Wild Australian Shorebirds as Reservoirs of Pathogenic Bacteria and Antimicrobial Resistance

by

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Abstract

Many existing and emerging diseases of humans are of zoonotic origin. In addition, the development of antimicrobial resistance (AMR) presents a serious risk for hospitals, agriculture, and the community. Habitat loss and degradation are forcing many wild animal populations into closer contact with human populations, presenting opportunities for the introduction and transmission of bacterial diseases. In Australia, many shorebird species undertake yearly migrations to and from breeding grounds in the high Artic, and during their migrations they pass over one-third of the human population. To determine if shorebirds are reservoirs of pathogenic bacteria the research presented in this thesis investigated the presence of three common enteric bacterial pathogens in twelve species of wild Australian shorebird; followed by AMR profiles of isolates, and genetic characterisation of selected isolates. In total, 1022 individual birds were sampled across three Australian states and tested for the presence of three potentially zoonotic pathogens; *Escherichia coli, Enterococcus* spp., and *Salmonella* spp. Two-hundred and six *E. coli*, 266 *Enterococcus*, and 20 *Salmonella* isolates were recovered, with AMR observed in 42% of *E. coli* isolates, 85% of *Enterococcus* isolates, and 10% of *Salmonella* isolates. Sedentary birds were more likely to carry AMR bacteria than migratory birds. A selection of *E. coli* isolates (n=16) underwent whole genome sequencing, and analysis of their genomes indicated a high level of genetic diversity with each isolate having a unique serotype. A total of 33 recognised virulence genes and eight AMR genes were detected. An important food-borne pathogen, *Salmonella enterica* serovar Hvittingfoss, was also recovered from one species of bird. This study shows that wild shorebirds can carry pathogenic enteric bacteria. While migratory birds may be less likely to harbour AMR bacteria relative to sedentary birds, they pose the potential to act as vectors for enteric and foodborne pathogens.

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Statement of Authorship

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Signed:

Hannah Smith

Date: 31st December 2020

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List of Abbreviations

AK	Amikacin
AMC	Amoxicillin-clavulanic acid
AMP	Ampicillin
AMR	Antimicrobial resistance / Antimicrobial resistant
AWSG	Australasian Wader Study Group
C	Chloramphenicol
CAZ	Ceftazidime
CIP	Ciprofloxacin
CN	Gentamicin
CT	Colistin
CTX	Cefotaxime
DNA	Deoxyribonucleic acid
E	Erythromycin
EAAF	East Asian Australasian Flyway
EFT	Ceftiofur
ESBL	Extended-spectrum Beta Lactamase
HGT	Horizontal Gene Transfer
IMP	Imipenem
KF	Cefalothin
MDR	Multi drug resistance
MLST	Multilocus Sequence Typing
NA	Nalidixic acid
PCR	Polymerase chain reaction
RAMSAR	The Ramsar Convention on Wetlands of International Importance (especially as waterfowl habitat)
RNA	Ribonucleic acid
S	Streptomycin
SNP	Single nucleotide polymorphism

ST	Sequence Type
SXT	Sulfamethoxazole-trimethoprim
T	Tetracycline
VA	Vancomycin
VWSG	Victorian Wader Study Group
WGS	Whole genome sequencing

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Foreword to Chapter 1

This chapter consists of a broad-scale review of literature pertaining to zoonotic diseases in wildlife, focusing specifically on AMR bacteria in wild animals and the mechanisms of transmission into- and out of- these populations.

The overall goal of this chapter was to review previous research findings relating to this project, 'Wild shorebirds as reservoirs of pathogenic bacteria and antimicrobial resistance', to identify any current gaps in knowledge. His review aims to both justify this research, and clarify its place in the wider research community. This review consulted published literature, governmental documents, and media publications obtained through freely available online sources.

This chapter is arranged in sections, with each section focusing on a topic relating to zoonotic disease and AMR bacteria in wildlife.

1:

Literature review

1

1.1 Human and animal conflict- the emergence of disease and One Health

1.1.1 Human infectious diseases

At the beginning of the 20th century, illnesses caused by infectious agents ranked among the most common causes of death worldwide (Conly & Johnston, 2005). During the last two centuries, medical breakthroughs such as vaccination, antibiotics, and improved clinical and food hygiene practices have all lead to a massive reduction in deaths caused by infectious diseases. Despite these advances, many millions of people die each year from infectious disease, and tens of millions more are infected (Christou, 2011; Jones et al., 2008). In addition, new diseases emerge on a regular basis, and the increasing development of resistance to the drugs we use as treatment means that infectious diseases will remain a global challenge for many years to come (Gould, 2009).

Conflict between human populations and the environment is becoming increasingly common. Rapid population growth and urbanization sees the encroachment of people into previously isolated environments, and into close contact with species and populations to which humans have had limited or no prior exposure. Human activity, such as hunting, logging, mining, and the catching wild animals for trade and/or consumption all increase the risk of disease exposure, while also placing the animals and environments under threat of anthropogenic diseases. Additionally, climate change is altering the home ranges for many species, further increasing the risk of novel disease transmission between populations.

This literature review will summarise the conflict between human and animal populations and investigate how this leads to the emergence of new diseases. Specifically, it will: discuss how antimicrobial resistance (AMR) develops and how it spreads into the environment; focus on how clinically important enteric bacteria that are present in wild animals (birds, in particular) are harbouring antibiotic resistance; and address how the migratory patterns of wild birds may be a factor in the spread of both emerging diseases and AMR.

1.1.2 Disease emergence from animal populations

It can be advantageous for pathogens to have the ability to infect more than one species. While some diseases only affect one host, many affect multiple. A zoonotic disease is one that is transmissible from animals to a human (Brown, 2004). Such diseases are often transferred from animal populations with which we have constant or very regular contact, such as domesticated livestock and pets (Brown, 2004). More recently, we have a gained an appreciation for the potential for wild animals to act as reservoirs and vectors of zoonotic diseases (Burroughs, Knobler, & Lederberg, 2002).

As the world becomes more globalised, the emergence and prevalence of zoonotic diseases increases; it is estimated that 75% of all emerging diseases that have occurred in the past decade have been zoonotic in origin (Allen et al., 2010). Movement of animals (anthropogenically-mediated or otherwise), ecological disruption, and an increasing human population result in greater chances for diseases to come into contact with new hosts (Burroughs et al., 2002).

Livestock workers are often on the frontline of new or emerging diseases, due to their increased contact with animals (Armand-Lefevre, Ruimy, & Andremont, 2005). However, zoonotic diseases can enter a population at any point in the food chain. Infections such as salmonellosis can arise from the direct handling of sick animals, from handling contaminated corpses, or through eating tainted food products (Abo-Amer & Shobrak, 2015). Consumption of infectious items is one of the main methods of zoonotic disease transmission into human populations. Outbreaks of zoonotic diseases such as Ebola are often traced back to the consumption of tainted bush meat (Brown, 2004). Wet markets in particular are of particular concern with regards to novel infectious diseases. SARS and COVID-19 emerged from such markets, where the risk comes not from the trade of farmed animals, but from wild animals (Albers et al., 2020).

1.1.3 Zoonosis and its impacts

Zoonotic diseases have a large impact on human health: 61% of all human diseases have an animal origin (Allen et al., 2010). Zoonotic diseases can be acquired through contact with infected animals, consumption of infected animal products (meat, milk, eggs etc.) or contaminated water and contact with disease vectors. Treating, preventing and creating strategies to deal with zoonotic diseases is often more difficult than single-host diseases; with diseases that only infect one species, such as those restricted to humans, treatment can be provided direct to the population, and prevention methods can be tailored around clinical or laboratory-based surveillance programs (Burroughs et al., 2002; Haydon et al., 2002). Zoonotic diseases, however, often require much more diverse approaches to surveillance and prevention. Treatment and prevention options for such diseases are also far more complex, especially when wild animal populations are involved in an outbreak (Haydon et al., 2002).

As previously mentioned, zoonotic infections are a constant burden on the general public. The current COVID-19 pandemic proves the danger viral diseases can pose to the global community, but it would be remiss to ignore the impact that bacterial diseases have on the community each year. Salmonellosis, caused by *Salmonella* spp. is endemic in different livestock species and is a global health issue (Forshell & Wierup, 2006). Overall, there are around 93 million enteric infections and 155,000 diarrheal deaths a year caused by nontyphoidal *Salmonella* infections from the consumption of raw beef, fish, shellfish and milk (Abo-Amer & Shobrak, 2015; Ao et al., 2015). This is not counting other gastrointestinal pathogens such as *E. coli*, *Campylobacter*, *Yersinia* and *Staphylococcus* spp., all of which are major contributors to gastroenteritis cases globally (Christou, 2011).

There is more to consider than simply the loss of lives experienced from the burden of zoonotic infections - there is also the economic impact. This can manifest through the direct loss of livestock to infections, and can have implications as far reaching as country-wide trade bans. One such example is the export ban on British beef during the BSE outbreak in the 1980s; combined with the cost of culling infected or exposed cattle

and other control measures, costs soared to approximately 4.9 billion pounds sterling (adjusted for inflation) during and after the outbreak (Burroughs et al., 2002; Bank of England., 2021).

Of great importance are those zoonotic bacteria that aside from having the potential to pass from animals to humans, also have the potential to bear resistance to the antimicrobials used to treat such infections. Bacteria such as *Enterococcus, E. coli, Salmonella, Staphylococcus*, and *Pseudomonas* are all capable of transmission to humans from domestic pets, livestock, or wildlife, but these bacteria are also recognised for their capacity to develop AMR (Werner et al., 2013). Due to the prolific use of antibiotics by humanity, resistance has already become endemic in bacterial populations in domestic animals and livestock, and there is evidence that resistant strains of bacteria are infiltrating wild populations (Christou, 2011; Gilchrist et al., 2007). The problems these bacteria pose in a changing world is one that will be explored in more depth further into this review (Section 1.2.4).

1.1.4 Emerging infectious diseases and habitat modification

Emerging zoonotic diseases are increasingly more common due to a heightened association with human populations and wildlife: this can be through habitat alteration, and the ensuing interactions between people and previously undisturbed wildlife (Loaiza et al., 2017). The alteration of farming practices is also another important avenue through which zoonoses enter wildlife populations (Gortazar et al., 2014). Traditional farming has mostly given way to intensive farming in developed countries, with the aim of fitting as many animals into as small a space as possible (Burroughs et al., 2002). When these farming practices are combined with factors such as poor disease control and disturbed habitat, it can bring about ideal conditions for the emergence of new diseases, as seen with the emergence of Nipah virus (Chua, Chua, & Wang, 2002). Originally found in fruit bats, Nipah spread to pigs as a result of habitat destruction and the encroach of piggeries into fruit bat habitats. Fruit bats would feed in cultivated fruit orchards where piggeries were located, and both their excrement and half-eaten fruit would fall into the pig pens. Workers would then interact with the pigs, and contract

Nipah themselves. (Chua, Chua & Wang, 2002). By the time the original outbreak was contained, a total of 265 cases of encephalitis were recorded with 105 deaths, while over one million pigs had been culled to curb the spread of infection (Field et al., 2001).

There are many other avenues through which zoonotic infections may enter human populations; poaching of wildlife for bushmeat or the exotic pet trade, changing human behaviours around wildlife (such as the rise of inappropriate wildlife tourism, and wildlife trade and translocation to name a few (Chomel, Belotto, & Meslin, 2007). These diseases have a large impact, both through direct losses (such as loss of human life) or indirect losses (monetary loss through death of livestock, closing of trade routes and loss of working hours due to sickness) and are likely to remain a significant problem into the future.

1.2 Virulence, resistance, and the spread of AMR genes into the wild

1.2.1 Overview of antibiotic use and AMR

The discovery of antibiotics revolutionised how we live. Without antibiotics, the probability of premature death due to infection would be 40% higher (da Costa, Loureiro, & Matos, 2013). The increase in AMR prevalence threatens to return us to a time before antibiotics and all the benefits they provide. Already, an extra 700,000 deaths annually are reported worldwide due to antibiotic resistance, with that number estimated to rise to 10 million annually by 2050 (de Kraker, Stewardson, & Harbarth, 2016; O'Niell, 2014). It is understood that AMR in general results in poorer outcomes for patients in terms of morbidity and mortality (Albrechtova et al., 2014; Shahcheraghi et al., 2013).

AMR bacteria present a great problem, with ‘superbugs’ representing one of the greatest clinical challenges. A ‘superbug’ is a highly virulent, multi-drug resistant and highly transmissible strain of a bacteria (Amabile-Cuevs, 2013) and can be found in both hospital and community settings. One such example of a superbug is methicillin-resistant *Staphylococcus aureus* (MRSA) – a pathogen estimated to kill roughly 19,000 American patients annually, and has a mortality rate of 20% in Australia and New

Zealand (Coombs et al., 2019). Its incidence is increasing, both in hospital settings and in the community (Alkasir et al., 2013; Boucher & Corey, 2008).

The main source of high concentrations of AMR bacteria and their associated genes is from anthropological sources, such as hospitals, wastewater treatment plants, and farms (da Costa, Loueiro, & Matos, et al., 2013). The waste that comes from hospitals can act as a source of contamination- some antibiotics are not broken down in the body, and so are excreted and end up in wastewater systems where they will interact with a multitude of other bacteria (da Costa et al., 2013). High concentrations of antibiotic compounds are often found in and downstream of wastewater treatment plants, which harbour (indeed often require) large bacterial populations (Jarnheimer et al., 2004; Ma et al., 2013; Zurfluh et al., 2015). These areas are often reservoirs of AMR bacteria and AMR genes (Ma et al., 2013).

Aside from AMR, virulence is a serious concern in clinical settings. Virulence mechanisms are a bacteria's ability to cause damage to a host, and are vital for a successful infection. Such mechanisms include toxin production, building physical barriers to prevent access by the hosts immune system, or attacking the hosts own immune cells (Casadevall & Pirofski, 2001; Lapierre et al., 2016). Increased virulence, while resulting in a more pathogenic organism, does not guarantee a more infectious organism. Virulence factors carry a fitness cost (the organism's ability to replicate and survive in a competitive environment), and some genes are more energetically expensive than others. The production of adhesion molecules, colonization enzymes, toxins and capsules require energy, and if it does not allow an organism to be as competitive as other bacteria with the potential to fill that niche, that organism will be outcompeted and potentially die out (Fauci, 2005). As such, virulence is often limited by bacterial competition, preventing bacteria from growing increasingly deadly as time goes by (Beceiro, Tomas, & Bou, 2013).

