PLOS ONE

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

Mariel Fulham[1], Fiona McDougall[2], Michelle Power[2], Rebecca R. McIntosh[3], Rachael Gray[1] *

1 Faculty of Science, Sydney School of Veterinary Science, The University of Sydney, Sydney, New South Wales, Australia, 2 Department of Biological Sciences, Macquarie University, North Ryde, Sydney, New South Wales, Australia, 3 Research Department, Cowes, Victoria, Australia

* rachael.gray@sydney.edu.au

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.

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

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.*
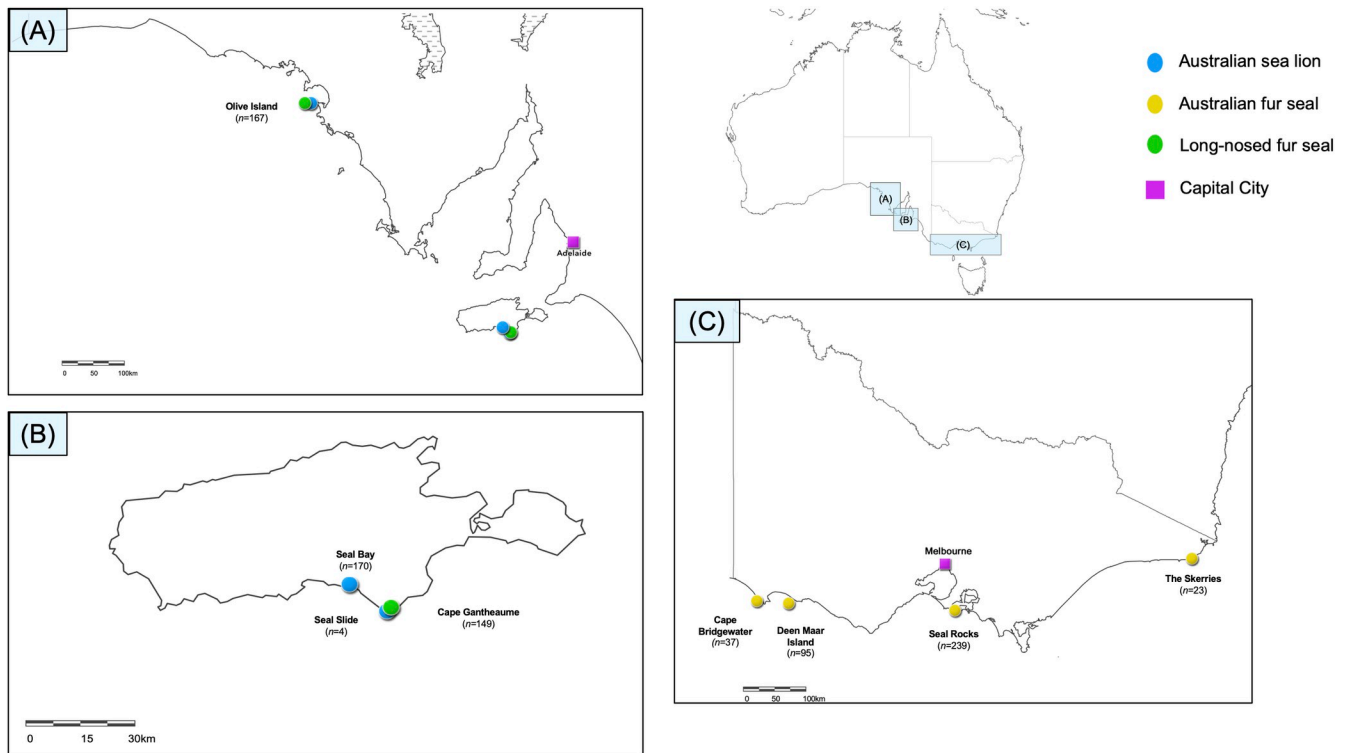
Fig 1. Map of the geographical locations of breeding colonies and pinniped species sampled. Breeding colonies in South Australia (A) include Olive Island, (B) Seal Bay, Seal Slide and Cape Gantheaume on Kangaroo Island, and (C) Cape Bridgewater, Deen Maar Island, Seal Rocks and The Skerries in Victoria. 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.

https://doi.org/10.1371/journal.pone.0258978.g001

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.

https://doi.org/10.1371/journal.pone.0258978.t001

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.

