher relative abundance in 2020/21.

Three families, *Fusobacteriaceae*, *Clostridiaceae* and *Ruminococcaceae* were present in pups in both breeding seasons. There was a significant difference in the relative abundance of *Fusobacteriaceae* ($p = 0.001$) and *Clostridiaceae* ($p < 0.001$) between the 2019 and 2020/21 breeding seasons. In 2019, the relative abundance of *Fusobacteriaceae* and *Clostridiaceae* were significantly higher than in 2020/21 in both untreated (control) pups ($p = 0.001$ and $p < 0.001$) and treated pups ($p = 0.018$ and $p = 0.041$). For both treatment groups, there was no significant difference in the relative abundance of *Ruminococcaceae* ($p > 0.05$).

Alpha diversity

Comparison of alpha diversity between treatment groups and breeding seasons

Two measures of alpha diversity (Chao1 and Shannon-Wiener Index) were used to analyse differences in within-sample diversity between treatment groups and breeding seasons. To determine any immediate differences post-treatment, alpha diversity was compared between capture 1 and capture 2 for each cohort (2019 and 2020/21) as well as within each treatment group.

TABLE 3 Mean Chao1 index and Shannon-Wiener indices for untreated (control) and treated groups, and cohorts as a whole (combined) in both the 2019 and 2020/21 breeding seasons.

Alpha diversity metric	Mean	Standard deviation	Treatment group	Breeding season
Chao1 Index	92.66	49.04	Combined	2019
	89.33	50.51	Untreated (control)	2019
	97.51	47.61	Treated	2019
	69.48	33.87	Combined	2020
	62.33	32.91	Untreated (control)	2020
	76.51	33.64	Treated	2020
Shannon-Wiener Index	3.77	0.68	Combined	2019
	3.85	0.63	Untreated (control)	2019
	3.68	0.74	Treated	2019
	3.96	0.76	Combined	2020
	3.66	0.86	Untreated (control)	2020
	4.22	0.54	Treated	2020

In the 2019 cohort, the mean diversity, as indicated by the Shannon-Wiener index, was higher in the untreated (control) group compared to the treated group, whilst richness, measured through the Chao1 index, was higher in treated pups (Table 3 and Figure 5). There was no significant difference in alpha diversity between treatment groups based on the Chao1 index ($p = 0.157$) and the Shannon-Wiener index ($p = 0.159$). No significant differences in alpha diversity were observed between capture 1 and capture 2 in the untreated (control) and treated groups in the Chao1 index ($p = 0.318$ and $p = 0.558$) and the Shannon-Wiener index ($p = 1.00$ and $p = 0.644$).

The mean diversity in the 2020/21 cohort was higher in the treated group (Table 3 and Figure 5), which was reflected in the significant difference in the Shannon-Wiener index ($p = 0.008$). Richness was also, on average, higher in the treated group (Table 3), however, there was no significant difference in the Chao1 index between treatment groups ($p = 0.104$) or in alpha diversity between capture 1 and capture 2 for both the untreated (control) and treated groups for the Chao1 index ($p = 0.250$ and $p = 0.193$) and the Shannon-Wiener index ($p = 0.693$ and $p = 0.977$).

There was a significant difference in both the Chao1 index ($p = 0.001$) and the Shannon-Wiener index ($p = 0.017$) between the 2019 and 2020/21 breeding seasons. On average, richness was greater in the 2019 cohort whilst diversity was greater in the 2020/21 cohort (Table 3). Diversity and richness were also compared in treatment groups between breeding seasons. In the untreated (control) groups, there was no significant difference in diversity ($p = 0.215$) between seasons, however, there was a

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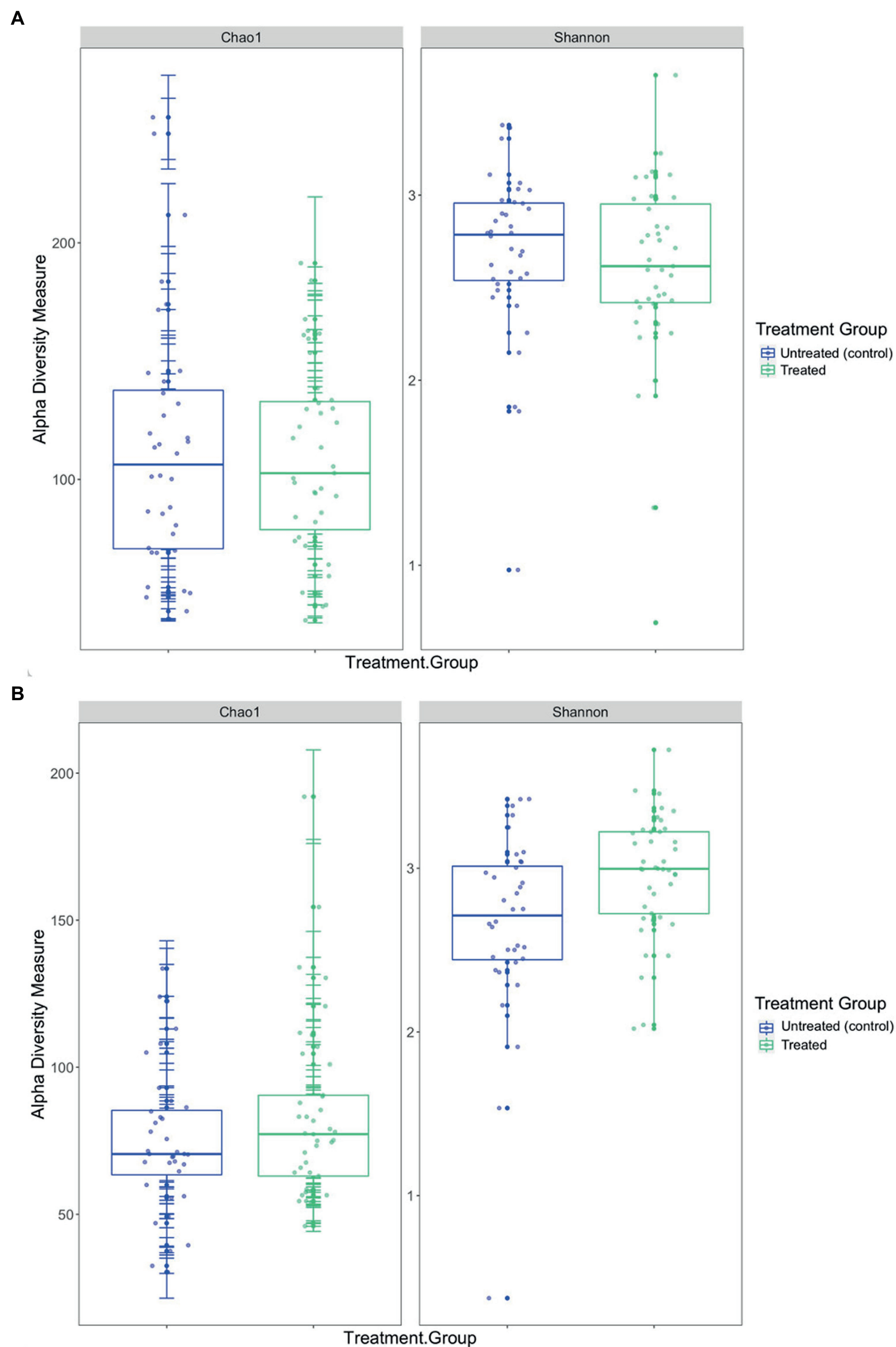

FIGURE 5
Comparison of the two alpha diversity measures, Chao1 index and the Shannon-Wiener index between treatment groups in the **(A)** 2019 breeding season and **(B)** 2020/21 breeding season.

significant difference in richness ($p = 0.013$), with higher richness observed in the 2019 untreated (control) group (Table 3). There was a significant difference in both the Chao1 index ($p = 0.025$) and the Shannon-Wiener index ($p = 0.001$) between the treated groups between breeding seasons, with higher diversity in 2020/21.

Effect of treatment and pup morphometrics on alpha diversity

When analysing Australian sea lion pups sampled during the 2019 breeding season as a cohort using the first LMM, there was a significant correlation between capture and Chao1 ($R^2 = 0.134$, $p = 0.048$; Supplementary Table 1). The richness of the gut microbiota fluctuated across captures, with the highest mean richness observed in samples collected during capture 3 (101 ± 5.21). No significant correlations between any of the variables included in the first model and the Shannon-Wiener index were identified (Supplementary Table 1). The correlation between pup morphometrics and alpha diversity was also investigated within each treatment group using the third LMM, which included the same factors as model 1 with treatment group excluded. In untreated (control) pups, there was no significant correlation between the Chao1 index and pup morphometrics, however, there was a significant correlation between capture and the Shannon-Wiener index ($R^2 = 0.176$, $p = 0.011$; Supplementary Table 2). The mean diversity varied across captures and was highest after capture 3 (4.11 ± 0.38) before decreasing by capture 4 (3.74 ± 0.61). In Australian sea lion pups treated in the 2019 breeding season, there was no significant correlation between pup morphometrics and both the Chao1 index and the Shannon-Wiener index (Supplementary Table 2).

In the 2020/21 cohort, there was a significant correlation between treatment group and both the Chao1 index ($R^2 = 0.107$, $p = 0.043$) and the Shannon-Wiener index ($R^2 = 0.198$, $p = 0.002$; Supplementary Table 1). The mean values of richness and diversity were higher in the treated compared to the untreated (control) group (Supplementary Table 1). However, within the untreated (control) and treated groups there was no significant correlation between pup morphometrics and alpha diversity (Supplementary Table 2).

Relationship between treatment group, haematological parameters and alpha diversity

In the 2019 cohort, there was a significant correlation between capture and the Chao1 index ($R^2 = 0.134$, $p = 0.013$) in the second LMM (Supplementary Table 1); significant correlations between the Chao1 index and any of the other variables, including treatment group, were not identified. As with the results of model 1, there was no significant correlation with any factors and the Shannon-Wiener index. In the fourth model, which determined correlations between haematological parameters and alpha diversity within each treatment group, there were no significant correlations observed for the Chao1 index in either treatment group (Supplementary Table 2). There was a significant correlation between capture and the Shannon-Wiener index in the untreated (control) group ($R^2 = 0.257$, $p = 0.019$); there were no significant correlations for the Shannon-Wiener index in the treated group. There was no significant correlation or relationship between haematological parameters and alpha diversity in either treatment group in the 2019 breeding season.

Analysis using the second fitted LMM found a significant correlation between treatment and both the Chao1 index ($R^2 = 0.135$, $p = 0.015$) and the Shannon-Wiener index ($R^2 = 0.200$, $p = 0.04$) in the 2020/21 breeding season (Supplementary Table 1). Within the untreated (control) and treated groups during the 2020/21 season there was no significant correlation between any factors included in model four and richness or diversity (Supplementary Table 1).

Beta diversity

Beta diversity in the 2019 breeding season

Based on analysis of the differences in ASV abundance through the Bray–Curtis dissimilarity, 13.8% of the variation in the spread of the data could be explained by the first axis, and 8.7% could be explained by the second axis (Figure 6). There was no clear clustering of the data based on treatment group, indicating that dissimilarity between samples was not based on the treatment status of the individual (Figure 6). The results from the PERMANOVA models indicate that in pups sampled during the 2019 breeding season, pup ID was the most significant predictor of microbial similarity and accounted for most of the variation (Table 4). The capture event was also a significant predictor ($p = 0.02$), along with pup ID ($p = 0.001$) in the second model. The results from all models, presented in Table 4, determined that treatment group, hookworm status, age, pup weight, standard length and haematological parameters (TPP, TNCC, absolute neutrophil count, absolute lymphocyte count, absolute monocyte count and absolute eosinophil count), were all non-significant predictors of microbial similarity.

There was a difference in the number of bacterial families identified between treatment groups, with five and seven in untreated (control) and treated groups, respectively. However, ANCOM analysis indicated that these unique features were not present at a high enough abundance to be detected as differentially abundant at the family or genus level between treatment groups.

Beta diversity in the 2020/21 breeding season

The analysis of the beta diversity of the gut microbial community of pups sampled during the 2020/21 breeding season revealed that the first and second axis explained 13.1 and 7.6% of the variation, respectively (Figure 7). From the four PERMANOVA models, pup ID was the most significant predictor of similarity and accounted for most of the variation in each model (Table 4). In the second model, both pup age and capture were also significant predictors ($p = 0.02$). The contribution of capture and age to similarity can be visualised in the Bray–Curtis dissimilarity matrix, with samples collected at similar captures from pups of closer age groups clustering together (Figure 7).

In the 2020/21 breeding season, seven and five bacterial families were detected in samples collected from untreated (control) and treated pups, respectively. Whilst there was a qualitative difference between the two groups, ANCOM analysis revealed that the features assigned to families only identified in

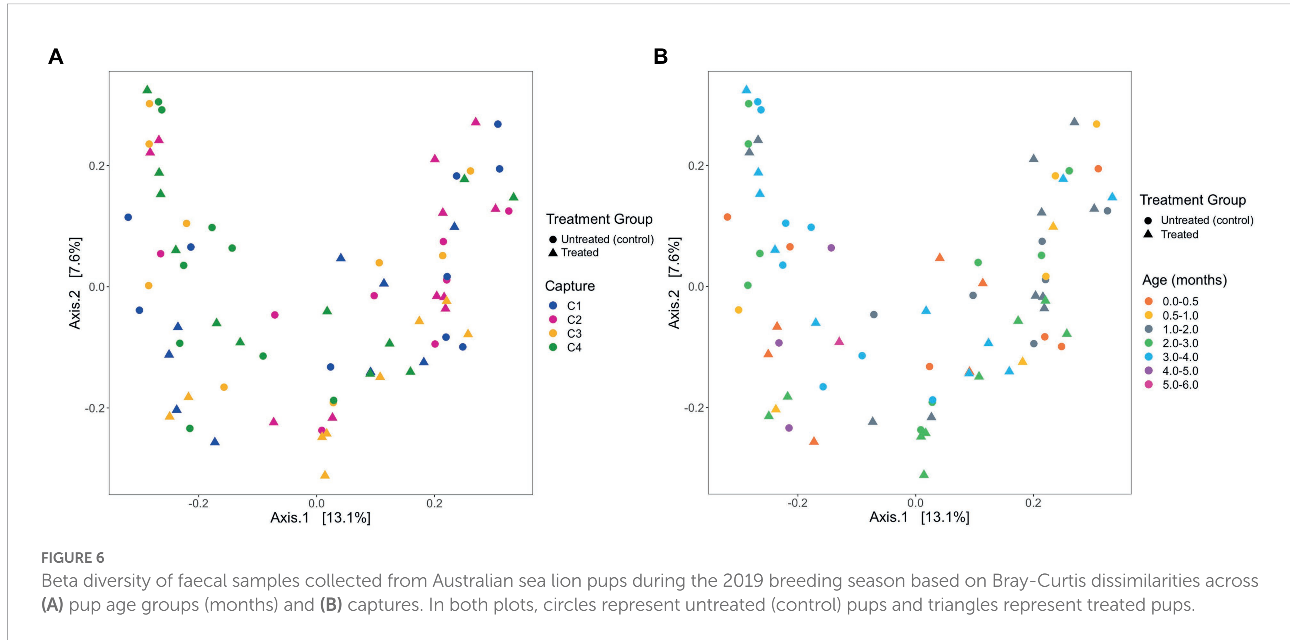

FIGURE 6
Beta diversity of faecal samples collected from Australian sea lion pups during the 2019 breeding season based on Bray-Curtis dissimilarities across **(A)** pup age groups (months) and **(B)** captures. In both plots, circles represent untreated (control) pups and triangles represent treated pups.

untreated (control) pups were not present at a high enough abundance to be considered differentially abundant between treatment groups.