1.2.2 Clinical and economic impacts of AMR

Numerous species of bacteria are becoming difficult to treat, such as Gram negative bacteria like *E. coli, Klebsiella pneumoniae* and *Pseudomonas* spp. Resistance to one of the main antibiotic classes used to treat Gram negative infections, the carbapenems, is quickly increasing in prevalence due to the spread of beta-lactamases (enzymes that provide resistance to beta-lactam antibiotics) in such bacteria (Babic, Hujer, & Bonomo, 2006; Bonnedahl, 2011; Chua et al., 2014). The spread of such resistance is forcing the reissue of older and retired drugs as standard treatment options diminish. Many of these older antibiotics come with their own problems- colistin, one of the last effective antibiotics against Gram negative bacteria, is associated with significant nephrotoxicity and can be just as dangerous to the patient as the infection it treats (Chua et al., 2014; Cox, Koteva, & Wright, 2014).

In addition to direct health issues, drug resistance also increases costs associated with treatment, and more broadly healthcare provision worldwide. AMR is estimated to cost up to USD $1.6 million per year in Australia (Lee et al. 2020), €9 billion per year in Europe (Amabile-Cuevs, 2013; Aryee & Price, 2015), and is estimated to see a total cumulative loss of US$2.9 trillion by 2050 in OECD countries (Cecchini, Langer, & Slawomirski, 2015), while reducing profits in the agricultural sector through loss of product, decreased yields due to resistant infections, and the cost of purchasing antibiotics in bulk (Alkasir et al., 2013; Conly & Johnston, 2005; Nwankwo et al., 2014). In developing countries where money is already scarce, AMR bacteria can have a devastating impact by rendering cheap, widely available antibiotics useless (Biswas et al., 2014), effectively rendering an infection untreatable if the remaining options are too expensive for patients and hospitals to afford (Ahoyo et al., 2014).

A potential threat of AMR is that any new diseases that emerge may be caused by pathogens that are already resistant to numerous antibiotics, as AMR bacteria and their genes have been spreading into the environment for decades (French, 2010). It is not unthinkable that a once commensal organism could appear in hospitals and cause nosocomial infections, already prepared with a wide range of genes at its disposal- such

as has already happened with commensals turned major pathogens like *Enterococcus, Staphylococcus*, and *Pseudomonas*. Adaptive bacteria such as these have a propensity for acquiring AMR genes from other bacteria, potentially becoming untreatable (Bonnedahl, 2011; da Costa et al., 2013; Sellin et al., 2000).

1.2.3 Impacts of co-carriage of virulence and AMR

Virulence in clinical settings brings forth an obvious problem - the more virulent the bacteria, the more danger it poses to the infected host. However, the virulence of an organism is not the only factor in the outcome of an infection; the interactions of the hosts immune system with the infectious agent are equally as important. A usually avirulent bacterial strain can cause disease in impaired hosts, while virulent strains may not result in a detrimental outcome in hosts with robust (and/or previously primed) immune systems (Casadevall & Pirofski, 2001). As such, identifying virulent traits in an organism will not directly correlate with the outcome of a clinical infection; instead, by understanding an organism's virulence we can build an idea of what infection outcomes may occur (Casadevall & Pirofski, 2001).

A major concern in clinical settings is the co-carriage of virulence genes and AMR genes. As both factors make treating an infection more difficult, patients who are immunosuppressed, or who are colonised by an especially virulent, resistant strain of bacteria are at a higher risk for deleterious outcomes than those not (Beceiro et al., 2013). Virulence and AMR genes are spread between bacteria in similar manners (this will be explored further in Section 1.2.2), and as such both factors can be co-transmitted into new organisms. As such, in high-pressure environments such as hospital settings, or areas of high antibiotic pressure, virulence can be selected for alongside antibiotic resistance; a serious problem when treating patients (Baba et al., 2002; Beceiro et al., 2013; Gilmore, Lebreton, & van Schaik, 2013).

As previously mentioned, virulence factors all carry a fitness cost, and this is no less true when discussing their co-carriage with AMR genes. In environments without that same survival pressure (namely high levels of antibiotic usage and other clinical treatments) it

may be too energetically expensive for an organism to contain both AMR genes and virulence factors. As such, in these environments the co-carriage of these factors is often selected against, as less virulent and resistant bacteria are able to be more competitive (Beceiro et al., 2013; Kapperud et al., 1990).

1.2.4 How resistance develops

Antibiotics have revolutionised modern medicine. In the 1900s, before the advent of antibiotics, the three leading causes of death were pneumonia, tuberculosis and diarrhoea (Andersson & Hughes, 2012). Before antibiotics, infections were the cause of nearly one in three deaths across the globe (Spellberg, 2009). Antibiotics are so vital in the treatment and prevention of infections that they are the most commonly prescribed drug in hospitals (Biswas et al., 2014). The continued use of antibiotics is under pressure due to the emergence of AMR, whereby bacteria fail to be inhibited or killed by antibiotics when administered at concentrations tolerable by the patient (Aryee & Price, 2015). Such resistance has grown increasingly common over the past two decades in both clinical and community settings (McGettigan et al., 2019).

The main driving force behind the development of resistance is the transfer of genetic information between bacteria. There are two main mechanisms through which bacteria share or obtain genetic information; (1) horizontal gene transfer (HGT) or (2) mutation. HGT is the process by which bacteria obtain genetic information from an unrelated organism (Allen et al., 2010) often by sharing plasmids- circular, double-stranded DNA molecules that are distinct from a bacteria's chromosomal DNA (Lin et al., 2015). It is these processes that allow bacteria to instantly acquire AMR and related genes, rather than through slowly mutating resistance genes by themselves (Wasyl, 2014).

HGT is a natural process that bacteria undertake (Zhang et al., 2013; Zhang et al., 2012), and which provides bacteria with potentially beneficial genes. In areas of high bacterial population concentration (such as the human gut), HGT occurs around 25 times more frequently than in areas of low bacterial concertation (Amabile-Cuevs, 2013). HGT is critical to the dissemination of resistance, particularly within a mixed bacterial

population (da Costa et al., 2013). It can occur between different families of bacteria, and even occur between different kingdoms (such as between bacteria and archaea, yeast, and plants). HGT has been shown to be the main mechanism for the acquisition of AMR in Enterobacteriaceae and enterococci (Choi & Woo, 2013). Enterococci have acquired resistance genes that have taken them from being fully susceptible to an antibiotic, to fully resistant (Dixon, 2013). Many known AMR genes are found on mobile genetic elements such as plasmids, transposons and integrons that can be transferred quickly through HGT (Allen et al., 2010; Babic et al., 2006).

Plasmids can also be shared between different bacterial species with ease (Boucher & Corey, 2008). Such ease of transmission has ensured that AMR quickly passes into sensitive bacterial populations and species (Dotto et al., 2014), allowing for the fast dissemination of resistance through a population (Albrechtova et al., 2014; Bonnedahl et al., 2009; Zhao et al., 2014). This has been seen with the rapid spread of extended spectrum beta-lactamases in *E. coli* found in a population of wild gulls (*Larus michahellis*) in France (Babic et al., 2006). Coexistence of ESBLs with other resistance mechanisms is also frequent (Baez et al., 2015). So, we can understand how bacteria themselves pass AMR genes between each other- the next question is understanding where these bacterial populations are found.

1.2.5 How antibiotic misuse contributes to drug resistance

The main forces behind the emergence of drug resistance is the use, misuse and abuse of antimicrobials (Lubik, 2011), with the rate of AMR emergence being related to total consumption of antibiotics, appropriately used or not (Bonnedahl & Jarhult, 2014). It is only in recent decades, as the antibiotic pipeline has run dry, that we are now faced with the problem of antibiotics no longer working, with nothing on the horizon to replace them (Figure 1) (Amabile-Cuevs, 2013).

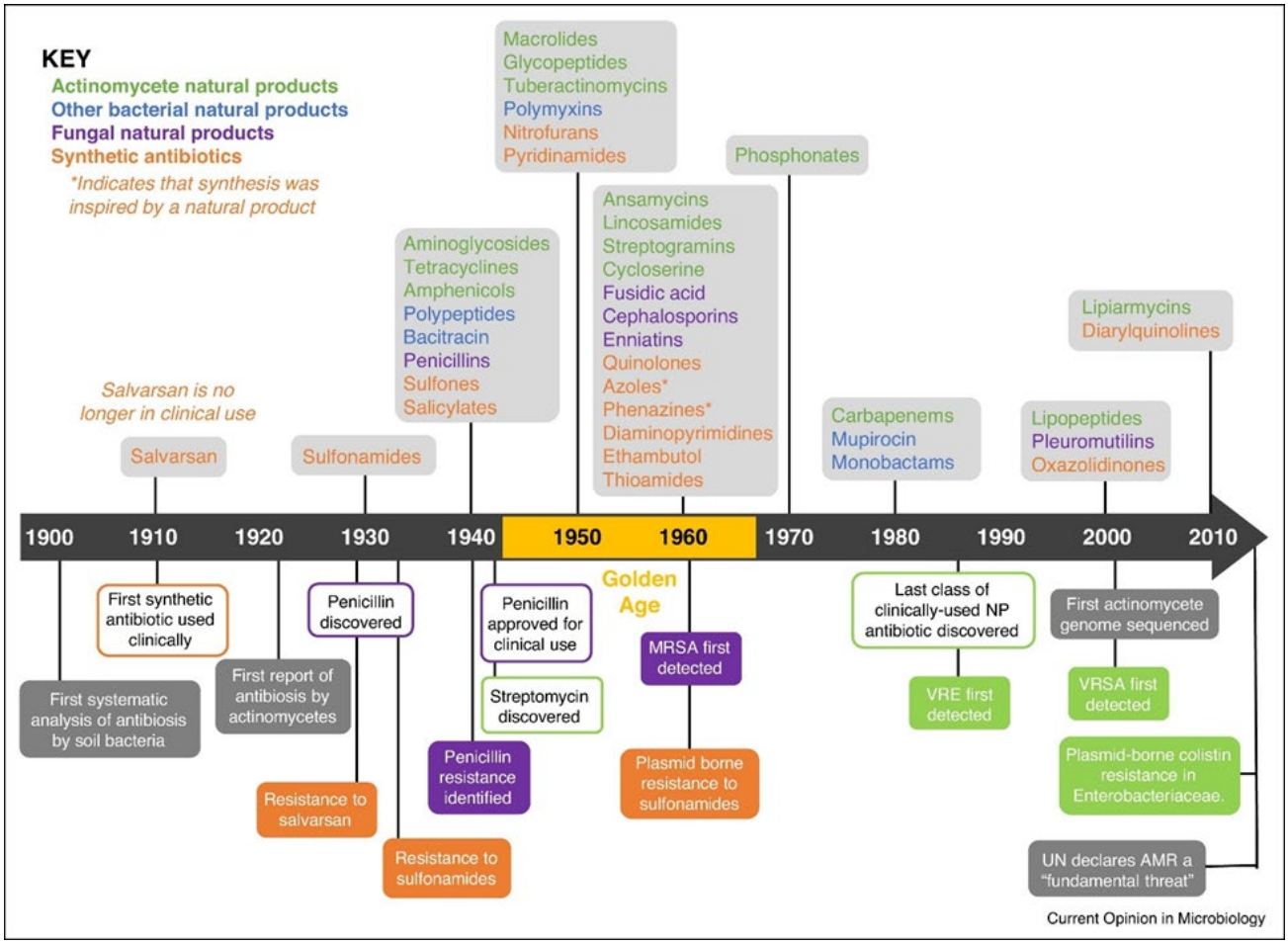

Figure 1. The timeline of antibiotic development and the emergence of antibiotic resistance. Despite many new resistance types developing, there have been few new antibiotics developed since the 1960s. Image is taken from Hutchings, Truman, & Wilkinson, 2019.

The U.S Food and Drug Administration has seen an approval of new antibacterial agents decrease by over 56% over the past 20 years (Conly & Johnston, 2005). In the past decade only two new antibacterial agents possessing Gram negative activity have been launched- these were tigecycline and doripenem, respectively, and these drugs are restricted to the most critical cases (Cox et al., 2014).

The over-prescription of broad-spectrum antibiotics (an antibiotic that acts against both Gram positive and Gram negative bacteria) in both agriculture and medicine has resulted in widespread resistance against commonly used drugs such as ampicillin and tetracycline (da Costa et al., 2013), with the frequency of resistance correlating with the per-capita antibiotic consumption of a country (Aryee & Price, 2015). This is exacerbated in developing countries, where antibiotic overuse is rife; due to dissemination of poor

quality or counterfeit antibiotics, self-medication, overdosing of antibiotics or the prescription of antibiotics where it is unnecessary or ineffective (Ahoyo et al., 2014; Aryee & Price, 2015; Biswas et al., 2014). In developing countries, the main goal may be to provide basic health under severe limitations (e.g. lack of diagnostic capabilities), with AMR control being a low priority (Biswas et al., 2014).

Even in developed nations, antibiotic usage is not always appropriate, with poor compliance to legislation noted. A report on Australian hospitals by Robinson, Robinson, and Whitby (2014) recorded compliance with antibiotic prescription legislation at 6.9%, while physicians were estimated to prescribe antibiotics unnecessarily 50% of the time (Biswas et al., 2014; Robinson et al., 2014). In developed nations, the problem is not in preventing the general community self-medicating or accessing the antibiotics at all; it is in convincing clinicians to change their prescription plans and working on the expectations of the community; in many countries a change is required in community expectations to prevent people from expecting antibiotics even if it is unlikely they have a bacterial infection, e.g. for a viral infection (McCullough et al., 2015).

