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A comparative study of anthropogenic antimicrobial pollution on Australia's little penguins

By

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A thesis submitted in fulfilment of the requirements for the degree of
Master of Research

On

The twelfth day of October 2018

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Declaration and Statement of Originality

I wish to acknowledge the following assistance in the research detailed in this report

I wish to acknowledge Associate Professor Michelle Power, my principal supervisor, for her great support, expert advice on laboratory procedures, providing PCR protocols, and thesis editing. Dr. Peter Dann, my secondary supervisor, and collaborator from Phillip Island Nature Parks, for expert advice on sample collection. Dr. Koa Webster, Macquarie University, for expert advice on Chapter 1 and thesis editing. Fiona McDougall, Macquarie University, for expert advice on microbiological work, developing microbiological protocols and sequence analysis.

All other research described in this report is my own original work.

This work has not previously been submitted for a degree or diploma in any university. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made in the thesis itself.

Ida Lundbäck
10th of October 2018

Acknowledgements

I wish to acknowledge Dr. Rachael Gray (BVSc, PhD), The University of Sydney for expert advice and thesis editing.

Marjolein Van Polanen Petel and Naomi Wells, Phillip Island Nature Parks, for teaching me how to handle penguins and conduct field sampling.
Dr. Gemma Carroll and Lachlan Phillips, Macquarie University; David Slip and volunteers, Taronga Conservation Society Australia; staff at Coffs Harbour Dolphin Marine Magic Park; Zoe Hogg and volunteers, St Kilda Earthcare; David Blyde, Sea World Queensland; David McLelland, Zoos South Australia; and Elizabeth Eyre, Sydney Sea Life Aquarium for assisting me with sample collection and providing samples from captive institutions.

My colleagues in the Power Parasitology Laboratory at Macquarie University, including: Daniel Russell and Mariel Fulham.

Lastly, I am immensely grateful to my husband, Shaun Clifford; and my parents, Gretel and Jimmy Lundbäck, for their support.

This research was funded by Sea World Research and Rescue Foundation, Australia.

I would like to thank Doug Gimesy, wildlife photographer, for documenting our fieldwork in St Kilda, February 2018.

Outline of Chapters

This thesis is written in the form of three journal articles, formatted according to the author guidelines of *Infection, Genetics and Evolution, Journal of Molecular Epidemiology and Evolutionary Genetics of Infectious Diseases (MEEGID)* modified to suit the Macquarie University Thesis Submission Guidelines. The first article is a review, while the second and third articles are original research papers. To meet the formatting standards of a Master of Research Thesis at Macquarie University all references are collated at the end of the thesis.

Chapter 1. Review: Physiological and ecological traits of wild birds affecting the acquisition of antimicrobial resistance

Chapter 1 consists of a review synthesising the literature on the role of wild birds in the dissemination of antimicrobial resistance, with a focus on enteric bacteria associated with horizontal gene transfer in wild birds. I was primarily interested in host traits of wild birds that are significant to the dissemination and retention of antimicrobial resistance, the species of resistant bacteria, and class 1 integron carriage.

I reviewed the literature and wrote this chapter with feedback provided by Michelle Power, my supervisor, and Dr. Koa Webster, a postdoctoral research fellow from my laboratory.

Chapter 2. Class 1 integrons in wild and captive little penguins (*Eudyptula minor*)

In Chapter 2 I investigate the presence of the class 1 integron, a genetic element conferring resistance to antimicrobial agents, in the faecal microbiota of wild and captive little penguins. Faecal samples were collected from wild and captive little penguins, DNA was extracted, and PCRs targeting the integron integrase class 1 gene (*IntI1*) were performed. Sequence analysis of PCR amplicons allowed cassette array and associated resistance genes analysis.

Michelle Power, my supervisor, and I, collected faecal samples from Phillip Island and St Kilda; Dr. Gemma Carroll and Lachlan Phillips, Macquarie University, collected faecal samples from Bowen Island and Montague Island. Faecal samples from captive individuals were collected and supplied by David Slip and volunteers, Taronga Conservation Society Australia; Volunteers at Coffs Harbour Marine Magic Park; David Blyde, Sea World Queensland; David McLelland, Zoos South Australia; and Elizabeth Eyre, Sydney Sea Life Aquarium. I performed all laboratory work, data analysis and statistics, and wrote this chapter with feedback from my supervisor, Michelle Power.

Chapter 3. Targeted screening for antibiotic resistance determinants in *Escherichia coli* and *Klebsiella pneumoniae* in wild and captive little penguins (*Eudyptula minor*)

In Chapter 2 I showed that class 1 integrons are present in wild and captive little penguins, with a significantly higher prevalence in captive individuals. In Chapter 3, I therefore aimed to elucidate if the bacterial species harbouring the class 1 integrons were associated with humans. I was specifically interested in isolating *Escherichia coli* and *Klebsiella pneumoniae*, two clinically relevant opportunistic pathogens, highly associated with the dissemination of antimicrobial resistance from human systems to wildlife.

Michelle Power, my supervisor, and I, collected faecal samples from Phillip Island and St Kilda; Dr. Gemma Carroll and Lachlan Phillips, Macquarie University, collected faecal samples from Bowen Island and Montague Island. Faecal samples from captive individuals were collected and supplied by David Slip with volunteers, Taronga Conservation Society Australia; Volunteers at Coffs Harbour Marine Magic Park; David Blyde, Sea World Queensland; David McLelland, Zoos South Australia; and Elizabeth Eyre, Sydney Sea Life Aquarium. I performed all laboratory work, data analysis and statistics, and wrote this chapter with feedback from my supervisor, Michelle Power.

Introduction to Thesis

This thesis presents a synthesis of the literature on antimicrobial resistance (AMR) in wild birds, in order to ascertain whether there are any traits, physiological or ecological, that are associated with the acquisition and retention of resistant organisms in wild birds. Major knowledge gaps were identified in types of bird species and bacterial species targeted for investigating AMR in wild birds. This was particularly evident in the Australian context.

In Australia, the little penguin (*Eudyptula minor*) is an iconic marine bird that spends the majority of its life at sea. The life history of the little penguin potentially increases the species' opportunity for exposure to resistant organisms present in the marine environment, making these birds an ideal study species for the investigation of the dissemination of antimicrobial resistance. Little penguin colonies in Australia are widely distributed, with mainland and island colonies ranging from New South Wales on the East coast to Western Australia. These colonies experience differing levels of human influences ranging from isolated off-shore islands to mainland sites highly frequented by tourists. There are also several little penguin colonies in captive institutions in Australia, enabling comparison of resistant determinants between wild and captive little penguins.

After identifying the little penguin as a key species to investigate in the dissemination of antimicrobial resistance from humans to wild birds, the aim of this study was to examine the bacterial community of wild and captive little penguins to determine if the class 1 integron, a mobile element that confers antibiotic resistance, was present in their faecal microbiota. In addition, two Gram-negative bacterial species of clinical relevance, *Escherichia coli* and *Klebsiella pneumoniae,* were genetically characterised and assessed for the presence of class 1 integrons to elucidate if the bacterial species harbouring the class 1 integrons are human associated.

Understanding the presence of organisms carrying resistance traits in the host is an important step towards understanding if human antimicrobial pollution is contributing to the decline seen in little penguin colonies across Australia, as such acquisition may affect little penguin health. Defining alterations caused by the acquisition of resistance determinants could thus help shape conservation practices for future management of the little penguin.

Chapter 1

Review: Physiological and ecological traits of wild birds affecting the acquisition of antimicrobial resistance

Abstract

The dissemination of antimicrobial resistance (AMR) is a significant global health priority, particularly given that antibiotic resistant bacteria have now been detected in taxonomically diverse wildlife species in diverse ecosystems. Wild birds have been recognised as crucial drivers, reservoirs, and vectors of resistant organisms and genetic determinants of resistance, due to several physiological and ecological host factors. Therefore, wild birds have been particularly important in the dissemination of AMR. In this review, the available data on AMR in enteric bacteria associated with horizontal gene transfer in wild birds worldwide was synthesised. Three ecological factors crucial to the dissemination of AMR through wild birds were recognised as being important: human association, migration and water association. Further, investigations into the role of birds in the dissemination of AMR have focused on specific bacterial group(s), and specific bird hosts, and as a consequence, knowledge gaps about the roles of other bacteria and bird hosts are evident. As a result of these gaps, current interpretation of the severity and dissemination of AMR through wild bird species is likely to be biased. The assessment of the literature revealed limited research in the Australian context. Yet, given the diversity and ecology of wild birds in Australia, opportunities to deeply address the issue of AMR and the role of wild birds in the dissemination of resistant organisms exist. Recommendations are given for addressing these gaps and questions for future areas are proposed.

1. Introduction

Antimicrobial resistance (AMR) is recognised as a significant global health priority (Prestinaci et al., 2015). The emergence of AMR is a result of natural selection, where attempts by humans to control bacterial growth placed a selective pressure on bacteria (Gillings, 2017). Such selective pressure conferred an advantage for those bacterial strains able to express a resistant genotype via mutation, or acquire resistance genes through lateral gene transfer (Gillings, 2017). It is likely that advantageous random mutations, or the acquisition of resistance genes only conferred a minor advantage in the pre-antibiotic era, however, when human use and development of antimicrobial compounds increased the exposure of bacteria to antibiotics, these mutations became fixed in bacterial lineages (Gillings, 2017).

Resistance within both Gram-negative and Gram-positive bacteria has dramatically increased over the last decade (Exner et al., 2017). Gram-negative

pathogens, which were once susceptible to many of the last lines of antimicrobial defences such as ureidopenicillins (piperacillin), third- and fourth-generation cephalosporins (cefotaxime and ceftazidime), carbapenems (imipenem and meropenem) and fluoroquinolones (ciprofloxacin), now demonstrate diverse acquired resistance profiles towards these antimicrobials, and multidrug resistant phenotypes continue to emerge (Exner et al., 2017). Opportunistic pathogens previously considered to be important in nosocomial (that is, originating in hospitals) infections including extended-spectrum β-lactamases (ESBL) in *Escherichia coli*, methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin resistant *Enterococcus species* (VRE), and carbapenemase type resistance in *Klebsiella pneumoniae* (KPC) (Otter and French, 2010), are now being increasingly detected in the community (Cantas et al., 2013). These bacteria are also increasingly being isolated from environmental systems, such as soil (Popowska et al., 2010), ground-water (Böckelmann et al., 2009), and waste-water (Schwartz et al., 2006).

Antibiotic resistance genes can be acquired through horizontal gene transfer (HGT) via transposons, plasmids or integrons carrying resistance genes (Gillings, 2014). Environments such as soil, water, sewage, raw meat, livestock, and the intestinal tracts of humans and animals have been identified as reservoirs of bacteria carrying antibiotic resistance traits (Kilonzo-Nthenge et al., 2013; Tacconelli et al., 2014). This widespread occurrence, and continuing emergence of AMR, further accentuates the dissemination of resistant bacteria and the threats posed to human and animal health.

Antibiotic resistant bacteria have been detected in taxonomically diverse wildlife species (Allen et al., 2010) in diverse ecosystems (Vittecoq et al., 2016). These bacteria show great diversity in resistance patterns, demonstrating that diverse genetic mechanisms that confer resistance traits have been disseminated into wildlife (Carroll et al., 2015). The presence of resistance traits in wildlife populations presents a measure of anthropogenic pollution as such traits are not normally found in the wildlife host (Dolejska et al., 2007) but have originated from a clinical context and are spread into the environment via human waste streams (Gillings, 2017). The impacts of the transmission of antibiotic resistant bacteria to wildlife hosts have not been empirically determined. However, the introduction of resistant bacterial species and associated resistance determinants into wildlife presents an opportunity to introduce resistance into novel genomes, altering the genetic composition of

commensal bacteria (Dolejska et al., 2007). If the introduced species is pathogenic, this may also lead to disease emergence in the wildlife host. In addition, the acquisition of resistant organisms has the potential to alter the complex community of the host gut microbiome. In captive individuals, antimicrobial administration affects the microbiome of the host by destabilizing the gut flora (Crémieux et al., 2003; Jernberg et al., 2010), and most HGT events between bacterial pathogens occur when the microbial composition is altered (Stecher et al., 2012). Such alterations can increase host susceptibility to infections, particularly gastrointestinal infections, caused by newly acquired pathogens or due to the pathogenic behaviour of opportunistic organisms (Francino, 2016).

When investigating the dissemination of AMR into Gram-negative bacteria of wildlife, *E. coli* is often targeted as a reference bacterium (Cole et al., 2005; Guenther et al., 2010; Kozak et al., 2009; Literák et al., 2007; Sacristán et al., 2014; Smith et al., 2014). *E. coli* is a Gram-negative bacterium belonging to the *Enterobacteriaceae* family, and can either function as a commensal of the intestinal tract (Abriouel et al., 2008; Shehabi et al., 2006), or a pathogen causing intestinal disease (Mainil, 2013; Riley, 2014). Large numbers of *E. coli* are passed in faeces and some strains can also persist in the environment outside of a host for extended periods (Salyers et al., 2004). These properties facilitate the significant role *E. coli* plays in the dissemination of AMR between humans and wildlife (Hammerum and Heuer, 2009; Sick, 1997; von Baum and Marre, 2005).

Wild birds are recognised as crucial drivers in the dissemination of AMR (Allen et al., 2010; Bonnedahl and Järhult, 2014; Literák et al., 2007). Birds can travel vast distances in short periods of time, and many species live in close association with human settlements. These traits along with migration and association of some bird species with water bodies may facilitate the dissemination of AMR to novel environments, ecosystems, and other wildlife (Bonnedahl and Järhult, 2014; Wang et al., 2017). As such, elucidating the role of wild birds in the dissemination of AMR from human-influenced systems into other environments is paramount.

In this review, the published literature on AMR in enteric bacteria associated with HGT in wild birds on a global scale will be synthesised. Of primary interest are the host traits of wild birds that are significant to the dissemination and retention of AMR, the species of resistant bacteria most commonly implicated, and class 1 integron carriage.

The literature review was conducted using the databases Elsevier and ProQuest to identify articles that documented the dissemination of AMR in wild birds. The search terms used were: “antimicrobial resistance” and “wild birds”. The search result generated 1,570 journal articles, however for the purpose of this review, 100 journal articles were examined. These journal articles were chosen as they were the first 100 articles to match the review search criteria described above. Out of the 100 journal articles chosen for abstract revision, 24 articles were chosen for whole article revision based on abstract content in relation to the aims of this review. After whole-article revision, 18 articles were included and information of bird host, resistance prevalence, bacterial species, and integron carriage were collated and presented in this review. Journal articles were only included in this review if they were an original study, and hence excluded if they were a review or summary article, or a communication piece.

2. AMR in birds

Many studies examining levels of AMR in wild birds utilise the method of disc diffusion (Bauer et al., 1966) to examine the susceptibility of isolated bacteria to antimicrobial agents. Figure 1 displays the number of cases of AMR in avian species grouped by bird order for a variety of antimicrobial agents based on disc diffusion. The majority of the examined literature used *E. coli* as the reference bacterium; 42% targeted *E. coli*, despite other Gram-negative bacteria such as *Klebsiella sp.*, *Salmonella sp.* and some Gram-positive species such as *Enterococcus sp.* displaying phenotypic resistance to antibiotics. *Klebsiella sp., Salmonella sp*. and *Enterococcus sp.* were represented in 6%, 14% and 25% of total examined publications respectively, with resistant strains of each reported in wild birds. Resistance towards penicillin, ampicillin, tetracycline, streptomycin, and sulphonamides is most commonly reported in *E. coli*, while tetracycline and ampicillin resistance is most commonly reported in *Klebsiella sp.* These antimicrobial resistance traits are often reported in high prevalence from a wide variety of birds (Figure 1). From the early 2000’s there appears to be an increasing trend in reports of multidrug resistant *E. coli* with complex resistance profiles showing resistance towards as many as eight antimicrobial agents. Figure 2 displays the number of birds demonstrating antimicrobial resistance grouped by avian family. Of significance is the presence of multidrug resistant *E. coli* isolated from gulls in the Arctic, previously considered to

be a relatively pristine environment, demonstrating the widespread nature of antimicrobial pollution (Figure 2; see Sjölund et al., 2008). Multidrug resistant *E. coli* isolates from birds of prey, passerine species and columbids in urban environments are also reported (Figure 2; see Guenther et al., 2010). The extent of multidrug resistant bacterial isolates in environments ranging from the Arctic to urban cities highlights the global scale of the dissemination of AMR and the potential role of wild birds as vectors and reservoirs of resistant bacteria.