Discussion

This study aimed to determine whether topical ivermectin treatment and elimination of *U. sanguinis* infection altered the gut microbiota in endangered Australian sea lion pups. The findings from this study indicate that topical ivermectin treatment does not result in compositional change in the gut microbiota of Australian sea lion pups, suggesting that treatment does not cause any significant change to the functional capacity of the gut microbiome. This knowledge is crucial when considering the safety and efficacy of antiparasitic treatment in a free-ranging population, given the profound impact that the gut microbiota can have on host development and health through the regulation of the immune system, digestion and by protecting the host against pathogens (Ley et al., 2008a, 2008b; Lozupone et al., 2012; Margaret et al., 2013).

It was hypothesised that treatment with topical ivermectin and subsequent elimination of *U. sanguinis* would alter the gut microbiota of Australian sea lion pups when compared pre- and post-treatment. However, in both the untreated (control) and treated groups in both breeding seasons, there were no significant differences in the composition of the gut microbiota between pre-treatment (capture 1) and post-treatment (capture 2) time points. Given the endemic nature of *U. sanguinis* infection in Australian sea lion pups at Seal Bay (Marcus et al., 2014), the composition of a 'healthy' gut microbiota or gut microbiota without parasite infestation is unknown. However, as pups are infected with *U. sanguinis via* the colostrum immediately after birth (Marcus et al., 2014), it is unlikely that the gut microbiota of an Australian sea lion without infection with *U. sanguinis* would be observed. There were no compositional changes in the gut microbiota seen in association with either the natural elimination of hookworm in untreated (control) pups or *via* administration of topical ivermectin in treated pups. This absence of a change in microbial composition could suggest that the gut microbiota of Australian sea lion pups has adapted to the presence of this endemic parasite over their long symbiotic association, with the same composition of gut microbiota attained regardless of the speed at which infection is cleared. Whilst understanding the gut microbiota of Australian sea lion pups has adapted to *U. sanguinis* is an important knowledge gap, it should be noted that the outcomes of *U. sanguinis* infection for Australian sea lion pups are either to succumb to infection or to naturally clear the parasite at approximately 2 months of age (Marcus et al., 2014). Additionally, no other macroscopic parasites have previously been observed in both live or dead Australian sea lion pups (Marcus et al., 2014).

For both treatment groups in 2019 and 2020/21, the gut microbial communities were characterised by five bacterial phyla: *Fusobacteria*, *Firmicutes*, *Proteobacteria*, *Actinobacteria*, and *Bacteroidetes*, and were dominated by *Fusobacteria*. These phyla have previously been identified as the main phyla in the gut of numerous pinniped species (Nelson et al., 2013; Smith et al., 2013; Bik et al., 2016; Delport et al., 2016; Medeiros et al., 2016; Pacheco-Sandoval et al., 2019; Stoffel et al., 2020; Tian et al., 2020; Toro-Valdivieso et al., 2021). The relative abundance of each bacterial phylum differs between species and is likely influenced by differences in life histories, geographical ranges, environmental conditions, diet, and sampling techniques.

The outcome of helminth treatment on the gut microbiota of the host has been studied in humans, mice, and numerous wildlife species with varying results (Cooper et al., 2013; Ramanan et al., 2016; He et al., 2018; Jenkins et al., 2018; Peachey et al., 2018; Moustafa et al., 2021). Some human studies observed no effect

TABLE 4 Results from PERMANOVA analysis from both 2019 and 2020/21 breeding seasons, with variables included in each model, variation accounted for by each variable (R^2) and *p*-value.

PERMANOVA Model	Variable	R^2 value	*p*-value	Breeding season
1	Pup ID	0.42	0.001*	2019
	Treatment group	0.015	0.12	
	Hookworm status	0.027	0.12	
2	Pup ID	0.42	0.001*	
	Treatment group	0.015	0.12	
	Capture	0.033	0.001*	
	Age	0.075	0.13	
3	Pup ID	0.41	0.001*	
	Treatment Group	0.015	0.11	
	Weight	0.065	0.12	
	Standard length	0.065	0.56	
	Sex	0.012	0.37	
4	Pup ID	0.32	0.002*	
	Treatment group	0.014	0.12	
	Total plasma protein	0.14	0.09	
	Total nucleated cell count	0.79	0.27	
	Absolute neutrophil count	0.049	0.28	
	Absolute lymphocyte count	0.045	0.26	
	Absolute monocyte count	0.043	0.43	
	Absolute eosinophil count	0.023	0.075	
1	Pup ID	0.35	0.001*	2020/21
	Treatment group	0.018	0.08	
	Hookworm status	0.023	0.048	
2	Pup ID	0.35	0.001*	
	Treatment group	0.016	0.074	
	Capture	0.055	0.02*	
	Age	0.72	0.02*	
3	Pup ID	0.35	0.001*	
	Treatment group	0.016	0.075	
	Weight	0.12	0.098	
	Standard length	0.088	0.19	
	Sex	0.016	0.073	
4	Pup ID	0.29	0.035*	
	Treatment group	0.014	0.074	
	Total plasma protein	0.022	0.32	
	Total nucleated cell count	0.092	0.23	
	Absolute neutrophil count	0.058	0.58	
	Absolute lymphocyte count	0.031	0.54	
	Absolute monocyte count	0.012	0.68	
	Absolute eosinophil count	0.023	0.75	

*Indicates significant result ($p < 0.05$).

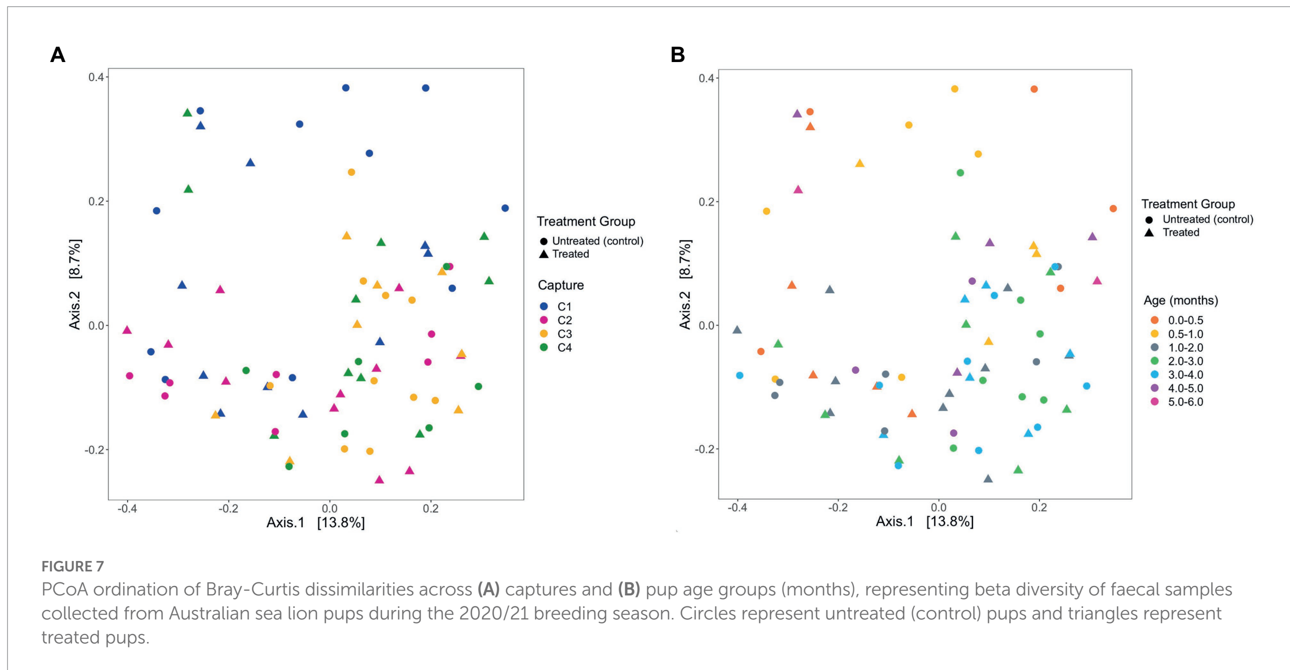

FIGURE 7
PCoA ordination of Bray-Curtis dissimilarities across **(A)** captures and **(B)** pup age groups (months), representing beta diversity of faecal samples collected from Australian sea lion pups during the 2020/21 breeding season. Circles represent untreated (control) pups and triangles represent treated pups.

between treated and untreated individuals pre- and post-treatment (Cooper et al., 2013; Ramanan et al., 2016; Rosa et al., 2018), whilst others found compositional changes and reduced alpha diversity in individuals' post-treatment (Jenkins et al., 2018). In this study, the only significant difference in alpha diversity correlated with treatment group was observed in Australian sea lion pups treated during the 2020/21 breeding season. Both the Chao1 and Shannon-Wiener indices were higher in treated pups compared to untreated (control) pups. However, in treated pups, there was no significant difference in alpha diversity between pre- and post-treatment time points (capture 1 versus capture 2), suggesting that the removal of hookworm infection after treatment is not the cause of the significant difference observed. Furthermore, hookworm status was not associated with significant changes in alpha diversity in either of the treatment groups.

Despite differences in richness and diversity of microbial community in Australian sea lion pups, there were no significant differences in microbial community composition between treatment groups, which is attributed to the topical method of administration. In Amur tigers (*Panthera tigris altaica*) treated with oral Fenbendazole and ivermectin, significant changes in both the gut microbiota and faecal metabolic phenotypes were seen, suggesting that treatment both disturbed the microbial community within the gut as well as metabolic homeostasis (He et al., 2018). Significant differences in gut microbial composition and diversity were also observed in Asian elephants (*Elephas maximus*) administered oral Albendazole treatment (Moustafa et al., 2021) and in humans treated with oral ivermectin for helminth infection (Jenkins et al., 2018).

Parasite presence in the gastrointestinal tracts of humans, livestock and wildlife species have been found to have differing impacts on gut microbial diversity, largely dependent on host and parasite species (Giacomin et al., 2015; Reynolds et al., 2015; Zaiss et al., 2015). In the present study, there was no clear association between the presence of *U. sanguinis* and taxonomic assignment of ASVs or microbial diversity in both treatment groups regardless of breeding season. Investigations into the relationship between parasite burden and the composition of the gut microbiota have produced mixed results. In western lowland gorillas (*Gorilla gorilla gorilla*), there was no relationship found between the number of eggs per gram (EPG) in faeces and the overall diversity and composition of the gut microbiota (Vlčková et al., 2018). In contrast, studies in both zebrafish (*Danio rerio*) and mice documented changes in both alpha diversity and the composition of the gut microbiome corresponding to parasite burden (Gaulke et al., 2019; Guiver et al., 2022). In minimally invasive and non-invasive studies it is difficult to determine the burden of parasite infection as the number of eggs observed in faeces is often not representative of the burden of adult worms in the intestine (Anderson and Schad, 1985; Warnick, 1992). In this study, a semi-quantitative evaluation of hookworm intestinal burden was used. The grading system suggested that the infection intensity of *U. sanguinis* was not significantly different between untreated (control) and treated pups, prior to the administration of treatment. Despite the lack of microbial composition changes associated with hookworm removal, it is possible that changes to the microbiota are occurring at the site of infection in the small intestine (Marcus et al., 2014) but these changes are not observed in the faecal microbiota. Furthermore, there is the potential for pups with higher parasite burdens to experience greater localised changes at the site of infection, such that the interpretation of these results should take this into consideration.

In pinnipeds, the relative abundance of *Fusobacteria* appears to be highest in pups and decreases with age (Nelson et al., 2013; Stoffel et al., 2020; Tian et al., 2020). For example, in southern elephant seals, there was a significantly higher abundance of

Fusobacteria in pups compared to sub-adults and adults (Nelson et al., 2013), and the relative abundance of *Fusobacteria* has been found to decrease steadily during the weaning period of Pacific harbour seals (Stoffel et al., 2020). Changes in bacterial phyla abundance was also reported with age in Australian fur seals, with significant shifts observed between pup, juvenile and adult life stages (Smith et al., 2013). In Australian sea lion pups, a shift in the relative abundance of bacterial phyla and families across capture events was observed, although this shift is likely an age-related change, with up to 5 months between first and fourth captures. The results from the Bray–Curtis dissimilarity matrix suggested that the gut microbiota was more similar in pups of the same age, regardless of treatment group. A higher relative abundance of *Fusobacteria* was also observed in the current study in both treatment groups between the 2019 and 2020/21 breeding seasons, where *Fusobacteria* was the most abundant bacterial phyla. Bacterial families belonging to the *Fusobacteria* phylum were also the most dominant in the gut microbiota of Australian sea lion pups between both breeding seasons. The trends observed in the 2020/21 breeding season also suggest that the relative abundance of *Fusobacteria* decreases with age, with the highest relative abundance occurring in samples collected during the first capture and steadily decreased across subsequent captures, irrespective of treatment group. There was no significant difference in the mean age of untreated (control) and treated pups at each capture event, suggesting that any differences in the composition or diversity of the gut microbiota is not due to differences in age structure between the two groups. The significant difference in the mean age of treated pups at the third capture event between 2019 and 2020/21 is likely due to more targeted sampling of younger pups, in order to maximise treatment effectiveness (Lindsay et al. unpublished).

The unique 18-month breeding cycle of the Australian sea lion (Higgins and Gass, 1993; Gales et al., 1997) means that the 2019 breeding season at Seal Bay occurred during the austral winter, whilst 2020/21 began during summer. As such, it was expected that seasonal and environmental variations could influence the gut microbiota of Australian sea lion pups. There were differences in the relative abundance of the more abundant phyla, *Fusobacteria*, *Firmicutes*, and *Actinobacteria*, and in two bacterial families that were present in both seasons, *Fusobacteriaceae* and *Clostridiaceae*. Within the 2019 and 2020/21 breeding seasons, there were no significant changes in the relative abundance of bacterial phyla or families across captures. However, qualitative changes in microbial community composition were observed; in 2019, the relative abundance of *Fusobacteria* remained relatively stable across all captures in both treatment groups, whilst the abundance of *Firmicutes* steadily decreased, and *Actinobacteria* and *Proteobacteria* increased. The relative abundance of *Fusobacteria* decreased more markedly in pups sampled during 2020/21 and was accompanied by a decrease in *Firmicutes* and an increase in *Proteobacteria*. This was also reflected at the family level, with the relative abundance of *Fusobacteriaceae*, a family belonging to the *Fusobacteria* phylum, decreasing from capture 1 to capture 4 in both treatment groups. Despite the significant differences between breeding seasons, the gut microbiota of both untreated (control) and treated Australian sea lion pups was dominated by the same bacteria phyla in 2019 and 2020/21, suggesting that the functional capacity of the gut microbial community is similar across treatment groups and breeding seasons. When comparing richness and diversity of the gut microbiota between seasons, there was again a significant difference; the richness of the gut microbiota was higher in Australian sea lion pups sampled in 2019, whilst diversity was, on average, higher in the 2020/21 cohort. The richness and diversity of the gut microbiota was significantly different in the treated group when compared between 2019 and 2020/21, and only richness differed significantly in the untreated (control) groups between seasons. The significant differences observed between seasons highlights the influence of external environmental conditions on the richness, diversity, and composition of the gut microbiota in Australian sea lion pups. Further investigations are required to determine the specific environmental characteristics contributing to these differences.

Host factors including pup weight, standard length, sex, and haematological parameters were also investigated to elucidate their relationship with treatment and the gut microbiota. In pups from both treatment groups sampled in the 2019 and 2020/21 breeding seasons, weight, standard length, and sex of the pup did not significantly influence the gut microbial composition. In sexually dimorphic pinniped species, differences in relative abundance of bacterial phyla and the relative abundance of ASVs or OTUs have been observed between male and females (Nelson et al., 2013; Stoffel et al., 2020). In adult southern elephant seals, differences between sexes were attributed to differences in diet rather than body mass (Nelson et al., 2013). When investigating the contribution of sex to the gut microbiome in weaned southern elephant seal pups (1–3 months old), Stoffel et al. (2020) found differences between sexes even though the male and female pups were indistinguishable, suggesting that some gut microbes are sex-specific and necessary for future adult feeding strategies. Whilst Australian sea lions are a sexually dimorphic species, Australian sea lion pups sampled as part of this study were a maximum of 4–6 months old and completely dependent upon their mothers for nutrition. Furthermore, Australian sea lion pups are not weaned until 18 months of age (Kirkwood and Goldsworthy, 2013). As a result, sampled pups were likely too young for any sex-associated differences in microbial composition to occur. The haematological parameters of Australian sea lion pups can be used as indicators of overall health and improvement in total leukocyte counts have previously been seen with ivermectin treatment (Marcus et al., 2015a,b; Lindsay et al., 2021). However, there was no significant association between any haematological parameter and alpha diversity, beta diversity, or the relative abundance of any bacterial phyla or family.