1.2.6 Antibiotic use in agriculture

The use of antibiotics as a preventative treatment rather than a curative one was once (and in some countries, still is) common practice. It is this avenue of preventative treatment (prophylaxis) that was discovered to be (seemingly) beneficial in the agricultural sector. In the 1950s, it was discovered that the addition of antibiotics to animal feed not only helped prevent sickness, but also increased growth in animals. Both the speed at which they grew, and the amount of weight they put on improved with regular doses of tetracycline in their food and water (Blanco et al., 2007; McEwen & Fedorka-Cray, 2002). This has led to the use of antibiotics in feed and water at sub-therapeutic levels (below the dosage required to treat disease), and is a common practice in some countries, including the U.S (Abo-Amer & Shobrak, 2015; Dotto et al., 2014). It is estimated that the amount of antibiotics used in agriculture is eight times higher than that used in human medicine (Bonnedahl, 2011). Such overuse has resulted in the rapid development of AMR in agriculture, the community, and in the wild (Allen et

al., 2010; Davies & Davies, 2010; McEwen & Fedorka-Cray, 2002). While many countries have made great strides in curbing the prophylactic use of antibiotics in animal feed, agricultural antibiotic use is still often poorly regulated and widely used in lower income settings (Topp et al., 2018).

Even in very low concentrations, the use of antibiotics as prophylaxis in agriculture can exert a selective pressure for AMR to develop (Zurfluh et al., 2015). In the bacteria of livestock populations that are regularly exposed to sub-lethal levels of antibiotics, drug resistance is either nearly or completely endemic (Ussui et al., 2013). Resistance is most common against older broad spectrum antibiotics that are less regulated for use, but as some countries allow for the use of clinically important antibiotics, resistance can be observed to our most important drugs (da Costa et al., 2013; Ussui et al., 2013). AMR extends down the food chain, and into the community when contaminated products are ingested (Glenn et al., 2013). This dispersal through the food chain results in communities acquiring AMR bacteria, even if the community itself has been consuming antibiotics in a responsible and medically appropriate manner.

1.2.7 Agricultural and wildlife populations as reservoirs and vectors of AMR

Livestock themselves act as reserviours for AMR bacteria to human populations, and vice versa. There is a high rate of bacterial exchange between farmers and livestock; farmers who worked with pigs had higher rates of *Staphylococcus aureus* colonization compared to those who had no contact with livestock at all (45% to 24%), regardless of the farms location. Considering that in Belgium, 44% of pigs carried MRSA, livestock have the ability to constantly disseminate high volumes of AMR bacteria to human populations (Crombe et al., 2012). Transmission of Enterobacteriaceae from animals to humans has been recorded numerous times (Armand-Lefevre et al., 2005).

The physical movement of both livestock and people can also aid in the transmission of AMR bacteria. With the advent of international travel, the chances for disease transmission have skyrocketed. An Australian study demonstrated that international travel increased the risk of colonisation by AMR *E. coli* from 8% to 49% (Kennedy &

Collignon, 2010). The international shipping of livestock also poses a risk for the spread of AMR, as such movement has been proven to introduce new diseases into countries (Greger, 2007).

It is perhaps unsurprising, then, that antibiotic resistance has been found in every human population studied with resistance prevalence increasing with population density (Allen et al., 2010). Environmental contamination of AMR bacteria and genes has most likely been occurring since the industrial production of antibiotics began, leading to how AMR spreads beyond hospital settings. Wild animal populations have been found to harbour AMR bacteria, and the prevalence of AMR seems to correlate with the degree of anthropogenic activity (Bonnedahl, 2011). Ninety percent of bacterial isolates from voles in the U.K carried AMR- in comparison, voles from Finland carried almost none (Allen et al., 2010). As there is a great difference in population density between the U.K and Finland, it can be inferred that human activity has an effect on AMR in wildlife.

1.2.8 Areas of leakage into the environment

AMR is present in every corner of the world and resistance can develop in nearly every setting. Yet how prevalent AMR is, and what resistance traits are present can vary depending on location. The drugs used in a hospital will differ from those used in agriculture, though there may be overlap (Berglund, 2015). Due to the often uncontrolled and unregulated use of antibiotics in both fields historically and in the present day, many tonnes of antibiotics have leaked out into the environment- through sewerage, farm run-off, or factory waste and leaks (Allen et al., 2010). This has created an unintended selective pressure on bacteria that we are often unaware are being affected by our waste. In this section the types of AMR found in different locations, and how such resistant bacteria and their genes are spread, will be further discussed.

Antibiotics and AMR genes have been present in environmental microbiota for millennia (da Costa et al., 2013). The difference now is that the concentrations of such genes are rapidly increasing in natural environments (Fick et al., 2009), and that we are more

aware of what dangers this spread may cause in the future (da Costa et al., 2013). AMR genes can persist in environments for years (Yau et al., 2010).

The spread of clinically important AMR bacteria into the natural environment is less studied than in clinical environments (Bonnedahl, 2011), but AMR bacteria and genes have been found in both aquatic and soil environments worldwide (Albrechtova et al., 2014; Allen et al., 2010; Igbinosa, 2016). Aquatic environments seem especially sensitive to AMR contamination, possibly due to wastewater, aquaculture and run-off (Figure 2), with AMR bacteria and genes having been detected in estuarine, riverine and coastal surface waters (Azevedo et al., 2013; Bonnedahl, 2011; Bonnedahl & Jarhult, 2014; Zurfluh et al., 2015). Livestock waste can also spread AMR from farm settings into the environment, when manure from treated animals is washed off fields and into waterways (Figure 2) (Sellin et al., 2000). As previously noted, many livestock populations harbour high prevalence of AMR bacteria (13% of *Salmonella* isolates from chicken and swine were resistant to five or more antimicrobials in Canada alone in 2007, Glenn et al. (2013)), so farms act as a constant source of AMR bacteria for waterways and environments close to farms. This environmental contamination has many ramifications; both from the spread of AMR from agriculture back into the community, and from dispersal of AMR into pristine habitats.

Figure 2. Manure on paddocks can be a source of AMR bacteria, and this waste can be washed into nearby waterways durings rains. Source: Photograph by Lynn Betts, U.S. Department of Agriculture, Natrual Resources Conservation Service, distributed under a CC0 license.

Wildlife, aside from acquiring AMR bacteria from human activity, may also act as vectors for distributing AMR into the wider natural environment (Alroy & Ellis, 2011; Wasyl, 2014). Highly mobile animals, such as bats and birds, have the potential to spread AMR into many different environments, with unknown consequences (Arnold, Williams, & Bennett, 2016; Greig et al., 2015). A cause for concern is the potential for disease transmission through interactions between wild animals and agriculture; both livestock and the land used (Figure 3). Mapping disease transfer between these populations is incredibly difficult, and often there is no direct transfer of a specific bacterium from one population to another. The interactions between wildlife, livestock and the environment are numerous and varied, and only a small number of livestock species and agricultural crops are able to be produced in entirely closed systems that prevent any circulation of disease (Van Schaik et al., 1998). Such intensive farm systems also bring their own problems by increasing pathogenicity of the bacteria already present in such farmed populations (Mennerat et al., 2010) Dynamic changes in livestock production, habitat

loss and agricultural intensification all increase the risks of disease and gene exchange between wild and agricultural populations (Hassell et al., 2017).

Figure 3. There are many avenues for cross-contamination between agriculture and wildlife. This includes contact with effluent, scavenging from food remains, and even direct contact.

1.3 Enteric bacteria and their importance in human, animal and environmental health

1.3.1 Enteric bacteria can be commensals and pathogens

When discussing bacterial pathogens, in both a clinical and environmental context, it is important to understand how ubiquitous many of these pathogens are. Also, to understand that bacterial pathogenicity often depends less on the individual bacteria in question, and more on its current location. Species such as *E. coli*, for example, are a

normal- and even beneficial- part of many organisms' gut microbiota and will cause no disease in this location. However, if the same bacteria manage to enter the urinary tract, they can cause serious bladder infections. Bacteria also do not need to be pathogenic to present a potential risk for animal and human health- as mentioned in the previous chapter, commensal organisms can still be reservoirs for genes of health importance.

The largest numbers of commensal bacteria can be found in the gut (Clemente et al., 2012). While it has previously been estimated that gut bacteria outnumber human cells 10:1, further calculations have put forward a more modest 1:1 ratio (Sender, Fuchs, & Milo, 2016). However, that is still estimated to be 38 trillion bacterial cells in the human gut alone (with a further trillion bacterial cells from skin and other microflora). The importance of gut microflora in the health and development of an organism is becoming more widely understood, with gut microbiota playing a role in digestive functions, metabolic functions, host growth, and having a large role in the functioning of the immune systems (Belkaid & Hand, 2014; Clemente et al., 2012). The more research is made into the functioning and composition of gut microbiota, the more links we find between regular bodily functions and the health of our microbiota.

Transmission of gut microbiota is a regular and expected occurrence- whether this be through eating and drinking from sources contaminated with faecal bacteria, behavioural habits, or generally unsanitary conditions (Blaser & Falkow, 2009). Increased 'westernization' reduces transmission of gut bacteria between individuals due to improved sanitation practices, altered childbirth practices (i.e. caesarean sections), diets and antibiotic use (Martínez et al., 2015). The altered microbiomes in such westernized individuals tend to have low bacterial diversity, high individual variation, and little transmission between community members. Interestingly, these altered microbiomes are also being increasingly linked to non-infectious diseases that are becoming more common in developed countries- autoimmune diseases, allergies, inflammatory bowel disease and certain cancers (Blaser & Falkow, 2009; Martínez et al., 2015). Outside of these populations, such non-infectious diseases remain low; however, infectious bacterial, viral and parasitical diseases instead place a large burden on the health of populations (Christou, 2011; Greenhill et al., 2014). These next sections will

discuss the role of enteric bacteria as reservoirs of genes of importance in populations, and the role of enteric bacteria as pathogens- in both humans and wild animals.

1.3.2 Enteric bacteria as reservoirs of resistance

Enteric bacteria have great potential to act as reservoirs for various resistance genes; both in clinical and environmental settings. Some of the more common enteric bacteria (such as *E. coli*, *Enterococcus* and *Salmonella*) are able to survive for long periods outside of a host in environmental settings such as waterways, sewerage treatment plants, and soil (Cizek et al., 1994; Jin et al.,2004; E. Mourkas et al., 2019; Tien et al., 2017). In these environments, they are able to interact with multitudes of organisms and share genetic material- the perfect recipe for acquiring novel genes. The majority of the time, it is this transfer of new genes, rather than spontaneous mutation that leads to the emergence of new pathogens (Ochman & Moran, 2001).

AMR bacteria and genes have become commonplace in both clinical and community settings in the present day. Antibiotic resistance- both in populations directly exposed to antibiotics, and those indirectly exposed- has increased dramatically in enteric populations in recent decades (Allen et al., 2010). Resistance has been found in enteric bacteria even in remote settings (Bartoloni et al., 2009; Pallecchi et al., 2008; Sjolund et al., 2008). As previously mentioned, the 'modern' lifestyle seen in many parts of the world has drastically changed the human microbiome. A large part of this change is due to antibiotic usage at multiple points of our lives, from birth to death. The usage of antibiotics directly exposes much of our gut microbiota to the selective pressures these drugs cause, providing opportunities for microbiota to being to develop resistance to said drugs (even if they are not the target organism of the antibiotics).

1.3.3 Changing gut flora and AMR

As mentioned in Section 1.2, antibiotic usage in clinical, agricultural and veterinary settings have seen antibiotic compounds and resistant bacteria spread throughout the environment (Fernández-Delgado & Suárez, 2009)- and enteric bacteria are no exception. Similar to the alteration of diet and lifestyle seen in developed societies,

livestock populations have also experienced a rapid change in rearing practices. Agricultural intensification- the use of antibiotic prophylaxis, the intensive crowding of animals into small areas for maximum profit (as seen in feed lots and barn-raised chickens), and the change to high- energy diets designed for quickest growth have also altered the microbiomes of livestock (Evangelos Mourkas et al., 2020). While many countries are now working to limit what the amounts and types of antibiotics that can be used in agricultural uses, AMR enteric bacteria are still widespread, with studies finding resistance to older drugs like tetracycline being found in cattle in over 80% of farms investigated (Sawant et al., 2007), and resistance to clinically important drugs such as colistin being discovered in up to 21% of pigs investigated (Morales et al., 2012). Importantly, livestock can directly transfer enteric bacteria carrying resistance to farm workers- studies as early as Levy, Fitzgerald, and Macone (1976) demonstrated that farm workers were being colonised with tetracycline-resistant bacteria from the chickens they worked with. These changes in traditional farming methods leave livestock populations (and consequently, human populations) at risk of being rapidly colonised by AMR and pathogenic bacteria.

Wild animals are not unaffected by this- both acquiring AMR enteric bacteria from livestock (Gaukler et al., 2009; LeJeune et al., 2008) and from humans (Camarda et al., 2006; Hernandez et al., 2012). A study of urban pigeons in Italy discovered that nearly 50% of them carried *Campylobacter* (Gargiulo et al., 2014), and sweeping reviews of the literature have shown that not only can wildlife harbour enteric AMR bacteria, but they can also contribute to further environmental pollution of such bacteria (Greig et al., 2015). Even enteric bacteria isolated from environmental systems have been found to be contaminated with AMR bacteria descended from anthropogenic activity (Wright, 2010). Even when understanding that many current antibiotic resistance genes evolved in enteric bacteria before the advent of the antibiotic revolution, the appearance of resistance to wholly synthetic drugs (Blanco et al., 2007) and the sheer concentration of resistance genes found in non-human enteric bacteria (Gaukler et al., 2009) is a condemnation for how anthropogenic activity has altered the natural systems found in the gut microflora of many animals.

1.3.4 Enteric bacteria as pathogens

While enteric bacteria play many roles in host functioning and are mostly beneficial for their hosts continued survival, they also pose many disadvantages to their host. Although most enteric bacteria are commensal, or even beneficial for the host, some can be pathogenic; and when the gut microbiota is disturbed in certain circumstances, even commensal enterics can become pathogens. This can be seen with *Clostridium difficile*- normally, a regular part of human gut microflora, but under the right conditions (usually, after antibiotic use) this bacterium can overgrow and cause severe diarrhoea and pseudomembranous colitis (Lessa, Gould, & McDonald, 2012). Other common commensal enterics such as *E. coli* can cause long-term colonisation if pathogenic strains enter the gut (Batt, Rutgers, & Sancak, 1996).