Disc diffusion sensitivity assays have long been the preferred method to test for antimicrobial resistance due to ease of methodology, low cost, and flexibility in the type and numbers of antimicrobial agents which can be evaluated simultaneously (Reller et al., 2009). More recently, the increased understanding of the genetic basis of resistance and dissemination of AMR has seen the development of molecular detection methods. Resistance is now often measured by the presence of genes encoding resistance and the genetic elements associated with HGT (Pérez-Pérez and Hanson, 2002; Strommenger et al., 2003; Volkmann et al., 2004). Molecular methods to detect AMR are increasingly being applied to birds and other wildlife species.

Figure 1. Cases of resistance reported from various bird orders based on disc diffusion assays (Following page).

Antibiotic abbreviations: AMC, Amoxicillin-clavulanate; AMK, Amikacin; AMP, Ampicillin; AMS, Ampicillin/Sulbactam; AMX, Amoxicillin; AUG, Amoxicillin/Clavulanic Acid; AZT, Aztreonam; BAC, Bacitracin; C, Chloramphenicol; CDR, Cefadroxil; CEP, Cephalothin; CF, Cephalotin; CFZ, Cefazolin; CHL, Chloramphenicol; CP, Ciprofloxacin; CPD, Cefpodoxime; CXM, Cefuroxime; DIF, Difloxacin; ENR, Enrofloxacin; ERY, Erythromycin; FEP, Cefepime; FLU, Flumequine; FOS, Fosfomycin; FOX, Cefoxitin; GM, Gentamicin; IPM, Imipenem; KM, Kanamycin; LIN, Lincomycin; MEC, Mecillinam; NAL, Nalidixic acid; NET, Netilmicin NIT, Nitrofurantoin; NM, Neomycin; OTC, Oxytetracycline; PEN, Penicillin; PIP, Piperacillin; PTZ, Piperacillin/Tazobactam; QD, Quinupristin–dalfopristin; RIF, Rifampin; RL, Sulfamethoxazole; S, Streptomycin; SP, Spectinomycin; SU, Sulphonamides; SXT, Trimethoprim/Sulfamethoxazole; TE, Tetracycline; TEC, Teicoplanin; TMP, Trimethoprim; TO, Tobramycin; VAN, Vancomycin.

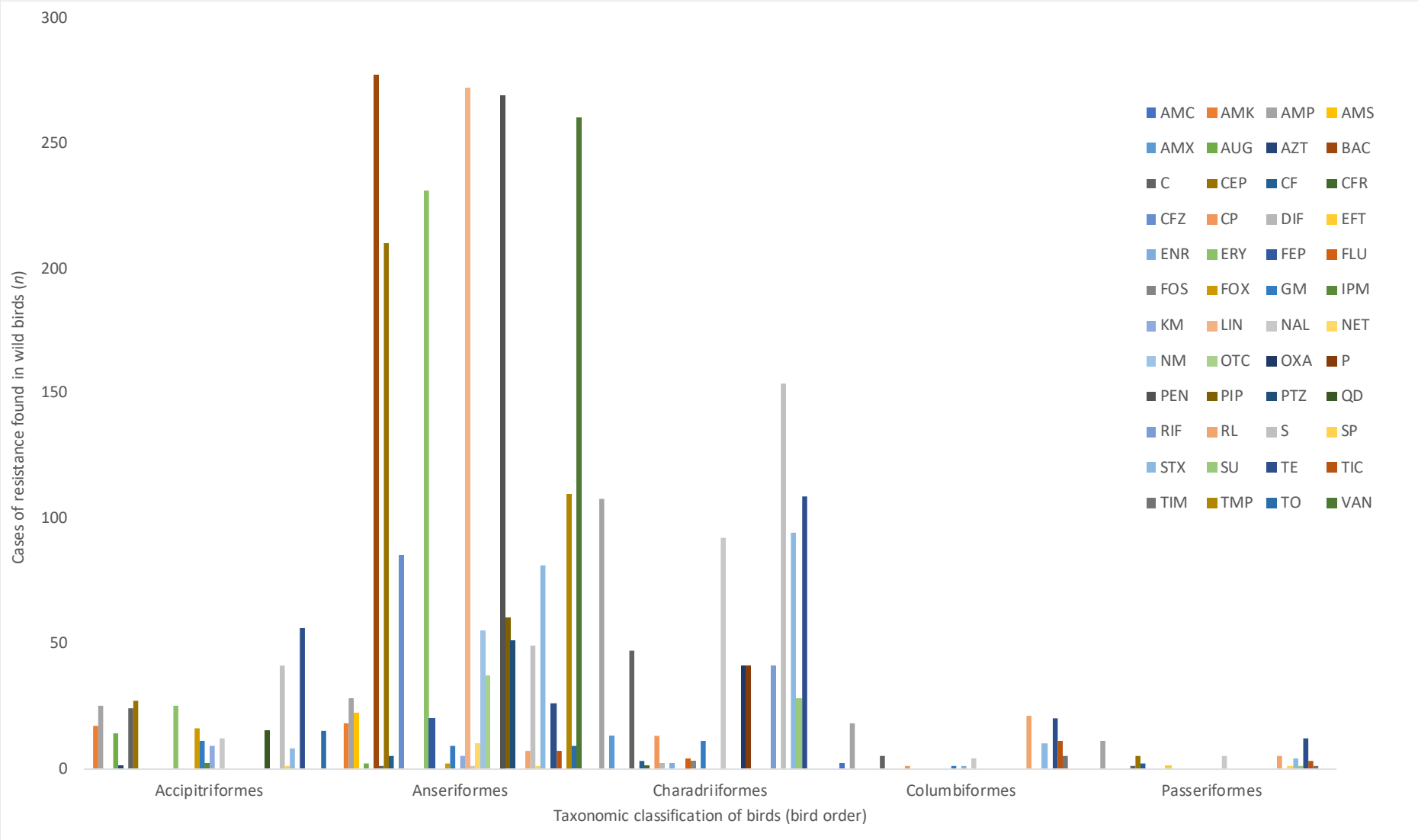
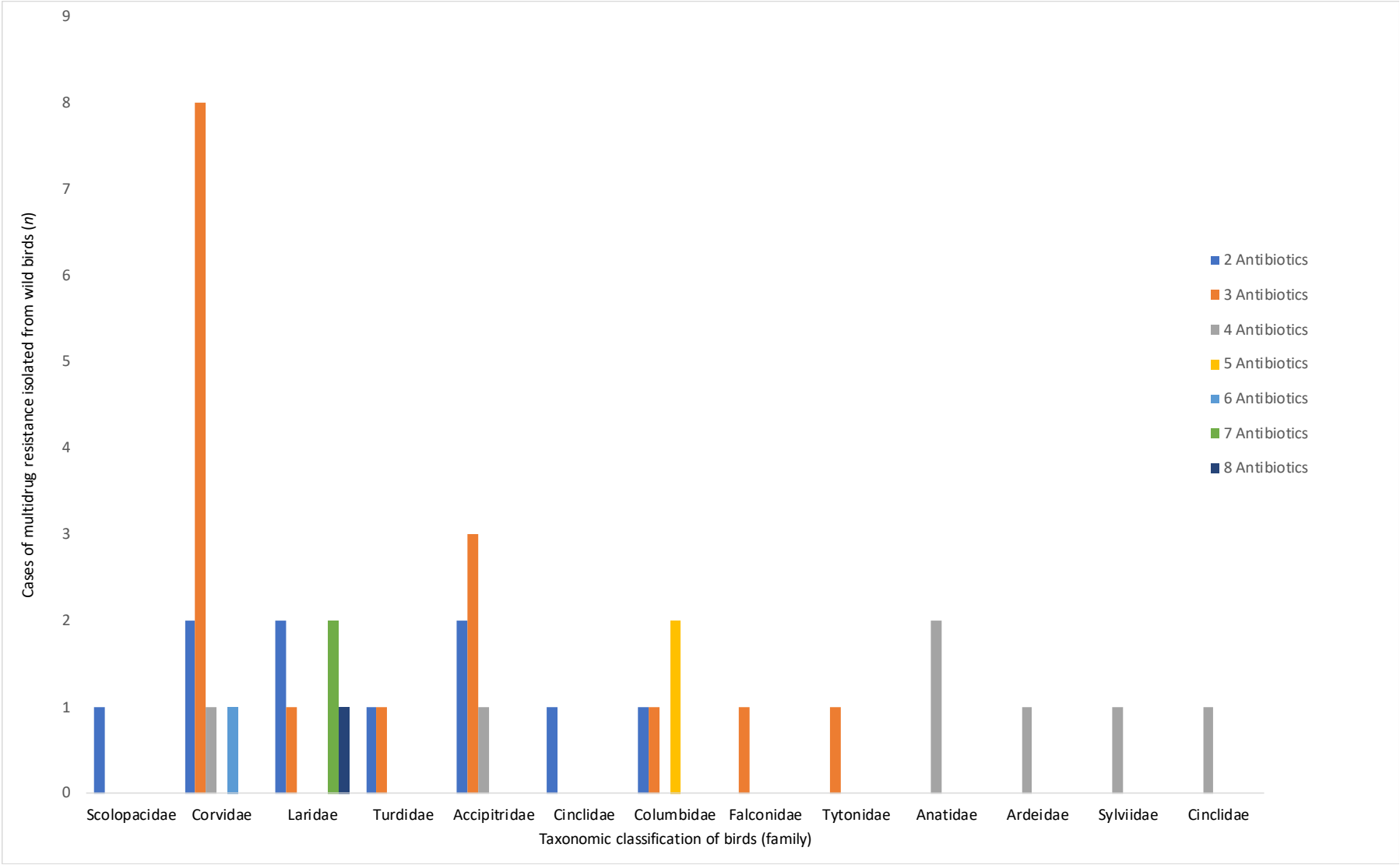

300
250
200
150
100
50
0
Cases of resistance found in wild birds (n)
AMC AMK AMP AMS
AMX AUG AZT BAC
C CEP CF CFR
CFZ CP DIF EFT
ENR ERY FEP FLU
FOS FOX GM IPM
KM LIN NAL NET
NM OTC OXA P
PEN PIP PTZ QD
RIF RL S SP
STX SU TE TIC
TIM TMP TO VAN
Accipitriformes
Anseriformes
Charadriiformes
Columbiformes
Passeriformes
Taxonomic classification of birds (bird order)

Figure 2. Number of times multidrug resistant bacteria have been isolated from different bird families in examined studies (Following page).
Methods of determining resistance differed between studies and included disc diffusion assays and PCRs detecting resistance genes. Graph shows number of cases of multidrug resistant bacteria have been isolated from. The colours indicate the number of antimicrobial agents the host showed resistance to within each bird family.


Cases of multidrug resistance isolated from wild birds (n)
0
1
2
3
4
5
6
7
8
9
Scolopacidae
Corvidae
Laridae
Turdidae
Accipitridae
Cinclidae
Columbidae
Falconidae
Tytonidae
Anatidae
Ardeidae
Sylviidae
Cinclidae
Taxonomic classification of birds (family)
2 Antibiotics
3 Antibiotics
4 Antibiotics
5 Antibiotics
6 Antibiotics
7 Antibiotics
8 Antibiotics

2.1 Horizontal gene transfer and acquired antibiotic resistance

While some bacterial species have an intrinsic ability to resist antibiotics, HGT has been pivotal for the rapid emergence of AMR. HGT allows bacterial strains to 'capture' DNA from other bacterial strains present in the environment (Gillings, 2014), allowing bacteria to incorporate advantageous genes from several different bacterial species and lineages. Some bacteria also have the ability to recombine the 'captured' DNA into their own genome (Gillings, 2014). However, recombination within the genome is limited to sharing of DNA between similar species of bacteria due to DNA repair processes (Thomas and Nielsen, 2005). Genes that can integrate into a plasmid or any other mobile DNA element can spread without recombination into the bacterial genome (Thomas and Nielsen, 2005). Exchange of resistance between bacterial strains therefore increases if the resistance genes are located on a mobile DNA element, such as a plasmid or integron (Machado et al., 2005).

2.2 Integrons

Integrons are mobile genetic elements commonly present in bacterial genomes (Gillings, 2014). All integrons share three features: (1) the presence of an integrase gene (*int1*) that facilitates gene capture from the environment, (2) a recombination site (*attI*), which facilitates insertion of a gene cassette(s), and (3) an adjacent promotor site (P_c) which enables expression of inserted gene cassette(s) (Messier and Roy, 2001). The integron system has two key advantages; firstly, the new genetic material is integrated into the bacterial genome at a specific recombination site, so it does not disturb existing genes. Secondly, the newly integrated gene is expressed via the integron promoter and therefore is instantly primed for natural selection (Gillings, 2014). Class 1 integrons have been assembled under selective pressure due to human use of antibiotics and these elements have played a major role in the dissemination of antibiotic resistance (Gillings, 2014). They are geographically and biologically widespread, arise from a human clinical context, and are spread into the environment via human waste streams (Stokes and Gillings, 2011). These attributes support their use as an indicator of anthropogenic pollution (Gillings et al., 2015).

2.3 Class 1 integrons in wild birds

The presence of class 1 integrons in birds has almost exclusively been examined in *E. coli*. A bias towards the species of birds examined for class 1 integrons is also evident with birds of prey, columbids and water associated species the most commonly investigated. Figure 3 displays the number of times class 1 integrons have been isolated from different bird orders.

The prevalence of class 1 integrons in birds is generally low, with the highest reported prevalence in gull species and diurnal birds of prey (Figure 3; see Dolejská et al., 2009). Genes encoding resistance to aminoglycosides (*aadA*), tetracycline (*tetA*), sulphonamides (*sul*), and trimethoprim (*dfrA*) are commonly detected in wild birds (Cole et al., 2005; Dolejska et al., 2007; Dolejská et al., 2009; Gionechetti et al., 2008; Pinto et al., 2010). The gene cassette arrays in class 1 integrons can also contain multiple genes encoding resistance to different antibiotics, leading to multidrug resistant profiles.

Integron resistance profiles in gulls appear to be diverse (Dolejská et al., 2009; Gionechetti et al., 2008). Cassettes in class 1 integrons containing the resistance genes *aadA1, aadA2,* and *dhfr-aadA* combinations have been reported in addition to the extended spectrum β-lactamases (*bla*) genes combined with *aadA* genes (Dolejská et al., 2009). Other frequently isolated profiles reported are the *dfrA1–aadA1a* and *aadB–aadA2* cassette arrays as well as the *estX* cassette, alone or in combination with other cassettes (Gionechetti et al., 2008). Studies on waterfowl have reported *aadA*, *sul* and *bla* genes (Cole et al., 2005). In birds of prey, resistance profiles such as *tetA-sul*, *aadA-tetA-sul* (Pinto et al., 2010), and *sul-tetA-aadA* or *sul-aadA* combinations (Literák et al., 2007) have been reported.

Reports on resistance in birds and integron carriage are limited to studies of a few species, leaving important ecological knowledge gaps. For example, are there certain traits, ecological or physiological, associated with these bird species that may affect the acquisition of resistant bacteria carrying class 1 integrons? Comparative studies of class 1 integron carriage across different bird species could address this knowledge gap. Furthermore, as the class 1 integron has been proposed an indicator of anthropogenic antimicrobial pollution of wildlife, wider ranging studies addressing integron carriage in wild birds would also identify if the class 1 integron is a suitable indicator of anthropogenic antimicrobial pollution in wild birds.