Topical ivermectin treatment of Australian sea lion pups has been identified as a potential management strategy to improve the health of free-ranging pups in a declining population (Lindsay et al., 2021), however, unexpected consequences of treatment need to be explored to ensure deleterious outcomes do not ensue. Characterisation of the

gut microbiota of free-ranging untreated (control) and treated Australian sea lion pups revealed that topical ivermectin treatment and elimination of *U. sanguinis* infection did not alter the composition of the gut microbial community. Despite some minor differences, the absence of statistically significant alterations to the composition of the gut microbial community, together with the known benefits of ivermectin treatment for Australian sea lion pup health and growth, suggest that topical ivermectin could be an effective management strategy to mitigate the impact of endemic hookworm disease on population growth in this species.

Data availability statement

The data presented in this study are deposited in the Dryad repository, https://doi.org/10.5061/dryad.ngf1vhhxh. Raw sequences are deposited in the European Nucleotide Archive, accession number PRJEB56630.

Ethics statement

The animal study was reviewed and approved by the Animal Ethics Committee at the University of Sydney.

Author contributions

MF, RG, and MP contributed to the conceptualization and design of the study. MF and RG collected all samples from pinniped pups. MF completed laboratory analysis of samples and data analysis and drafted the manuscript. All authors participated in revising the manuscript, contributed to the manuscript, and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.1048013/full#supplementary-material

References

Aivelo, T., and Norberg, A. (2018). Parasite–microbiota interactions potentially affect intestinal communities in wild mammals. *J. Anim. Ecol.* 87, 438–447. doi: 10.1111/1365-2656.12708

Anderson, R. M., and Schad, G. A. (1985). Hookworm burdens and faecal egg counts: an analysis of the biological basis of variation. *Trans. R. Soc. Trop. Med. Hyg.* 79, 812–825. doi: 10.1016/0035-9203(85)90128-2

Bates, D., Mächler, M., Bolker, B., and Walker, S. (2015). Fitting linear mixed-model effects using lme4. *J. Stat. Softw.* 1, 1–48. doi: 10.18637/jss.v067.i01

Bäumler, A. J., and Sperandio, V. (2016). Interactions between the microbiota and pathogenic bacteria in the gut. *Nature* 535, 85–93. doi: 10.1038/nature18849

Bik, E. M., Costello, E. K., Switzer, A. D., Callahan, B. J., Holmes, S. P., Wells, R. S., et al. (2016). Marine mammals harbor unique microbiotas shaped by and yet distinct from the sea. *Nat. Commun.* 7:10516. doi: 10.1038/ncomms10516

Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C. C., Al-Ghalith, G. A., et al. (2019). Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat. Biotechnol.* 37, 852–857. doi: 10.1038/s41587-019-0209-9

Bradshaw, C. J. A., Davis, L. S., Lalas, C., and Harcourt, R. G. (2000). Geographic and temporal variation in the condition of pups of the New Zealand fur seal (*Arctocephalus forsteri*): evidence for density dependence and differences

in the marine environment. *J. Zool.* 252, 41–51. doi: 10.1111/j.1469-7998.2000.tb00818.x

Brestoff, J. R., and Artis, D. (2013). Commensal bacteria at the interface of host metabolism and the immune system. *Nat. Immunol.* 14, 676–684. doi: 10.1038/ni.2640

Byard, R. W., and Machado, A. (2019). Characteristic "neck collar" injuries in Australian sea lions (*Neophoca cinerea*) caused by marine debris. *Forensic Sci. Med. Pathol.* 15, 631–634. doi: 10.1007/s12024-018-0060-1

Callahan, B. J., McMurdie, P. J., and Holmes, S. P. (2017). Exact sequence variants should replace operational taxonomic units in marker-gene data analysis. *ISME J.* 11, 2639–2643. doi: 10.1038/ismej.2017.119

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., and Holmes, S. P. (2016). DADA2: high-resolution sample inference from Illumina amplicon data. *Nat. Methods* 13, 581–583. doi: 10.1038/nmeth.3869

Castinel, A., Duignan, P. J., Pomroy, W. E., López-Villalobos, N., Gibbs, N. J., Chilvers, B. L., et al. (2007). Neonatal mortality in New Zealand sea lions (*Phocarctos hookeri*) at Sandy Bay, Enderby Island, Auckland Islands from 1998 to 2005. *J. Wildl. Dis.* 43, 461–474. doi: 10.7589/0090-3558-43.3.461

Cebra, J. J. (1999). Influences of microbiota on intestinal immune system development. *Am. J. Clin. Nutr.* 69, 1046–1051. doi: 10.1093/ajcn/69.5.1046s

Chao, A. (1984). Nonparametric estimation of the number of classes in a population. *Scand. J. Stat.* 11, 265–270.

Clemente, J. C., Ursell, L. K., Parfrey, L. W., and Knight, R. (2012). The impact of the gut microbiota on human health: an integrative view. *Cells* 148, 1258–1270. doi: 10.1016/j.cell.2012.01.035

Cooper, P., Walker, A. W., Reyes, J., Chico, M., Salter, S. J., Vaca, M., et al. (2013). Patent human infections with the whipworm, *Trichuris trichiura*, are not associated with alterations in the faecal microbiota. *PLoS One* 8:e76573. doi: 10.1371/journal.pone.0076573

Cortés, A., Peachey, L. E., Jenkins, T. P., Scotti, R., and Cantacessi, C. (2019). Helminths and microbes within the vertebrate gut – not all studies are created equal. *Parasitology* 146, 1371–1378. doi: 10.1017/S003118201900088X

DeLong, R. L., Orr, A. J., Jenkinson, R. S., and Lyons, E. T. (2009). Treatment of northern fur seal (*Callorhinus ursinus*) pups with ivermectin reduces hookworm-induced mortality. *Mar. Mamm. Sci.* 25, 944–948. doi: 10.1111/j.1748-7692.2008.00274.x

Delport, T. C., Power, M. L., Harcourt, R. G., Webster, K. N., and Tetu, S. G. (2016). Colony location and captivity influence the gut microbial community composition of the Australian sea lion (*Neophoca cinerea*). *Appl. Environ. Microbiol.* 82, 3440–3449. doi: 10.1128/AEM.00192-16

Ebert, D., and Fields, P. D. (2020). Host–parasite co-evolution and its genomic signature. *Nat. Rev. Genet.* 21, 754–768. doi: 10.1038/s41576-020-0269-1

Fulham, M., McDougall, F., Power, M., McIntosh, R. R., and Gray, R. (2022). Carriage of antibiotic resistant bacteria in endangered and declining Australian pinniped pups. *PLoS One* 17:e0258978. doi: 10.1371/journal.pone.0258978

Fulham, M., Power, M., and Gray, R. (2018). Comparative ecology of *Escherichia coli* in endangered Australian sea lion (*Neophoca cinerea*) pups. *Infect. Genet. Evol.* 62, 262–269. doi: 10.1016/j.meegid.2018.05.002

Fulham, M., Power, M., and Gray, R. (2020). Diversity and distribution of *Escherichia coli* in three species of free-ranging Australian pinniped pups. *Front. Mar. Sci.* 7:571171. doi: 10.3389/fmars.2020.571171

Gales, N. J., Shaughnessy, P. D., and Dennis, T. E. (1994). Distribution, abundance and breeding cycle of the Australian sea lion *Neophoca cinerea* (Mammalia: Pinnipedia). *J. Zool.* 234, 353–370. doi: 10.1111/j.1469-7998.1994.tb04853.x

Gales, N. J., Williamson, P., Higgins, L. V., Blackberry, M. A., and James, I. (1997). Evidence for a prolonged post implantation period in the Australian sea lion (*Neophoca cinerea*). *J. Reprod. Fertil.* 111, 159–163. doi: 10.1530/jrf.0.1110159

Gaulke, C. A., Martins, M. L., Watral, V. G., Humphreys, I. R., Spagnoli, S. T., Kent, M. L., et al. (2019). A longitudinal assessment of host-microbe-parasite interactions resolves the zebrafish gut microbiome's link to *Pseudocapillaria tomentosa* infection and pathology. *Microbiome* 7:10. doi: 10.1186/s40168-019-0622-9

Giacomin, P., Croese, J., Krause, L., Loukas, A., and Cantacessi, C. (2015). Suppression of inflammation by helminths: a role for the gut microbiota? *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 370, 1–6. doi: 10.1098/RSTB.2014.0296

Glöckner, F. O., Yilmaz, P., Quast, C., Gerken, J., Beccati, A., Ciuprina, A., et al. (2017). 25 years of serving the community with ribosomal RNA gene reference databases and tools. *J. Biotechnol.* 261, 169–176. doi: 10.1016/j.jbiotec.2017.06.1198

Goldsworthy, S. D., Gales, N. J., and Chilvers, B. L. (2015). *Neophoca cinerea. IUCN Red List Threat. Species 2015 e.T14549A45228341*. Gland: IUCN.

Goldsworthy, S. D., Kennedy, C., and Lashmar, K. (2014). Monitoring the status, trends in abundance and key demographic rates of the Australian sea lion population at Seal Bay - Kangaroo Island. *SARDI Publication No. F2014/000787-1 SARDI Research Report Series No. 815*.

Goldsworthy, S. D., Lowther, A. D., Shaughnessy, P. D., McIntosh, R. R., and Page, B. (2007). *Assessment of pup production and the maternal investment strategies of the Australian sea lion Neophoca cinerea at dangerous reef in the 2006-07 breeding season*. Adelaide, Australia: SARDI Aquatic Sciences Publication.

Goldsworthy, S. D., Shaughnessy, P. D., Mackay, A. I., Bailleul, F., Holman, D., Lowther, A. D., et al. (2021). Assessment of the status and trends in abundance of a coastal pinniped, the Australian sea lion *Neophoca cinerea*. *Endanger. Species Res.* 44, 421–437. doi: 10.3354/ESR01118

Goldsworthy, S. D., Shaughnessy, P. D., Smart, J., Mackay, A. I., Bailleul, F., Reinhold, S.-L., et al. (2019). Monitoring of Seal Bay and other populations on Kangaroo Island: 2017/2018. Report to the Department for Environment and Water. *SARDI Publication No. F2014/000322-5 SARDI Research Report Series No. 1018*.

Guinane, C. M., and Cotter, P. D. (2013). Role of the gut microbiota in health and chronic gastrointestinal disease: understanding a hidden metabolic organ. *Ther. Adv. Gastroenterol.* 6, 295–308. doi: 10.1177/1756283X13482996

Guiver, E., Galan, M., Lippens, C., Bellenger, J., Faivre, B., and Sorci, G. (2022). Increasing helminth infection burden depauperates the diversity of the gut microbiota and alters its composition in mice. *Curr. Res. Parasitol. Vector Borne Dis.* 2:100082. doi: 10.1016/j.crpvbd.2022.100082

Hall, M., and Beiko, R. G. (2018). "16S rRNA gene analysis with QIIME2" *Meth. Mol. Biol.* 1849, 113–129. doi: 0.1007/978-1-4939-8728-3_8

Hamer, D. J., Goldsworthy, S. D., Costa, D. P., Folwer, S., Page, M., and Sumner, M. D. (2013). The endangered Australian sea lion extensively overlaps with and regularly becomes by-catch in demersal shark gill-nets in South Australian shelf waters. *Biol. Conserv.* 157, 386–400. doi: 10.1016/j.biocon.2012.07.010

He, F., Zhai, J., Zhang, L., Liu, D., Ma, Y., Rong, K., et al. (2018). Variations in gut microbiota and fecal metabolic phenotype associated with Fenbendazole and Ivermectin tablets by 16S rRNA gene sequencing and LC/MS-based metabolomics in Amur tiger. *Biochem. Biophys. Res. Commun.* 499, 447–453. doi: 10.1016/j.bbrc.2018.03.158

Higgins, L. V., and Gass, L. (1993). Birth to weaning: parturition, duration of lactation, and attendance cycles of Australian sea lions (*Neophoca cinerea*). *Can. J. Zool.* 71, 2047–2055. doi: 10.1139/z93-290

Hooper, L. V., Littman, D. R., and Macpherson, A. J. (2012). Interactions between the microbiota and the immune system. *Science* 336, 1268–1273. doi: 10.1126/science.1223490

Hooper, L. V., Midtvedt, T., and Gordon, J. I. (2002). How host-microbial interactions shape the nutrient environment of the mammalian intestine. *Annu. Rev. Nutr.* 22, 283–307. doi: 10.1146/annurev.nutr.22.011602.092259

Jenkins, T. P., Formenti, F., Castro, C., Piubelli, C., Perandin, F., Buonfrate, D., et al. (2018). A comprehensive analysis of the faecal microbiome and metabolome of *Strongyloides stercoralis* infected volunteers from a non-endemic area. *Sci. Rep.* 8:15651. doi: 10.1038/s41598-018-33937-3

Johansson, M. A., Saghafian-Hedengren, S., Haileselassie, Y., Roos, S., Troye-Blomberg, M., Nilsson, C., et al. (2012). Early-life gut bacteria associate with IL-4−, IL-10− and IFN-γ production at two years of age. *PLoS One* 7:e49315. doi: 10.1371/journal.pone.0049315

Kirkwood, Roger., and Goldsworthy, Simon. (2013). *Fur Seals and Sea Lions*. Collingwood, VIC: CSIRO Publishing.