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$_2$O) 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.
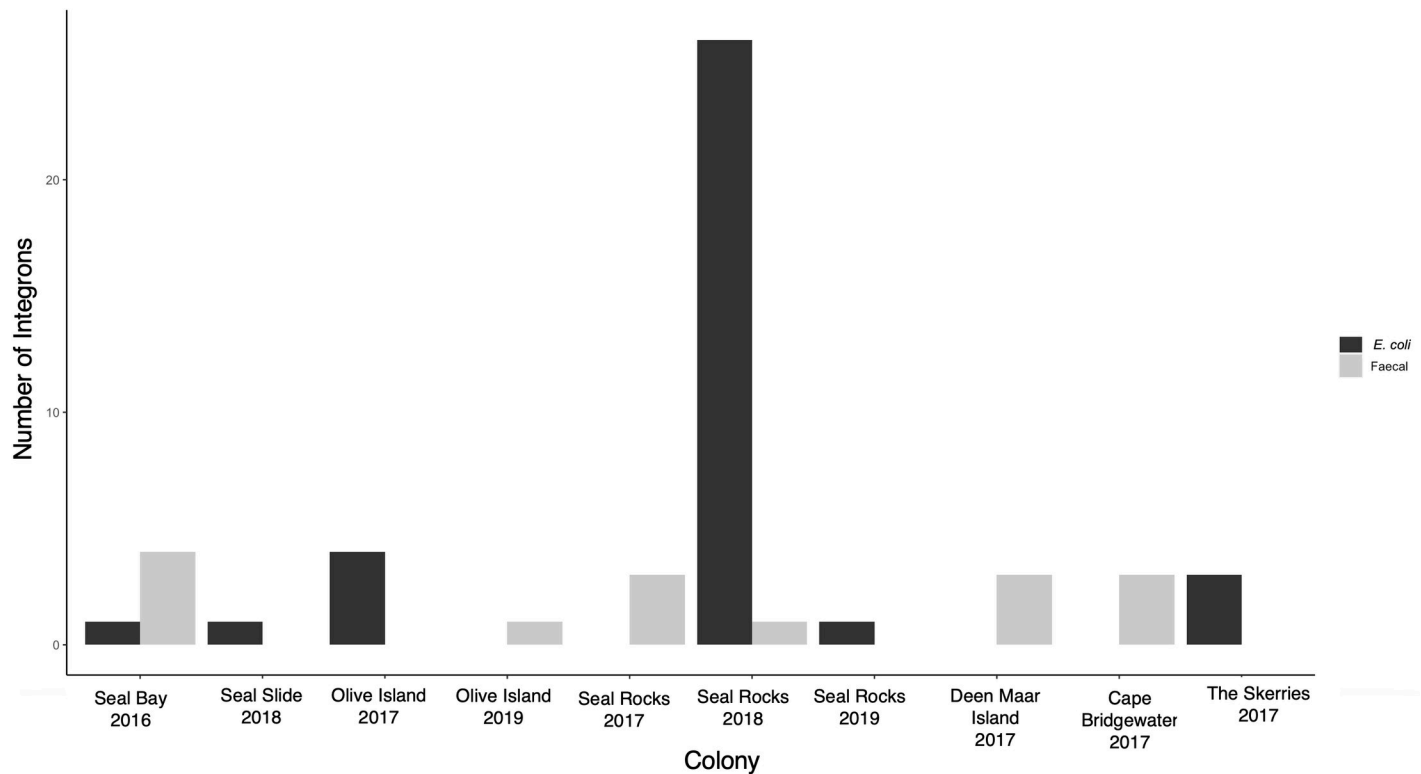
Fig 2. Graph of class 1 integrons detected in pinniped pups. Total number of class 1 integrons detected in faecal and *E. coli* isolate DNA in pups at each colony where integrons were detected during each breeding season sampled.