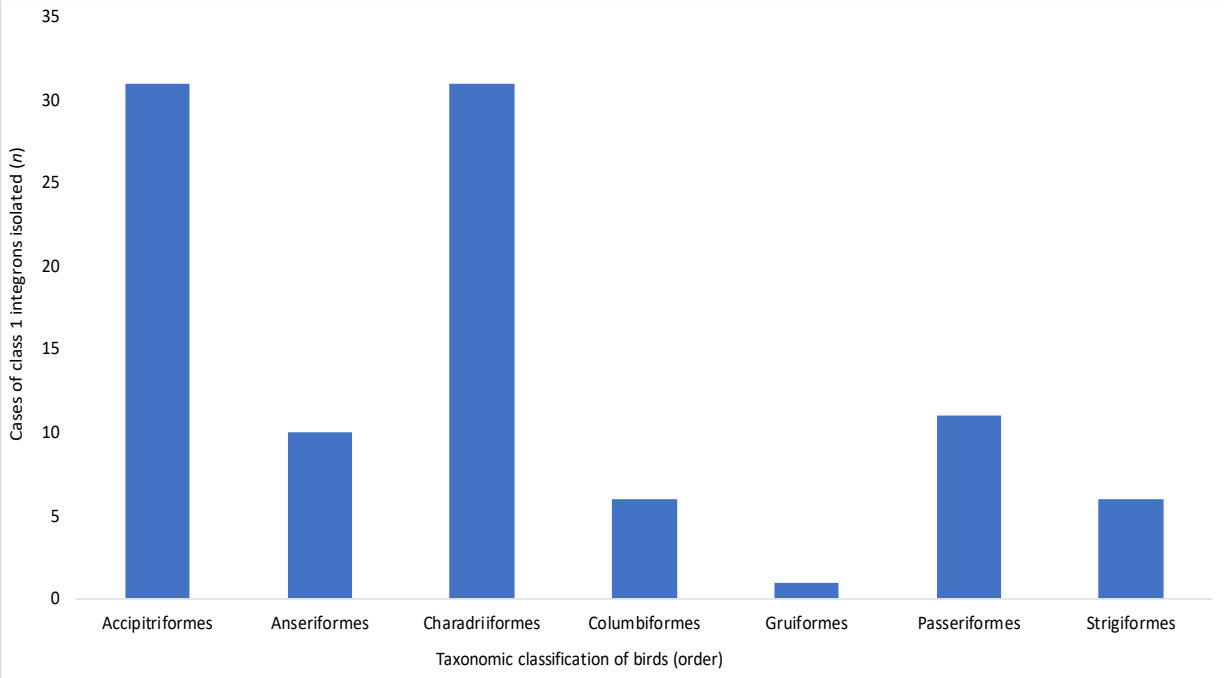


Figure 3. Number of times class 1 integrons have been detected from different bird orders across examined studies.

The highest prevalence of class 1 integrons were reported in the orders Accipitriformes (birds of prey) and Charadriiformes (gulls).

3. Factors affecting AMR prevalence in wild birds

When examining acquired resistance in wild birds the majority of studies focus on waterfowl, birds of prey and passerine species (Figures 1 to 3). These bird species share similar ecological niches and are taxonomically related or in the same taxonomic order; therefore, the dissemination of AMR is well documented in some orders of birds while many others have not been examined. This is particularly evident in the Southern Hemisphere, and specifically in Australia. It is possible that wild bird hosts in orders not yet investigated may harbour traits that affect the dissemination of AMR. To understand the ecology of antibiotic resistance in the environment or outside of clinical contexts using birds as indicators, wider ranging investigations of different orders are essential. At present, the bias in bird host species examined is coupled with the bias towards reference bacteria used to elucidate the dissemination of AMR in wild birds. It is also likely that the bird hosts investigated share certain host traits and habitats, which further increases the likelihood of the colonisation of resistant organisms. However, research to date highlights three factors that seem particularly important in the acquisition and retention of resistant organisms in wild birds: the degree of

human association, water association, and migratory behaviour (Bonnedahl and Järhult, 2014).

3.1 Human association

Synthesis of the literature reveals a relationship between the degree of human association and the acquisition of resistant organisms in wild birds. Wild birds with high levels of human association such as columbids (pigeons and doves) and larids (gulls) in city centres (Figure 1; see Radimersky et al., 2010; Sacristán et al., 2014), or on frequently visited beaches (Figure 1; see Gionechetti et al., 2008; Dolejská et al., 2009) tend to have a high prevalence of resistant bacteria. Multidrug resistance (3 or more antibiotics) has been reported in 32.9%, 31% and 28%, respectively, of isolated *E. coli* strains in different gull species that frequently associate with humans (Figure 2; see Dolejská et al., 2009; Radimersky et al., 2010; Sacristán et al., 2014). In contrast, only 6.5% of *E. coli* strains isolated from Audouin's gulls present on frequently visited beaches have been reported resistant to two or more antibiotics (Camarda et al., 2006). Audouin's gulls differ from other gull species as they tend to avoid scavenging from human waste, and prefer fish as their main food source (Camarda et al., 2006), resulting in reduced potential exposure to human-associated bacteria.

Bird species with moderate human association, typically passerine species and birds of prey, present in environments adjacent to towns and in reserves, generally harbour a lower prevalence of resistant bacteria. For example, 13.7% of bacteria from Russian rooks (*Corvus frugilegus*) were found to be resistant to at least one antibiotic compound (Figure 1; see Literák et al., 2007), while multidrug resistant bacteria were seen in 4.8% of passerine species and birds of prey (Figure 2; see Guenther et al., 2010). The presence of resistant organisms in passerine species and birds of prey increases if the wild bird is near farms or agricultural lands. For example, 75% of *E. coli* strains isolated from common buzzards (*Bueto bueto*) located close to farming and agriculture were resistant to at least one antimicrobial compound (Figure 1; see Radhouani et al., 2012). Additionally, insects and small rodents living on farms or land adjacent to farms can acquire resistance genes and resistant organisms from waste and sewage released from the farms (Kozak et al., 2009). Thus, dietary prey items may act as reservoirs of resistance genes eventually accumulating in a wild bird host. A relationship between diet and prevalence of resistance has been considered in passerine birds (Gaukler et al., 2009), although it can be argued that livestock effluent and a global overuse of antibiotics in agricultural settings could also be driving this relationship (Wei et al., 2011).

Use and production of antibiotics has created a 'zone' of antibiotic residue present around all major cities and major human activity areas (Gillings et al., 2015). Thus, human influences greatly affect the dissemination of AMR into the environment in two ways; 1) through environmental pollution with antibiotics and 2) through excretion of resistant bacteria. The zone of antibiotics further selects for those bacteria that have resistance traits. The dissemination of bacteria and antibiotic residue further increases if the human activity area is located within close proximity to water systems such as rivers and harbours (Baquero et al., 2008).

3.2 Water association

Water is well recognised as a dissemination medium for resistant bacteria between human, domestic animal, and wildlife populations (Baquero et al., 2008). Resistant bacteria are continuously being released into the environment through human waste streams (Gillings, 2014), and sewage treatment plants rarely remove all bacteria from treated sewage, resulting in contaminated water being disseminated into the natural environment (Rizzo et al., 2013). Furthermore, antibiotic residues are also present in sewage treatment plants and spread into the environment through contaminated water (Gillings et al., 2015). The presence of resistant organisms in water sources thus increases the potential for exchange of bacterial strains between humans and wildlife. Consequently, levels of resistant bacteria have drastically increased over the last decade (Taylor et al., 2011).

Although water is a crucial dissemination medium for AMR, it can also act as a barrier. In the Southern Hemisphere, large water bodies between continents and large distances between landmasses along avian migratory routes have resulted in Southern Hemisphere birds migrating almost exclusively within the Southern Hemisphere (Dingle, 2008). Water can therefore act as a barrier to the dissemination of AMR, with major implications for the spread of AMR between the Northern and Southern Hemispheres (Dingle, 2008).

3.3 Migration

Migration has been recognised as a crucial host trait in the dissemination of AMR (Bonnedahl and Järhult, 2014). Migration allows birds that have acquired AMR bacteria to disseminate both genes and the AMR bacteria they carry into environments far from where they were originally acquired (Bonnedahl and Järhult, 2014). Seasonal variation in migration is evident in the Northern and Southern Hemispheres which may influence the spread of

AMR (Dingle, 2008). The mild winters of the Southern Hemisphere may have driven the selection for diverse responses to environmental variation, whereas Northern Hemisphere migrants are commonly driven by cold winter temperatures resulting in migration to warmer breeding grounds (Newton and Dale, 1996). In the Southern Hemisphere, migration is associated with the level of aridity of the continent, with migratory patterns often complex and dependent on rainfall (Alerstam, 1993). Northern Hemisphere migrants are most commonly detected in Africa and South America, and less in Australasia (Dingle, 2008). Only one Northern Hemisphere migrant, the barn swallow (*Hirundo rustica*) is present in all Southern Hemisphere continents (Dingle, 2008), although AMR has not been reported nor studied in this species in the literature examined in this review. No raptors or birds of prey are known to migrate from Asia to Australasia (Bildstein, 2006) which is likely due to the water barrier between the landmasses. As birds of prey commonly carry resistant organisms (Figures 1 and 2), this could result in a slower dissemination of resistance.

3.4 Wild birds in Australia

Australia is an island continent rich in birdlife and diversity. Approximately 345 species of birds live in close proximity to major cities (Clements et al., 2018), resulting in Australian cities supporting more species than all other non-urban areas on a unit-area basis (Ives et al., 2016). Most Australian cities are located < 25 km from the coastline, which has experienced major anthropogenic changes that have increased interactions between humans and wild birds through shared habitat, urban adaptation of birds, and tourism. The increased interaction, as well as the modifications of the natural environment due to anthropogenic development, has driven behavioural change and adaptation in many bird species. Many species and numbers of wild birds frequently associate with humans along beaches and in cities, often consuming human foodstuff via scavenging in rubbish bins, and drinking and foraging within city wastewater streams and canals. Psittaciformes (parrots and their allies) have increased in their distribution around Australia due to increased water availability from agricultural development in arid Australia (Dingle, 2004). Wetland species, such as the Australian white ibis (*Threskiornis molucca*), have become increasingly dependent on artificial habitats and human associated landscapes due to the loss of natural wetland areas (Murray et al., 2013). The modified home ranges, behaviours, and integrations seen in these species may therefore increase the chances of acquiring resistant organisms.

Australia also provides a unique opportunity to investigate the impact of within-continent migration on the dissemination of AMR, and for comparison with Northern

Hemisphere migratory host species. Psittaciformes would be ideal targets to examine AMR prevalence and dissemination within Australia, as at least 14 out of the 52 Australian Psittaciformes species are migratory, and confined to migration on the Australian continent (Dingle, 2004). Investigating the prevalence of AMR in Psittaciformes would therefore aid in elucidating the potential spread of AMR due to migration within Australia, from cities to arid and rural environments. Additionally, Procellariiformes (Shearwaters) are present in large numbers in Australia and are known to travel to Alaskan coastlines during their winter migration thus making an excellent species to investigate the potential dissemination of AMR from the Southern to the Northern Hemisphere.

4. Concluding remarks

Factors such as migratory status and proximity to water are important elements that need to be considered when assessing the dissemination of AMR through wild birds. Diet appears to contribute to the dissemination of AMR in wild birds, although further investigation is needed to determine the impact of diet on the acquisition and retention of resistant organism in wild birds. However, the proximity to humans, and the level of human association the host is subjected to, are the most significant factors affecting the acquisition and retention of AMR bacteria.

In studies to date on the dissemination of AMR by wild birds, the focus on bacterial group(s) and bird host groups has resulted in knowledge gaps about the roles of other bacterial species and bird hosts in the dissemination of AMR. This may affect our understanding and interpretation of the severity and dissemination of AMR through wild bird species. Further, there has been limited research in an Australian context, with only one known study on AMR in birds in Australia.

An approach to addressing gaps in Australia may be to use key bird species and urban adapted species to examine the dissemination of AMR across the Australian landscape via wild birds. Orders such as Suliformes (Gannets and Shags), Pelecaniformes (Pelicans, Boobies, Cormorants, and Frigatebirds) and Charadriiformes (Shorebirds) would be ideal study species. Species within these orders frequently associate with humans and water at beaches; such significant associations influence the likelihood of acquiring resistance organisms. The Australian white ibis would be a particularly interesting target given its proximity to, and high association with humans, as well as its dependence on artificial water sources. Orders such as Procellariiformes (Shearwaters) and Sphenisciformes (Penguins) have not yet been investigated, even though they are highly associated with water, have a

long-distance migration (Shearwaters), and experience human association due to anthropogenic development of the Australian coastline. These are also traits conducive to being sentinel species of ocean ecosystem health. Sphenisciformes (Penguins) live along the Eastern coastline and offshore islands of Australia in a gradient of human association from remote to intensely occupied and are also impacted by tourist visitation. Penguins could therefore act as a sentinel species for the spread of AMR as well as a target to investigate the implications of human association to AMR ecology. In areas other than Australia, targeting host orders of ecological significance could also be applied to investigate AMR dissemination by wild birds globally.

In summary, although wild birds have been considered being particularly important to the dissemination of AMR worldwide, major knowledge gaps still remain, as evidence from literature. Future studies should focus on comparative examinations of genetic elements such as the class 1 integron and bacterial carriage across different bird species to address outstanding questions such as; i) are we interpreting the degree (and severity) of the dissemination of AMR through wild birds correctly by relying on only a few species?; ii) are we focusing on the most appropriate bacterial species in the host?; iii) what are the key host species to investigate to aid our understanding of the dissemination of AMR through wild birds? Given the likely role that avian species play in the dissemination of AMR, further investigation of their importance as a sentinel species for understanding the dissemination of AMR is paramount.

Chapter 2

Class 1 integrons in wild and captive little penguins (*Eudyptula minor*)

Abstract

The dissemination of antimicrobial resistance in wildlife species has been greatly facilitated by horizontal gene transfer of genetic elements such as class 1 integrons. Class 1 integrons can acquire resistance genes from the environment and rapidly express these advantageous genes, a process that has driven the evolution of multidrug resistance. The presence of class 1 integrons in wildlife hosts presents a measure of anthropogenic pollution, as the class 1 integron was derived in clinical settings and then spread into the environment and non-human hosts via human waste-streams. This study investigated the prevalence and diversity of class 1 integrons in wild and captive little penguins (*Eudyptula minor*), an Australian seabird undergoing decline. PCR screening of little penguin faecal samples (n = 448) revealed a significant difference ($p < 0.05$) in the prevalence of class 1 integrons in wild and captive populations, 3.2% and 44.7%, respectively. Genes that confer resistance to streptomycin, spectinomycin and trimethoprim were detected in both wild and captive little penguins, indicating that both wild and captive little penguins serve as reservoirs of antibiotic resistance determinants. Defining alterations of the little penguin gut microbiome caused by the acquisition of bacteria carrying resistance determinants could aid in shaping conservation practices for future management of the little penguin.

1. Introduction

Antibiotic resistant bacteria and associated genetic determinants of resistance have now been disseminated to diverse wildlife species across all ecosystems (Allen et al., 2010). The widespread occurrence and continuing emergence of antimicrobial resistance (AMR) accentuates the threat posed by the dissemination of AMR on both human and animal health. The dissemination of AMR from human sources to wildlife has been greatly facilitated by anthropogenic influences (Marti et al., 2014). Humans broadly drive AMR ecology in two ways; firstly, through environmental pollution with antibiotics and secondly, through the dissemination of resistant bacteria (Gillings et al., 2015). Both of these mechanisms work together, with the 'zone of antibiotics' that is now present around cities (Gillings et al., 2015), creating a selective force for those bacteria that possess resistance traits. This puts a selective pressure on bacterial traits conferring resistance to antimicrobial agents, in this manner such genes can become fixed in bacterial lineages.

Human associated resistance determinants are commonly disseminated into the environment via human waste-streams (Gillings, 2017). Consequently, the dissemination of bacteria and antibiotic residues further increases if the human activity area is located within

close proximity to water systems such as rivers and harbours. Once present in the environment, these bacteria could colonise and/or introduce resistance determinants into the microbiota of wildlife genomes, therefore altering the microbial composition of wildlife faecal microbiota. The impacts of AMR to wildlife species are unknown (Pellegrini et al., 2009).