Lane, D. (1991). "Nucleic acid techniques in bacterial systematics" in *Nucleic Acid Techniques in Bacterial Systematics*. ed. E. Stackebrandt (Hoboken, NJ: John Wiley and Sons)

Lee, C. Y., Peralta-Sánchez, J. M., Martínez-Bueno, M., Møller, A. P., Rabelo-Ruiz, M., Zamora-Muñoz, C., et al. (2020). The gut microbiota of brood parasite and host nestlings reared within the same environment: disentangling genetic and environmental effects. *ISME J.* 14, 2691–2702. doi: 10.1038/s41396-020-0719-y

Ley, R. E., Hamady, M., Lozupone, C., Turnbaugh, P. J., Ramey, R. R., Bircher, J. S., et al. (2008a). Evolution of mammals and their gut microbes. *Science* 320, 1647–1651. doi: 10.1126/science.1155725

Ley, R. E., Lozupone, C. A., Hamady, M., Knight, R., and Gordon, J. I. (2008b). Worlds within worlds: evolution of the vertebrate gut microbiota. *Nat. Rev. Microbiol.* 6, 776–788. doi: 10.1038/nrmicro1978

Li, R. W., Wu, S., Li, W., Navarro, K., Couch, R. D., Hill, D., et al. (2012). Alterations in the porcine colon microbiota induced by the gastrointestinal nematode *Trichuris suis*. *Infect. Immun.* 80, 2150–2157. doi: 10.1128/IAI.00141-12

Lindsay, S. A., Caraguel, C. G. B., and Gray, R. (2021). Topical ivermectin is a highly effective seal 'spot-on': a randomised trial of hookworm and lice treatment in the endangered Australian sea lion (*Neophoca cinerea*). *Int. J. Parasitol. Parasites Wildl.* 16, 275–284. doi: 10.1016/j.ijppaw.2021.11.002

Ling, J. K. (1999). Exploitation of fur seals and sea lions from Australian, New Zealand and adjacent subantarctic islands during the eighteenth, nineteenth and twentieth centuries. *Aust. Zool.* 31, 323–350. doi: 10.7882/AZ.1999.036

Lozupone, C. A., Stombaugh, J. I., Gordon, J. I., Jansson, J. K., and Knight, R. (2012). Diversity, stability and resilience of the human gut microbiota. *Nature* 489, 220–230. doi: 10.1038/nature11550

Lyons, E. T., DeLong, R. L., Nadler, S. A., Laake, J. L., Orr, A. J., DeLong, B. L., et al. (2011). Investigations of peritoneal and intestinal infections of adult hookworms (*Uncinaria* spp.) in northern fur seal (*Callorhinus ursinus*) and California sea lion (*Zalophus californianus*) pups on San Miguel Island, California (2003). *Parasitol. Res.* 109, 581–589. doi: 10.1007/s00436-011-2289-4

Mandal, S., van Treuren, W., White, R. A., Eggesbø, M., Knight, R., and Peddada, S. D. (2015). Analysis of composition of microbiomes: a novel method for studying microbial composition. *Microb. Ecol. Health Dis.* 26:27663. doi: 10.3402/mehd.v26.27663

Marcus, A. D., Higgins, D. P., and Gray, R. (2014). Epidemiology of hookworm (*Uncinaria sanguinis*) infection in free-ranging Australian sea lion (*Neophoca cinerea*) pups. *Parasitol. Res.* 113, 3341–3353. doi: 10.1007/s00436-014-3997-3

Marcus, A. D., Higgins, D. P., and Gray, R. (2015a). Health assessment of free-ranging endangered Australian sea lion (*Neophoca cinerea*) pups: effect of haematophagous parasites on haematological parameters. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* 184, 132–143. doi: 10.1016/j.cbpa.2015.02.017

Marcus, A. D., Higgins, D. P., and Gray, R. (2015b). Ivermectin treatment of free-ranging endangered Australian sea lion (*Neophoca cinerea*) pups: effect on hookworm and lice infection status, haematological parameters, growth, and survival. *Parasitol. Res.* 114, 2743–2755. doi: 10.1007/s00436-015-4481-4

Margaret, M.-N., Michael, G. H., Thomos, G. B. C., Hannah, V. C., Tomislav, D.-L., Angela, E. D., et al. (2013). Animals in a bacterial world, a new imperative for the life sciences. *Proc. Natl. Acad. Sci. U. S.A.* 110, 3229–3236. doi: 10.1073/pnas.1218525110

Martin, I., Djuardi, Y., Sartono, E., Rosa, B. A., Supali, T., Mitreva, M., et al. (2018). Dynamic changes in human-gut microbiome in relation to a placebo-controlled anthelminthic trial in Indonesia. *PLoS Negl. Trop. Dis.* 12:e0006620. doi: 10.1371/journal.pntd.0006620

Martin, I., Kaisar, M. M. M., Wiria, A. E., Hamid, F., Djuardi, Y., Sartono, E., et al. (2019). The effect of gut microbiome composition on human immune responses: an exploration of interference by helminth infections. *Front. Genet.* 10:1028. doi: 10.3389/fgene.2019.01028

McIntosh, R. R., and Kennedy, C. W. (2013). Morphology, sex ratio and cause of death in Australian sea lion (*Neophoca cinerea*) pups. *Aust. Mammal* 35, 93–100. doi: 10.1071/AM12037

McMurdie, P. J., and Holmes, S. (2013). phyloseq: An R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS One* 8:e61217. doi: 10.1371/journal.pone.0061217

Medeiros, A. W., Giongo, A., Valdez, F. P., Blaese de Amorin, D., Tavares, M., d'Azevedo, P. A., et al. (2016). Characterization of the faecal bacterial community of wild young South American (*Arctocephalus australis*) and Subantarctic fur seals (*Arctocephalus tropicalis*). *FEMS Microbiol. Ecol.* 92, 1–8. doi: 10.1093/femsec/fiw029

Michael, S. A., Hayman, D. T. S., Gray, R., and Roe, W. D. (2021). Risk factors for New Zealand sea lion (*Phocarctos hookeri*) pup mortality: Ivermectin improves survival for conservation management. *Front. Mar. Sci.* 8:680678. doi: 10.3389/fmars.2021.680678

Michael, S. A., Hayman, D. T. S., Gray, R., Zhang, J., Rogers, L., and Roe, W. D. (2019). Pup mortality in New Zealand sea lions (*Phocarctos hookeri*) at Enderby Island, Auckland Islands, 2013-18. *PLoS One* 14:e0225461. doi: 10.1371/journal.pone.0225461

Moustafa, M. A. M., Chel, H. M., Thu, M. J., Bawm, S., Htun, L. L., Win, M. M., et al. (2021). Anthropogenic interferences lead to gut microbiome dysbiosis in Asian elephants and may alter adaptation processes to surrounding environments. *Sci. Rep.* 11:741. doi: 10.1038/s41598-020-80537-1

Nelson, T. M., Rogers, T. L., Carlini, A. R., and Brown, M. (2013). Diet and phylogeny shape the gut microbiota of Antarctic seals: a comparison of wild and captive animals. *Environ. Microbiol.* 15, 1132–1145. doi: 10.1111/1462-2920.12022

Oksanen, J., Simpson, G. L., Guillaume Blanchet, F., Kindt, R., Legendre, P., Minchin, P. R., et al. (2022). *vegan: Community Ecology Package. R package version 2.6-2.*

Pacheco-Sandoval, A., Schramm, Y., Heckel, G., Brassea-Pérez, E., Martínez-Porchas, M., and Lago-Lestón, A. (2019). The Pacific harbor seal gut microbiota in Mexico: its relationship with diet and functional inferences. *PLoS One* 14:e0221770. doi: 10.1371/journal.pone.0221770

Page, B., McKenzie, J., McIntosh, R., Baylis, A., Morrissey, A., Calvert, N., et al. (2004). Entanglement of Australian sea lions and New Zealand fur seals in lost fishing gear and other marine debris before and after government and industry attempts to reduce the problem. *Mar. Pollut. Bull.* 49, 33–42. doi: 10.1016/j.marpolbul.2004.01.006

Peachey, L. E., Molena, R. A., Jenkins, T. P., di Cesare, A., Traversa, D., Hodgkinson, J. E., et al. (2018). The relationships between faecal egg counts and gut microbial composition in UK thoroughbreds infected by cyathostomins. *Int. J. Parasitol.* 48, 403–412. doi: 10.1016/j.ijpara.2017.11.003

Preheim, S. P., Perrotta, A. R., Martin-Platero, A. M., Gupta, A., and Alm, E. J. (2013). Distribution-based clustering: using ecology to refine the operational taxonomic unit. *Appl. Environ. Microbiol.* 79, 6593–6603. doi: 10.1128/AEM.00342-13

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., et al. (2013). The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res.* 41, D590–D596. doi: 10.1093/nar/gks1219

Ramanan, D., Bowcutt, R., Lee, S. C., Tang, M. S., Kurtz, Z. D., Ding, Y., et al. (2016). Helminth infection promotes colonization resistance via type 2 immunity. *Science* 352, 608–612. doi: 10.1126/science.aaf3229

Rausch, S., Held, J., Fischer, A., Heimesaat, M. M., Kühl, A. A., Bereswill, S., et al. (2013). Small intestinal nematode infection of mice is associated with increased enterobacterial loads alongside the intestinal tract. *PLoS One* 8:e74026. doi: 10.1371/journal.pone.0074026

Reynolds, L. A., Finlay, B. B., and Maizels, R. M. (2015). Cohabitation in the intestine: interactions among helminth parasites, bacterial microbiota, and host immunity. *J. Immunol.* 195, 4059–4066. doi: 10.4049/jimmunol.1501432

Rognes, T., Flouri, T., Nichols, B., Quince, C., and Mahé, F. (2016). VSEARCH: a versatile open source tool for metagenomics. *PeerJ* 4:e2584. doi: 10.7717/peerj.2584

Rosa, B. A., Supali, T., Gankpala, L., Djuardi, Y., Sartono, E., Zhou, Y., et al. (2018). Differential human gut microbiome assemblages during soil-transmitted helminth infections in Indonesia and Liberia. *Microbiome* 6:33. doi: 10.1186/s40168-018-0416-5

Schroeder, B. O., and Bäckhed, F. (2016). Signals from the gut microbiota to distant organs in physiology and disease. *Nat. Med.* 22, 1079–1089. doi: 10.1038/nm.4185

Seguel, M., Muñoz, F., Navarrete, M. J., Paredes, E., Howerth, E., and Gottdenker, N. (2016). Hookworm infection in South American fur seal (*Arctocephalus australis*) pups: pathology and factors associated with host tissue damage and mortality. *Vet. Pathol.* 54, 288–297. doi: 10.1177/0300985816677151

Seguel, M., Pavés, H., Paredes, E., and Schlatter, R. (2013). Causes of mortality in south American fur seal pups (*Arctophoca australis gracilis*) at Guafo Island, southern Chile (2004–2008). *Mar. Mamm. Sci.* 29, 36–47. doi: 10.1111/j.1748-7692.2011.00534.x

Shannon, C. E. (1948). A mathematical theory of communication. *Bell. Syst. Tech. J.* 27, 379–423. doi: 10.1002/j.1538-7305.1948.tb01338.x

Smith, S. C., Chalker, A., Dewar, M. L., and Arnould, J. P. Y. (2013). Age-related differences revealed in Australian fur seal *Arctocephalus pusillus doriferus* gut microbiota. *FEMS Microbiol. Ecol.* 86, 246–255. doi: 10.1111/1574-6941.12157

Spraker, T. R., DeLong, R. L., Lyons, E. T., and Melin, S. R. (2007). Hookworm enteritis with bacteremia in California sea lion pups on San Miguel Island. *J. Wildl. Dis.* 43, 179–188. doi: 10.7589/0090-3558-43.2.179

Spratt, D. M., and Beveridge, I. (2019). Wildlife parasitology in Australia: past, present and future. *Aust. J. Zool.* 66, 286–305. doi: 10.1071/ZO19017

Stoffel, M. A., Acevedo-Whitehouse, K., Morales-Durán, N., Grosser, S., Chakarov, N., Krüger, O., et al. (2020). Early sexual dimorphism in the developing gut microbiome of northern elephant seals. *Mol. Ecol.* 29, 2109–2122. doi: 10.1111/mec.15385

Stokes, A. L., Terkildsen, M., Lindsay, S. A., Stonnill, M., Fulham, M., Taylor, S., et al. (2020). *Growth Curves, Age Prediction and a Body Condition Index in Ivermectin-Treated Neophoca cinerea Pups*. Sydney: Doctor of Veterinary Medicine Professionally Focused Project, The University of Sydney.

Taylor, S., Terkildsen, M., McQuilty, R., Lee, D., Wing-Simpson, A., and Gray, R. (2022). Non-essential heavy metals and protective effects of selenium against mercury toxicity in endangered Australian sea lion (*Neophoca cinerea*) pups with hookworm disease. *Environ. Int.* 107521, 1–14. doi: 10.1016/j.envint.2022.107521

Taylor, S., Terkildsen, M., Stevenson, G., de Araujo, J., Yu, C., Yates, A., et al. (2021). Per and polyfluoroalkyl substances (PFAS) at high concentrations in neonatal Australian pinnipeds. *Sci. Total Environ.* 786:147446. doi: 10.1016/j.scitotenv.2021.147446

Threatened Species Scientific Committee (2020). *Conservation Advice Neophoca cinerea Australian Sea Lion*. Canberra: Department of Agriculture, Water and the Environment.

Thursby, E., and Juge, N. (2017). Introduction to the human gut microbiota. *Biochem. J.* 474, 1823–1836. doi: 10.1042/BCJ20160510

Tian, J., Du, J., Han, J., Song, X., and Lu, Z. (2020). Age-related differences in gut microbial community composition of captive spotted seals (*Phoca largha*). *Mar. Mamm. Sci.* 36, 1231–1240. doi: 10.1111/mms.12728

Toro-Valdivieso, C., Toro, F., Stubbs, S., Castro-Nallar, E., and Blacklaws, B. (2021). Patterns of the fecal microbiota in the Juan Fernández fur seal (*Arctocephalus philippii*). *Microbiol. Open* 10:e1215. doi: 10.1002/mbo3.1215

Ursell, L. K., Metcalf, J. L., Parfrey, L. W., and Knight, R. (2012). Defining the human microbiome. *Nutr. Rev.* 70, S38–S44. doi: 10.1111/j.1753-4887.2012.00493.x

Vlčková, K., Pafčo, B., Petrželková, K. J., Modrý, D., Todd, A., Yeoman, C. J., et al. (2018). Relationships between gastrointestinal parasite infections and the fecal microbiome in free-ranging western lowland gorillas. *Front. Microbiol.* 9:1202. doi: 10.3389/FMICB.2018.01202/FULL

Walk, S. T., Blum, A. M., Ewing, S. A.-S., Weinstock, J. V., and Young, V. B. (2010). Alteration of the murine gut microbiota during infection with the parasitic helminth *Heligmosomoides polygyrus*. *Inflamm. Bowel Dis.* 16, 1841–1849. doi: 10.1002/ibd.21299

Warnick, L. (1992). Daily variability of equine fecal strongyle egg counts. *Cornell Vet.* 82, 453–463.

Yatsunenko, T., Rey, F. E., Manary, M. J., Trehan, I., Dominguez-Bello, M. G., Contreras, M., et al. (2012). Human gut microbiome viewed across age and geography. *Nature* 486, 222–227. doi: 10.1038/nature11053

Yilmaz, P., Parfrey, L. W., Yarza, P., Gerken, J., Pruesse, E., Quast, C., et al. (2014). The SILVA and "All-species Living Tree Project (LTP)" taxonomic frameworks. *Nucleic Acids Res.* 42, D643–D648. doi: 10.1093/nar/gkt1209

Zaiss, M. M., Rapin, A., Lebon, L., Dubey, L. K., Mosconi, I., Sarter, K., et al. (2015). The intestinal microbiota contributes to the ability of helminths to modulate allergic inflammation. *Immunity* 43, 998–1010. doi: 10.1016/j.immuni.2015.09.012

Supplementary Table 1. Results from linear mixed models one and two, analysing the correlations between pup morphometric and haematological data and alpha diversity in the 2019 and 2020/21 breeding seasons.