Perhaps unsurprisingly, enteric pathogens cause the bulk of diarrhoea and enteric cases; Gram-negative enteric pathogens are estimated to cause more than three million deaths each year (Donnenberg, 2000). These deaths are mostly concentrated in low to middle-income countries, where access to adequate healthcare may be scarce (Khalil et al., 2018). In the US alone, there are estimated to be 76 million cases of foodborne illness, resulting in 325,000 hospitalizations and 5,200 deaths a year (Buzby & Roberts, 2009). While the costs of enteric disease worldwide have not been calculated, it is almost certainly substantial (Buzby & Roberts, 2009). Despite this, mortality rates from diarrhoeal diseases have been decreasing since 1990, and most such illnesses are preventable, given adequate sanitation, hygiene and food production practices (Buzby & Roberts, 2009; Khalil et al., 2018): the main obstacle to these improvements in many developing countries is the cost of setting up the required infrastructure. As a result, enteric pathogens still present a major risk to developing nations and may take priority over longer term problems such as AMR.

1.3.5 Enteric bacteria- commensals to pathogens

A major challenge emerging in clinical settings over the last few decades has been previously commensal enteric bacteria becoming pathogens. One such example of this is *Enterococcus*; a commensal organism that has become a serious threat in clinical and

community settings due to their ability to acquire AMR genes (Gilmore et al., 2013). The switch between commensal and pathogen can occur near-instantaneously through HGT (Wallace, Fishbein, & Dantas, 2020), as covered in Chapter 2. This presents a problem as commensal bacteria, aside from the many functions they have been noted to play previously, also play a role in protecting the hosts against pathogen encroachment; by competing with pathogenic bacteria for space and resources, they help prevent successfully colonisations. If the commensal bacteria themselves become pathogenic, they can cause serious infections that the body struggles to fight (Abt & Artis, 2013; David, 2012). This problem is intensified further when those new pathogens have also acquired AMR genes, and are resistant to clinical treatment (Gao, Howden, & Stinear, 2018).

It is not only the clinical and agricultural sector that contributes to the burden of enteric disease; interactions with wildlife present ample opportunities for outbreaks of enteric disease. For *Salmonella* alone, multiple wild populations have been shown to have caused disease outbreaks: wild birds in the United Kingdom have been implicated in spreading enteric *Salmonella* by contaminating food sources (Lawson et al., 2014), and hedgehogs in Sweden were also implicated in Salmonellosis outbreaks in local communities (Handeland et al., 2002). The incidences of enteric diseases originating in and being transmitted from wild animals is increasing as the world becomes more urbanised and wild populations are brought into conflict more with human populations (Wiethoelter et al., 2015), and such diseases threaten not only human populations, but the populations of the animals themselves- especially if those populations are already endangered (Hiltunen, Virta, & Laine, 2017; Power, Emery, & Gillings, 2013). In this next chapter, we will explore the role that wild birds play in the spread of bacterial disease and AMR, and how we can predict how they may transmit these diseases across multiple populations.

1.4 Birds and the spread of disease

1.4.1 Birds as mechanisms for the of spread of disease

It is well established that AMR is present in environmental bacteria, and that such bacteria are also becoming prevalent in wild animal populations (Allen et al., 2010). It is still unknown what effect this introduction of AMR bacteria has on wild animal populations. It is hypothesised that this introduction of AMR into pristine populations facilitates the emergence of 'new' diseases that will be drug resistant before they emerge (Brown, 2004).

AMR can be disseminated throughout natural environments by animal populations (Section 1.2.3). Highly mobile animals such as birds are adept at covering large distances and are well known vectors for pathogenic microorganisms that can cause disease in humans. Diseases such as Lyme disease, avian influenza, and West Nile virus are dispersed across large geographical areas by mobile hosts (Abulreesh, Goulder, & Scott, 2007; Dixon, 2013). Birds have also been implicated in outbreaks of enteritis (Abulreesh et al., 2007; Bonnedahl, 2011). Both free living and domestic bird populations have been found to be reservoirs of AMR bacteria (Cole et al., 2005), potentially acting as vectors for AMR dispersal.

The diversity of bird species means that birds can be found in many environmental niches and can tolerate different levels of human disturbance. Many species forage on agricultural land, while some scavenge directly off landfill sites and sewerage outlets (Figure 4), resulting in such species rapidly becoming colonised with pathogenic and AMR bacteria (Alroy & Ellis, 2011; Blanco et al., 2007; Cizek et al., 2007; Sellin et al., 2000). Whole orders such as waterfowl, passerines (perching birds) and birds of prey have been found to harbour AMR bacteria (Bonnedahl, 2011; Cizek et al., 2007; Nwankwo et al., 2014). Wild populations can transmit infectious disease from these areas of high bacterial diversity and contaminate land, human environments and water sources (Cole et al., 2005).

Figure 4. Birds such as Gulls, Crows, and Ibis will readily forage on rubbish dumps and sewerage outlets and habituate to living in areas of high human population density, increasing their chances of acquiring pathogenic and AMR bacteria (Gearin, 2016).

It is perhaps this crossover between intensely contaminated environments and living in areas of high human density that resulted in AMR first being isolated from wild pigeons in 1975 (Bonnedahl & Jarhult, 2014). The intrusions of humans into natural habitats of wild birds have facilitated the development and carriage of zoonoses, and birds can carry infectious agents asymptomatically. The global distribution, annual migrations and habitual communal roosting in environments shared by other species further enhance the mixing and dissemination of zoonotic diseases among wild populations (Abulreesh et al., 2007; Chan et al., 2015; Sellin et al., 2000). There are three major potential routes of disease movements through birds- the movement of poultry, the movement of caged birds and the movements of wild birds (Bennun, 2006).

Additionally, some species are potentially more prone to spreading bacteria than others. Groups such as waterbirds and shorebirds are particularly vulnerable to disease transfer due to their gregarious flocking behaviours and tendency to defecate in the same waters

they forage in (Rogers & Hulzebosch., 2014). Monitoring the health of all three of these routes in imperative in understanding and tracking the spread of disease from birds.

1.4.2 Canaries in the coalmine for disease presence

Surveillance is the cornerstone of our understanding of AMR and provides a starting point for designing strategies to combat any potential emerging infectious disease (Albrechtova et al., 2014). Some populations are more accessible than others. Urban populations of gulls, crows and pigeons, for example, can be captured and monitored more closely than can a reclusive or highly mobile wild population, such as groups such as shorebirds which are highly wary and utilise open habitats. In addition, it is these urban populations that will have first contact with novel AMR bacteria due to their feeding habits and interactions with human settlements. These populations can be used as indicators of pollution in urban, near urban and agricultural settings (Bonnedahl, 2011; Camarda et al., 2006).

Surveillance studies are already underway in some wild populations for diseases such as avian influenza; at-risk wild populations are monitored, and susceptible sentinel birds such as chickens are used to monitor if the disease has entered a country (Wille, Latorre-Margalef, & Waldenström, 2017). However, there is little comparable work being done for bacterial diseases or for AMR. This provides a large gap in our awareness of if a new strain of AMR bacteria has entered the country, and as such leaves us exposed to the risk of entry and dissemination of bacterial diseases.

1.4.3 Birds as disease reservoirs

Both wild and domestic birds act as reservoirs for many zoonotic pathogens. Birds are reservoirs for many pathogens, in particular *Salmonella* spp., *Campylobacter* spp. and *E. coli.* These and other pathogens can pass easily to people through direct contact or contamination of food and water sources (Baez 2015, Abulreesh et al. 2007). Birds such as gulls are particularly important as reservoirs: aside from carrying zoonotic enteric bacteria, they have also been found to harbour high levels of AMR bacteria, with

resistant genes against beta-lactams, aminoglycosides and tetracycline antibiotics (Alroy & Ellis, 2011; Baez et al., 2015).

AMR has been found even in remote and isolated populations. For example, 8% of *E. coli* isolates collected from Artic birds displayed AMR (Allen et al., 2010), while birds of prey were noted to carry bacteria with resistance to streptomycin and tetracycline at high frequency (Dixon, 2013). In addition, the AMR profiles of *E. coli* in populations of Yellow-Legged Gulls (*Larus michahellis*) sampled in the south of France were genetically heterogenous, reflecting that these AMR genes had been acquired from a diverse population of *E. coli* (Bonnedahl, 2011). Similarly, Black-Headed Gulls (*Chroicocephalus ridibundus*) have been found to carry AMR *Salmonella* sp., with the prevalence of resistant bacteria increasing from 2 – 3% before 1994 to 13% in 2005. Gulls that were not colonised by *Salmonella* were also found to be re-infected upon returning to their nesting grounds, providing a constant source of infection for the gulls (Cizek et al., 2007). This shows that not only can wild birds be able to act as reservoirs for pathogens, they are also capable of keeping such bacteria circulating in their own populations.

1.4.4 The implications of birds in disease dissemination

One of the main concerns surrounding birds being colonised by AMR and zoonotic bacteria is their ability to act as a vector. A vector is any agent that carries and transmits an infectious pathogen into another living organism (Allen et al., 2010). Due to their ability to range over large distances, wild birds can act as a vehicle for both direct disease dissemination to human populations, and indirect disease dissemination through secondary vectors such as insects. One such example of indirect dissemination is the introduction of West Nile virus in the United States. Infected birds returning from their migrations were fed on by resident mosquito populations. These populations would acquire the virus from the birds they fed off, and transmit the disease to the local communities through their feeding, establishing the disease in the area (Abulreesh et al., 2007; Chan et al., 2015; Hartemink et al., 2007). Through the movements of migratory birds, a novel disease was introduced into a country while bypassing any biosecurity measures in place.

There are two main concerns regarding birds as vectors. There is the spread of disease in a local environment, by resident birds that may pose a constant threat of infecting those that live in the nearby area. Resident birds will defecate in local waterways as they feed (Abulreesh et al., 2007), or may contaminate local food sources with zoonotic and AMR bacteria. Outbreaks of campylobacteriosis occurred in the United Kingdom, caused by wild birds (Magpies, *Pica pica* and Jackdaws, *Corvus monedula*) picking open milk bottles that had been delivered to people's doorsteps (Abulreesh et al., 2007). The second area of concern revolves around migratory birds. The migratory movements of birds varies depending on species, and can vary from small movements in a single country (Kirby et al., 2008) to intercontinental movements that cross hemispheres (Beiring, 2013). While intercontinental migrants are not a year-round infection risk, they pose the possibility of dispersing novel pathogens and AMR between countries. The risks of both migratory and non-migratory birds in dispersing pathogens will be discussed in the next two sections.

1.4.5 Non-migratory birds

Non-migratory populations may still make large movements in response to adverse conditions in their home range. Mute Swans (*Cygnus olor*) dispersed a strain of avian influenza into Europe as they made short-distance movements to avoid adverse weather in their home range (Abulreesh et al., 2007). Even non-mobile populations can contribute to disease dispersal, with captive populations of Bar-Headed Geese (*Anser indicus*) transmitting avian influenza to livestock workers and resulting in mass mortality events in the captive geese populations (Abulreesh et al., 2007).

As resident populations remain in an area year-round, they act as both a reservoir and a constant potential source of disease dissemination. Water reservoirs that supply towns and cities are vulnerable to contamination, and faeces from large flocks of water birds can be a major contributor to faecal coliform levels (Dixon, 2013). This problem of bird faecal contamination making its way into water reservoirs is exacerbated by the fact that birds breeding in urbanized areas are more likely to carry *E. coli* than more remote birds,

and are more likely to carry AMR as well (Bonnedahl, 2011; Sellin et al., 2000). There is evidence that some bacteria, *E. coli* especially, is shared between human and urban bird populations. Yellow-legged Gulls in France have been documented to carry *E. coli* similar to that found in the nearby human populations, with similar results found in Sweden (Bonnedahl, 2011; Bonnedahl et al., 2009; Cizek et al., 2007). Even without direct interactions with wild birds (such as through hunting), these populations can still transmit bacteria to nearby communities.

1.4.6 Migratory birds

Migration carries the risk of introducing or re-introducing disease into an environment. The introduction of West Nile virus into the U.S.A has been mentioned in Section 1.4, but migrating birds have also been implicated in the introduction of West Nile virus into the Middle East via migrating White Storks, and as the cause of an outbreak of eastern equine encephalomyelitis in Jamaica in 1962 (Chatterjee, Ghosh, & Chattopadhyay, 2006; Rappole, Derrickson, & Hubalek, 2000).

When migratory birds travel, they stop at specific stopover or 'staging sites' along their migratory routes in order to rest and refuel. These staging sites are areas in which birds will feed and rest, and can hold hundreds of thousands of birds, often of different species, as they refuel for the next leg of their flight. These staging sites provide ample opportunity for disease transference amongst birds. Not only are species from different habitats and countries intermingling in dense flocks, they are also often immunocompromised due to the pressures of migration on the birds (Risely, Klaassen & Hoye., 2018). While sick individuals will most likely die during the migration, individuals with mild infections or who are asymptomatic will complete the journey to their breeding or wintering grounds (Boere & Stroud, 2006; Bonnedahl, 2011). It is these birds that pose the biggest risk of disease dissemination, and which shall be discussed in the next section.

1.5 Migratory birds and disease

1.5.1 Costs and benefits of migration

Migration is essential to the survival of many species of bird (Altizer, Bartel, & Han, 2011). Millions of migratory birds travel between breeding and wintering grounds annually around the globe and migrate along well-established routes known as flyways (Altizer et al., 2011; Boere & Stroud, 2006; Yong et al., 2018). Section 1.5.2 provides details of important migratory routes.

One of the benefits of migration is that it allows migratory populations to escape from areas of high pathogen and parasite density. Migrating individuals reduce their infection chances compared to those that stay in locations of high pathogen density (Risely, Klaassen, & Hoye, 2018). Migration also provides an indirect benefit in that it lowers pathogen prevalence in the general population (Altizer et al., 2011; Risely et al., 2018). This is due to the fact that sick individuals are less likely to undergo migration (migratory separation), and those sick individuals that do migrate are more likely to perish during migration (migratory culling). This acts to remove reservoirs of disease from a population (Altizer et al., 2011; Risely et al., 2018).