Antibiotic resistance genes can be acquired through horizontal gene transfer (HGT) (Gillings, 2014) or acquisition of resistance determinants via mobile DNA elements, such as plasmids, transposons or integrons (Machado et al., 2005). The dissemination of resistance has therefore been greatly facilitated by these mobile genetic elements, particularly integrons (Rowe-Magnus et al., 2001). While there are three different types of integrons, the class 1 integron has been instrumental in the dissemination of AMR, both in clinical settings and in the environment (Partridge et al., 2009). Once in the environment, the class 1 integron has the ability to 'sample' its surroundings for advantageous genes, enabling new genetic resistance profiles, affecting the pathogenicity, transmission and virulence of the bacteria (Domingues et al., 2012; Lupo et al., 2012). The integron recombination site (*attI*) and adjacent promotor (P_c) allow the newly integrated gene(s) to be immediately expressed and subjected to natural selection (Collis et al., 1998; Hall and Collis, 1995), thus enabling rapid emergence of new resistance profiles.

Class 1 integrons have now been reported in diverse hosts including wild birds (Nebbia et al., 2008), marsupials (Power et al., 2013), marine mammals (Delport et al., 2015; Fulham et al., 2018), domestic animals (Murinda et al., 2005), and reptiles (Thaller et al., 2010), with no empirical knowledge on the implications of the acquisition or retention of these resistance determinants to the health of the host. Due to the origin of class 1 integrons, the presence of bacteria carrying class 1 integrons in wildlife can be seen as a measure of anthropogenic pollution (Dolejska et al., 2007), with marine birds and vertebrates being proposed as useful indicators of the spread of AMR (Aguirre and Tabor, 2004).

In birds, the proximity to humans, and exposure to anthropogenic influences affects the acquisition of class 1 integrons (Cole et al., 2005; Dolejská et al., 2009; Gionechetti et al., 2008; Literák et al., 2007; Pinto et al., 2010). However, the prevalence of the class 1 integron is generally low, with the highest prevalence of class 1 integrons reported in species that associate with water sources (Chapter 1; see Dolejská et al., 2009; Gionechetti et al., 2008). Studies investigating integron carriage in wild birds tend to focus on species in close proximity to humans and water sources, with class 1 integrons having previously been isolated from diurnal birds of prey, pigeons and doves, and waterfowl (Cole et al., 2005;

Dolejská et al., 2009; Gionechetti et al., 2008; Literák et al., 2007; Pinto et al., 2010). However, reports on integron carriage are limited to a few species leaving significant knowledge gaps, particularly on integron carriage in marine birds.

The little penguin (*Eudyptula minor*) is the only penguin species inhabiting mainland Australia, with coastal and island colonies ranging from New South Wales to Western Australia and Tasmania. Little penguins spend a majority of their lives at sea (Boersma, 2008), which potentially increases their exposure to resistant organisms present in the marine environment. These factors, coupled with their widespread coastal distribution makes the little penguin an ideal sentinel species and indicator of marine ecosystem health (Boersma, 2008). The introduction of predators, invasive plant species, development of coastal areas, and other anthropogenic influences have caused little penguin colonies to decline over the last 50 years (Fortescue, 1995). Human association and influences posed on Australia's little penguin colonies occur in a gradient, from remote areas experiencing little contact to intensely occupied areas that are frequented by tourists. The differing levels of human influences exerted on Australia's little penguin colonies thus create a naturally occurring gradient ideal for measuring anthropogenic influences.

This study aimed to investigate the presence of class 1 integrons in faecal microbiota of wild and captive little penguins. By doing so, this study will i) determine if wild little penguins serve as reservoirs of class 1 integrons and associated gene cassettes; and ii) contrast the prevalence of class 1 integrons and the diversity of gene cassettes in wild and captive little penguins.

2. Methodology

2.1 Ethics statement

This research was conducted under approval of the Macquarie University Animal Ethics Committee, animal research authority 2017/014 and 2014/057-11, and with approvals from the Department of Environment, Land, Water and Planning, Victoria, permit number 10008371, Office of Environment and Heritage, New South Wales National Parks and Wildlife Services, permit number SL100746 and SL100746, and appropriate approvals from collaborating zoos and marine parks.

2.2 Sampling sites and faecal sample collection

Little penguin faecal samples (n = 448) were collected from four coastal and island colonies in New South Wales, the Australian Capital Territory and Victoria, and five captive colonies in New South Wales, Victoria, Queensland, and South Australia (Table 1). Faecal samples were collected from little penguins at Bowen Island, St Kilda Breakwater and Phillip Island using cloacal-swabs or by setting a paper card in the burrow entrance and sampling faeces indirectly; samples collected from Montague Island were swabs of paper cards placed inside the nest. Faecal samples from the five captive colonies were collected opportunistically from the enclosure substrate. All samples were collected using FecalSwab™ (Copan, Brescia, Italy) and transported at room temperature to Macquarie University. Samples were stored at 4°C until further processing.

Table 1. Sample sites, intensity of human influence, and geographical coordinates of sample sites.

Sample site	State	Human impact	Geographical coordinates
Phillip Island	VIC	High	38°30'39.5"S 145°08'42.1"E
St Kilda	VIC	High	37°51'53.4"S 144°57'54.6"E
Montague Island	NSW	Low	36°15'03.4"S 150°13'33.4"E
Bowen Island	ACT	Low	35°07'07.1"S 150°46'06.2"E
Taronga Zoo	NSW	High	33°50'36.7"S 151°14'28.9"E
Dolphin Marine Magic	NSW	High	30°17'54.4"S 153°08'11.0"E
Sea World	QLD	High	27°57'24.7"S 153°25'32.5"E
Adelaide Zoo	SA	High	34°54'45.8"S 138°36'25.0"E
Sea Life Sydney	NSW	High	33°52'12.1"S 151°12'09.3"E

2.3 DNA extraction and PCR screening

Genomic DNA was extracted from faecal samples (150-200 mg) or from faecal swab media (200 μL). Extraction was performed using the ISOLATE Fecal DNA Kit (Bioline, Sydney, Australia) as per manufacturer's instructions. To determine DNA competency, a 16S rDNA PCR was performed using the universal eubacteria primers F27 and R1492 (Appendix 1) (Lane, 1991). The PCR was performed in 25 μL reactions using GoTaq® Green 2X (Promega, Madison, Wisconsin, USA), 50 μM of each primer and 2 μL DNA. The PCR protocol included an initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 58°C for 30 sec, extension 72°C for 1.5 min, with a final extension of 72°C for 5 min.

DNA samples positive for 16S rDNA were further screened for the presence of the integron integrase class 1 gene (*intI1*), using the primers HS463a and HS464 (Appendix 1) (Holmes et al., 2003). The PCR reaction was performed with 25 μL reactions using GoTaq® colourless mastermix (Promega, Madison, Wisconsin, USA), 50 μM of each primer and 2 μL DNA. The PCR protocol included an initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C 30 for sec, annealing at 55°C for 30 sec, extension 72°C for 1.5 min, with a final extension of 72°C for 5 min (Waldron and Gillings, 2015). The primers HS463a and HS464 amplify an internal fragment of the *intI1* gene, which results in an approximately 473bp product. Samples generating a band of 473bp were therefore deemed *intI1* positive (Holmes et al., 2003).

Samples containing *intI1* amplicons were further amplified for identification of the gene cassette array using the primers HS458 and HS459 (Sequences in Appendix 1) (Holmes et al., 2003). The PCR was performed using 25 μL reactions using GoTaq® colorless mastermix (Promega, Madison, Wisconsin, USA), 50 μL of each primer and 2 μL DNA (Waldron and Gillings, 2015). The protocol was performed as described for the HS463a/HS464 PCR above.

All reactions included a positive sample (*E. coli* KC2) and negative control (PCR H_2O) and were resolved using agarose gel electrophoresis (16S 2% agarose w/v, HS463/464 and HS458/459 3% agarose w/v). Electrophoresis was conducted at 100V for 25 minutes in 1x TBE for 16S, and 40 min for HS463/463 and HS458/459 (pH 8) with SYBR safe DNA stain (Invitrogen, Australia). Product size was approximated against a HyperLadderII 50bp DNA marker (Bioline, Sydney, Australia).

2.4 DNA Cloning, sequencing, and analyses

Positive amplicons identified from the HS458/HS459 PCRs were purified using the MinElute PCR Purification Kit (Qiagen, Melbourne, Australia), as per manufacturer's instructions. Amplicons containing a single band were sequenced directly from the PCR product. Amplicons with multiple bands, indicating the presence of more than one gene, were cloned using the TOPO TA cloning kit (Invitrogen, Australia) and transformed into One Shot® TOP10F' Chemically Competent *E. coli* cells (Thermo-Fisher, Australia) as per manufacturer's instructions. Plasmid extractions were performed using the Qiagen Plasmid Mini Kit (Qiagen, Melbourne, Australia) as per manufacturer's instructions.

Overlapping sequence fragments were obtained using the primers HS458 and HS459 (Holmes et al., 2003) for sequencing of PCR products and plasmids. DNA sequencing was performed at The Ramaciotti Centre for Genomics (Sydney, NSW, Australia) using Big Dye Terminator chemistry version 3.1 and ABI 3730/3730xl Capillary Sequencers (Applied Biosystems, Foster City, CA, USA). Sequences were manually checked for quality, assembled using Geneious R11 (version 11.1.5) software (Biomatters Limited, Auckland, New Zealand), and analysed for the presence antibiotic resistance genes using Integrall (http://integrall.bio.ua.pt). Annotation was performed manually using the BlastN suite and BlastX (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Class 1 integron gene cassette arrays were confirmed by the presence of the 3' conserved region containing *qacE*Δ. For arrays containing multiple gene cassettes, *attC* recombination sites located between cassettes were identified using the highly conserved sequence GTTRRRY (Stokes et al., 2001).

2.5 Statistical analysis

RStudio (V 1.0.143, Boston, Massachusetts, USA) software was employed for all statistical analyses. Fisher's exact test was used to test for differences in class 1 integron prevalence between wild and captive individuals with all wild and all captive sites pooled for this analysis. Pearson's chi-squared test was used to test class 1 integron prevalence and distribution between wild and captive sites, respectively. For the captive site comparison, post-hoc comparison was conducted by using adjusted residuals to calculate a critical z value (Bonferroni corrections $p < 0.005$). Significance was determined when $p < 0.05$.

3. Results

3.1 Class 1 integron frequency

Faecal DNA (*n* = 448) was deemed competent for downstream PCR analysis by 16S rDNA, with all samples then tested for the presence of the class 1 integron integrase gene. The prevalence of class 1 integrons was significantly higher (Fisher's Exact Test, Two-sided, $p < 0.001$) in captive little penguins (44.7%; *n* = 103) compared to wild little penguins (3.2%; *n* = 345) (Figure 1). A significant difference ($\chi^2_{4, 103} = 36.64$, $p < .001$) in class 1 integron prevalence was evident between captive sampling sites (Figure 2). The highest prevalence was seen in samples collected from Sea World. The observed *intI1* frequency at Taronga Zoo was lower than expected (z = 3.23), while higher than expected at Sea World (z = -5.93) (critical z = -2.81).

For samples from wild sites, the highest prevalence of *intI1* genes was found at Montague Island (9.1%; 6/66), followed by St Kilda Breakwater (2.1%; 1/48) and Phillip Island (1.8%; 4/228). No samples collected from Bowen Island (0/3) were positive for the presence of the *intI1* gene. The between site comparison for wild little penguins revealed a non-significant difference ($\chi^2_{4, 345} = 9.26$, $p > 0.05$) in class 1 integron prevalence.

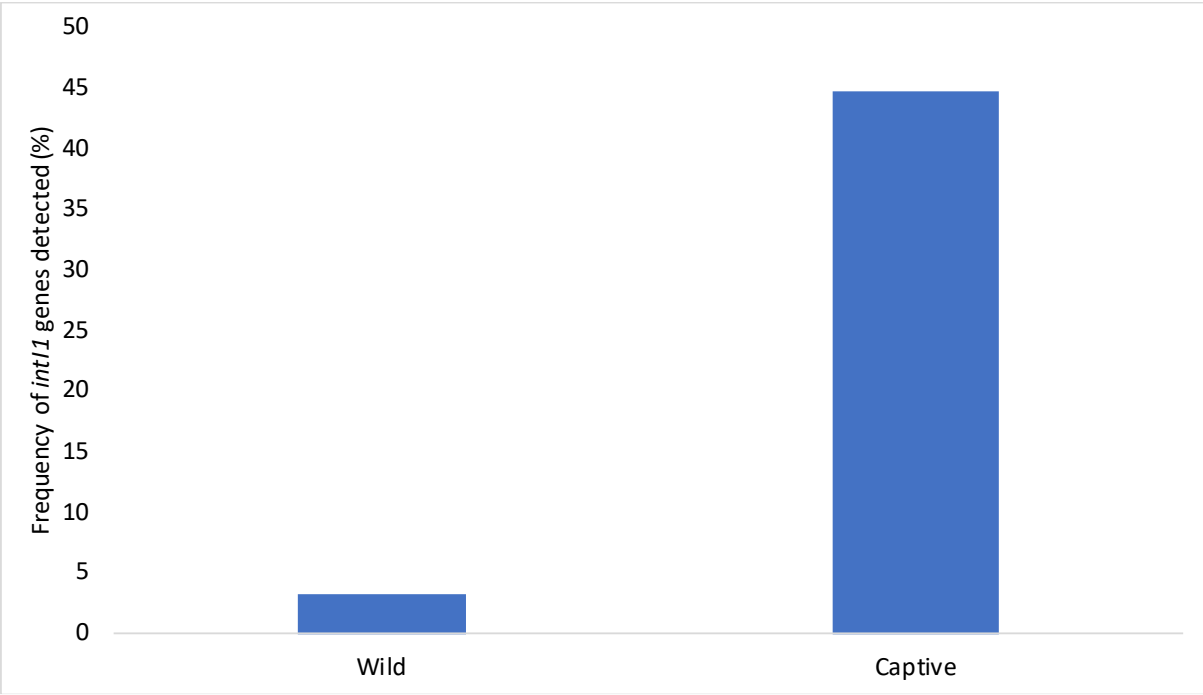


Figure 1. Frequency of *intI1* genes (percentage) detected in wild and captive little penguins. A total of 448 faecal samples from wild (n = 345) and captive (n = 103) little penguins were tested.

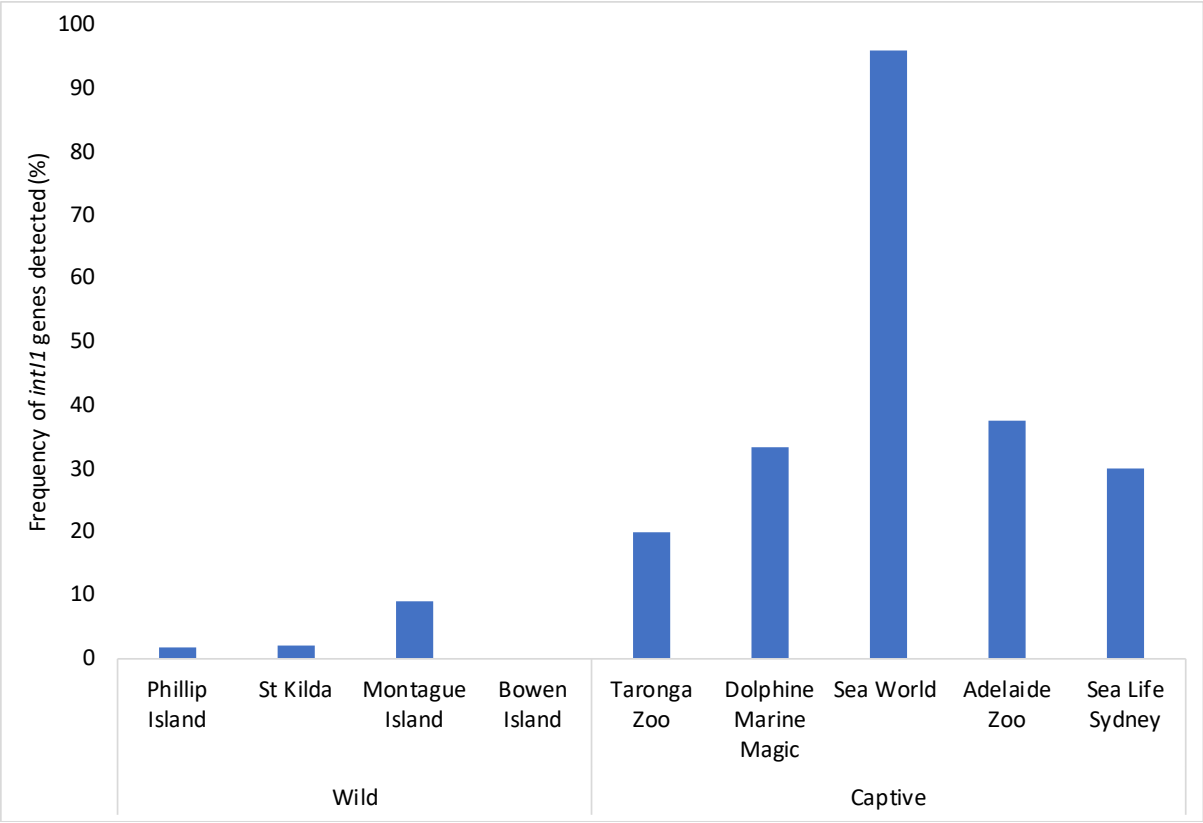


Figure 2. Frequency of *intI1* genes (percentage) detected in wild and captive little penguins at each sampling site.