Alpha diversity metric	Model	Term	Est	S.E.	Lower CI	Upper CI	p-value	R^2 Value	Breeding Season
Chao1	1	Treatment group	8.65	10.12	-11.61	28.78	0.395		
		Capture	-23.9	12.28	-48.24	0.51	0.048*		
		Age	17.01	9.50	-3.49	35.83	0.076	0.086	2019
		Weight	4.46	3.48	-2.43	11.36	0.203		
		Standard length	-1.99	1.96	-5.87	1.89	0.312		
		Sex	-4.58	10.73	-25.9	16.80	0.670		
Chao1	2	Treatment group	18.785	11.848	-4.685	42.255	0.14		
		Capture	-35.451	13.094	-61.390	-9.513	0.013*		
		Age	20.959	9.358	2.422	39.496	0.08		
		Hookworm status	10.621	19.949	-28.894	50.138	0.5		
		Total plasma protein	1.745	0.894	-0.026	3.517	0.09	0.134	2019
		Total nucleated cell count	-1.860	7.959	-17.627	13.906	0.82		
		Absolute neutrophil count	0.595	8.262	-15.770	16.969	0.95		
		Absolute lymphocyte count	3.841	9.265	-14.505	22.189	0.69		
		Absolute monocyte count	5.565	17.119	-28.344	39.475	0.76		
		Absolute eosinophil count	-6.883	12.341	-31.330	17.563	0.61		
Shannon-Wiener Index	1	Treatment group	-2.430	0.153	-0.553	0.069	0.124		
		Capture	0.119	0.173	-0.234	0.455	0.527		
		Age	-0.127	0.137	-0.410	0.145	0.357	0.097	2019
		Weight	0.101	0.049	-0.004	0.199	0.072		
		Standard length	-0.049	0.027	-0.104	0.005	0.076		
		Sex	-0.279	0.162	-0.607	0.051	0.096		
Shannon-Wiener Index	2	Treatment group	-0.154	0.201	-0.530	0.218	0.436		
		Capture	-0.199	0.204	-0.633	0.127	0.225		
		Age	0.041	0.149	-0.235	0.320	0.783		
		Hookworm status	-0.247	0.301	-0.812	0.310	0.414		
		Total plasma protein	-0.021	0.013	-0.005	0.045	0.148	0.089	2019
		Total nucleated cell count	-0.093	0.118	-0.312	0.130	0.430		
		Absolute neutrophil count	0.077	0.122	-0.152	0.304	0.527		
		Absolute lymphocyte count	0.125	0.139	-0.134	0.386	0.372		
		Absolute monocyte count	0.112	0.255	-0.394	0.586	0.662		
		Absolute eosinophil count	0.018	0.184	-0.335	0.358	0.919		

Chao1	1	Treatment group	13.320	7.086	-0.723	27.360	0.043*	0.107	2020
		Capture	6.494	9.333	-12.00	24.989	0.488		
		Age	1.366	8.018	-26.673	5.106	0.182		
		Weight	0.863	2.490	-4.072	5.799	0.729		
		Standard length	0.840	1.511	-2.155	8.384	0.579		
		Sex	1.366	9.230	-16.924	19.657	0.882		
Chao1	2	Treatment group	19.575	7.918	3.883	35.267	0.015*		
		Capture	16.512	8.061	0.548	32.485	0.435		
		Age	-10.890	7.008	-24.777	2.996	0.123		
		Hookworm status	15.412	10.484	-5.361	36.187	0.145		
		Total plasma protein	0.833	0.713	-0.579	2.246	0.245	0.135	2020
		Total nucleated cell count	-1.813	2.515	-6.799	3.171	0.472		
		Absolute neutrophil count	1.565	2.638	-3.661	6.793	0.554		
		Absolute lymphocyte count	2.536	5.076	-7.522	12.596	0.618		
		Absolute monocyte count	0.278	12.016	-23.532	24.089	0.981		
		Absolute eosinophil count	3.259	7.232	-11.071	17.590	0.653		
Shannon-Wiener Index	1	Treatment group	0.573	0.151	0.272	0.876	0.002*		
		Capture	-0.428	0.200	-0.826	-0.031	0.350		
		Age	0.035	0.171	-0.307	-0.374	0.846	0.198	2020
		Weight	0.027	0.053	-0.078	0.133	0.610		
		Standard length	0.033	0.032	-0.305	0.097	0.301		
		Sex	0.158	0.197	-0.233	0.551	0.425		
Shannon-Wiener Index	2	Treatment group	0.590	0.172	0.249	0.935	0.04*		
		Capture	-0.204	0.175	-0.553	0.142	0.28		
		Age	0.198	0.152	-0.103	0.508	0.23		
		Hookworm status	0.180	0.228	-0.271	0.632	0.45		
		Total plasma protein	-0.009	0.015	-0.040	0.021	0.55	0.200	2020
		Total nucleated cell count	-0.066	0.054	-0.175	0.041	0.26		
		Absolute neutrophil count	0.083	0.057	-0.029	0.197	0.17		
		Absolute lymphocyte count	0.115	0.110	-0.103	0.334	0.34		
		Absolute monocyte count	0.210	0.261	-0.313	0.728	0.47		
		Absolute eosinophil count	0.007	0.157	-0.304	0.319	0.98		

Supplementary Table 2. Results from linear mixed models three and four, analysing the correlations between pup morphometric and haematological data and alpha diversity within each treatment group in 2019 and 2020/21.

Alpha diversity metric	Model	Term	Est	S.E.	Lower CI	Upper CI	p-value	R^2 Value	Treatment group	Breeding Season
Chao1	3	Capture	0.030	0.202	-0.377	0.441	0.879			
		Age	0.050	0.180	-0.411	0.413	0.782			
		Weight	0.060	0.076	-0.093	0.234	0.435	0.038	Untreated (control)	2019
		Standard length	-0.036	0.037	-0.112	0.039	0.344			
		Sex	-0.143	0.207	-0.583	0.323	0.507			
Chao1	4	Capture	-0.226	0.225	-0.676	0.229	0.319			
		Age	0.045	0.154	-0.274	0.353	0.768			
		Hookworm status	0.204	0.285	-0.367	0.776	0.477			
		Total plasma protein	0.022	0.014	-0.005	0.051	0.118			
		Total nucleated cell count	-0.360	0.281	-0.925	0.202	0.206	0.268	Untreated (control)	2019
		Absolute neutrophil count	0.303	0.287	-0.270	0.877	0.295			
		Absolute lymphocyte count	0.306	0.293	-0.28	0.893	0.301			
		Absolute monocyte count	0.007	0.360	-0.712	0.741	0.983			
		Absolute eosinophil count	0.248	0.322	-0.395	0.902	0.443			
Shannon-Wiener Index	3	Capture	-39.300	14.929	-69.169	-9.432	0.011*			2019
		Age	31.339	13.346	4.634	58.039	0.231			
		Weight	8.328	5.675	-3.025	19.681	0.148	0.176	Untreated (control)l	
		Standard length	-4.191	2.797	-9.786	1.405	0.140			
		Sex	20.705	15.281	-9.871	51.276	0.181			
Shannon-Wiener Index	4	Capture	-43.716	18.096	-79.920	-7.510	0.019*			
		Age	21.668	12.389	-3.118	46.453	0.086			
		Hookworm status	13.920	22.988	-32.071	59.911	0.544			
		Total plasma protein	2.620	1.155	0.308	4.931	0.028			
		Total nucleated cell count	9.467	22.661	-35.890	54.804	0.678	0.257	Untreated (control)	2019
		Absolute neutrophil count	-12.294	23.069	-58.448	33.86	0.596			
		Absolute lymphocyte count	-14.615	23.604	-61.838	32.609	0.538			
		Absolute monocyte count	-13.617	28.956	-71.549	44.314	0.640			
		Absolute eosinophil count	-14.801	25.897	-66.614	37.011	0.570			
Chao1	3	Capture	-7.996	22.199	-52.655	37.272	0.720			
		Age	4.020	13.585	-25.681	31.609	0.769			
		Weight	3.184	4.363	-5.594	12.023	0.471	0.105	Treated	2019
		Standard length	-1.256	0.293	-7.505	4.868	0.671			
		Sex	-26.477	15.211	-58.206	7.457	0.110			

Chao1	4	Capture	-14.720	18.031	-52.840	23.586	0.419			
		Age	11.611	13.451	-16.746	39.684	0.393			
		Hookworm status	-22.551	35.06	-93.422	47.645	0.523			
		Total plasma protein	-0.922	1.723	-4.669	2.773	0.595			
		Total nucleated cell count	-6.196	7.816	-22.181	10.651	0.433	0.158	Treated	2019
		Absolute neutrophil count	5.723	8.369	-12.581	22.917	0.498			
		Absolute lymphocyte count	14.236	10.378	-6.798	25.104	0.177			
		Absolute monocyte count	28.268	23.496	-20.780	76.071	0.236			
		Absolute eosinophil count	-9.502	16.635	-43.031	23.966	0.571			
Shannon-Wiener Index	3	Capture	0.209	0.324	-0.450	0.874	0.526			
		Age	-0.303	0.198	-0.700	0.099	0.135			
		Weight	0.153	0.063	0.021	0.280	0.075	0.203	Treated	2019
		Standard length	-0.073	0.043	-0.162	0.014	0.097			
		Sex	-0.418	0.217	-0.871	0.048	0.075			
Shannon-Wiener Index	4	Capture	-0.191	0.264	-0.722	0.343	0.473			
		Age	0.036	0.197	-0.360	0.341	0.854			
		Hookworm status	-0.998	0.524	-2.166	0.086	0.064			
		Total plasma protein	-0.003	0.024	-0.054	0.046	0.883			
		Total nucleated cell count	-0.056	0.116	-0290	0.180	0.629	0.089	Treated	2019
		Absolute neutrophil count	0.073	0.125	-0.178	0.324	0.558			
		Absolute lymphocyte count	0.159	0.153	-0.148	0.468	0.303			
		Absolute monocyte count	0.413	0.349	-0.334	1.138	0.242			
		Absolute eosinophil count	0.072	0.249	-0.430	0.597	0.771			
Chao1	3	Capture	12.171	12.632	-13.261	37.494	0.340			
		Age	-26.853	11.555	-50.016	-3.665	0.249			
		Weight	4.099	3.135	-2.187	1.384	0198	0.260	Untreated (control)	2020
		Standard length	0707	1.894	-3.089	4.53	0.710			
		Sex	3.958	12.470	-21.028	-3.665	0.752			
Chao1	4	Capture	37.173	10.998	17.887	56.470	0.186			
		Age	-33.676	10.308	-51.778	-15.570	0.254			
		Hookworm status	32.174	12.566	10.107	-54.242	0.152			
		Total plasma protein	1.551	0.918	-0.060	3.163	0.100			
		Total nucleated cell count	-7.047	4.985	-15.802	1.707	0.166	0.341	Untreated (control)	2020
		Absolute neutrophil count	8.247	4.245	0.791	15.703	0.060			
		Absolute lymphocyte count	5.7773	8.495	-9.141	20.696	0.501			
		Absolute monocyte count	-18.983	24.659	-62.269	24.302	0.466			

		Absolute eosinophil count	-1.135	11.937	-22.098	19.826	0.924			
Shannon-Wiener Index	3	Capture	-0.668	0.343	-1.370	0.019	0.0681	0.177	Untreated (control)	2020
		Age	-0.079	0.314	-0.709	9.557	0.802			
		Weight	0.063	0.085	-0.107	0.234	0.457			
		Standard length	0.518	0.051	-0.051	0.155	0.319			
		Sex	0.162	0.314	-0.519	0.842	0802			
Shannon-Wiener Index	4	Capture	-0.116	0.288	-0.775	0.462	0.690	0.208	Untreated (control)	2020
		Age	0.105	0.270	-0.440	0.764	0.701			
		Hookworm status	0.505	0.326	-0.157	1.185	0.129			
		Total plasma protein	-0.028	0.023	-0.079	.0205	0.247			
		Total nucleated cell count	-0.149	0.129	-0.411	0.120	0.256			
		Absolute neutrophil count	0.216	0.110	-0.010	0.439	0.056			
		Absolute lymphocyte count	0.095	0.220	-0.361	0.543	0.668			
		Absolute monocyte count	0.347	0.640	-0.938	1.632	0.590			
		Absolute eosinophil count	0.104	0.310	-0.545	0.734	0.737			
Chao1	3	Capture	1.536	13.313	-25.098	28.171	0.115	0.045	Treated	2020
		Age	1.082	10.480	-19.885	22.049	0.103			
		Weight	-3.236	3.553	-10.345	3.872	-0.911			
		Standard length	2.134	2.264	-2.394	6.663	0.943			
		Sex	4.127	10.480	-20.874	29.126	0.103			
Chao1	4	Capture	2.748	12.8-8	-22.877	28.375	0.831	0.084	Treated	2020
		Age	1.272	11.554	-21.843	24.388	0.913			
		Hookworm status	-23.428	21.450	-66.344	19.488	0.280			
		Total plasma protein	-0.363	1.454	-3.274	2.546	0.804			
		Total nucleated cell count	-2.375	3.657	-9.693	2.942	0.519			
		Absolute neutrophil count	2.822	4.206	-5.594	11.238	0.506			
		Absolute lymphocyte count	8.471	7.407	-6.348	23.292	0.259			
		Absolute monocyte count	-0.859	15.121	-31.113	29.394	0.955			
		Absolute eosinophil count	10.682	9.412	-8.149	29.513	0.262			
Shannon-Wiener Index	3	Capture	-0.209	0.218	-0.640	0.234	0.358	0.029	Treated	2020
		Age	0.173	0.172	-0.170	0.517	0.319			
		Weight	-0.020	0.058	-0.137	0.096	0.725			
		Standard length	0.017	0.037	-0.057	0.091	0.643			
		Sex	0.035	0.205	-0.374	0.448	0.863			

Shannon-Wiener Index	4	Capture	-0.247	0.203	-0.655	0.160	0.231			
		Age	0.216	0.183	-0.151	0.593	0.244			
		Hookworm status	-0.161	0.341	-0.833	0.520	0.637			
		Total plasma protein	0.023	0.023	-0.022	0.070	0.307			
		Total nucleated cell count	-0.036	0.058	-0.152	0.080	0.534	0.127	Treated	2020
		Absolute neutrophil count	0.038	0.066	-0.095	0.172	0.568			
		Absolute lymphocyte count	0.119	0.117	-0.116	0.355	0.315			
		Absolute monocyte count	0.140	0.240	-0.348	0.621	0.561			
		Absolute eosinophil count	0.066	0.149	-0.233	0.365	0.661			

Chapter 6.
General Discussion

Australian sea lion pups at Seal Bay, Kangaroo Island, 2019.

6.1. General discussion, limitations, and future directions

Marine mammals are continuously threatened by anthropogenic processes (Avila et al., 2018; Davidson et al., 2012; Gales et al., 2003; Pompa et al., 2011; P. Reijnders et al., 2009; Schumann et al., 2013), with increased anthropogenic utilisation of coastal ecosystems resulting in widespread contamination and degradation of the marine environment (Ansari and Matondkar, 2014; Beiras, 2018; Crain et al., 2009; Marcovecchio et al., 1994; Pommepuy et al., 2006). The threats faced by marine mammals include interactions with fisheries and entanglement in marine debris (Gales et al., 2003; Hamer and Goldsworthy, 2006; McIntosh et al., 2015; McLeay et al., 2015; Page et al., 2004), habitat degradation (Poloczanska et al., 2007; Schumann et al., 2013), and exposure to a multitude of pollutants from anthropogenic origins (Beiras, 2018; Helm et al., 2014; P. Reijnders et al., 2009; Taylor et al., 2021; Tuholske et al., 2021). Many marine mammal populations are impacted by these threats with 11% of all marine mammals considered endangered and 3% critically endangered (Pompa et al., 2011). Many pinniped species inhabit breeding colonies located within highly productive coastal areas (Kovacs et al., 2012) and rely on both marine and terrestrial environments to forage and raise their offspring. Given the close proximity to the coast and the reliance on the marine/terrestrial interface, pinnipeds could be at greater risk of exposure to anthropogenic impacts and pollutants, including the spill-over of potentially pathogenic bacteria from human sources (Ashley et al., 2020; Goldsworthy et al., 2021; Kovacs et al., 2012; Kretzmann et al., 2010; Stoddard et al., 2008).

Australian fur seals (*Arctocephalus pusillus doriferus*), long-nosed fur seals (*Arctocephalus forsteri*) and Australian sea lions (*Neophoca cinerea*) are vulnerable to a number of common threats (Kirkwood and Goldsworthy, 2013; McIntosh et al., 2015; Page et al., 2004). While the population trend for *A. forsteri* continues to increase (Shaughnessy et al., 2015, 2014; Shaughnessy and Goldsworthy, 2015), both *A. p. doriferus* and *N. cinerea* exhibit decreasing population trends (Goldsworthy et al., 2021; McIntosh et al., 2022). The causes of these declines are multifactorial, with climate change (McLean et al., 2018), fisheries interactions (Hamer et al., 2013; McLeay et al., 2015; Shaughnessy et al., 2003), exposure to anthropogenic pollutants (Fulham et al., 2018; Taylor et al., 2021, 2018) and disease (Marcus et al., 2015a, 2014) all thought to be contributors. However, one key knowledge gap is the presence of human-associated microbes and antimicrobial resistant bacteria (ARB) in free-ranging Australian pinniped populations.