Migration is an extremely strenuous process that shorebirds spend months preparing for by building up the fat reserves for their journey to fuel their flight (Piersma, Gudmundsson, & Lilliendahl, 1999). When undergoing their migrations, many species will focus their energetic resources on what is essential, primarily flight muscles (Oldland et al., 2009). Non-essential processes such as gut function, non-essential muscles and immune function are all diminished during the bird's flight (Altizer et al., 2011; Oldland et al., 2009; Risely et al., 2018).

1.5.2 Migratory routes

Australia's migratory birds live in the East-Asian Australasian Flyway (EAAF) (Figure 5). This flyway extends from the high Artic down to the southern limits of Australia and New Zealand. The EAAF encompasses all Southeast Asia, as well as covering large parts of East Asia and India. Over 50 million migratory birds migrate through the EAAF, with

many of Australia's 8 million shorebirds migrating along the entire breadth of the flyway (Yong et al., 2018).

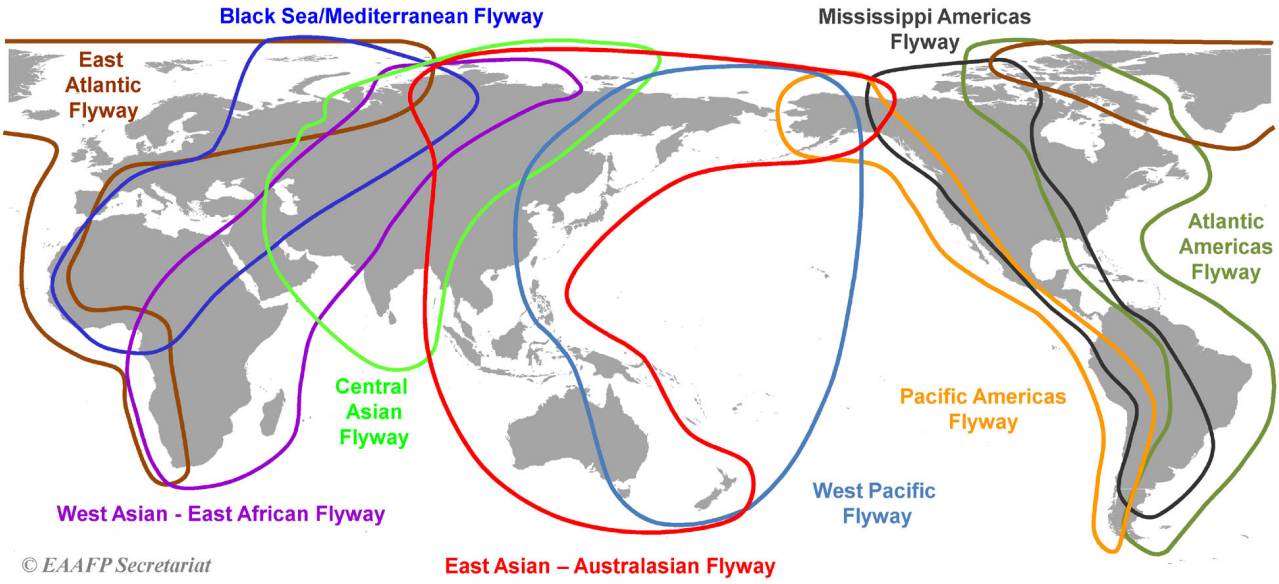

Figure 5. The flyways of the world for migratory birds. Image is reproduced from Boere & Stroud.,2006.

While migratory birds have adapted over millions of years to be able to cope with the demands of their annual journeys, their migrations are coming under threat and may be opening new pathways to disease. It is likely that many migratory species are altering their migration routes or stopover selections in response to habitat loss. Habitat degradation and destruction greatly impact wetlands and coastal zones, with some of these habitats disappearing entirely- almost 50% of intertidal areas in China and Korea have been destroyed in the last 30 years, and similar levels of destruction of important habitat is likely to have occurred in many other countries over the same timespan (Oldland et al., 2009; Murray et al., 2014). Shorebirds are very sensitive to habitat disruption, often being reliant on specific 'staging areas' (locations birds will stop to feed and rest along their migration routes) (Yong et al., 2018).

Some of these stopover sites can support hundreds of thousands of birds; only 20km of the shoreline at Bohai Bay, China, in the EAAF supports over 45% of the flyways entire population of Red Knots (*Calidris canutus*). Over half the flyway's population of Bar-tailed Godwit (*Limosia lappiconica*) rely on the nature reserve of Yalu Jiang, China

(Aharon-Rotman, 2015; Choi et al., 2014 Rogers et al., 2010), and extreme site fidelity amongst some species prevents them from being able to move to new staging grounds (Aharon-Rotman, 2015; Yong et al., 2018). The loss of important staging sites can have disastrous impacts on entire species: the loss of the Saemangeum estuary after the construction of a 33-km long seawall saw observed counts of shorebirds drop from ~181,000 in 2006 before completion of the seawall to 3 birds observed in 2014 after completion (Moores et al., 2016). Importantly, there have been no corresponding increases in shorebird populations at nearby sites after the seawall was built (Lee et al., 2018); the affected birds most likely starved to death.

The EAAF is one of the most threatened flyways in the world (Aharon-Rotman, 2015). It has the highest number of threatened shorebird species and has experienced some of the most staggering population's declines. Seventeen of the nineteen wader species found in the southern half of Australia have experienced population declines in the past 15 years, with the highest declines seen in species that are the most reliant on the Yellow Sea for their migratory stop overs (Clemens et al., 2016, Studds et al., 2017). The EAAF contains over 45% of the world's human population and some of the fastest growing economies, and it is predicted that by 2032 over 75% of the Asia Pacific land surface will have been impacted by infrastructure development (Yong et al., 2018). In addition to habitat loss (Figure 6), habitat degradation due to aquaculture, pollution, the spread of invasive Spartina and hunting along the flyway also places pressure on migrating birds (Melville, Chen & Ma., 2016; Gallo-Cajiao et al., 2020; Kirby et al., 2008).

Figure 6. One of the main threats facing shorebirds along the EAAF is habitat loss and degradation. Here, thousands of Godwit rest on mudflats that are in the process of being reclaimed for industrial construction (Lowen, 2016).

Climate change also has an impact on birds (Aharon-Rotman, 2015). The migration of Bar-tailed Godwits advanced six days from 2008-2020 (Conklin, Lisovski & Battley., 2021)

as artic-breeding birds have attempted to keep pace with changes in climate and the impact it has on seasonality (Oldland et al., 2009). Warmer environments are also affecting shorebird populations physically. Red Knots (*Calidris canutus*) that hatch in warmer summers are smaller and have shorter bills; a feature that results in lowered survival rates when the juveniles reach their wintering grounds and are unable to reach deep enough into the mud to prey on their preferred species of bivalves (Van Girls et al., 2016).

Due to the factors impacting shorebirds, their populations are restricted to increasingly degraded staging grounds that are filled to capacity with, with birds that are not as physiologically fit as their parents, interacting in times of great stress with decreased immune function. These factors all increase the chances of birds contracting novel AMR strains and diseases, transmitting them to other species, and dispersing them to new locations along their migratory routes.

1.5.3 Biosecurity and migration

It is well established that birds can carry and disperse zoonotic and AMR bacteria, and migratory birds are no exception. Migratory birds are particularly prone to periodic outbreaks of infectious disease at congregation sites. One example is an outbreak of avian botulism in Taiwan in 2002-2003 which killed more than 7% of the global population of Black-faced Spoonbills (*Platalea minor*) (Yong et al., 2018). In addition, the prevalence of some diseases has been linked with the movement of migratory birds, with Buggy Creek virus increasing in prevalence in mosquito hosts when Cliff Swallows (*Petrochelidon pyrrhonota*) returned to breed (Brown et al., 2007).

Commensal bacteria that are carried by birds can also cause disease in humans. Various *Salmonella* strains have been widely reported in wild birds, and thermotolerant campylobacters are a normal part of bird gut microflora. Even a few carriers of these bacteria can transport them long distances, and migratory birds can excrete bacteria acquired in their country of origin along their migration route, disseminating bacteria along the entire flyway (Baez et al., 2015).

1.5.4 Potential pathogen development in wild migratory birds

The main concern regarding birds and disease transmission is that of novel pathogen development and emerging infectious diseases. A major threat is the possibility of a pathogens acquiring resistance genes and entering the community. Many of the attributes of migratory wild birds, such as their tendency to gather in large flocks, the stresses they face along their migration route, and their interactions with a wide range of geographically distant habitats mean that they have the potential to act as incubators for the intermixing of geographically distinct bacteria and AMR genes.

Diverse groups of birds such as migratory gulls have been found to carry *E. coli* isolates with a great diversity of AMR traits, even in countries with a relatively low consumption of antibiotics (Bonnedahl et al., 2009; Bonnedahl et al., 2010; Camarda et al., 2006). The dense breeding colonies of these birds allows for the ease of transmission between individual birds, and provide opportunity for genetic recombination (Bonnedahl, 2011). Such circumstances provide the chance for bacteria in wild birds to intermingle with a) bacteria from geographically distant locations, b) with environmental AMR genes from industrial pollution and run-off and c) human-associated bacteria. The changes experienced in the home ranges of migratory populations can bring with them diseases that were once confined to different regions of Australia or were kept out of Australia entirely. This has been seen with mosquito-borne diseases such as West Nile virus, avian influenza, Murray Valley encephalitis virus and Japanese encephalitis virus (Epstein et al., 2006). Thus, it is feasible that a similar occurrence could occur in the future; with or without the interaction of outside vectors as occurred with West Nile virus in the USA.

1.5.5 Current lack of studies on migrating birds

To date, three studies have surveyed the presence of AMR *E. coli, Enterococcus* and *Salmonella* in non-migratory Silver Gulls (*Chroicocephalus novaehollandiae*) and passerines (Blyton et al., 2015; Dolejska et al., 2016; Oravcova, Svec, & Literak, 2017). A single study by Hoque et al. (2012) reported Salmonella enterica serovar Virchow and Hvittingfoss in a single Australian White Ibis (*Threskiornis molucca*) and two Plumed

Whistling Ducks (*Dendrocygna eytoni*). A previous study by this team surveyed a small sample of migratory and non-migratory birds (Smith et al., 2019), but no study to date has undertaken a large-scale investigation into what clinically important and AMR bacteria present in Australian shorebirds. There are also no long-term studies investigating if migratory birds can introduce and disseminate bacteria into Australia. In order to properly prepare for any new disease emergence coming from our migratory birds, we need to have a better understand of what potential pathogens are circulating in our migratory populations. Such knowledge can be of benefit to both humans and migratory bird populations.

1.6 Conclusion

The prevalence of AMR bacteria in the environment is exacerbated by anthropological activities, and is spreading into isolated animal populations. Once a population of resistant bacteria is in a wild animal host such as migratory birds it is out of our control, and unable to be regulated through methods such as reducing antibiotic usage or utilizing combinations of antibiotics that slow the development of resistance. Currently it is unknown what effect this spread is having on human and wild populations.

AMR is a global problem, and should be tackled in a coordinated approach, rather than country by country. In the current age of globalisation, the world is so heavily interconnected that it no longer matters where a new bacterial strain originates. Within 24 hours it can be carried to any corner of the globe and its dispersal will not be noticed until a patient is dying in hospital. Such has already been seen with the current COVID-19 pandemic- and it will not be the last pandemic disease that the world will face. Conceivably, by way of example, farm run-off in China may result in a new strain of AMR *E. coli* in Australia; while habitat fragmentation along Australia's shorelines may lead to mass mortality events in birds in Taiwan. In addition, the misuse of antibiotics, both in the clinical and agricultural sector, must be curbed. Antibiotic compounds, AMR bacteria and genes, and pathogenic bacteria leak from areas of use, thus it is vital that pollutants that may facilitate AMR are dealt with appropriately.

With these issues in mind, this study set out to determine what pathogenic and AMR *E. coli, Enterococcus* and *Salmonella* is present in a variety of wild Australian shorebirds. This study also aimed to investigate the genetics of the target bacteria recovered from these shorebird populations, to attempt to determine if Australian shorebirds can act as vectors for the introduction of disease and AMR into Australia.

1.7 References:

Abo-Amer, A. E., & Shobrak, M. Y. (2015). Isolation and Molecular Characterization of Multidrug-Resistant *Salmonella, Shigella* and *Proteus* from Domestic Birds. *Thai Journal of Veterinary Medicine, 45*(1), 23-34.

Abt, M. C., & Artis, D. (2013). The dynamic influence of commensal bacteria on the immune response to pathogens. *Current Opinion in Microbiology, 16*(1), 4-9.

Abulreesh, H., Goulder, R., & Scott, G. (2007). Wild birds and human pathogens in the context of ringing and migration. *Ringing & Migration, 23*(4), 193-200.

Aharon-Rotman, Y. (2015). Challenges within the annual cycle of long-distance migratory waders along the East Asian-Australasian flyway. PhD thesis, Deakin University.

Ahoyo, T., Bankole, H., Adeoti, F., Gbohoun, A., Assavedo, S., Amoussou-Guenou, M., Kinde-Gazard, D., Pittet, D. (2014). Prevelance of nosocomial infections and anti-infective therapy in Benin: results of the first nationwide survery in 2012. *Antimicrobial Resistance and Infection Control, 3*(17), 1-16.

Albers, H. J., Lee, K. D., Rushlow, J. R., & Zambrana-Torrselio, C. (2020). Disease risk from human-environment interactions: environment and development economics for joint conservation-health policy. *Environmental and Resource Economics, 76*(4), 929-944.

Albrechtova, K., Kubelova, M., Mazancova, J., Dolejska, M., Literak, I., & Cizek, A. (2014). High prevelence and variability of CTX-M-15-Producing and Fluoroquinolone-Resistant *Escherichia coli* observed in stray dogs in rural Angola. *Microbial Drug Resistance, 20*(4), 372-5.