3.2 Gene cassette array analysis

Based on the presence of a 473 bp product detected in the HS463/HS464a PCR, 57 samples were determined to be *intI1* positive and were subsequently analysed for gene cassette arrays. Class 1 integrons contained genes within the cassette arrays encoding aminoglycoside adenyltransferases (*aadA*) and dihydrofolate reductases (*dfrA*). In wild samples, gene cassette arrays contained only *aadA*, and in captive samples both *aadA* and *dfrA* were detected, as well as an array that contained both genes (Figure 3; Table 2).

Table 2. Cassette arrays and resistance genes identified in wild and captive little penguins.

Site type	**Sample site**	**Samples with cassette arrays (*n*)**	**Resistance profile**
Wild	Montague Island	6	No genes
	Phillip Island	2	No genes
		2	*aadA2*
	St Kilda	1	No genes
Captive	Taronga Zoo	1	*aadA1*
		2	*aadA2, dfrA16*
		2	*aadA2*
		1	No genes
	Dolphin Marine Magic	10	*aadA1*
	Sea World	24	No genes
	Adelaide Zoo	2	*aadA1*
		1	*dfrA5*
	Sydney Sea Life	3	No genes

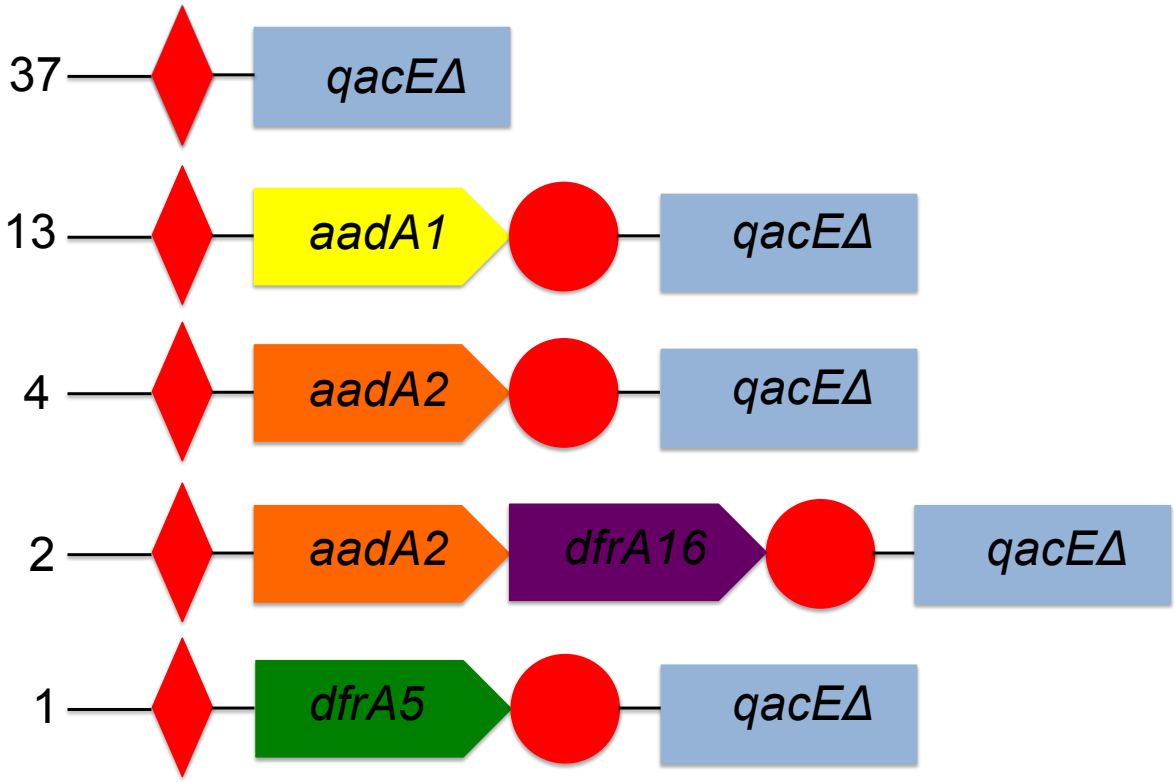


Figure 3. Schematic maps of integron cassette arrays recovered from 57 little penguins. The numbers of little penguins with each array is shown to the left. Red diamonds correspond to the primary integron recombination site, *attI1*; red circles correspond to the gene cassette recombination sites, *attC*; and arrows correspond to each gene. Gene symbols are as follows: *aadA* genes encode aminoglycoside adenyltransferases that confer resistance to streptomycin and spectinomycin; *qacE*Δ confers resistance to quaternary ammonium compounds, *dfrA* genes encode dihydrofolate reductases that confer resistance to trimethoprim.

4. Discussion

This study found class 1 integrons and associated gene cassettes in both wild and captive little penguins; however, the occurrence of class 1 integrons was significantly higher in captive individuals. This finding suggests that the acquisition of class 1 integrons could result from conditions experienced in captivity, and that there is an exchange of class 1 integrons at this interface. In both wild and captive populations genes encoding resistance to streptomycin, spectinomycin and trimethoprim were detected. Bacterial isolates with class 1 integrons and similar gene cassette arrays are commonly isolated from environmental sources, human clinical samples (Partridge et al. 2009), wastewater (Czekalski et al., 2014), captive marine mammals (Delport et al., 2015; Stoddard et al., 2008) and Australian native animals (McDougall, in review; Power et al., 2013). This study further supports the effects of

human influences on the dissemination of antibiotic resistance to wildlife species both in the wild and in captivity.

The prevalence of class 1 integrons and cassette array diversity differed between captive sites. Although the highest prevalence of class 1 integrons was detected at Sea World, none of these arrays contained resistance genes. At Coffs Harbour Dolphin Marine Magic Park, all faecal samples were opportunistically collected from the enclosure substrate and the same individual could have been sampled more than once. Alternatively, the class 1 integron containing the *aadA1* gene may have been disseminated to all individuals inhabiting the enclosure.

The variation in integron diversity seen across the captive populations could be due to several factors, such as the number of animals in the enclosure, co-habituation of the enclosure with other species, administration of medications (antibiotics or other), vitamin and mineral supplements, dietary source, as well as supplier, shipment, storage, and possible freeze-thaw processes of feed, and water source quality. Of particular importance is water availability, water source, treatment and filtration of the water in the captive enclosures. Some captive institutions source water for the enclosures from adjacent harbours and coastal areas (pers. comm. Sydney Sea Life Aquarium). As harbours and water sources in close proximity to human activity play a significant role in the dissemination of resistant organisms and are potential sources of organisms carrying resistance traits, this could affect the acquisition of resistance traits in bacteria within captive individuals. Additionally, resistant organisms have been shown to survive treatment and wastewater filtration processes (Prado et al., 2008; Tennstedt et al., 2005) serving as a potential source of these bacteria and their resistance traits. Lastly, population demographics and origin of captive individuals can affect the exposure to, and therefore, acquisition of resistant traits. For example, whether individuals were captive or wild bred, or acquired from another captive site, could drive the dissemination of AMR within and between captive populations.

Class 1 integrons were detected in wild little penguins across multiple sites, illustrating that the transfer of organisms harbouring resistance traits is occurring in both wild and captive settings. This finding also shows the potential for microbial flow from human associated settings, such as coastal sources, through to the marine environment. The little penguin colonies on Phillip Island and St Kilda Breakwater are both exposed to high levels of anthropogenic influences. The Summerland peninsula where the Phillip Island colony is located was previously used as a housing estate, and remnants of roads, tracks, and sewage systems are still present throughout the colony. The land was bought back as a conservation

effort between 1985-2010, and in 2011 the last road was removed from the colony (Phillip Island Nature Parks). The Phillip Island colony is now one of Australia's largest tourist attractions and intensely visited by up to one million tourists each year (Phillip Island Nature Parks). Similarly, the St Kilda little penguin colony is located along the breakwater of St Kilda Marina, adjacent to a Melbourne beach (Victoria) and is also a popular tourist site.

Montague Island and Bowen Island are far more isolated than both Phillip Island and St Kilda Breakwater, and penguin colonies are exposed to minimal disturbance by humans. Both islands are within 10 km of mainland Australia where run-off from agricultural settings could affect the spread of resistance determinants into marine ecosystems. While faecal samples from Bowen Island were negative for the class 1 integron, Montague Island had the highest prevalence among the wild sampling sites. Montague Island has research facilities and national parks houses that use septic tanks for removal of effluent. The sewage system was last updated in 1994 (Department of Environment and Climate Change NSW, 2009). A study investigated the presence of human associated resistant organisms and class 1 integrons at a research station in Antarctica (Power et al., 2016) where human effluent is macerated and disposed of into seawater. The study found class 1 integrons present in marine invertebrates, soil and seawater; however, no class 1 integrons were detected in marine birds or mammals. Therefore, the presence of human effluent on Montague Island could explain why this site had the highest prevalence of class 1 integrons. Further, tourist boats operate around Montague Island and ballast or sewage may also be transferred to this site (Department of Environment and Climate Change NSW, 2009).

In this study, the presence of the class 1 integron was detected in the faecal microbiota of both wild and captive little penguins. The identification of the bacterial species harbouring the class 1 integrons was beyond the scope of this study. However, future investigations identifying the bacteria harbouring these resistance determinants would undoubtedly add to the understanding of the mechanisms by which both wild and captive populations of little penguins acquire and carry class 1 integrons.

5. Conclusion

This study identified class 1 integrons in both wild and captive little penguins, with a significantly higher prevalence in captive individuals. These findings suggest that captive environments play a major role in the establishment (and dissemination) of antimicrobial-resistance genes. Health effects posed by the carriage and integration of class 1 integrons have not yet been empirically determined, and its effects on little penguin health remain

unknown. While the mechanisms by which the class 1 integron are disseminated into wildlife microbiota remain unknown, human association and water sources appear to be strong drivers. Understanding the effects of acquiring and retaining resistant traits on the host is an important step towards understanding if human antimicrobial pollution is contributing to the decline seen in little penguin colonies across Australia, as such acquisition may affect little penguin health. Defining alterations caused by the acquisition of resistance determinants could thus help shape conservation practices for future management of the little penguin.

Chapter 3

Targeted screening for antibiotic resistance determinants in *Escherichia coli* and *Klebsiella pneumoniae* in wild and captive little penguins (*Eudyptula minor*)

Abstract

Antibiotic resistant bacteria in wildlife is being increasingly reported. Several terrestrial and aquatic bird species have been proposed as sentinels for the spread and dispersal of resistant bacteria, but few have been considered sentinels in the marine context. The little penguin (*Eudyptula minor*) is a top order predator that is found along coastal areas of Australia. The species' proximity to human habitation exposes it to anthropogenic influences, such as wastewater run-off, making it susceptible to exposure to human-associated bacteria. Class 1 integrons (genetic determinants of antibiotic resistance) have recently been detected in little penguin faecal DNA (Chapter 2). Here I aim to determine if class 1 integrons are present in two Gram-negative opportunistic pathogens, *Escherichia coli* and *Klebsiella pneumoniae,* and to genetically characterise these bacteria from captive and wild little penguins. Little penguin faecal samples were tested for the presence of *E. coli* (n = 569) and *K. pneumoniae* (n = 361). The distribution of *E. coli* and *K. pneumoniae* differed between captive and wild little penguins. There was a significantly higher prevalence of *E. coli* in captive little penguins than in wild, 29.1% and 16.3%, respectively ($p < 0.05$), and although the prevalence of *K. pneumoniae* was also higher in captive individuals (16.7% compared to 4.4%), the observed difference was not statistically significant. Further analysis of *E. coli* identified isolates assigned to phylogroup B1 to be the most frequently isolated in captive little penguins (40%), followed by D (30%) and A (20%); the remaining 10% consisted of phylogroups B2 and F. In comparison, the most frequently isolated phylogroup from wild little penguins were B2 (55%) followed by B1 (22%) and D (9%), with the remaining 14% made up of phylogroups A, C, F, and E. No class 1 integrons were detected in the either *E. coli* or *K. pneumoniae* isolates suggesting that the class 1 integrons previously found in the faecal DNA from little penguins may be integrated in their endemic microbiota or other acquired bacterial species.

1. Introduction

Antibiotic resistant bacteria have now spread to diverse wildlife species across all ecosystems (Allen et al., 2010). The spread of resistant bacteria to wildlife is facilitated by the dissemination of human associated resistance organisms via human waste-streams into the environment (Gillings, 2017). Once in the environment, wildlife may frequently encounter antibiotic resistant organisms, facilitating colonisation and introduction of resistance determinants into the microbiota of wildlife (Pellegrini et al., 2009). As a result, the microbial

composition of wildlife microbiota can be altered, with potential consequences for wildlife health mediated by the introduction of novel disease agents (Pellegrini et al., 2009).

The Gram-negative coliform bacteria, *E. coli* and *K. pneumoniae*, are commonly used as indicator species for the detection of resistant bacteria in wildlife (Guenther et al., 2010; Literak et al., 2010; Sacristán et al., 2014; Wang et al., 2017). These bacterial species are used as indicators of sanitary quality of foodstuff and water sources due to their ubiquitous nature in soil, water, vegetation, and in the lower intestinal tract of humans and other animals (Johnson, 2011). *E. coli* and *K. pneumoniae* are commensal species in the intestinal tract of vertebrates (Abriouel et al., 2008; Shehabi et al., 2006), and some strains can act as intestinal pathogens (Mainil, 2013; Riley, 2014). Large numbers of *E. coli* and *K. pneumoniae* are passed in faeces, and some strains persist in the environment outside of the host for extended periods of time (Salyers et al., 2004). For these reasons, Gram-negative coliform bacteria play a significant role in the dissemination of antibiotic resistance from humans to wildlife (Hammerum and Heuer, 2009; Sick, 1997; von Baum and Marre, 2005).

E. coli strains can be characterised into eight phylogroups (A, B1, B2, C, D, E, F, and clade I) based on genetic substructure and the mechanisms by which they cause disease (Clermont et al., 2013, 2014). Phylogenetic studies of *E. coli* have increased our understanding of the distribution of *E. coli* strain types (Tenaillon et al., 2010), and it is now understood that the different phylogroups are non-randomly dispersed in the environment, being distinctly linked to their source of origin (Clermont et al., 2013). Pathogenic strains of *E. coli* are mainly assigned to phylogroups B2 and D (Clermont et al., 2013, 2014), while generalist non-pathogenic strains in ectotherms, birds, and the environment belong to phylogroup B1 (Gordon and Cowling, 2003).