Chapter 2 of this thesis provided the first report of class 1 integrons, determinants of antimicrobial resistance (AMR), in free-ranging *A. p. doriferus* and in the faecal microbiota of *N. cinerea* pups, identifying five different gene cassettes encoding resistance to four classes of antibiotics. Class 1 integrons have been proposed as useful indicators of anthropogenic pollution, occurring at a higher abundance in environments and individuals that are exposed to greater levels of anthropogenic impact (Gillings et al., 2014). The presence of class 1 integrons and antimicrobial resistance genes (ARGs) in free-ranging pinniped pups indicates that pinniped populations in Australia are exposed to genetic and microbial anthropogenic pollution. Given that pups sampled as part of this study were less than two months of age and therefore restricted to their breeding colonies, the acquisition of resistant bacteria could be via maternal microbial transfer (Nelson et al., 2013), or from terrestrial contamination of the breeding colony itself. This highlights the potential role of juvenile and adult pinnipeds (Delport et al., 2015), along with other wildlife species (for example, sea birds) as carriers of resistant bacteria that facilitate the dissemination of AMR within Australia (Dolejska et al., 2007; Dolejska and Literak, 2019).

The findings presented in **Chapter 2** indicate that *A. p. doriferus* pups are exposed to higher levels of anthropogenic pollution compared *to N. cinerea* and *A. forsteri* pups, which could be consistent with one of its main colonies being geographically proximate to Australia's second largest city. The colony at Seal Rocks, one of the key breeding colonies for this species (McIntosh et al., 2022, 2018), had the highest abundance of class 1 integrons across three breeding seasons. Furthermore, the ARGs and integron variants identified in both *E. coli* and in the faecal microbiota suggest microbes are of anthropogenic origin (Baysarowich et al., 2008; Bie et al., 2018; Dawes et al., 2010; Hastak et al., 2020). The IS*26* variant of the class 1 integron, identified in *A. p. doriferus* pups at Seal Rocks is commonly associated with *E. coli* from domestic livestock (Dawes et al., 2010; Reid et al., 2017; Zingali et al., 2020) and human clinical cases (Hastak et al., 2020). The *arr3* gene cassette, an ARG that confers resistance to rifampicin, was also detected in *A. p. doriferus* pups at Seal Rocks and is associated with domestic animal and human pathogens (Baysarowich et al., 2008; Bie et al., 2018; Xia et al., 2016). Together, the presence of the *arr3* gene cassette and the IS*26* variant suggest that free-ranging individuals at the Seal Rocks breeding colony are exposed to microbial pollution of human and domestic animal origin.

The absence of class 1 integrons and ARGs in *A. forsteri* pups was unexpected, given that ARGs were found in *N. cinerea* pups in breeding colonies within close proximity to *A. forsteri* colonies. These differences between species and breeding colonies highlights the need for further investigations to better understand the factors that influence acquisition of ARB and ARGs in free-ranging pinniped pups, including the source of these ARBs and ARGs. However, the key finding is that *A. p. doriferus* and *N. cinerea* populations in southern Australia are presumably being exposed to numerous sources of anthropogenic pollution, facilitating the acquisition of class 1 integrons and resistant bacteria.

The relationship between ARB, ARGs and heavy metals in *A. p. doriferus* pups was also explored in **Chapter 2.** The association between high levels of ARGs and heavy metals has been demonstrated in environmental sources, such as soil and sediments (de Vincente et al., 1990; Dickinson et al., 2019; Nguyen et al., 2019; Rosewarne et al., 2010), where heavy metals act as selective agents that promote the evolution of AMR (Pal et al., 2015). While no significant relationship was found between ARGs and concentrations of trace elements and heavy metals in *A. p. doriferus* pups, this aspect of the study was limited by a small sample size. High concentrations of heavy metals, including mercury and lead, have previously been detected in *A. p. doriferus* and *N. cinerea* pups (Cobb-Clark and Gray, 2018; Taylor et al. in review). It is possible that increased concentrations of heavy metals could be contributing to the higher carriage of class 1 integrons in these species, and given the gap in our current understanding of which environmental factors are contributing to the increased levels of ARB in *A. p. doriferus* pups, this relationship warrants further exploration.

Escherichia coli is commonly used as an indicator of faecal contamination of water sources (Ahmed et al., 2016; Beversdorf et al., 2007) and its presence in numerous wildlife species has been associated with exposure to faecal bacteria from human and domestic livestock sources (Goldberg et al., 2007; McDougall et al., 2021; Pesapane et al., 2013; Rwego et al., 2008b, 2008a; Skurnik et al., 2006). However, the majority of studies to date have focused on terrestrial species. The characterisation of *E. coli* diversity in free-ranging Australian pinniped pups in **Chapter 3** identified a widespread presence of *E. coli* and human-associated phylotypes (including phylotype B2), addressing a significant knowledge gap regarding the presence of human-associated bacteria in pinnipeds in Australia. While it was originally hypothesised that *E. coli* and human-associated phylotypes would be more abundant in pinniped pups in breeding colonies located in closer geographical proximity to

human populations, there was no significant difference in the prevalence of *E. coli* or phylotype distribution across breeding colonies.

It has been suggested that *E. coli* is infrequently isolated from wild mammals inhabiting pristine environments, including pinnipeds (Delport et al., 2015; Stoddard et al., 2009, 2008). The prevalence of *E. coli* in *A. p. doriferus, A. forsteri*, and *N. cinerea* pups in this study (87.3%) is similar to what has been reported in Antarctic pinnipeds (83%) (Mora et al., 2018), but higher than in adult free-ranging (7.7%) and captive (84%) *N. cinerea* (Delport et al., 2015). In adult *N. cinerea*, the prevalence of *E. coli* was higher in captive individuals compared to free-ranging individuals, suggesting that the occurrence of *E. coli* in this species is strongly influenced by exposure to anthropogenic pressures experienced in captivity (Delport et al., 2015). Isolation of *E. coli* from terrestrial wildlife species in Australia further supports the suggestion of *E. coli* being associated with exposure to anthropogenic influences, with wildlife species living in close association with humans more likely to carry *E. coli* compared to those living in undisturbed habitats (Gordon and Cowling, 2003). The prevalence of *E. coli* in free-ranging Australian pinniped pups observed in this study is higher than what has previously been reported in animals that are closely associated with humans, suggesting that populations in both South Australia and Victoria are frequently exposed to *E. coli* and faecal pollution from anthropogenic sources, regardless of breeding colony proximity to human activities.

The human-associated B2 phylotype was the most dominant phylotype across the eight breeding colonies and multiple breeding seasons from 2016-2019. Strains of *E. coli* belonging to the B2 phylotype carry more genes that encode virulence factors than strains belonging to other phylotypes (Johnson and Russo, 2002; Picard et al., 1999), and are frequently implicated as causative agents of extraintestinal disease in humans and avian species (Denamur et al., 2021; Kaper et al., 2004; Manges et al., 2019; Reid et al., 2000). The abundance of the B2 phylotype in Australian pinniped pups sampled in this study (73.7%) was higher than previously reported in Antarctic pinnipeds (~50%) (Mora et al., 2018; Power et al., 2016) and in captive adult *N. cinerea* (67%) (Delport et al., 2015). It has been suggested that the B2 phylotype is more adapted to the intestinal environment, which could contribute to the prevalence identified in free-ranging Australian pinniped pups (Gordon and Cowling, 2003; Nowrouzian et al., 2006, 2005). Analysis of B2 isolates from pinniped pups identified sub-types I, II, and IX, which have previously been associated with extraintestinal disease in

humans and avian species (Clermont et al., 2014; Riley, 2014). While assigning *E. coli* isolates to specific strain types was beyond the scope of this study, the high prevalence of the B2 phylotype observed in free-ranging pinniped pups highlights the need for further analysis to incorporate strain-typing and characterisation of virulence genes. Knowledge of the pathogenicity of *E. coli* strains carried by free-ranging pups will provide enhanced understanding of the potential risk that these human-associated microbes pose to pinniped pup health. Conversely, a greater understanding of the pathogenicity of *E. coli* strains carried by free-ranging pups could also assist in determining whether free-ranging pinnipeds could have a potential role as reservoirs of human-associated bacteria for other free-ranging marine and terrestrial species.

Analysis of single *E. coli* isolates has previously been utilised in Australian wildlife species to characterise the diversity of *E. coli* (Delport et al., 2015; Fulham et al., 2018; Lundbäck et al., 2020), however, whether a single isolate is representative of overall diversity within each host has not been previously explored and represents a key knowledge gap. **Chapter 4** characterised the intra-individual (within) and inter-species (between species) diversity of *E. coli* in *A. p. doriferus, A. forsteri* and *N. cinerea* pups and addressed this gap in knowledge. The intra-individual diversity of *E. coli* phylotypes was not significantly different in each of the three species, with the majority of pups (23/30; 76.7%) carrying *E. coli* isolates belonging to only one phylotype.

The B2 phylotype was again the most dominant phylotype among repeat isolates and was present in 26/30 pups. In pups carrying the B2 phylotype, it was rare for other phylotypes to be present, supporting what has previously been reported in *E. coli* isolated from humans; strains belonging to the B2 phylotype appear to be more well adapted to the intestinal tract (Blyton et al., 2013; Nowrouzian et al., 2006) and outcompete strains belonging to other phylotypes (Smati et al., 2013). Analysis of B2 isolates revealed that there was greater intra-individual and inter-species diversity of B2 sub-types, with most pups carrying two or three sub-types and only three pups carrying one sub-type. Given the intra-individual B2 sub-type diversity, it was determined that a single isolate would not be representative of the overall diversity of B2 sub-types within *A. p. doriferus, A. forsteri* or *N. cinerea* pups.

Although a single isolate appears to be representative of *E. coli* phylotype diversity when strains belonging to the B2 phylotype are present, this approach does have limitations, for example, it is less likely that rarer phylotypes will be detected. However, the results

presented in the preceding chapter **(Chapter 3)** show that phylotypes C, E and F, which are globally less prevalent (Touchon et al., 2020), are infrequently identified in *A. p. doriferus*, *N. cinerea* and *A. forsteri* pups. The results from this study suggest that when *E. coli* isolates are assigned to the B2 phylotype, it is likely that it is representative of phylotype diversity within the individual. While a single isolate may not be representative of *E. coli* B2 sub-type diversity within free-ranging Australian pinniped pups, being able to utilise a single *E. coli* isolate as a representative of diversity is an efficient and cost-effective method to investigate the presence of human-associated bacteria in free-ranging wildlife species.

Chapter 5 characterised the gut microbiota of untreated (control) and treated neonatal *N. cinerea* pups pre- and post-treatment with topical ivermectin and addressed a crucial knowledge gap in the understanding of the safety and efficacy of topical ivermectin treatment. The impact of endemic infection with hookworm (*Uncinaria sanguinis*) in *N. cinerea* pups has previously been reported (Marcus et al., 2014) and recent studies have considered the use of topical ivermectin as a management strategy to aid in the conservation of this endangered species (Lindsay et al., 2021; Marcus et al., 2015b). Treatment for hookworm infection has also been utilised to assist in the management of other pinniped species, including New Zealand sea lion (Castinel et al., 2007; Michael et al., 2021), northern fur seal, and South American fur seal (DeLong et al., 2009; Seguel et al., 2016, 2013) pups. While treatment with ivermectin in all of these species resulted in clearance of hookworm infection and improved pup health and survival (Castinel et al., 2007b; DeLong et al., 2009; Michael et al., 2021; Seguel et al., 2016, 2013), none of these prior studies considered the impact of antiparasiticide administration on the gut microbiota.

The gut microbial community is essential for gut development (Cebra, 1999; Ottman et al., 2012), regulation of immune responses (Brestoff and Artis, 2013), is critical for food digestion (Hooper et al., 2002), and the synthesis of essential minerals and vitamins (Yatsunenko et al., 2012). The microbiota can also assist in preventing pathogens from colonising the intestinal environment (Bäumler and Sperandio, 2016). During early life stages, the gut microbiota undergoes continual change while becoming established (Koenig et al., 2011; Nelson et al., 2013; Palmer et al., 2007; Tian et al., 2020). Disturbances to this establishment, such as helminth infection (Martin et al., 2019; Walk et al., 2010), can cause shifts in microbial community composition resulting in potentially lifelong dysbiosis (Carding et al., 2015; Kriss et al., 2018). Given the importance of the gut microbiota for development

and regulation of both innate and adaptive immune responses (Cebra, 1999; Hill and Artis, 2010; Honda and Littman, 2016; Hooper et al., 2012), understanding the potential impact of parasite removal on the microbial community in the gut of endangered *N. cinerea* pups was a critical component of the evaluation of topical ivermectin as a treatment for endemic hookworm infection.

The characterisation of the gut microbiota of *N. cinerea* pups revealed that the microbial community in both untreated (control) and treated pups was dominated by five bacterial phyla: *Fusobacteria*, *Firmicutes*, *Proteobacteria*, *Actinobacteria* and *Bacteroidetes*. An important finding from this study was that alterations in the composition of the gut microbiota were not associated with hookworm elimination or administration of treatment. The absence of a significant difference in the composition of the gut microbiota associated with treatment and *U. sanguinis* elimination was unexpected and suggests that the gut microbiota has potentially co-evolved or co-adapted with this endemic parasite. In other wildlife species, treatment for parasites has resulted in changes to the gut microbiota composition and overall diversity (He et al., 2018; Moustafa et al., 2021), however, in these previous studies, oral antiparasitic treatment was administered. It is possible that the administration of topical rather than oral ivermectin limits this disturbance to the gut microbiota. For this reason, it could be an effective and safer form of management intervention for the elimination of hookworm in *N. cinerea* pups.

A significant difference in the composition of the gut microbiota and the diversity of the microbial community was seen between pups sampled during the 2019 and 2020/21 breeding seasons at Seal Bay Conservation Park. However, there were no significant differences between the untreated (control) and treated cohorts when compared across seasons, suggesting that these differences were unrelated to treatment. Due to the asynchronous breeding cycle of *N. cinerea*, the 2019 season occurred during the austral winter, while the 2020/21 breeding season began during the austral summer. However, elucidating the environmental conditions that contributed to the significant differences observed were beyond the scope of this study.

While there was no evident impact of topical ivermectin treatment and elimination of *U. sanguinis* infection on the gut microbiota of *N. cinerea* pups, changes in the relative abundance of bacterial phyla were observed between repeated captures of individual pups. These changes were not unexpected, given that the microbial community in young mammals

undergoes continuous change in the first years of life while becoming established (Jeremy E Koenig et al., 2011; Nelson et al., 2013; Palmer et al., 2007; Smith et al., 2013; Tian et al., 2020). It is likely that the gut microbiota in *N. cinerea* pups continues to change until pups are weaned and begin independent foraging, as has been observed in *A. p. doriferus* pups, with significant differences observed between pup and juvenile gut microbial communities (Smith et al., 2013). *Neophoca cinerea* pups are not weaned until they are at least 18-months old (Higgins and Gass, 1993), and *N. cinerea* pups sampled as part of this study were a maximum of six months old. While characterisation of the gut microbiota of adult free-ranging *N. cinerea* has been undertaken (Delport et al., 2016), the gut microbiota of juvenile *N. cinerea* has not yet been characterised. Collecting samples from older pups (between 6-18 months of age) and juvenile *N. cinerea* is a vital next step for future investigation.

Future studies should involve ongoing monitoring of untreated (control) and treated *N. cinerea* pups as they transition into juvenile and adult life stages. Opportunistic faecal sampling of these older age cohorts could be utilised to characterise the gut microbiota and determine how these microbial communities have developed during the period of transition from pup to juvenile and adult life stages.

6.2. Conclusion

The findings of this thesis address key knowledge gaps concerning the presence of human-associated bacteria and antimicrobial resistance genes in free-ranging pinniped populations in Australia and provides evidence to suggest that these populations are exposed to anthropogenic microbial and faecal pollution. A baseline of data is presented, providing a comparison point for future investigations, a critical step for ongoing monitoring of anthropogenic impacts on pinniped populations in Australia. The finding of a high prevalence of human-associated *E. coli* phylotypes and antimicrobial resistance genes in these free-ranging pinniped populations highlights the imperative for ongoing monitoring of anthropogenic pollution in these species to mitigate any future microbial impacts that could threaten the health and viability of free-ranging pinniped populations in Australia, particularly the endangered Australian sea lion.