Alkasir, R., Liu, X., Zahra, M., Ferreri, M., Su, J., & Han, B. (2013). Characteristics of *Staphylococcus aureus* Small Colony Variant and its parent strain isolated from chrinic mastitis at a dairy farm in Beijin, China. *Microbial Drug Resistance, 19*(2), 138-145.

Allen, H. K., Donato, J., Wang, H. H., Cloud-Hansen, K. A., Davies, J., & Handelsman, J. (2010). Call of the wild: antibiotic resistance genes in natural environments. *Nature Reviews Microbiology, 8*(4), 251-259. doi:10.1038/nrmicro2312

Alroy, K., & Ellis, J. C. (2011). Pilot study of antimicrobial-resistant *Escherichia coli* in herring gulls (*Larus argentatus*) and wastewater in the northeastern United States. *Journal of Zoo and Wildlife Medicine, 42*(1), 160-163. doi:10.1638/2010-0130.1

Altizer, S., Bartel, R., & Han, B. A. (2011). Animal migration and infectious disease risk. *Science, 331*(6015), 296-302. doi:10.1126/science.1194694

Amabile-Cuevs, C. (2013). Antibiotic resistance: From Darwin to Lederberg to Keynes. *Microbial Drug Resistance, 19*(2), 73-87.

Andersson, D. I., & Hughes, D. (2012). Evolution of antibiotic resistance at non-lethal drug concentrations. *Drug Resistance Updates, 15*(3), 162-172. doi:10.1016/j.drup.2012.03.005

Ao, T. T., Feasey, N. A., Gordon, M. A., Keddy, K. H., Angulo, F. J., & Crump, J. A. (2015). Global burden of invasive nontyphoidal *Salmonella* disease, 2010. *Emerging Infectious Diseases, 21*(6), 941.

Armand-Lefevre, L., Ruimy, R., & Andremont, A. (2005). Clonal comparison of *Staphylococcus aureus* isolates from healthy pig farmers, human controls, and pigs. *Emerging Infectious Diseases, 11*(5), 711-714. doi:10.3201/eid1105.040866

Arnold, K. E., Williams, N. J., & Bennett, M. (2016). 'Disperse abroad in the land': the role of wildlife in the dissemination of antimicrobial resistance. *Biology Letters, 12*(8), 20160137.

Aryee, A., & Price, N. (2015). Antimicrobial stewardship - can we afford to do without it? *British Journal of Clincal Pharmacology, 79*(2), 173-181. doi:10.1111/bcp.12417

Azevedo, J., Araujo, S., Oliveira, C., Correia, A., & Henriques, I. (2013). Analysis of antibiotic resistance in bacteria isolated from the surface microlayer and underlying water of an estuarine enviroment. *Microbial Drug Resistance, 19*(1), 64-71.

Baba, T., Takeuchi, F., Kuroda, M., Yuzawa, H., Aoki, K., Oguchi, A., Nagai, Y., Iwama, N., Asano, K., Naimi, T., Kuroda, H., Cui, L., Yamamoto, K., & Hiramatsu, K. (2002). Genome and virulence determinants of high virulence community-acquired MRSA. *Lancet, 359*(9320), 1819-1827. doi:10.1016/s0140-6736(02)08713-5

Babic, M., Hujer, A. M., & Bonomo, R. A. (2006). What's new in antibiotic resistance? Focus on beta-lactamases. *Drug Resistance Updates, 9*(3), 142-156. doi:10.1016/j.drup.2006.05.005

Baez, J., Hernandez-Garcia, M., Guamparito, C., Diaz, S., Olave, A., Guerrero, K., Canton, R., Baquero, F., Gahona, J., Valenzuela, N., Del Campo, R., & Silva, J. (2015). Molecular characterization and genetic diversity of ESBL-producing *Escherichia coli* colonizing the migratory Franklin's gulls (*Leucophaeus pipixcan*) in Antofagasta, North of Chile. *Microbial Drug Resistance, 21*(1), 111-116. doi:10.1089/mdr.2014.0158

Bank of England. (2021). Inflation calculator. https://www.bankofengland.co.uk/monetary-policy/inflation/inflation-calculator

Bartoloni, A., Pallecchi, L., Rodríguez, H., Fernandez, C., Mantella, A., Bartalesi, F., Strohmeyer, M., Kristiansson, C., Gotuzzo, E., & Paradisi, F. (2009). Antibiotic resistance in a very remote Amazonas community. *International Journal of Antimicrobial Agents, 33*(2), 125-129.

Batt, R., Rutgers, H., & Sancak, A. (1996). Enteric bacteria: friend or foe? *Journal of Small Animal Practice, 37*(6), 261-267.

Beceiro, A., Tomas, M., & Bou, G. (2013). Antimicrobial resistance and virulence: a successful or deleterious association in the bacterial world? *Clinical Microbiology Reviews, 26*(2), 185-230. doi:10.1128/CMR.00059-12

Beiring, M. (2013). Determination of valuable areas for migratory songbirds along the East-Asian Australasian Flyway (EEAF), and an approach for strategic conservation planning. Masters Thesis, University of Natural Resources and Life Sciences Vienna.

Belkaid, Y., & Hand, T. W. (2014). Role of the microbiota in immunity and inflammation. *Cell, 157*(1), 121-141. doi:10.1016/j.cell.2014.03.011

Bennun, L. (2006). Migratory birds as scapegoats. *Multinational Monitor, 27*(2), 21.

Berglund, B. (2015). Environmental dissemination of antibiotic resistance genes and correlation to anthropogenic contamination with antibiotics. *Infection Ecology & Epidemiology, 5*(1), 28564. doi:10.3402/iee.v5.28564

Biswas, M., Roy, D. N., Tajmim, A., Rajib, S. S., Hossain, M., Farzana, F., & Yasmen, N. (2014). Prescription antibiotics for outpatients in Bangladesh: a cross-sectional health survey conducted in three cities. *Annals of Clinical Microbiology and Antimicrobials, 13*(15), 15. doi:10.1186/1476-0711-13-15

Blanco, G., Lemus, J. A., Grande, J., Gangoso, L., Grande, J. M., Donazar, J. A., Arroyo, B., Frias, O., & Hiraldo, F. (2007). Geographical variation in cloacal microflora and bacterial antibiotic resistance in a threatened avian scavenger in relation to diet and livestock farming

practices. *Environmental Microbiology, 9*(7), 1738-1749. doi:10.1111/j.1462-2920.2007.01291.x

Blaser, M. J., & Falkow, S. (2009). What are the consequences of the disappearing human microbiota? *Nature Reviews Microbiology, 7*(12), 887-894.

Blyton, M. D., Pi, H., Vangchhia, B., Abraham, S., Trott, D. J., Johnson, J. R., & Gordon, D. M. (2015). Genetic Structure and Antimicrobial Resistance of *Escherichia coli* and Cryptic Clades in Birds with Diverse Human Associations. *Applied and Environmental Microbiology, 81*(15), 5123-5133. doi:10.1128/AEM.00861-15

Boere, G., & Stroud, D. (2006). *Waterbirds around the world: A global overview of the conservation, management and research of the world's waterbird flyways*. The Stationery Office, 71 Lothian Road, Edinburgh, UK: Scottish Natural Heritage.

Bonnedahl, J. (2011). Antibiotic resistance in *Enterobacteriacea* isolated from wild birds. *Digital Comprehensive Summaries of Uppsala Dissertations, 641*, 1-62.

Bonnedahl, J., Drobni, M., Gauthier-Clerc, M., Hernandez, J., Granholm, S., Kayser, Y., Melhus, A., Kahlmeter, G., Waldenstrom, J., Johansson, A., & Olsen, B. (2009). Dissemination of *Escherichia coli* with CTX-M type ESBL between humans and yellow-legged gulls in the south of France. *PLoS ONE, 4*(6), e5958. doi:10.1371/journal.pone.0005958

Bonnedahl, J., Drobni, P., Johansson, A., Hernandez, J., Melhus, A., Stedt, J., Olsen, B., & Drobni, M. (2010). Characterization, and comparison, of human clinical and black-headed gull (*Larus ridibundus*) extended-spectrum beta-lactamase-producing bacterial isolates from Kalmar, on the southeast coast of Sweden. *Journal of Antimicrobial Chemotherapy, 65*(9), 1939-1944. doi:10.1093/jac/dkq222

Bonnedahl, J., & Jarhult, J. D. (2014). Antibiotic resistance in wild birds. *Upsala Journal of Medical Sciences, 119*(2), 113-116. doi:10.3109/03009734.2014.905663

Boucher, H. W., & Corey, G. R. (2008). Epidemiology of methicillin-resistant *Staphylococcus aureus*. *Clinical Infectious Diseases, 46 Suppl 5*(5), S344-349. doi:10.1086/533590

Brown, C. (2004). Emerging zoonoses and pathogens of public health significance - an overview. *Revue Scientifique Et Technique De L Office International Des Epizooties, 23*(2), 435-442. doi:DOI 10.20506/rst.23.2.1495

Brown, C. R., Brown, M. B., Moore, A. T., & Komar, N. (2007). Bird movement predicts Buggy Creek virus infection in insect vectors. *Vector Borne Zoonotic Diseases, 7*(3), 304-314. doi:10.1089/vbz.2006.0646

Burroughs, T., Knobler, S., & Lederberg, J. (2002). *The emergence of zoonotic diseases: Understanding the impact on animal and human health: workshop summary*. National Academy Press, Washington.

Buzby, J. C., & Roberts, T. (2009). The economics of enteric infections: human foodborne disease costs. *Gastroenterology, 136*(6), 1851-1862.

Camarda, A., Circella, E., Pennelli, D., Madio, A., Bruni, G., Lagrasta, V., Marzano, G., Mallia, E., & Campagnari, E. (2006). Wild birds as biological indicators of environmental pollution: biotyping and antimicrobial resistance patterns of *Escherichia coli* isolated from Audouin's gulls (*Larus audouinii*) living in the Bay of Gallipoli (Italy). *Italian Journal of Animal Science, 5*(3), 287-290.

Casadevall, A., & Pirofski, L. (2001). Host-pathogen interactions: the attributes of virulence. *Journal of Infectious Disease, 184*(3), 337-344. doi:10.1086/322044

Cecchini, M., Langer, J., & Slawomirski, L. (2015). Antimicrobial resistance in G7 countries and beyond: economic issues, policies and options for action. *Paris: Organization for Economic Co-operation and Development*, 1-75.

Chan, J. F., To, K. K., Chen, H., & Yuen, K. Y. (2015). Cross-species transmission and emergence of novel viruses from birds. *Current Opinion in Virology, 10*, 63-69. doi:10.1016/j.coviro.2015.01.006

Chatterjee, S., Ghosh, A. K., & Chattopadhyay, J. (2006). Controlling disease in migratory bird population: a probable solution through mathematical study. *Dynamical Systems: An International Journal, 21*(3), 265-288. doi:10.1080/14689360500456279

Choi, J. M., & Woo, G. J. (2013). Molecular characterization of high-level gentamicin-resistant *Enterococcus faecalis* from chicken meat in Korea. *International Journal of Food Microbiology, 165*(1), 1-6. doi:10.1016/j.ijfoodmicro.2013.02.016

Choi, C. Y., Battley, P. F., Potter, M. A., Rogers, K. G., & Ma, Z. (2015). The importance of Yalu Jiang coastal wetland in the north Yellow Sea to Bar-tailed Godwits Limosa lapponica and Great Knots Calidris tenuirostris during northward migration. *Bird Conservation International*, *25*(1), 53-70.

Chomel, B. B., Belotto, A., & Meslin, F. X. (2007). Wildlife, exotic pets, and emerging zoonoses. *Emerging Infectious Diseases, 13*(1), 6-11. doi:10.3201/eid1301.060480

Christou, L. (2011). The global burden of bacterial and viral zoonotic infections. *Clinical Microbiology and Infection, 17*(3), 326-330. doi:10.1111/j.1469-0691.2010.03441.x

Chua, K. B., Chua, B. H., & Wang, C. W. (2002). Anthropogenic deforestation, El Niño and the emergence of Nipah virus in Malaysia. *Malaysian Journal of Pathology, 24*(1), 15-21.

Chua, K. Y., Grayson, M. L., Burgess, A. N., Lee, J. Y., & Howden, B. P. (2014). The growing burden of multidrug-resistant infections among returned Australian travellers. *Medical Journal of Australia, 200*(2), 116-118. doi:10.5694/mja13.10592

Cizek, A., Dolejska, M., Karpiskova, R., Dedicova, D., & Literak, I. (2007). Wild black-headed gulls (*Larus ridibundus*) as an environmental reservoir of *Salmonella* strains resistant to antimicrobial drugs. *European Journal of Wildlife Research, 53*(1), 55-60. doi:10.1007/s10344-006-0054-2

Cizek, A., Literak, I., Hejlicek, K., Treml, F., & Smola, J. (1994). *Salmonella* contamination of the environment and its incidence in wild birds. *Journal of Veterinary Medicine Series B-Zentralblatt Fur Veterinarmedizin Reihe B-Infectious Diseases and Veterinary Public Health, 41*(5), 320-327.

Clemente, J. C., Ursell, L. K., Parfrey, L. W., & Knight, R. (2012). The impact of the gut microbiota on human health: an integrative view. *Cell, 148*(6), 1258-1270. doi:10.1016/j.cell.2012.01.035

Clemens, R., Rogers, D. I., Hansen, B. D., Gosbell, K., Minton, C. D., Straw, P., ... & Fuller, R. A. (2016). Continental-scale decreases in shorebird populations in Australia. *Emu-Austral Ornithology*, *116*(2), 119-135.

Cole, D., Drum, D. J., Stalknecht, D. E., White, D. G., Lee, M. D., Ayers, S., Sobsey, M., & Maurer, J. J. (2005). Free-living Canada geese and antimicrobial resistance. *Emerging Infectious Diseases, 11*(6), 935-938. doi:10.3201/eid1106.040717

Conklin, J. R., Lisovski, S., & Battley, P. F. (2021). Advancement in long-distance bird migration through individual plasticity in departure. *Nature communications*, *12*(1), 1-9.

Conly, J., & Johnston, M. (2005). Where are all the new antibiotcs? The new antibiotic paradox. *Infectious Diseases and Medical Microbiology, 16*(3), 159-160.