K. pneumoniae is a well distributed ubiquitous pathogen (Castinel et al., 2008), however increasing reports of multidrug resistant *K. pneumoniae* in the environment are emerging (Wang et al., 2017). *K. pneumoniae* has historically been responsible for significant nosocomial outbreaks (Podschun and Ullmann, 1998) with cases of inter-hospital dissemination of resistant strains reported (Arlet et al., 1994). *K. pneumoniae* has been isolated from all stages of hospital sewage treatment (Prado et al., 2008), as well as in activated sludge, that is, biologically treated sewage (Tennstedt et al., 2005), likely due to its protective capsule enhancing its survival. More recently, antibiotic resistant *K. pneumoniae* have been reported in wild birds (Bonnedahl et al., 2014).

Bacteria have the capacity to exchange resistance determinants between strains present in the environment (Hammerum and Heuer, 2009; Sick, 1997; von Baum and Marre,

2005) via horizontal gene transfer (HGT) (Gillings, 2014). This exchange is enhanced if resistance determinants are located on mobile DNA elements, such as plasmids, transposons or integrons (Machado et al., 2005). The dissemination of resistance has been greatly facilitated by these mobile genetic elements, particularly integrons, which have been significant for bacterial evolution (Rowe-Magnus et al., 2001).

There are three classes of integrons which share three common features: (1) the presence of an integrase gene (*intI*) that facilitates gene capture from the environment, (2) a recombination site (*attI*), which facilitates insertion of a gene cassette(s), and (3) an adjacent promotor site (P_c) which enables expression of inserted gene cassette(s) (Messier and Roy, 2001). Integrons can 'capture' gene cassettes from the environment, and upon acquisition they are instantly subject to natural selection (Gillings, 2014). Hence, the integron is a crucial driver of the diversity seen in bacterial genomes (Gillings et al., 2008; Holmes et al., 2003; Rowe-magnus et al., 2003), and is considered to play a major role in the dissemination of antimicrobial resistance (AMR) (Gillings, 2014).

The class 1 integrons have been proposed as an indicator of anthropogenic pollution, due to their wide geographical and biological distribution, their origins from a human clinical context, as well as their potential to spread into and contaminate the environment via human waste streams (Stokes and Gillings, 2011). Increased coastal development has created a greater pathway for the transfer of resistant organisms from human systems to marine ecosystems (Fulham et al., 2018; Halpern et al., 2008), and antimicrobial resistant organisms are increasingly reported in the marine environment (Bogomolni et al., 2008; Fulham et al., 2018). Given that *E. coli* and *K. pneumoniae* are commonly found in contaminated water sources, are ubiquitous in the environment, and commonly carry class 1 integrons (Pinto et al., 2010; Wang et al., 2017), wildlife exposed to untreated wastewater runoff and contaminated coastal waters are therefore potentially at risk of acquiring *E. coli* and *K. pneumoniae* carrying class 1 integrons (Pellegrini et al., 2009). Marine birds and mammals can therefore be regarded as sentinels of the marine ecosystem and be useful indicators of the dissemination of AMR (Aguirre and Tabor, 2004).

In Australia, the little penguin (*Eudyptula minor*) is a potential sentinel indicator of marine health. The little penguin has a wide geographical distribution along the southern coast and offshore islands in New South Wales on the East coast to Western Australia, and the Eastern State of Tasmania. Little penguins spend around 80% of their lives at sea foraging, returning to land to moult, breed and rest (Boersma, 2008). During the last 50 years the introduction of predators, invasive plant species, and other anthropogenic influences have

resulted in the decline and movement of little penguin colonies to offshore islands where human influences are minimal (Fortescue, 1995). Many ecological studies have investigated factors driving the decline of the little penguin, however, anthropogenic pollution and infectious disease have not yet been considered.

Australia's little penguin colonies are located in a gradient of human association from remote sites on offshore islands to areas of high human population density as well as tourist sites. Colonies on Phillip Island and St Kilda Breakwater in Victoria are both exposed to high levels of human influences. The land where the Phillip Island colony is located was previously used as a housing estate, and remnants of roads, tracks, and sewage systems are still present throughout the colony. The estates were recovered for conservation efforts between 1985-2010, and the Phillip Island colony is now one of Australia's largest tourist attractions, visited by up to one million tourists annually (Phillip Island Nature Parks). Likewise, the little penguin colony on the St Kilda Breakwater in Melbourne, Victoria, is located in St Kilda Marina, a site of high human impact with significant tourist visitation given its proximity to Melbourne. In contrast, colonies on off-shore islands, such as Bowen Island in the Australian Capital Territory and Montague Island, in New South Wales are far more isolated from human influences, although their close proximity (< 10 km) to the mainland means that they are still susceptible to exposure of waste-water run-off from residential, industrial and agricultural settings. Little penguins are also held in numerous captive locations such as aquariums and zoos across Australia.

The differing levels of human influences experienced across Australia's little penguin colonies create a naturally occurring gradient ideal for measuring anthropogenic influences on Australia's little penguins. In chapter 2, I showed that class 1 integrons are present in the faecal microbiota of little penguins. Here I aim to determine what bacteria harbour the class 1 integrons by undertaking targeted screening of antimicrobial resistance determinants in *E. coli* and *K. pneumoniae,* two clinically relevant opportunistic pathogens, highly associated with the dissemination of AMR from human systems to wildlife.

2. Methods

2.1 Ethics statement

This research was conducted under approval of the Macquarie University Animal Ethics Committee, animal research authority 2017/014 and 2014/057-11, and with approvals from the Department of Environment, Land, Water and Planning, Victoria, permit number 10008371, Office of Environment and Heritage, New South Wales National Parks and Wildlife Services, permit number SL100746 and SL100746, and appropriate approvals from collaborating zoos and marine parks.

2.2 Sampling sites and faecal sample collection

Little penguin faecal samples (n = 569) were collected from four coastal and island colonies in NSW, ACT, and VIC and five captive colonies in NSW, VIC, QLD, and SA (Table 1). Faecal samples from Bowen Island, St Kilda Breakwater and Phillip Island were collected using cloacal-swabs and non-invasive sampling of droppings from paper cards placed in the burrow entrance. Faecal samples collected from Montague Island were burrow-swabs of paper cards placed inside the nest. Faecal samples from captive colonies were collected opportunistically from the enclosure substrate. All faecal samples were collected using FecalSwab™ (Copan, Brescia, Italy). Following collection, swabs were transported at room temperature to Macquarie University and were stored at 4°C until processing for bacterial culturing.

2.3 Bacterial culture

2.3.1 E. coli

Faecal samples (n = 569) were inoculated onto Chromocult® Coliform agar (Merck Millipore, Australia) and incubated at 37°C for 24 h based on the methodology of Finney et al. (2003). Samples that grew purple, undulate round colonies were considered positive for *E. coli* and sub-cultured to establish pure isolates for DNA extraction and genetic analysis.

Table 1. Sample sites, intensity of human influence, and geographical coordinates of sample sites.

Sample site	State	Human impact	Geographical coordinates
Phillip Island	VIC	High	38°30'39.5"S 145°08'42.1"E
St Kilda	VIC	High	37°51'53.4"S 144°57'54.6"E
Montague Island	NSW	Low	36°15'03.4"S 150°13'33.4"E
Bowen Island	ACT	Low	35°07'07.1"S 150°46'06.2"E
Taronga Zoo	NSW	High	33°50'36.7"S 151°14'28.9"E
Dolphin Marine Magic	NSW	High	30°17'54.4"S 153°08'11.0"E
Sea World	QLD	High	27°57'24.7"S 153°25'32.5"E
Adelaide Zoo	SA	High	34°54'45.8"S 138°36'25.0"E
Sea Life Sydney	NSW	High	33°52'12.1"S 151°12'09.3"E

2.3.2 K. pneumoniae

Faecal samples (n = 361) were tested for *Klebsiella sp.* by inoculating 200μl of faecal swab media into 5 mL Luria-Bertani broth (Sigma-Aldrich, Australia) with added amoxicillin (1 mL/100 mL broth) (Sigma-Aldrich, Australia) based on the methodology of F. McDougall (pers. comm.) followed by incubation at 37°C for 24 h. Samples were then streak-inoculated onto Simmons-Citrate Agar (Dutscher, France) with added myo-Inositol (Sigma-Aldrich, Australia) (SCAI plates) (Van Kregten et al., 1984), modified with the addition of amoxicillin (SCAIA plates) (1 mL/100 mL) to improve selection. Plates were incubated at 37°C for 48 h. Samples that grew small, yellow-orange undulate round colonies after 48 h were considered positive for *Klebsiella sp.* The SCA plate confirms *Klebsiella sp.* ability to metabolise citrate, the addition of myo-Inositol to the media (SCAI) changes the pH of the media, hence no colour change occurs. Therefore, to confirm citrate metabolism a single colony from *Klebsiella sp.* positive samples was inoculated into 5 mL Luria-Bertani broth and incubated for 24h at 37°C. Samples were then plated onto Simmons-Citrate Agar (SCA) (Dutscher, France) and dispensed (100 μl) into nutrient gelatin (NG) to confirm citrate- and gelatin metabolism. The nutrient gelatine was made up by dissolving 12 g commercial gelatine (Dr. Oetker, Bielefeld, Germany) and 2.5 g nutrient broth into 100 mL of water on a heat block which was then autoclaved (McDougall pers. comm.). Samples that were SCAIA+, SCA+

and NG- were deemed presumptive *Klebisella sp.* or *Enterobacter sp.* Strain specific *K. pneumoniae* PCRs where then performed as per section 2.7.

2.4 Extraction of DNA from isolated bacteria

Genomic DNA extraction was performed using the boil preparation method. Broth cultures were established by inoculating a single *E. coli* or *K. pneumoniae* colony from Chromocult or SCAIA agar into Luria-Bertani broth (5 mL) followed by incubation at 37°C for 24 h. Broth cultures were centrifuged for 10 min at 4000rpm (Eppendorf 5810R, rotor: A-4-62) and the bacterial pellet was re-suspended in 150 μL PCR water. Samples were heated for 15 min at 95°C and centrifuged for 10 min at 13000 rpm (Eppendorf 5430R, rotor FA-45-24-11-kit).

2.5 Phylotyping of E. coli isolates

To assign isolated *E. coli* to a phylogroup, extracted DNA was amplified using a quadruplex standard protocol (Clermont et al., 2013). The primer pairs used were ChuA.1b and ChuA.2, YjaA.1b and YjaA.2b, TspE4C2.1b and TspE4C2.2b, AceK.f and ArpA1.r (primer sequences are detailed in Appendix 1) (Clermont et al., 2013). The assignment of strains to phylogroups was determined by the presence and absence of the fragments associated with the primers described above (*chuA* 288bp, *yjaA* 211bp, *TspE4.C2* 152bp, and *arpA* 400bp). The PCR reaction was performed using GoTaq® Green 2X (Promega, Madison, Wisconsin, USA) (Clermont et al., 2013) using 50 μM primers (TspE4C2.1b and TspE4C2.2b 100 μM) and 2 μL DNA. The PCR protocol included an initial denaturation at 94°C for 4 min, followed by 35 cycles of denaturation at 94°C 20 sec, annealing at 59°C 20 sec, with a final extension of 72°C for 5 min.

2.6 Subtyping of phylogroup B2 E. coli isolates

An allele-specific PCR was used to determine subtypes from *E. coli* isolates in the B2 phylogroup (Clermont et al., 2014). The assignment of strains to the subgroups was determined by the presence of the following fragments *putP* 373bp (I), *pabB* 415bp (II), *trpA* 255bp (III), *trpA* 261bp (IV), *polB* 530bp (V), *dinB* 652bp (VI), *icd* 810bp (VII) *aes* 160bp (IX), *aes* 713bp (X), and *chuA* 1013bp (internal control) (primer sequences are detailed in Appendix 1) (Clermont et al., 2014). The PCR reaction was performed using GoTaq® Green 2X (Promega, Madison, Wisconsin, USA) using 20 μM primers and 2 μL DNA. The PCR protocol included an initial denaturation at 94°C for 4 min, followed by 35 cycles of

denaturation at 94°C for 20 sec, annealing at 57°C for 20 sec (panel 1) or 59°C (panel 2), and a final extension at 72°C for 5 min.

2.7 K. pneumoniae confirmation

To determine if presumptive *Klebsiella sp.* were *K. pneumoniae*, three strain-specific PCRs were performed (Diancourt et al., 2005). The assignment of strains as *K. pneumoniae* was determined by the presence of the gene fragments *gapA* 450bp, *mdh* 477bp, and *phoE* 420bp (primer sequences detailed in Appendix 1). The PCR reaction was performed using GoTaq® Colorless Mastermix (Promega, Madison, Wisconsin, USA) using 10 μM primers and 2 μL DNA. The PCR protocol included an initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 94°C for 40 sec, annealing at 50°C for 40 sec (for *mdh* and *phoE*) and 60°C (for *gapA*), and a final extension at 72°C for 1 min (Guo et al., 2015).

2.8 16S PCR, integron integrase screening and gene cassette identification

To determine DNA competency, a 16S rDNA PCR was performed using the universal eubacteria primers F27 and R1492 (primer sequences detailed in Appendix 1) (Holmes et al., 2003). The PCR reaction was performed using GoTaq® Green 2X (Promega, Madison, Wisconsin, USA) 50 μM primers and 2 μL DNA. The PCR protocol included an initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C 30 sec, annealing at 58°C 30 sec, extension 72°C 1.5 min, with a final extension of 72°C for 5 min.

DNA samples positive for 16S rDNA were further screened for the presence of the integron integrase class 1 gene (*intI1*), using the primers HS463a and HS464 (primer sequences detailed in Appendix 1) (Holmes et al., 2003). The PCR reaction was performed using 2 μl DNA and GoTaq® Colorless Mastermix (Promega, Madison, Wisconsin, USA) 50 μM primers and 2 μL DNA. The PCR protocol included an initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C 30 sec, annealing at 55°C 30 sec, extension 72°C 1.5 min, with a final extension of 72°C for 5 min (Waldron and Gillings, 2015). The primers HS463a and HS464 amplify an internal fragment of the class 1 integron integrase gene (*intI1*), which results in an approximately 473bp product. Samples generating a band of 473bp were therefore deemed *intI1* positive (Holmes et al., 2003).

All reactions described above included a positive (*E. coli* KC2, *K. pneumoniae*) and negative control (PCR H_2O) and were resolved using gel electrophoresis (16S 2% agarose w/v; and HS463a/HS464 and *K. pneumoniae* specific PCRs 3% agarose w/v). Electrophoresis

was conducted at 100V for 25 minutes in TBE for 16S, and 40 min for HS463a/HS464 and *K. pneumoniae* products (pH 8) with SYBR safe DNA stain (Invitrogen, Australia). Product size was approximated against a HyperLadderII 50bp DNA marker (Bioline, Sydney, Australia).

2.9 Statistical analysis

RStudio (V 1.0.143, Boston, Massachusetts, USA) software was employed for all statistical analyses. Fisher's exact test was used to test for differences in *E. coli* and *K. pneumoniae* prevalence between wild and captive little penguins, and 95% confidence intervals were calculated for the prevalence of *E. coli* and *K. pneumoniae*. A generalised linear model was utilised to analyse the potential relationship between phylogroup and subgroup prevalence with location and little penguin status (wild or captive). Factors were tested and included in the model based on AIC values. Significance was determined when $p < 0.05$.

3. Results

3.1 Prevalence of E. coli

E. coli was successfully isolated from 106 of 569 (18.6%) faecal samples from wild and captive little penguins (Table 2). The prevalence of *E. coli* differed significantly (Fisher's exact test, two-sided, χ^2 0.0048, $p < 0.05$) between wild and captive penguins, with a prevalence of 16.3% (76/466) in the wild samples compared to 29.1% (30/103) in captive samples (CI wild little penguins 20%, -1%; CI captive little penguins 57%, 2%).

Table 2. Number of samples tested for the presence of *E. coli* and *K. pneumoniae* at each sample site. ND, not detected; NT; not tested.