6.3. References

Ahmed, W., Sidhu, J. P. S., Smith, K., Beale, D. J., Gyawali, P., and Toze, S. (2016). Distributions of fecal markers in wastewater from different climatic zones for human fecal pollution tracking in Australian surface waters. *Appl Environ Microbiol* 82, 1316–1323. doi: 10.1128/AEM.03765-15.

Ansari, Z. A., and Matondkar, S. G. P. (2014). Anthropogenic activities including pollution and contamination of coastal marine environment. *J Ecophysiol Occup Health* 14, 71–78. doi: 10.15512/joeoh/2014/v14i1-2/50743.

Ashley, E. A., Olson, J. K., Raverty, S., Wilkinson, K., and Gaydos, J. K. (2020). Trace element concentrations in livers of pacific harbor seals (*Phoca vitulina richardii*) from San Juan County, Washington, USA. *J Wildl Dis* 56, 429–436. doi: 10.7589/2019-04-087.

Avila, I. C., Kaschner, K., and Dormann, C. F. (2018). Current global risks to marine mammals: taking stock of the threats. *Biol Conserv* 221, 44–58. doi: 10.1016/j.biocon.2018.02.021.

Bäumler, A. J., and Sperandio, V. (2016). Interactions between the microbiota and pathogenic bacteria in the gut. *Nature* 535, 85–93. doi: 10.1038/nature18849.

Baysarowich, J., Koteva, K., Hughes, D. W., Ejim, L., Griffiths, E., Zhang, K., et al. (2008). Rifamycin antibiotic resistance by ADP-ribosylation: structure and diversity of Arr. *Proc Natl Acad Sci U S A* 105, 4886–4891. doi: 10.1073/pnas.0711939105.

Beiras, R. (2018). *Marine pollution: sources, fate and effects of pollutants in coastal ecosystems*. Amsterdam, Netherlands: Elsevier doi: 10.1016/C2017-0-00260-4.

Beversdorf, L. J., Bornstein-Forst, S. M., and McLellan, S. L. (2007). The potential for beach sand to serve as a reservoir for *Escherichia coli* and the physical influences on cell die-off. *J Appl Microbiol* 102, 1372–1381. doi: 10.1111/j.1365-2672.2006.03177.x.

Bie, L., Fang, M., Li, Z., Wang, M., and Xu, H. (2018). Identification and characterization of new resistance-conferring SGI1s (*Salmonella* Genomic Island 1) in *Proteus mirabilis*. *Front Microbiol* 9, 3172. doi: 10.3389/fmicb.2018.03172.

Blyton, M. D. J., Banks, S. C., Peakall, R., and Gordon, D. M. (2013). High temporal variability in commensal *Escherichia coli* strain communities of a herbivorous marsupial. *Environ Microbiol* 15, 2162–2172. doi: 10.1111/1462-2920.12088.

Brestoff, J. R., and Artis, D. (2013). Commensal bacteria at the interface of host metabolism and the immune system. *Nat Immunol* 14, 676–684. doi: 10.1038/ni.2640.

Carding, S., Verbeke, K., Vipond, D. T., Corfe, B. M., and Owen, L. J. (2015). Dysbiosis of the gut microbiota in disease. *Microb Ecol Health Dis* 26, 26191. doi: 10.3402/mehd.v26.26191.

Castinel, A., Duignan, P. J., Pomroy, W. E., López-Villalobos, N., Gibbs, N. J., Chilvers, B. L., et al. (2007). Neonatal mortality in New Zealand sea lions (*Phocarctos hookeri*) at Sandy Bay, Enderby Island, Auckland Islands from 1998 to 2005. *J Wildl Dis* 43, 461–474. doi: 10.7589/0090-3558-43.3.461.

Cebra, J. J. (1999). Influences of microbiota on intestinal immune system development. *Am J Clin Nutr* 69, 1046–1051. doi: 10.1093/ajcn/69.5.1046s.

Clermont, O., Christenson, J. K., Daubié, A.-S., Gordon, D. M., and Denamur, E. (2014). Development of an allele-specific PCR for *Escherichia coli* B2 sub-typing, a rapid and easy to perform substitute of multilocus sequence typing. *J Microbiol Methods* 101, 24–27. doi: 10.1016/j.mimet.2014.03.008.

Cobb-Clark, A., and Gray, R. (2018). Concentration of trace elements and heavy metals in the fur and blood of Australian fur seal (*Arctocephalus pusillus doriferus*) pups.

Crain, C., Halpern, B., Beck, M., and Kappel, C. (2009). Understanding and managing human threats to the coastal marine environment. *Ann N Y Acad Sci* 1162, 39–62. doi: 10.1111/j.1749-6632.2009.04496.x.

Davidson, A. D., Boyer, A. G., Kim, H., Pompa-Mansilla, S., Hamilton, M. J., Costa, D. P., et al. (2012). Drivers and hotspots of extinction risk in marine mammals. *Proc Natl Acad Sci U S A* 109, 3395–3400. doi: 10.1073/pnas.1121469109.

Dawes, F. E., Kuzevski, A., Bettelheim, K. A., Hornitzky, M. A., Djordjevic, S. P., and Walker, M. J. (2010). Distribution of class 1 integrons with IS*26*-mediated deletions in their 3'-conserved segments in *Escherichia coli* of human and animal origin. *PLOS ONE* 5, e12754–e12754. doi: 10.1371/journal.pone.0012754.

de Vincente, A., Avilés, M., Codina, J. C., Borrego, J. J., and Romero, P. (1990). Resistance to antibiotics and heavy metals of *Pseudomonas aeruginosa* isolated from natural waters. *J Appl Microbiol* 68, 625–632. doi: 10.1111/j.1365-2672.1990.tb05228.x.

DeLong, R. L., Orr, A. J., Jenkinson, R. S., and Lyons, E. T. (2009). Treatment of northern fur seal (*Callorhinus ursinus*) pups with ivermectin reduces hookworm-induced mortality. *Mar Mamm Sci* 25, 944–948. doi: 10.1111/j.1748-7692.2008.00274.x.

Delport, T. C., Harcourt, R. G., Beaumont, L. J., Webster, K. N., and Power, M. L. (2015). Molecular detection of antibiotic-resistance determinants in *Escherichia coli* isolated from the

endangered Australian sea lion (*Neophoca cinerea*). *J Wildl Dis* 51, 555–563. doi: 10.7589/2014-08-200.

Delport, T. C., Power, M. L., Harcourt, R. G., Webster, K. N., and Tetu, S. G. (2016). Colony location and captivity influence the gut microbial community composition of the Australian sea lion (*Neophoca cinerea*). *Appl Environ Microbiol* 82, 3440–3449. doi: 10.1128/AEM.00192-16.

Denamur, E., Clermont, O., Bonacorsi, S., and Gordon, D. (2021). The population genetics of pathogenic *Escherichia coli*. *Nat Rev Microbiol* 19, 37–54. doi: 10.1038/s41579-020-0416-x.

Dickinson, A. W., Power, A., Hansen, M. G., Brandt, K. K., Piliposian, G., Appleby, P., et al. (2019). Heavy metal pollution and co-selection for antibiotic resistance: a microbial palaeontology approach. *Environ Int* 132, 105117. doi: 10.1016/j.envint.2019.105117.

Dolejska, M., Cizek, A., and Literak, I. (2007). High prevalence of antimicrobial-resistant genes and integrons in *Escherichia coli* isolates from Black-headed Gulls in the Czech Republic. *J Appl Microbiol* 103, 11–19. doi: 10.1111/j.1365-2672.2006.03241.x.

Dolejska, M., and Literak, I. (2019). Wildlife is overlooked in the epidemiology of medically important antibiotic-resistant bacteria. *Antimicrob Agents Chemother* 8, e01167-19. doi: 10.1128/AAC.01167-19.

Fulham, M., Power, M., and Gray, R. (2018). Comparative ecology of *Escherichia coli* in endangered Australian sea lion (*Neophoca cinerea*) pups. *Infect Genet Evol* 62. doi: 10.1016/j.meegid.2018.05.002.

Gales, Nick., Hindell, M., and Kirkwood, Roger. (2003). *Marine mammals: fisheries, tourism and management issues*. Collingwood, Vic: CSIRO Publishing.

Gillings, M. R., Gaze, W. H., Pruden, A., Smalla, K., Tiedje, J. M., and Zhu, Y.-G. (2014). Using the class 1 integron-integrase gene as a proxy for anthropogenic pollution. *ISME J* 9. doi: 10.1038/ismej.2014.226.

Goldberg, T. L., Gillespie, T. R., Rwego, I. B., Wheeler, E., Estoff, E. L., and Chapman, C. A. (2007). Patterns of gastrointestinal bacterial exchange between chimpanzees and humans involved in research and tourism in western Uganda. *Biol Conserv* 135, 511–517. doi: 10.1016/j.biocon.2006.10.048.

Goldsworthy, S. D., Shaughnessy, P. D., Mackay, A. I., Bailleul, F., Holman, D., Lowther, A. D., et al. (2021). Assessment of the status and trends in abundance of a coastal pinniped, the

Australian sea lion *Neophoca cinerea*. *Endanger Species Res* 44, 421–437. doi: 10.3354/ESR01118.

Gordon, D. M., and Cowling, A. (2003). The distribution and genetic structure of *Escherichia coli* in Australian vertebrates: host and geographic effects. *Microbiology* 149, 3575–3586. doi: 10.1099/mic.0.26486-0.

Hamer, D. J., and Goldsworthy, S. D. (2006). Seal-fishery operational interactions: identifying the environmental and operational aspects of a trawl fishery that contribute to by-catch and mortality of Australian fur seals (*Arctocephalus pusillus doriferus*). *Biol Conserv* 130, 517–529. doi: 10.1016/j.biocon.2006.01.014.

Hamer, D. J., Goldsworthy, S. D., Costa, D. P., Folwer, S., Page, M., and Sumner, M. D. (2013). The endangered Australian sea lion extensively overlaps with and regularly becomes by-catch in demersal shark gill-nets in South Australian shelf waters. *Biol Conserv* 157, 386–400. doi: 10.1016/j.biocon.2012.07.010.

Hastak, P., Cummins, M. L., Gottlieb, T., Cheong, E., Merlino, J., Myers, G. S. A., et al. (2020). Genomic profiling of *Escherichia coli* isolates from bacteraemia patients: a 3-year cohort study of isolates collected at a Sydney teaching hospital. *Microb Genom* 6, e000371. doi: 10.1099/mgen.0.000371.

He, F., Zhai, J., Zhang, L., Liu, D., Ma, Y., Rong, K., et al. (2018). Variations in gut microbiota and fecal metabolic phenotype associated with Fenbendazole and Ivermectin tablets by 16S rRNA gene sequencing and LC/MS-based metabolomics in Amur tiger. *Biochem Biophys Res Commun* 499, 447–453. doi: 10.1016/j.bbrc.2018.03.158.

Helm, R. C., Costa, D. P., DeBruyn, T. D., O'Shea, T. J., Wells, R. S., and Williams, T. M. (2014). "Overview of effects of oil spills on marine mammals," in *Handbook of Oil Spill Science and Technology*, ed. M. Fingas.

Higgins, L. V., and Gass, L. (1993). Birth to weaning: parturition, duration of lactation, and attendance cycles of Australian sea lions (*Neophoca cinerea*). *Can J Zool* 71, 2047–2055. doi: 10.1139/z93-290.

Hill, D. A., and Artis, D. (2010). Intestinal bacteria and the regulation of immune cell homeostasis. *Annu Rev Immunol* 28, 623–667. doi: 10.1146/annurev-immunol-030409-101330.

Honda, K., and Littman, D. R. (2016). The microbiota in adaptive immune homeostasis and disease. *Nature* 535, 75–84. doi: 10.1038/nature18848.

Hooper, L. V., Littman, D. R., and Macpherson, A. J. (2012). Interactions between the microbiota and the immune system. *Science* 336, 1268–1273. doi: 10.1126/science.1223490.

Hooper, L. V., Midtvedt, T., and Gordon, J. I. (2002). How host-microbial interactions shape the nutrient environment of the mammalian intestine. *Annu Rev Nutr* 22, 283–307. doi: 10.1146/annurev.nutr.22.011602.092259.

Johnson, J. R., and Russo, T. A. (2002). Extraintestinal pathogenic *Escherichia coli*: "The other bad *E coli*." *Journal of Laboratory and Clinical Medicine* 139, 155–162. doi: 10.1067/mlc.2002.121550.

Kaper, J. B., Nataro, J. P., and Mobley, H. L. T. (2004). Pathogenic *Escherichia coli*. *Nat Rev Microbiol* 2, 123–140. doi: 10.1038/nrmicro818.

Kirkwood, Roger., and Goldsworthy, Simon. (2013). *Fur Seals and Sea Lions*. Collingwood, VIC: CSIRO Publishing.

Koenig, J. E., Spor, A., Scalfone, N., Fricker, A. D., Stombaugh, J., Knight, R., et al. (2011). Succession of microbial consortia in the developing infant gut microbiome. *Proc Natl Acad Sci U S A* 108 Suppl 1, 4578–4585. doi: 10.1073/pnas.1000081107.

Kovacs, K. M., Aguilar, A., Aurioles, D., Burkanov, V., Campagna, C., Gales, N., et al. (2012). Global threats to pinnipeds. *Mar Mamm Sci* 28, 414–436. doi: 10.1111/j.1748-7692.2011.00479.x.

Kretzmann, M., Rohrbach, L., Durham, K., Digiovanni, R., and Sañudo-Wilhelmy, S. (2010). Trace metal burdens in stranded seals from Long Island, New York: potential evidence for species differences in foraging. *Aquat Mamm* 36, 178–187. doi: 10.1578/AM.36.2.2010.178.

Kriss, M., Hazleton, K. Z., Nusbacher, N. M., Martin, C. G., and Lozupone, C. A. (2018). Low diversity gut microbiota dysbiosis: drivers, functional implications and recovery. *Curr Opin Microbiol* 44, 34–40. doi: 10.1016/j.mib.2018.07.003.

Lindsay, S. A., Caraguel, C. G. B., and Gray, R. (2021). Topical ivermectin is a highly effective seal 'spot-on': A randomised trial of hookworm and lice treatment in the endangered Australian sea lion (*Neophoca cinerea*). *Int J Parasitol Parasites Wildl* 16, 275–284. doi: 10.1016/j.ijppaw.2021.11.002.

Lundbäck, I. C., McDougall, F. K., Dann, P., Slip, D. J., Gray, R., and Power, M. L. (2020). Into the sea: Antimicrobial resistance determinants in the microbiota of little penguins (*Eudyptula minor*). *Infect Genet Evol*, 104697. doi: 10.1016/j.meegid.2020.104697.

Manges, A. R., Geum, H. M., Guo, A., Edens, T. J., Fibke, C. D., and Pitout, J. D. D. (2019). Global extraintestinal pathogenic *Escherichia coli* (ExPEC) lineages. *Clin Microbiol Rev* 32. doi: 10.1128/CMR.00135-18.

Marcovecchio, J. E., Gerpe, M. S., Bastida, R. O., Rodríguez, D. H., and Morón, S. G. (1994). Environmental contamination and marine mammals in coastal waters from Argentina: an overview. *Sci Total Environ* 154, 141–151. doi: 10.1016/0048-9697(94)90084-1.

Marcus, A. D., Higgins, D. P., and Gray, R. (2014). Epidemiology of hookworm (*Uncinaria sanguinis*) infection in free-ranging Australian sea lion (*Neophoca cinerea*) pups. *Parasitol Res* 113, 3341–3353. doi: 10.1007/s00436-014-3997-3.

Marcus, A. D., Higgins, D. P., and Gray, R. (2015a). Health assessment of free-ranging endangered Australian sea lion (*Neophoca cinerea*) pups: effect of haematophagous parasites on haematological parameters. *Comp Biochem Physiol A Mol Integr Physiol* 184, 132–143. doi: 10.1016/j.cbpa.2015.02.017.