Coombs, G. W., Daley, D. A., Lee, Y. T., & Pang, S. (2019). Australian Group on Antimicrobial Resistance (AGAR) Australian *Staphylococcus aureus* Sepsis Outcome Programme (ASSOP) Annual Report 2013. *Communicable Diseases Intelligence, 43*.

Cox, G., Koteva, K., & Wright, G. D. (2014). An unusual class of anthracyclines potentiate Gram-positive antibiotics in intrinsically resistant Gram-negative bacteria. *Journal of Antimicrobial Chemotherapy, 69*(7), 1844-1855. doi:10.1093/jac/dku057

Crombe, F., Willems, G., Dispas, M., Hallin, M., Denis, O., Suetens, C., Gordts, B., Struelens, M., & Butaye, P. (2012). Prevelance and antimicrobial susceptibility of methicillin-resistant *Staphylococcus aureus* among pigs in Belgium. *Microbial Drug Resistance, 18*(2), 125-131.

da Costa, P., Loureiro, L., & Matos, A. (2013). Transfer of Multidrug-Resistant Bacteria Between Intermingled Ecological Niches: The Interface Between Humans, Animals and the Environment. *International Journal of Environmental Research and Public Health, 10*(1), 278-294. doi:10.3390/ijerph10010278

David, R. (2012). Pathogens and commensals fight it out. *Nature Reviews Microbiology, 10*(7), 445-445.

Davies, J., & Davies, D. (2010). Origins and evolution of antibiotic resistance. *Microbiology and Molecular Biology Reviews, 74*(3), 417-433. doi:10.1128/MMBR.00016-10

de Kraker, M. E., Stewardson, A. J., & Harbarth, S. (2016). Will 10 million people die a year due to antimicrobial resistance by 2050? *PLoS Medicine, 13*(11), e1002184.

Dixon, B. (2013). Microbial ornithology? *Microbe, 8*(5), 196-197.

Dolejska, M., Masarikova, M., Dobiasova, H., Jamborova, I., Karpiskova, R., Havlicek, M., Carlile, N., Priddel, D., Cizek, A., & Literak, I. (2016). High prevalence of *Salmonella* and IMP-4-producing Enterobacteriaceae in the silver gull on Five Islands, Australia. *Journal of Antimicrobial Chemotherapy, 71*(1), 63-70. doi:10.1093/jac/dkv306

Donnenberg, M. S. (2000). Pathogenic strategies of enteric bacteria. *Nature, 406*(6797), 768-774.

Dotto, G., Giacomelli, M., Grilli, G., Ferazzi, V., Carattoli, A., Fortini, D., & Piccirillo, A. (2014). High prevelance of *oqx*AB in *Escherichia coli* isolates from domestic and wild lagomorphs in Italy. *Microbial Drug Resistance, 29*(2), 118-123.

Epstein, J. H., McKee, J., Shaw, P., Hicks, V., Micalizzi, G., Daszak, P., Kilpatrick, A., & Kaufman, G. (2006). The Australian White Ibis (*Threskiornis molucca*) as a Reservoir of Zoonotic and Livestock Pathogens. *EcoHealth, 3*(4), 290-298. doi:10.1007/s10393-006-0064-2

Fauci, A. S. (2005). Emerging and reemerging infectious diseases: the perpetual challenge. *Annals of the Academy of Medicine, 80*(12), 1079-1085. doi:10.1097/00001888-200512000-00002

Fernández-Delgado, M., & Suárez, P. (2009). Multiple antibiotic resistances of enteric bacteria isolated from recreational coastal waters and oysters of the Caribbean Sea. *Annals of Microbiology, 59*(3), 409.

Fick, J., Soderstrom, H., Lindberg, R. H., Phan, C., Tysklind, M., & Larsson, D. G. (2009). Contamination of surface, ground, and drinking water from pharmaceutical production. *Environmental Toxicology and Chemistry, 28*(12), 2522-2527. doi:10.1897/09-073.1

Field, H., Young, P., Yob, J. M., Mills, J., Hall, L., & Mackenzie, J. (2001). The natural history of Hendra and Nipah viruses. *Microbes and Infection, 3*(4), 307-314.

Forshell, L. P., & Wierup, M. (2006). *Salmonella* contamination: a significant challenge to the global marketing of animal food products. *Revue Scientifique et Technique-Office International des Epizooties, 25*(2), 541-554.

French, G. L. (2005). Clinical impact and relevance of antibiotic resistance. *Advanced Drug Delivery Reviews, 57*(10), 1514-1527. doi:10.1016/j.addr.2005.04.005

French, G. L. (2010). The continuing crisis in antibiotic resistance. *International Journal of Antimicrobial Agents, 36 Suppl 3*(0), S3-7. doi:10.1016/S0924-8579(10)70003-0

Gallo-Cajiao, E., Morrison, T. H., Woodworth, B. K., Lees, A. C., Naves, L. C., Yong, D. L., Choi, C., Mundkur, T., Bird, J., & Jain, A. (2020). Extent and potential impact of hunting on migratory shorebirds in the Asia-Pacific. *Biological Conservation, 246*, 108582.

Gao, W., Howden, B. P., & Stinear, T. P. (2018). Evolution of virulence in *Enterococcus faecium*, a hospital-adapted opportunistic pathogen. *Current Opinion in Microbiology, 41*, 76-82. doi:10.1016/j.mib.2017.11.030

Gargiulo, A., Russo, T. P., Schettini, R., Mallardo, K., Calabria, M., Menna, L. F., Raia, P., Pagnini, U., Caputo, V., Fioretti, A, & Dipineto, L. (2014). Occurrence of enteropathogenic bacteria in urban pigeons (*Columba livia*) in Italy. *Vector Borne Zoonotic Diseases, 14*(4), 251-255. doi:10.1089/vbz.2011.0943

Gaukler, S. M., Linz, G. M., Sherwood, J. S., Dyer, N. W., Bleier, W. J., Wannemuehler, Y. M., Nolan, L. K., & Logue, C. M. (2009). *Escherichia coli, Salmonella*, and *Mycobacterium avium* subsp *paratuberculosis* in Wild European Starlings at a Kansas Cattle Feedlot. *Avian Diseases, 53*(4), 544-551. doi:DOI 10.1637/8920-050809-Reg.1

Gearin, C. (2016). Detective gulls sniff out illegally dumped trash. *New Scientist*(3085), 22.

Gilchrist, M. J., Greko, C., Wallinga, D. B., Beran, G. W., Riley, D. G., & Thorne, P. S. (2007). The potential role of concentrated animal feeding operations in infectious disease epidemics and antibiotic resistance. *Environmental Health Perspective, 115*(2), 313-316. doi:10.1289/ehp.8837

Gilmore, M. S., Lebreton, F., & van Schaik, W. (2013). Genomic transition of enterococci from gut commensals to leading causes of multidrug-resistant hospital infection in the antibiotic era. *Current Opinions in Microbiology, 16*(1), 10-16. doi:10.1016/j.mib.2013.01.006

Glenn, L., Lindsey, R., Folster, J., Pecic, G., Boerlin, P., Gilmour, M., Harbottle, H., Zhao, S., McDermott, P., Fedorka-Cray, P., & Fyre, J. (2013). Antimicrobial resistance genes in multidrug-resistant *Salmonella enterica* isolated from animals, retail meats, and humans in the United States and Cnada. *Microbial Drug Resistance, 19*(3), 175-184.

Gortazar, C., Reperant, L. A., Kuiken, T., de la Fuente, J., Boadella, M., Martinez-Lopez, B., Ruiz-Fons, F., Estrada-Pena, A., Drosten, C., Medley, G., Ostfeld, R., Peterson, T., VerCauteren, K. C., Menge, C., Artois, M., Schultsz, C., Delahay, R., Serra-Cobo, J., Poulin, R., Keck, F., Aguirre, A. A., Henttonen, H., Dobson, A., Kutz, S., Lubroth, J., & Mysterud, A. (2014). Crossing the interspecies barrier: opening the door to zoonotic pathogens. *PLoS Pathogens, 10*(6), e1004129. doi:10.1371/journal.ppat.1004129

Gould, I. M. (2009). Antibiotic resistance: the perfect storm. *International Journal of Antimicrobial Agents, 34 Suppl 3*(0), S2-5. doi:10.1016/S0924-8579(09)70549-7

Greenhill, A. R., Guwada, C., Siba, V., Michael, A., Yoannes, M., Wawarie, Y., Ford, R., Siba, P., & Horwood, P. F. (2014). Antibiotic resistant *Shigella* is a major cause of diarrhoea in the Highlands of Papua New Guinea. *Journal of Infection in Developing Countries, 8*(11), 1391-1397. doi:10.3855/jidc.4396

Greger, M. (2007). The long haul: risks associated with livestock transport. *Biosecurity and Bioterrorism, 5*(4), 301-311. doi:10.1089/bsp.2007.0028

Greig, J., Rajic, A., Young, I., Mascarenhas, M., Waddell, L., & LeJeune, J. (2015). A scoping review of the role of wildlife in the transmission of bacterial pathogens and antimicrobial resistance to the food Chain. *Zoonoses Public Health, 62*(4), 269-284. doi:10.1111/zph.12147

Handeland, K., Refsum, T., Johansen, B. S., Holstad, G., Knutsen, G., Solberg, I., Schulze, J., & Kapperud, G. (2002). Prevalence of *Salmonella typhimurium* infection in Norwegian hedgehog populations associated with two human disease outbreaks. *Epidemiology and Infection, 128*(3), 523-527. doi:10.1017/s0950268802007021

Hartemink, N. A., Davis, S. A., Reiter, P., Hubalek, Z., & Heesterbeek, J. A. (2007). Importance of bird-to-bird transmission for the establishment of West Nile virus. *Vector Borne Zoonotic Diseases, 7*(4), 575-584. doi:10.1089/vbz.2006.0613

Hassell, J. M., Begon, M., Ward, M. J., & Fèvre, E. M. (2017). Urbanization and Disease Emergence: Dynamics at the Wildlife–Livestock–Human Interface. *Trends in Ecology & Evolution, 32*(1), 55-67.

Haydon, D., Cleaveland, S., Taylor, L., & Laurenson, M. (2002). Identifying reservious of infection: A conceptual and practical challenge. *Emerging Infectious Diseases, 8*(12), 1468-1473. doi:10.3201/eid0812.010317

Hernandez, J., Stedt, J., Bonnedahl, J., Molin, Y., Drobni, M., Calisto-Ulloa, N., Gomez-Fuentes, C., Astorga-Espana, M., Gonzalez-Acuna, D., Waldenstrom, J., Blomqvist, M., & Olsen, B. (2012). Human-associated extended-spectrum beta-lactamase in the Antarctic. *Applied and Environmental Microbiology, 78*(6), 2056-2058. doi:10.1128/AEM.07320-11

Hiltunen, T., Virta, M., & Laine, A. L. (2017). Antibiotic resistance in the wild: an eco-evolutionary perspective. *Philosphical Transactions of the Royal Society of London B Biological Science, 372*(1712). doi:10.1098/rstb.2016.0039

Hoque, M. A., Burgess, G. W., Greenhill, A. R., Hedlefs, R., & Skerratt, L. F. (2012). Causes of morbidity and mortality of wild aquatic birds at Billabong Sanctuary, Townsville, North Queensland, Australia. *Avian Diseases, 56*(1), 249-256. https://doi.org/10.1637/9863-072611-Case.1

Hutchings, M. I., Truman, A. W., & Wilkinson, B. (2019). Antibiotics: Past, present and future. *Current Opinion in Microbiology, 51*, 72-80.

Igbinosa, E. O. (2016). Detection and Antimicrobial Resistance of *Vibrio* Isolates in Aquaculture Environments: Implications for Public Health. *Microbial Drug Resistance, 22*(3), 238-245. doi:10.1089/mdr.2015.0169

Jarnheimer, P. A., Ottoson, J., Lindberg, R., Stenstrom, T. A., Johansson, M., Tysklind, M., Winner, M., & Olsen, B. (2004). Fluoroquinolone antibiotics in a hospital sewage line; occurrence, distribution and impact on bacterial resistance. *Scandanavian Journal of Infectious Diseases, 36*(10), 752-755. doi:10.1080/00365540410021027

Jin, G., Englande, A., Bradford, H., & Jeng, H. W. (2004). Comparison of *E. coli*, enterococci, and fecal coliform as indicators for brackish water quality assessment. *Water Environment Research, 76*(3), 245-255.

Jones, K. E., Patel, N. G., Levy, M. A., Storeygard, A., Balk, D., Gittleman, J. L., & Daszak, P. (2008). Global trends in emerging infectious diseases. *Nature, 451*(7181), 990-993. doi:10.1038/nature06536

Kapperud, G., Gustavsen, S., Hellesnes, I., Hansen, A. H., Lassen, J., Hirn, J., Jahkola, M., Montenegro, M., & Helmuth, R. (1990). Outbreak of *Salmonella typhimurium* infection traced to contaminated chocolate and caused by a strain lacking the 60-megadalton virulence plasmid. *Journal of Clinical Microbiology, 28*(12), 2597-2601. doi:0095-1137/90/122597-05$02.00/0

Kennedy, K., & Collignon, P. (2010). Colonisation with *Escherichia coli* resistant to "critically important" antibiotics: a high risk for international travellers. *European Journal of Clinical Microbiology and Infectious Diseases, 29*(12), 1501-1506.

Khalil, I. A., Troeger, C., Blacker, B. F., Rao, P. C., Brown, A., Atherly, D. E., Brewer, T., Engmann, C., Houpt, E., & Kang, G. (2018). Morbidity and mortality due to *Shigella* and enterotoxigenic *Escherichia coli* diarrhoea: the Global Burden of Disease Study 1990–2016. *The Lancet Infectious Diseases, 18*(11), 1229-1240.