Sample site	***E. coli* isolates (*n* faecal samples)**	**Presumptive *Klebsiella sp.* (*n* faecal samples)**	**Presumptive *K. pneumoniae***
Phillip Island	65 (265)	52 (265)	15
St Kilda	3 (48)	2 (48)	1
Montague Island	8 (141)	ND (30)	ND
Bowen Island	0 (12)	NT	NT
Taronga Zoo	5 (30)	NT	NT
Dolphin Marine Magic	0 (30)	NT	NT
Sea World	20 (25)	NT	NT
Adelaide Zoo	1 (8)	4 (8)	3
Sea Life Sydney	4 (10)	2 (10)	ND

3.2 E. coli phylotyping and subtyping

Phylogroup analysis showed uneven distribution between wild and captive little penguins (Figure 1). There was a significant difference in phylogroup distribution between wild and captive little penguins ($p = 0.0452$), as well as between sampling sites (Figure 2) ($p = 0.0042$). The most frequent phylogroup present in captive little penguins was B1 (40%), followed by D (30%) and A (20%) with the remaining 10% comprising phylogroups B2 (7%) and F (3%). In comparison, B2 was the most frequent phylogroup in wild little penguins (55%), followed by B1 (22%) and D (9%), with the remaining 14% made up of phylogroups A, C, F and E. Phylogroup C and clade E were only present in wild little penguins. The uneven sample sizes present across both wild and captive colonies in this study needs to be considered when phylogroup distribution is interpreted. The large confidence intervals reported on the prevalence of *E. coli* suggests that the sample size is not large enough to accurately interpret the phylogroup distribution, and additional sampling and analysis is required. However, phylogroup B1 and B2 prevalence was higher in wild little penguins, while phylogroups A, B1 and D were more prevalent in captive individuals, irrespective of sample size.

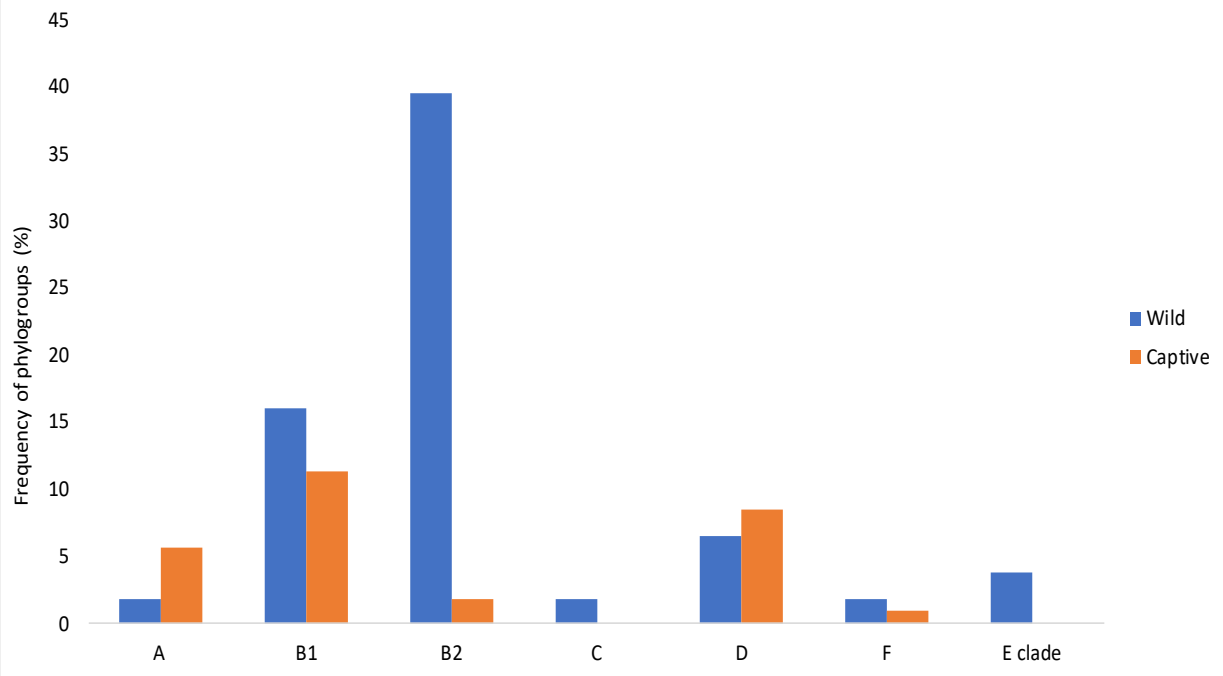


Figure 1. Phylogroup distribution of isolated *E. coli* in wild and captive little penguins (*n* = 106). Phylogroup distribution differed between wild and captive little penguins, with a greater phylogroup diversity seen in wild little penguins.

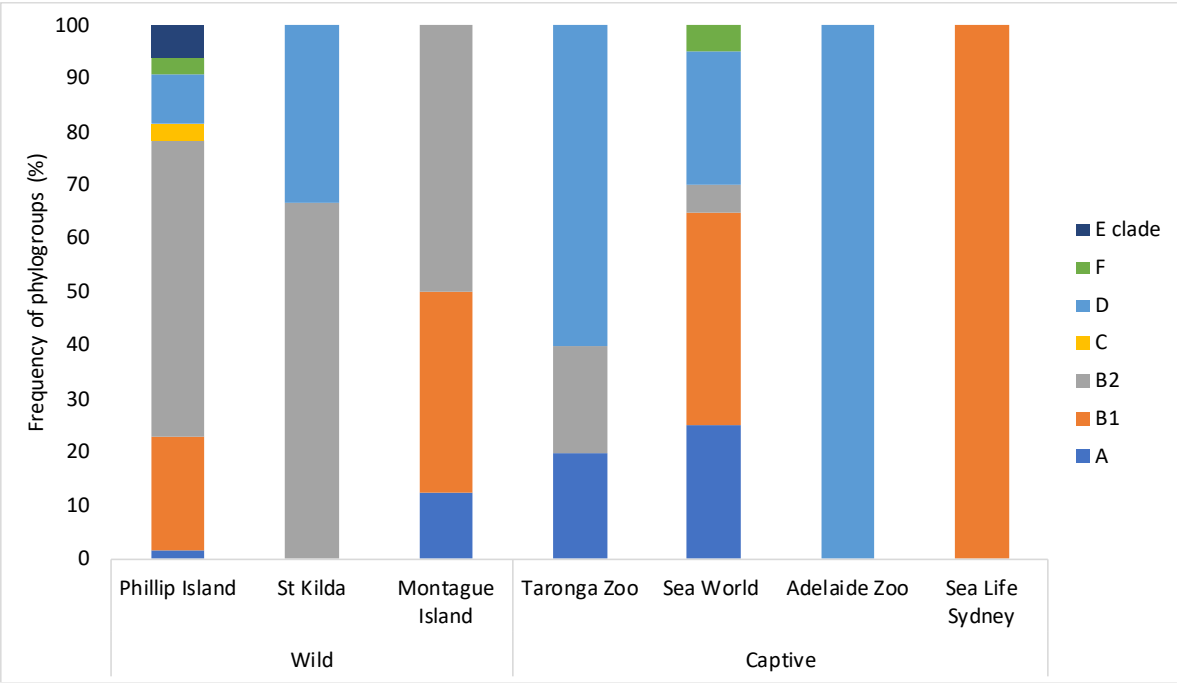


Figure 2. Phylogroup distribution of isolated *E. coli* across sampling sites. Phillip Island was the most diverse in phylogroup distribution with all phylogroups present.

Isolates that were assigned to phylogroup B2 were further characterised into one of nine B2 subgroups of *E. coli* involved in extra-intestinal infections. There was a significant difference in subgroup distribution between wild and captive little penguins ($p = 0.04882$; Figure 3). Subgroup distribution was more diverse in wild individuals than captive (Figure 3 and 4). The two isolates obtained from captive little penguin samples were assigned to subgroups VI (Taronga Zoo) and I (Sea World). The most prevalent subgroups obtained from the wild little penguin isolates were subgroups III (53%), followed by unassigned (30%), II (12%), and subgroups IV and VI (5%), the remaining subtypes were not detected (Figure 3).

Subgroup distribution also differed significantly between sampling sites ($p = 0.01671$; Figure 4). The highest diversity of subgroups was present at Phillip Island. All isolates from Montague Island, Taronga Zoo and Sea World contained only one subgroup; subgroup II, subgroup VI and subgroup I, respectively.

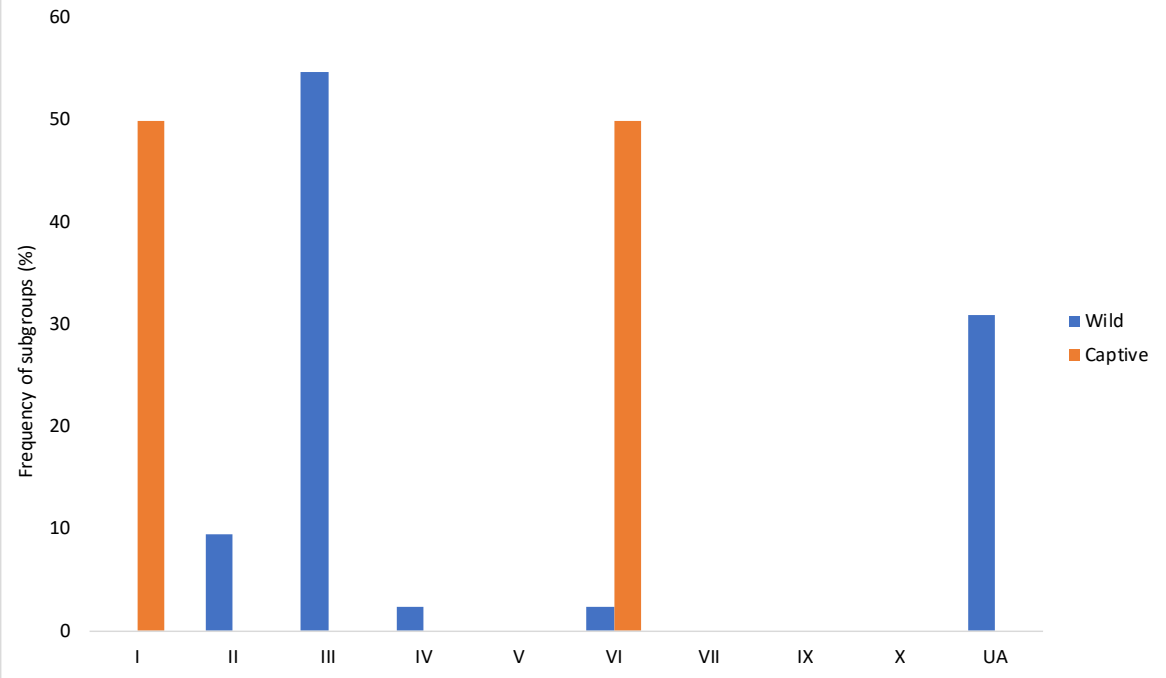


Figure 3. Subgroup distribution of isolated *E coli* belonging to phylogroup B2 ($n = 42$) in wild and captive LPs.

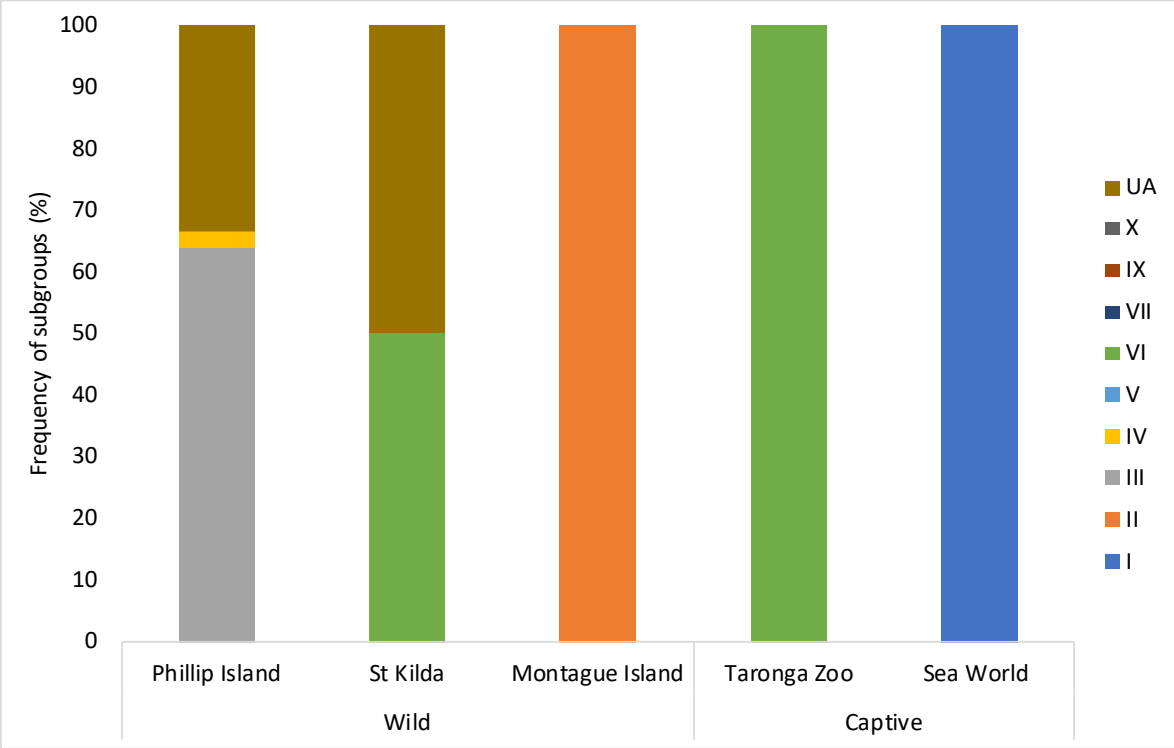


Figure 4. Subgroup distribution of isolated *E. coli* based on sample site.

3.3 Prevalence of K. pneumoniae

K. pneumoniae was successfully isolated from 19 of 361 (5.3%) faecal samples, from wild and captive little penguins. The prevalence of *K. pneumoniae* was 4.4% in wild little penguins compared to 16.7% in captives (Table 2). The prevalence of *K. pneumoniae* differed between wild and captive little penguins, however this difference was not statistically significant (Fisher's exact test, two-sided, χ^2 0.061, $p > 0.05$, CI wild little penguins 6%, -1%; CI captive little penguins 56%, -18%).

3.4 Integron integrase screening and gene cassette identification

Screening of all bacterial isolates resulted in detection of no class 1 integron integrase genes in any of the *E. coli* or *K. pneumoniae* isolates.

4. Discussion

This study examined the ecology and distribution of the Gram-negative opportunistic pathogens *E. coli* and *K. pneumoniae* in wild and captive little penguins. Both *E. coli* and *K. pneumoniae* were isolated from wild and captive little penguins, with a significantly higher prevalence of *E. coli* present in captive individuals. The prevalence of *K. pneumoniae* differed between wild and captive individuals; however, the difference was not significant. Previous research has established that *E. coli* is less commonly isolated from wild birds compared to mammals, with only one-quarter of avian individuals carrying *E. coli* (Gordon and Cowling, 2003). In contrast, birds that live in close association with humans are more likely to carry *E. coli* (Blyton et al., 2015; Gordon and Cowling, 2003). Hence, human association influences the frequency of *E. coli* in wild birds, as shown here with greater prevalence of *E. coli* in captive compared to wild little penguins. Human connectivity appears to influence phylogroup distribution in wild birds. In Australian wild birds exposed to differing levels of human contact, a significant effect on the phylogenetic assignment of isolated *E. coli* was evident (Blyton et al., 2015). Similarly, our findings in the little penguin showed differences in phylogroup distribution.

Pathogenic strains of *E. coli* belong to the same phylogroups as isolates from the little penguin. Phylogroups A, B1, B2 and D were isolated from both wild and captive little penguins. The B2 phylogroup includes extra-intestinal pathogens of many vertebrates including pathogens of birds (Johnson et al., 2008). In pigeons (*Columba livia*) multidrug resistant enteropathogenic *E. coli* (EPEC) and Shiga-toxin producing *E. coli* (STEC) strains were assigned to phylogroups A, B1, and D (Ghanbarpour and Daneshdoost, 2012).