Marcus, A. D., Higgins, D. P., and Gray, R. (2015b). Ivermectin treatment of free-ranging endangered Australian sea lion (*Neophoca cinerea*) pups: effect on hookworm and lice infection status, haematological parameters, growth, and survival. *Parasitol Res* 114, 2743–2755. doi: 10.1007/s00436-015-4481-4.

Martin, I., Kaisar, M. M. M., Wiria, A. E., Hamid, F., Djuardi, Y., Sartono, E., et al. (2019). The effect of gut microbiome composition on human immune responses: an exploration of interference by helminth infections. *Front Genet* 10, 1028. doi: 10.3389/fgene.2019.01028.

McDougall, F. K., Boardman, W. S. J., and Power, M. L. (2021). Characterization of beta-lactam-resistant *Escherichia coli* from Australian fruit bats indicates anthropogenic origins. *Microb Genom* 7, 000571. doi: 10.1099/mgen.0.000571.

McIntosh, R. R., Kirkman, S. P., Thalmann, S., Sutherland, D. R., Mitchell, A., Arnould, J. P. Y., et al. (2018). Understanding meta-population trends of the Australian fur seal, with insights for adaptive monitoring. *PLOS ONE* 13, e0200253. doi: 10.1371/journal.pone.0200253.

McIntosh, R. R., Kirkwood, R., Sutherland, D. R., and Dann, P. (2015). Drivers and annual estimates of marine wildlife entanglement rates: a long-term case study with Australian fur seals. *Mar Pollut Bull* 101, 716–725. doi: 10.1016/j.marpolbul.2015.10.007.

McIntosh, R. R., Sorrell, K. J., Thalmann, S., Mitchell, A., Gray, R., Schinagl, H., et al. (2022). Sustained reduction in numbers of Australian fur seal pups: implications for future population monitoring. *PLOS ONE* 17, e0265610. doi: 10.1371/journal.pone.0265610.

McLean, L. J., George, S., Ierodiaconou, D., Kirkwood, R. J., and Arnould, J. P. Y. (2018). Impact of rising sea levels on Australian fur seals. *PeerJ* 6, e5786–e5786. doi: 10.7717/peerj.5786.

McLeay, L., Tsolos, A., and Boyle, M. K. (2015). Operational interactions with threatened, endangered or protected species in South Australian managed fisheries data summary: 2007/08-2013/14. *SARDI Publication No. F2009/000544-5 SARDI Research Report Series No. 851*.

Michael, S. A., Hayman, D. T. S., Gray, R., and Roe, W. D. (2021). Risk factors for New Zealand sea lion (*Phocarctos hookeri*) pup mortality: Ivermectin improves survival for conservation management. *Front Mar Sci* 8. doi: 10.3389/fmars.2021.68.

Mora, A., García-Peña, F., Alonso, M., Pedraza-Diaz, S., Ortega-Mora, L., Garcia-Parraga, D., et al. (2018). Impact of human-associated *Escherichia coli* clonal groups in Antarctic pinnipeds: presence of ST73, ST95, ST141 and ST131. *Sci Rep* 8, 4678. doi: 10.1038/s41598-018-22943-0.

Moustafa, M. A. M., Chel, H. M., Thu, M. J., Bawm, S., Htun, L. L., Win, M. M., et al. (2021). Anthropogenic interferences lead to gut microbiome dysbiosis in Asian elephants and may alter adaptation processes to surrounding environments. *Sci Rep* 11, 741. doi: 10.1038/s41598-020-80537-1.

Nelson, T. M., Rogers, T. L., Carlini, A. R., and Brown, M. v (2013). Diet and phylogeny shape the gut microbiota of Antarctic seals: a comparison of wild and captive animals. *Environ Microbiol* 15, 1132–1145. doi: 10.1111/1462-2920.12022.

Nguyen, C. C., Hugie, C. N., Kile, M. L., and Navab-Daneshmand, T. (2019). Association between heavy metals and antibiotic-resistant human pathogens in environmental reservoirs: a review. *Front Environ Sci Eng* 13, 46. doi: 10.1007/s11783-019-1129-0.

Nowrouzian, F. L., Adlerberth, I., and Wold, A. E. (2006). Enhanced persistence in the colonic microbiota of *Escherichia coli* strains belonging to phylogenetic group B2: role of virulence factors and adherence to colonic cells. *Microbes Infect* 8, 834–840. doi: 10.1016/j.micinf.2005.10.011.

Nowrouzian, F. L., Wold, A. E., and Adlerberth, I. (2005). *Escherichia coli* strains belonging to phylogenetic group B2 have superior capacity to persist in the intestinal microflora of infants. *J Infect Dis* 191, 1078–1083. doi: 10.1086/427996.

Ottman, N., Smidt, H., de Vos, W. M., and Belzer, C. (2012). The function of our microbiota: who is out there and what do they do? *Front Cell Infect Microbiol* 2, 104. doi: 10.3389/fcimb.2012.00104.

Page, B., McKenzie, J., McIntosh, R., Baylis, A., Morrissey, A., Calvert, N., et al. (2004). Entanglement of Australian sea lions and New Zealand fur seals in lost fishing gear and other marine debris before and after Government and industry attempts to reduce the problem. *Mar Pollut Bull* 49, 33–42. doi: 10.1016/j.marpolbul.2004.01.006.

Pal, C., Bengtsson-Palme, J., Kristiansson, E., and Larsson, D. G. J. (2015). Co-occurrence of resistance genes to antibiotics, biocides and metals reveals novel insights into their co-selection potential. *BMC Genomics* 16. doi: 10.1186/S12864-015-2153-5.

Palmer, C., Bik, E. M., Digiulio, D. B., Relman, D. A., and Brown, P. O. (2007). Development of the human infant intestinal microbiota. *PLoS Biol* 5, 177. doi: 10.1371/journal.pbio.

Pesapane, R., Ponder, M., and Alexander, K. A. (2013). Tracking pathogen transmission at the human–wildlife interface: banded mongoose and *Escherichia coli*. *Ecohealth* 10, 115–128. doi: 10.1007/s10393-013-0838-2.

Picard, B., Garcia, J. S., Gouriou, S., Duriez, P., Brahimi, N., Bingen, E., et al. (1999). The link between phylogeny and virulence in *Escherichia coli* extraintestinal infection. *Infect Immun* 67, 546. doi: 10.1128/iai.67.2.546-553.1999.

Poloczanska, E. S., Babcock, R. C., Butler, A., Hobday, A. J., Hoegh-Guldberg, O., Kunz, T. J., et al. (2007). "Climate change and Australian marine life," in *Oceanography and Marine Biology: An Annual Review*, eds. R. N. Gibson, R. J. A. Atkinson, and J. D. M. Gordon, 407–478.

Pommepuy, M., Hervio-Heath, D., Caprais, M. P., Gourmelon, M., le Saux, J. C., and le Guyader, F. (2006). "Fecal Contamination in Coastal Areas: An Engineering Approach," in *Oceans and Health: Pathogens in The Marine Environment*, eds. B. S. and R. R. Colwell (Boston, MA: Springer), 331–359. doi: 10.1007/0-387-23709-7_14.

Pompa, S., Ehrlich, P. R., and Ceballos, G. (2011). Global distribution and conservation of marine mammals. *Proc Natl Acad Sci U S A* 108, 13600–13605. doi: 10.1073/pnas.1101525108.

Power, M. L., Samuel, A., Smith, J. J., Stark, J. S., Gillings, M. R., and Gordon, D. M. (2016). *Escherichia coli* out in the cold: Dissemination of human-derived bacteria into the Antarctic microbiome. *Environ Pollut* 215, 58–65. doi: 10.1016/j.envpol.2016.04.013.

Reid, C., Wyrsch, E., Zingali, T., Liu, M., Darling, A., Chapman, T., et al. (2017). Porcine commensal *Escherichia coli*: a reservoir for class 1 integrons associated with IS*26*. *BioRxiv* 3, e000143. doi: 10.1099/mgen.0.000143.

Reid, S. D., Herbelin, C. J., Bumbaugh, A. C., Selander, R. K., and Whittam, T. S. (2000). Parallel evolution of virulence in pathogenic *Escherichia coli*. *Nature* 406, 64–67. doi: 10.1038/35017546.

Reijnders, P., Aguilar, A., and Borrell, A. (2009). "Pollution and marine mammals," in *Encyclopedia of Marine Mammals*, 890–898.

Riley, L. W. (2014). Pandemic lineages of extraintestinal pathogenic *Escherichia coli*. *Clin Microbiol Infect* 20, 380–390. doi: /10.1111/1469-0691.12646.

Rosewarne, C. P., Pettigrove, V., Stokes, H. W., and Parsons, Y. M. (2010). Class 1 integrons in benthic bacterial communities: abundance, association with Tn402-like transposition modules and evidence for coselection with heavy-metal resistance. *FEMS Microbiol Ecol* 72, 35–46. doi: 10.1111/j.1574-6941.2009.00823.x.

Rwego, I. B., Gillespie, T. R., Isabirye-Basuta, G., and Goldberg, T. L. (2008a). High rates of *Escherichia coli* transmission between livestock and humans in rural Uganda. *J Clin Microbiol* 46, 3187–3191. doi: 10.1128/JCM.00285-08.

Rwego, I. B., Isabirye-Basuta, G., Gillespie, T. R., and Goldberg, T. L. (2008b). Gastrointestinal bacterial transmission among humans, mountain gorillas, and livestock in Bwindi Impenetrable National Park, Uganda. *Conserv Biol* 22, 1600–1607. doi: 10.1111/j.1523-1739.2008.01018.x.

Schumann, N., Gales, N. J., Harcourt, R. G., and Arnould, J. P. Y. (2013). Impacts of climate change on Australian marine mammals. *Aust J Zool* 61, 146–159. doi: 10.1071/ZO12131.

Seguel, M., Muñoz, F., Navarrete, M. J., Paredes, E., Howerth, E., and Gottdenker, N. (2016). Hookworm infection in South American fur seal (*Arctocephalus australis*) pups: pathology and factors associated with host tissue damage and mortality. *Vet Pathol* 54, 288–297. doi: 10.1177/0300985816677151.

Seguel, M., Pavés, H., Paredes, E., and Schlatter, R. (2013). Causes of mortality in South American fur seal pups (*Arctophoca australis gracilis*) at Guafo Island, southern Chile (2004–2008). *Mar Mamm Sci* 29, 36–47. doi: 10.1111/j.1748-7692.2011.00534.x.

Shaughnessy, P. D., and Goldsworthy, S. D. (2015). Increasing abundance of pups of the long-nosed fur seal (*Arctocephalus forsteri*) on Kangaroo Island, South Australia, over 26 breeding seasons to 2013–14. *Wildl Res* 42, 619–632. doi: 10.1071/WR14209.

Shaughnessy, P. D., Goldsworthy, S. D., and Mackay, A. I. (2014). Status and trends in abundance of New Zealand fur seal populations in South Australia. *SARDI Publication Number F2014/000338-1. SARDI Research Report Series No. 781 (Adelaide, South Australia)*.

Shaughnessy, P. D., Goldsworthy, S. D., and Mackay, A. I. (2015). The long-nosed fur seal (*Arctocephalus forsteri*) in South Australia in 2013–14: abundance, status and trends. *Aust J Zool* 63, 101. doi: 10.1071/ZO14103.

Shaughnessy, P., Kirkwood, R., Cawthorn, M., Kemper, C., and Pemberton, D. (2003). "Pinnipeds, cetaceans and fisheries in Australia: a review of operational interactions," in *Marine mammals: fisheries, tourism and management issues*, eds. N. J. Gales, M. A. Hindell, and R. Kirkwood (Melbourne: CSIRO Publishing), 136–152.

Skurnik, D., Ruimy, R., Andremont, A., Amorin, C., Rouquet, P., Picard, B., et al. (2006). Effect of human vicinity on antimicrobial resistance and integrons in animal faecal *Escherichia coli*. *J Antimicrob Chemother* 57, 1215–1219. doi: 10.1093/jac/dkl122.

Smati, M., Clermont, O., Gal, F. le, Schichmanoff, O., Jauréguy, F., Eddi, A., et al. (2013). Real-Time PCR for quantitative analysis of human commensal *Escherichia coli* populations reveals a high frequency of subdominant phylogroups. *Appl Environ Microbiol* 79, 5005. doi: 10.1128/AEM.01423-13.

Smith, S. C., Chalker, A., Dewar, M. L., and Arnould, J. P. Y. (2013). Age-related differences revealed in Australian fur seal *Arctocephalus pusillus doriferus* gut microbiota. *FEMS Microbiol Ecol* 86, 246–255. doi: 10.1111/1574-6941.12157.

Stoddard, R. A., Atwill, E. R., Conrad, P. A., Byrne, B. A., Jang, S., Lawrence, J., et al. (2009). The effect of rehabilitation of northern elephant seals (*Mirounga angustirostris*) on antimicrobial resistance of commensal *Escherichia coli*. *Vet Microbiol* 133, 264–271. doi: 10.1016/j.vetmic.2008.07.022.

Stoddard, R. A., Atwill, E. R., Gulland, F. M. D., Miller, M. A., Dabritz, H. A., Paradies, D. M., et al. (2008). Risk factors for infection with pathogenic and antimicrobial-resistant fecal bacteria in northern elephant seals in California. *Public Health Rep* 123, 360–370. doi: 10.1177/003335490812300316.

Taylor, S., Lynch, M., Terkildsen, M., Stevenson, G., Yates, A., Piro, N., et al. (2018). Utility of fur as a biomarker for persistent organic pollutants in Australian fur seals (*Arctocephalus pusillus doriferus*). *Sci Total Environ* 610–611, 1310–1320. doi: 10.1016/j.scitotenv.2017.08.204.

Taylor, S., Terkildsen, M., Stevenson, G., de Araujo, J., Yu, C., Yates, A., et al. (2021). Per and polyfluoroalkyl substances (PFAS) at high concentrations in neonatal Australian pinnipeds. *Sci Total Environ* 786, 147446. doi: 10.1016/j.scitotenv.2021.147446.

Tian, J., Du, J., Han, J., Song, X., and Lu, Z. (2020). Age-related differences in gut microbial community composition of captive spotted seals (*Phoca largha*). *Mar Mamm Sci* 36, 1231–1240. doi: 10.1111/mms.12728.

Touchon, M., Perrin, A., de Sousa, J. A. M., Vangchhia, B., Burn, S., O'Brien, C. L., et al. (2020). Phylogenetic background and habitat drive the genetic diversification of *Escherichia coli*. *PLoS Genet* 16, e1008866–e1008866. doi: 10.1371/journal.pgen.1008866.

Tuholske, C., Halpern, B. S., Blasco, G., Villasenor, J. C., Frazier, M., and Caylor, K. (2021). Mapping global inputs and impacts from of human sewage in coastal ecosystems. *PLOS ONE* 16, e0258898. doi: 10.1371/journal.pone.0258898.

Walk, S. T., Blum, A. M., Ewing, S. A.-S., Weinstock, J. v, and Young, V. B. (2010). Alteration of the murine gut microbiota during infection with the parasitic helminth *Heligmosomoides polygyrus*. *Inflamm Bowel Dis* 16, 1841–1849. doi: 10.1002/ibd.21299.

Xia, W., Xu, T., Qin, T., Li, P., Liu, Y., Kang, H., et al. (2016). Characterization of integrons and novel cassette arrays in bacteria from clinical isolates in China, 2000-2014. *J Biomed Res* 30, 292–303. doi: 10.7555/JBR.30.20150153.

Yatsunenko, T., Rey, F. E., Manary, M. J., Trehan, I., Dominguez-Bello, M. G., Contreras, M., et al. (2012). Human gut microbiome viewed across age and geography. *Nature* 486, 222–227. doi: 10.1038/nature11053.

Zingali, T., Reid, C., Chapman, T., Gaio, D., Liu, M., Darling, A., et al. (2020). Whole genome sequencing analysis of porcine faecal commensal *Escherichia coli* carrying class 1 integrons from sows and their offspring. *Microorganisms* 8, 843. doi: 10.3390/microorganisms8060843.