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.
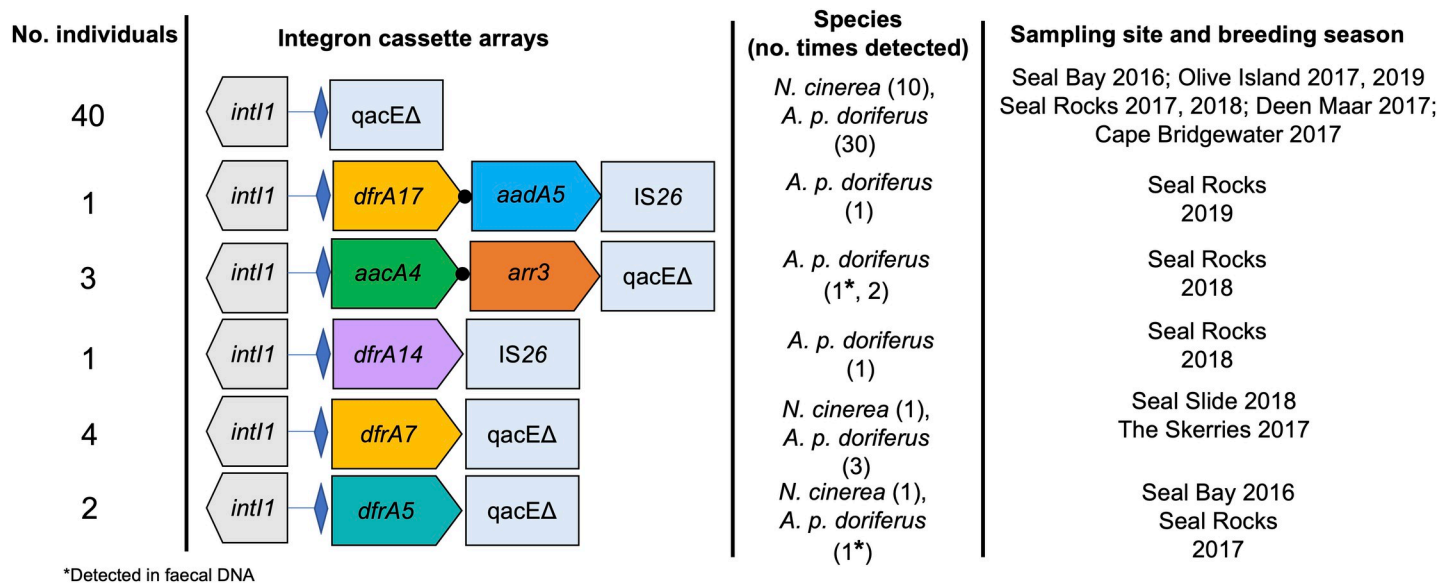
Fig 3. Schematic map of class 1 integron gene cassette arrays identified in *N. cinerea* and *A. p. doriferus* across all sampling sites and breeding seasons. Number of individuals with each array are listed on the left-hand side. Gene cassettes are represented as broad arrows. Blue diamonds represent 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.

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.

Acknowledgments

marine charters. We thank Robert McQuilty, Royal Prince Alfred Hospital NSW, for his assistance in trace and heavy metal analysis.

Author Contributions

Conceptualization: Mariel Fulham, Michelle Power, Rachael Gray.

Data curation: Mariel Fulham, Fiona McDougall.

Formal analysis: Mariel Fulham, Fiona McDougall.

Funding acquisition: Mariel Fulham, Rebecca R. McIntosh, Rachael Gray.

Investigation: Mariel Fulham, Michelle Power, Rachael Gray.

Methodology: Mariel Fulham, Michelle Power, Rachael Gray.

Project administration: Rachael Gray.

Resources: Michelle Power, Rachael Gray.

Supervision: Michelle Power, Rachael Gray.

Writing – original draft: Mariel Fulham.

Writing – review & editing: Mariel Fulham, Fiona McDougall, Michelle Power, Rebecca R. McIntosh, Rachael Gray.

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