Infections by STEC strains of *E. coli*, as well as typical and atypical enteropathogenic *E. coli* (aEPEC) have been reported in captive Psittaciformes (parrots and allies), Columbiformes (pigeons and doves) and Strigiformes (nocturnal birds of prey) (Sanches et al., 2017). Whilst *E. coli* strains from little penguins were not classified beyond phylogroups and B2 subgroups, the potential pathogenicity warrants further study into potential health impacts to this species.

This investigation into *E. coli* and *K. pneumoniae* aimed to determine if the class 1 integrons detected in the faecal microbiota of wild and captive little penguins (Chapter 2) were associated with these bacteria. However, class 1 integrons were not detected in any *E. coli* or *K. pneumoniae* isolated from penguins, leaving the question as to what bacteria are carrying the class 1 integrons?

Potential reservoirs of these class 1 integrons include the commensal bacterial species within the penguin faecal microbiota or other bacterial species that may have colonised the little penguins. Investigations of the gut microbiome of the little penguin have shown that the most dominant phyla in little penguins are Proteobacteria, followed by Firmicutes, Bacteroidetes, Planctomycetes, and Actinobacteria (Dewar et al., 2013). These major phyla present in the little penguin gut microbiota (Dewar et al., 2013) are also common in wastewater (Zhang et al., 2011) and can potentially carry class 1 integrons. Some species within Firmicutes and Bacteroidetes are not easily cultivated (Zhang et al., 2011), thus attempting to investigate if Firmicutes and Bacteroidetes contain the class 1 integrons would be challenging. An emerging technique epicPCR (Emulsion, Paired Isolation and Concatenation PCR) (Spencer et al., 2016) could potentially be used to identify integron-bacterial species associations. epicPCR can link functional genes with phylogenetic markers, trace horizontal gene transfer networks and map ecological interactions between microbial cells (Spencer et al., 2016). Further investigation is required to elucidate the role of 'unculturable' bacterial species on the maintenance and dissemination of antimicrobial resistance (Novo et al., 2013) in the little penguin.

5. Conclusion

This study identified human-associated phylogroups of *E. coli* and isolates of *K. pneumoniae* in both wild and captive little penguins. These findings demonstrate the transfer of organisms from human systems to little penguins occurs in both captive and wild settings. As the human-associated bacterial species were present in both captive and wild settings, the little penguin appears to be a useful indicator species of marine ecosystem health. The health impacts posed by the acquisition of such bacterial strains have not yet been determined, and

the effects on little penguin health remain unknown. Given that class 1 integrons were not detected in the bacterial isolates, further investigation is required to elucidate what bacterial species have acquired the class 1 integrons detected in the faecal microbiota of the little penguin. A better understanding of the little penguin microbiome and the microbial transfer from human-associated systems to the little penguins will improve our understanding of the effects of acquiring human associated and resistant organisms on little penguin health and aid management strategies of this declining, yet iconic, marine species.

Concluding statement

In this thesis, I synthesised the available literature regarding antimicrobial resistance in wild birds, identifying key knowledge gaps, particularly within the Australian context. Based on this review, I proposed the little penguin to be a key species for investigating the dissemination of AMR from human systems to the marine environment in Australia. I examined the faecal microbiota of wild and captive little penguins and was particularly interested in the presence of the class 1 integron and the human associated opportunistic pathogens *E. coli* and *K. pneumoniae.* In Chapter 2, it was determined that both wild and captive little penguins serve as reservoirs the resistance determinants, with a significantly higher prevalence of the class 1 integron found in captive individuals. The conditions experienced in captive settings are likely a driver of the high prevalence of class 1 integrons and acquired resistance genes found in these populations. Wild little penguins were also found to carry organisms harbouring class 1 integrons. These findings demonstrate that organisms carrying the mechanisms needed to acquire resistant traits have integrated into the faecal microbiota of wild little penguins. Wild little penguins therefore have the potential to acquire resistance genes should they come in contact with such genes in the environment. In Chapter 3, I isolated *E. coli* and *K. pneumoniae* to investigate if the organisms carrying the class 1 integrons belong to these bacterial species. Although both *E. coli* and *K. pneumoniae* are highly associated with the dissemination if AMR from human systems to the environment, no isolates contained the class 1 integron. Thus, one question remains unanswered, where are the class 1 integrons? Future studies could utilise new techniques such as epicPCR to elucidate which bacterial species the class 1 integrons have integrated into. Defining alterations caused by the acquisition of resistance determinants and human associated bacterial species could aid in shaping conservation practices for future management of the little penguin.

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Appendix

Appendix 1. Primers used in thesis experiments to amplify 16S rDNA, integron components and bacterial specific primers.

Primer	Sequence	Target	Reference
F27	AGAGTTTGATCMTGGCTCAG	16S rDNA	Lane 1991
R1492	TACGGYTACCTTGTTACGACTT	16S rDNA	Lane 1991
HS463a	CTGGATTTCGATCACGGCACG	*IntI1*	Holmes et al. 2003
HS464	ACATGCGTGTAAATCATCGTCG	*IntI1*	Holmes et al. 2003
HS458	GTTTGATGTTATGGAGCAGCAACG	*attI1*	Holmes et al. 2003
HS459	GCAAAAAGGCAGCAATTATGA GCC	*qacE*Δ	Holmes et al. 2003
ChuA.1b	ATGGTACCGGACGAACCAAC	*ChuA*	Clermont et al. 2013
ChuA.2	TGCCGCCAGTACCAAAGACA	*ChuA*	Clermont et al. 2013
YjaA.1b	CAAACGTGAAGTGTCAGGAG	*YjaA*	Clermont et al. 2013
YjaA.2b	AATGCGTTCCTCAACCTGTG	*YjaA*	Clermont et al. 2013
TspE4C2.1b	CACTATTCGTAAGGTCATCC	*TspE4.C2*	Clermont et al. 2013
TspE4C2.2b	AGTTTATCGCTGCGGGTCGC	*TspE4.C2*	Clermont et al. 2013
AceK.f	AACGCTATTCGCCAGCTTGC	*arpA*	Clermont et al. 2013
ArpA1.r	TCTCCCCATACCGTACGCTA	*arpA*	Clermont et al. 2013
pabBgpII.f	GAGTCACTGCCAGAAATTGCA	*pabB*	Clermont et al. 2014
pabBgpII.r	GGCGAAAGGCTTAAAATTGCACT	*pabB*	Clermont et al. 2014
trpAgpIII.f	GACGCGCTGGAATTAGGCTC	*trpA*	Clermont et al. 2014
trpAgpIII.r	ATCGGCAACCAGCACCGAAT	*trpA*	Clermont et al. 2014
dinBgpVI.f	CAGCGGTGGAGATGCGCGAT	*dinB*	Clermont et al. 2014
dinBgpVI.r	TCGTCAATGCCCTGACTACA	*dinB*	Clermont et al. 2014
icdgpVII.f	GCGGTATTCGCTCTCTGAAT	*icd*	Clermont et al. 2014
icdgpVII.r	CAATTAAATCAGCCGCTTCG	*icd*	Clermont et al. 2014
aesgpIX.f	CCTGGCCTGCAACGGGAG	*aes*	Clermont et al. 2014
aesgpIX.r	TCTGGCTGCGGATAAAAGAG	*aes*	Clermont et al. 2014
chuAgene.1	CGATACGGTCGATGCAAAAG	*chuA*	Clermont et al. 2014
chuAgene.2	TTGGACAACATCAGGTCATC	*chuA*	Clermont et al. 2014
putPgpI.f	GGTATCGCTTACTTTAACGG	*putP*	Clermont et al. 2014

putPgpI.r	ACCACCGGACCAAACGCC	*putP*	Clermont et al. 2014
trpAgpIV.f	TGCCAGTGGAAGAGTCCGCT	*trpA*	Clermont et al. 2014
trpAgpIV.r	CCGGGGCGGAAATACCAAAG	*trpA*	Clermont et al. 2014
polBgpV.f	GCCGTTTCGCCGAAGATAAA	*polB*	Clermont et al. 2014
polBgpV.r	TAATGATCTTCAGCGCCTGT	*polB*	Clermont et al. 2014
aesgpX.f	GACCGTTGTGAATACTCTTCA	*aes*	Clermont et al. 2014
aesgpX.r	TATAACAGGGCGGCACATTT	*aes*	Clermont et al. 2014
fgapA173	TGAAATATGACTCCACTCACGG	*gapA*	Diancourt et al. 2015
rgapA181	CTTCAGAAGCGGCTTTGATGGCTT	*gapA*	Diancourt et al. 2015
mdh130f	CCCAACTCGCTTCAGGTTCAG	*mdh*	Diancourt et al. 2015
mdh867r	CCGTTTTTCCCCAGCAGCAG	*mdh*	Diancourt et al. 2015
phoE604.1f	ACCTACCGCAACACCGACTTCTTCGG	*phoE*	Diancourt et al. 2015
phoE604.2r	TGATCAGAACTGGTAGGTGAT	*phoE*	Diancourt et al. 2015

MACQUARIE University

ANIMAL RESEARCH AUTHORITY (ARA)

AEC Reference No.: 2017/014-6 **Date of Expiry:** 21 May 2019

***Full Approval Duration:** 22 May 2017 to 15 May 2020* (36 Months)

This ARA remains in force until the Date of Expiry (unless suspended, cancelled or surrendered) **and will only be renewed upon receipt of a satisfactory Progress Report before expiry (see Approval email for submission details).**

Principal Investigator:
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Others Participating:
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In case of emergency, please contact:
the Principal Investigator / Associate Investigator, or the
Animal Welfare Officer - 9850 7758 / 0439 497 383

The above-named are authorised by MACQUARIE UNIVERSITY ANIMAL ETHICS COMMITTEE to conduct the following research:

Title of the project: **Parasite diversity and disease risk in the little penguin, *Eudyptula minor***

Purpose: **5 - Research: Human or Animal Health and Welfare**

Aims:
1. **Conduct a broad scale, comparative survey of prevalence and diversity of parasites in the little penguin across the species' geographical range, to determine variation in host parasite interactions.**
2. **Elucidate transmission pathways using parasite host specificity, to define impacts of parasites to conservation management of the little penguin.**
3. **Assess *E. coli* diversity across little penguin colonies to identify integration of human derived strains and correlate this with proximity to humans, domestic animals and other wildlife inhabitants near penguin colonies.**
4. **Assess prevalence and diversity of haemoprotozoa using existing tissue bank collections**

Surgical Procedures category: **3 - Minor Conscious Intervention**

All procedures must be performed as per the AEC-approved protocol, unless stated otherwise by the AEC and/or AWO.

Maximum numbers approved (for the Full Approval Duration):

Species	Strain	Age/Weight/Sex	Total	Supplier/Source
18 - Native Captive	Little penguin - *Eudyptula minor*	Any/Any/Any	200	Captive
20 - Native Wild	Little penguin - *Eudyptula minor*		1000	Wild
20 - Native Wild	Short-tailed shearwater - *Puffinus tenuirostris*		1000	
			2200	

Location of research:

Location	*Full street address*
See Appendix 1	

Amendments approved by the AEC since initial approval:
1. Amendment #1 - Add Rachael Gray as Associate (Executive approved. Ratified by AEC 22 June 2017).
2. Amendment #2 - Add Ida Lundback as Postgraduate Student (Executive approved. Ratified by AEC 22 June 2017).
3. Amendment – 17/06/2018 – Addition of captive samples and one wild site in NSW (Executive approved. Ratified by AEC 19 July 2018).

Conditions of Approval:
1. **Care must be taken to collect only the minimum amount of blood necessary for analysis from each individual**

Being animal research carried out in accordance with the Code of Practice for a prescribed research purpose and in connection with animals (other than exempt animals) that have been obtained from the holder of an animal suppliers licence.

A/Prof. Nathan Hart (Chair, Animal Ethics Committee) **Approval Date:** 19 July 2018

Adapted from Form C (issued under part IV of the Animal Research Act, 1985)

ANIMAL RESEARCH AUTHORITY (ARA)

AEC Reference No.: 2014/057-11 **Date of Expiry:** 23 December 2018

Full Approval Duration: *23 December 2014 to 23 December 2020*

This ARA remains in force until the Date of Expiry (unless suspended, cancelled or surrendered) **and will only be renewed upon receipt of a satisfactory Progress Report before expiry (see Approval email for submission details).**

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Michelle Power	0414 350 344
Lachlan Phillips	0411 237 831
Pierre Atherton	0402734637

The above-named are authorised by MACQUARIE UNIVERSITY ANIMAL ETHICS COMMITTEE to conduct the following research:

Title of the project : Marine predators, marine parks and marine hotspots: is zoning an effect conservation tool in a changing environment?

Purpose: 7 - Research: Environmental Study

Aims: 1. Quantify foraging and movements of seals and seabirds, using GPS, accelerometers and other loggers 2. Characterise the spatio-temporal distribution of prey density and associated environmental drivers through in-situ sampling and acoustic surveys 3. Establish the sensitivity of seals and penguins to DMS concentrations in controlled and natural settings 4. Determine the relationship between DMS concentrations and the distribution/density of prey relevant to little penguins and other mid-trophic level predators 5. Experimentally assess the influence of DMS on prey tracking and foraging behaviour of predators 6. Integrate predator behaviour, environmental and prey density information to infer how foraging strategies and associated energetic demands may respond to future environmental change. 7. To examine the sensitivity of little penguins to DMS. 8. Measure the energy requirements of nesting little penguins.

Surgical Procedures category: 4 - Minor Surgery With Recovery

All procedures must be performed as per the AEC-approved protocol, unless stated otherwise by the AEC and/or AWO.

Maximum numbers approved (for the Full Approval Duration):

Species	Strain	Sex/Age/Weight	Total	Supplier/Source
4E Pinnipedia	Australian and New Zealand Fur Seal	Any	120	Wild
20 native Wild	little penguin, silver gull, crested tern, short-tailed shearwater, wedge-tailed shearwater	Any	900	Wild
20 native Wild	little penguins – Metabolic studies	Any	36	Wild
20 native Wild	little penguins – DMS Observational	Any	10,000	Wild
20 native Wild	little penguins – Scent recognition	Any – Chicks	20	Wild
20 native Wild	little penguins – Scent recognition	Any - Adults	20	Wild
		TOTAL	11,096	

Location of research:

Location	*Full street address*
In - Situ / Wild	Montague Island Nature Reserve, Montague Island via Narooma NSW, Jervis Bay, NSW Booderee National Park (Parks Australia), Jervis Bay Territory ACT, Jervis Bay Marine Park, NSW Batemans Marine Park, Batemans Bay, NSW
Sydney Harbour	

Amendments approved by the AEC since initial approval:

1. Amendment #1 – Amend experimental design and number of animals (Approved by AEC 16 July 2015).
2. Amendment #2 – Addition of Dr Clive McMahon as Associate Investigator (Executive approved, ratified by AEC 16 July 2015)
3. Amendment #3 – Add location of research – Sydney Harbour (Executive approved, ratified by AEC 14 July 2016).
4. Amendment #4(a) – Amend experimental design, Amendment #4(b) – Amend procedure and Amendment #4(c) – Amend number and type of animals (Approved by AEC 18 August 2016).
5. Amendment #5 – Amend Technique (Executive approved, ratified by AEC 15 September 2016).
6. Amendment #6 – Add Michelle Power as Associate Investigator (Executive approved, ratified by AEC 12 April 2017).
7. Amendment #7 - Add Lachlan Phillips as Associate Investigator (Executive approved. Ratified by AEC 19 October 2017).
8. Amendment #8 - Add Pierre Atherton as Associate Investigator (Executive approved. Ratified by AEC 19 October 2017).
9. Amendment #9 - Extension to approval duration for three years (Approved by AEC 15 February 2018).

AEC Reference No.: 2014/057-11 **Date of Expiry:** 23 December 2018

Conditions of Approval:

1. Amendment #2 – Dr Clive McMahon is to complete the Research Animal Care and Ethics (RACE) Training 2015

Being animal research carried out in accordance with the Code of Practice for a recognised research purpose and in connection with animals (other than exempt animals) that have been obtained from the holder of an animal suppliers licence.

A/Prof. Nathan Hart (Chair, Animal Ethics Committee) **Approval Date:** 15 February 2018