Kirby, J. S., Stattersfield, A. J., Butchart, S. H. M., Evans, M. I., Grimmett, R. F. A., Jones, V. R., O'Sullivan, J., Tucker, G., & Newton, I. (2008). Key conservation issues for migratory land- and waterbird species on the world's major flyways. *Bird Conservation International, 18*(S1), S49-S73. doi:10.1017/s0959270908000439

Lapierre, L., Gatica, M. A., Riquelme, V., Vergara, C., Yanez, J. M., San Martin, B., Saenz, L., Vidal, M., Martinez, M., Araya, P., Flores, R., Duery, O., & Vidal, R. (2016). Characterization of Antimicrobial Susceptibility and Its Association with Virulence Genes Related to Adherence, Invasion, and Cytotoxicity in *Campylobacter jejuni* and *Campylobacter coli* Isolates from Animals, Meat, and Humans. *Microbial Drug Resistance, 22*(5), 432-444. doi:10.1089/mdr.2015.0055

Lawson, B., de Pinna, E., Horton, R. A., Macgregor, S. K., John, S. K., Chantrey, J., Duff, J., Kirkwood, J., Simpson, V., Robinson, R., Wain, J., & Cunningham, A. A. (2014). Epidemiological evidence that garden birds are a source of human salmonellosis in England and Wales. *PLoS ONE, 9*(2), e88968. doi:10.1371/journal.pone.0088968

Lee, J. K., Jin-Young, P., Hwa-Jung, K., Hur, W.-H., Sung-Hyun, K., & Jin-Han, K. (2018). Effects of the Saemangeum Reclamation Project on migratory shorebird staging in the

Saemangeum and Geum Estuaries, South Korea. *Bird Conservation International, 28*(2), 238-250.

Lee, X. J., Stewardson, A. J., Worth, L. J., Graves, N., & Wozniak, T. M. (2020). Attributable length of stay, mortality risk, and costs of bacterial health care-associated infections in Australia: A retrospective case-cohort study. *Infectious Diseases Society of America, 2021*(72), e506-14.

LeJeune, J., Homan, J., Linz, G., & Pearl, D. (2008). Role of the European Starling in the transmission of *E. coli* O157 on dairy farms. *Vertebrate Pest Conference, 23*, 31-34.

Lessa, F. C., Gould, C. V., & McDonald, L. C. (2012). Current status of *Clostridium difficile* infection epidemiology. *Clinical Infectious Diseases, 55*(suppl_2), S65-S70.

Levy, S., Fitzgerald, G., & Macone, A. (1976). Changes in intestinal flora of farm personnel after introduction of a tetracycline-supplement feed on a farm. *The New England Journal of Medicine, 295*(11), 583-588.

Lin, J., Nishino, K., Roberts, M. C., Tolmasky, M., Aminov, R. I., & Zhang, L. (2015). Mechanisms of antibiotic resistance. *Frontiers in Microbiology, 6*(34), 34. doi:10.3389/fmicb.2015.00034

Loaiza, J. R., Dutari, L. C., Rovira, J. R., Sanjur, O. I., Laporta, G. Z., Pecor, J., Foley, D., Eastwood, G., Kramer, L., Radtke, M., & Pongsiri, M. (2017). Disturbance and mosquito diversity in the lowland tropical rainforest of central Panama. *Scientific Reports, 7*(1), 7248. doi:10.1038/s41598-017-07476-2

Lowen, J. (2016). Reclamation of Yellow Sea causing serious declines in migratory shorebirds. Retrieved from https://www.birdlife.org/asia/news/reclamation-yellow-sea-causing-serious-declines-migratory-shorebirds

Lubik, N. (2011). Tools for tracking antibiotic resistance. *Environmental Health Perspectives, 119*(5), A214-A217.

Ma, L., Zhang, X., Zhao, F., Wu, B., Cheng, S., & Yang, L. (2013). Sewage treatment plant serves as a hot-spot reserviour of intergrons and gene cassettes. *Journal of Enviromental Biololgy, 34*(2 Suppl), 391-399.

Martínez, I., Stegen, J. C., Maldonado-Gómez, M. X., Eren, A. M., Siba, P. M., Greenhill, A. R., & Walter, J. (2015). The gut microbiota of rural papua new guineans: composition, diversity patterns, and ecological processes. *Cell Reports, 11*(4), 527-538.

McCullough, A. R., Rathbone, J., Parekh, S., Hoffmann, T. C., & Del Mar, C. B. (2015). Not in my backyard: a systematic review of clinicians' knowledge and beliefs about antibiotic resistance. *Journal of Antimicrobial Chemotherapy, 70*(9), 2465-2473. doi:10.1093/jac/dkv164

McEwen, S. A., & Fedorka-Cray, P. J. (2002). Antimicrobial use and resistance in animals. *Clinical Infectious Diseases, 34*(3), S93-S106. doi:10.1086/340246

McGettigan, P., Roderick, P., Kadam, A., & Pollock, A. (2019). Threats to global antimicrobial resistance control: Centrally approved and unapproved antibiotic formulations sold in India. *British Journal of Clinical Pharmacology, 85*(1), 59-70. doi:10.1111/bcp.13503

Melville, D. S., Chen, Y., & Ma, Z. (2016). Shorebirds along the Yellow Sea coast of China face an uncertain future—a review of threats. *Emu-Austral Ornithology*, *116*(2), 100-110.

Mennerat, A., Nilsen, F., Ebert, D., & Skorping, A. (2010). Intensive farming: evolutionary implications for parasites and pathogens. *Evolutionary Biology, 37*(2-3), 59-67.

Moores, N., Rogers, D. I., Rogers, K., & Hansbro, P. M. (2016). Reclamation of tidal flats and shorebird declines in Saemangeum and elsewhere in the Republic of Korea. *Emu-Austral Ornithology, 116*(2), 136-146.

Morales, A. S., Fragoso de Araujo, J., de Moura Gomes, V. T., Reis Costa, A. T., dos Prazeres Rodrigues, D., Porfida Ferreira, T. S., de Lima, F., Felizardo, M., & Micke Moreno, A. (2012). Colistin resistance in *Escherichia coli* and *Salmonella enterica* strains isolated from swine in Brazil. *Scientific World Journal, 2012*, 109795. doi:10.1100/2012/109795

Mourkas, E., Florez-Cuadrado, D., Pascoe, B., Calland, J. K., Bayliss, S. C., Mageiros, L., Meric, G., Hitchings, M., Quesada, A., Porrero, C., Ugarte-Ruiz, M., Guiterrez-Fernandez, J., Dominguez, L., & Sheppard, S. K. (2019). Gene pool transmission of multidrug resistance among *Campylobacter* from livestock, sewage and human disease. *Environmental Microbiology, 21*(12), 4597-4613. doi:10.1111/1462-2920.14760.

Mourkas, E., Taylor, A. J., Méric, G., Bayliss, S. C., Pascoe, B., Mageiros, L., Calland, J., Hitchings, M., Ridley, A., & Vidal, A. (2020). Agricultural intensification and the evolution of host specialism in the enteric pathogen *Campylobacter jejuni*. *Proceedings of the National Academy of Sciences, 117*(20), 11018-11028.

Murray, N. J., Clemens, R. S., Phinn, S. R., Possingham, H. P., & Fuller, R. A. (2014). Tracking the rapid loss of tidal wetlands in the Yellow Sea. *Frontiers in Ecology and the Environment*, *12*(5), 267-272.

Nwankwo, C., Ayogu, T., Iroha, I., Ejikeugwu, C., Nwakaeze, E., Oji, A., & Ilang, D. (2014). Cloacal feacal carriage and occurrence of antibiotic resistant *Escherichia coli* in chicken grown with and without antibiotic supplemented feed. *Journal of Veterinary Medicine and Animal Health, 6*(3), 91-94. doi:10.5897/jvmah2013.0267

O'Niell, J. (2014). Antimicrobial resistance: Tackling a crisis for the health and wealth of nations. *Review on Antimicrobial Resistance*, 1-16.

Ochman, H., & Moran, N. A. (2001). Genes lost and genes found: evolution of bacterial pathogenesis and symbiosis. *Science, 292*(5519), 1096-1099.

Oldland, J., Rogers, D., Clemens, R., Berry, L., Maguire, G., & Gosbell, K. (2009). Shorebird conservation in Australia. *Birds Australia Conservation Statment,* (14).

Oravcova, V., Svec, P., & Literak, I. (2017). Vancomycin-resistant enterococci with *vanA* and *vanB* genes in Australian gulls. *Environmental Microbiology Reports, 9*(3), 316-318. doi:10.1111/1758-2229.12542

Pallecchi, L., Bartoloni, A., Paradisi, F., & Rossolini, G. M. (2008). Antibiotic resistance in the absence of antimicrobial use: mechanisms and implications. *Expert Review of Anti-Infective Therapy, 6*(5), 725-732.

Piersma, T., Gudmundsson, G. A., & Lilliendahl, K. (1999). Rapid changes in the size of different functional organ and muscle groups during refueling in a long-distance migrating shorebird. *Physiological and Biochemical Zoology, 72*(4), 405-415. doi:10.1086/316680

Power, M. L., Emery, S., & Gillings, M. R. (2013). Into the wild: dissemination of antibiotic resistance determinants via a species recovery program. *PLoS ONE, 8*(5), e63017. doi:10.1371/journal.pone.0063017

Rappole, J. H., Derrickson, S. R., & Hubalek, Z. (2000). Migratory birds and spread of West Nile virus in the Western Hemisphere. *Emerging Infectious Diseases, 6*(4), 319-328. doi:10.3201/eid0604.000401

Risely, A., Klaassen, M., & Hoye, B. J. (2018). Migratory animals feel the cost of getting sick: A meta-analysis across species. *Journal of Animal Ecology, 87*(1), 301-314. doi:10.1111/1365-2656.12766

Robinson, H. L., Robinson, P. C., & Whitby, M. (2014). Poor compliance with community-acquired pneumonia antibiotic guidelines in a large Australian private hospital emergency department. *Microbial Drug Resistance, 20*(6), 561-567. doi:10.1089/mdr.2014.0064

Rogers, D. I., & Hulzebosch, M. (2014). *Use of non-tidal ponds by shorebirds at the Western Treatment Plant*: Arthur Rylah Institute for Environmental Research.

Rogers, D. I., Yang, H.-Y., Hassell, C. J., Boyle, A. N., Rogers, K. G., Chen, B., Zhang, Z., & Piersma, T. (2010). Red Knots (*Calidris canutus piersmai* and *C. c. rogersi*) depend on a small threatened staging area in Bohai Bay, China. *Emu-Austral Ornithology, 110*(4), 307-315.

Sawant, A. A., Hegde, N. V., Straley, B. A., Donaldson, S. C., Love, B. C., Knabel, S. J., & Jayarao, B. M. (2007). Antimicrobial-resistant enteric bacteria from dairy cattle. *Applied and Environmental Microbiology, 73*(1), 156-163.

Sellin, M., Palmgren, H., Broman, T., Bergstrom, S., & Olsen, B. (2000). Involving ornithologists in the surveillance of vancomycin-resistant enterococci. *Emerging Infectious Diseases, 6*(1), 87-88. doi:10.3201/eid0601.000119

Sender, R., Fuchs, S., & Milo, R. (2016). Revised estimates for the number of human and bacteria cells in the body. *PLoS Biology, 14*(8), e1002533.

Shahcheraghi, F., Nobari, S., Rahmati Ghezelgeh, F., Nasiri, S., Owlia, P., Nikbin, V. S., & Imani Fooladi, A. A. (2013). First report of New Delhi metallo-beta-lactamase-1-producing *Klebsiella pneumoniae* in Iran. *Microbial Drug Resistance, 19*(1), 30-36. doi:10.1089/mdr.2012.0078

Sjolund, M., Bonnedahl, J., Hernandez, J., Bengtsson, S., Cederbrant, G., Pinhassi, J., Kahlmeter, G., & Olsen, B. (2008). Dissemination of multidrug-resistant bacteria into the Arctic. *Emerging Infectious Diseases, 14*(1), 70-72. doi:10.3201/eid1401.070704

Smith, H. G., Clarke, R. H., Larkins, J.-A., Bean, D. C., & Greenhill, A. R. (2019). Wild Australian birds and drug-resistant bacteria: characterisation of antibiotic-resistant *Escherichia coli* and *Enterococcus* spp. *Emu-Austral Ornithology, 119*(4), 384-390.

Spellberg, B. (2009). *Rising Plague: The global threat from deadly bacteria and our dwindling arsenal to fight them*. Amherst, New York, USA: Spellberg Prometheus Books.

Studds, C. E., Kendall, B. E., Murray, N. J., Wilson, H. B., Rogers, D. I., Clemens, R. S., ... & Fuller, R. A. (2017). Rapid population decline in migratory shorebirds relying on Yellow Sea tidal mudflats as stopover sites. *Nature communications*, *8*(1), 1-7.

Tien, Y. C., Li, B., Zhang, T., Scott, A., Murray, R., Sabourin, L., Marti, R., & Topp, E. (2017). Impact of dairy manure pre-application treatment on manure composition, soil dynamics of antibiotic resistance genes, and abundance of antibiotic-resistance genes on vegetables at harvest. *The Science of the Total Environment, 581*, 32-39. doi:10.1016/j.scitotenv.2016.12.138

Topp, E., Larsson, D. J., Miller, D. N., Van den Eede, C., & Virta, M. P. (2018). Antimicrobial resistance and the environment: assessment of advances, gaps and recommendations for agriculture, aquaculture and pharmaceutical manufacturing. *FEMS Microbiology Ecology, 94*(3).

Ussui, M., Iwasa, T., Fukuda, A., Sato, T., Okubo, T., & Tamura, Y. (2013). The role of flies in spreading the extended-Spectrum β-lactamase gene from cattle *Microbial Drug Resistance, 19*(5), 415-420.

Van Gils, J. A., Lisovski, S., Lok, T., Meissner, W., Ożarowska, A., De Fouw, J., ... & Klaassen, M. (2016). Body shrinkage due to Arctic warming reduces red knot fitness in tropical wintering range. *Science*, *352*(6287), 819-821.

Van Schaik, G., Dijkhuizen, A., Benedictus, G., Barkema, H., & Koole, J. (1998). Exploratory study on the economic value of a closed farming system on Dutch dairy farms. *Veterinary Record, 142*(10), 240-242.

Wallace, M., Fishbein, S., & Dantas, G. (2020). Antimicrobial resistance in enteric bacteria: current state and next-generation solutions. *Gut Microbes, 12*(1), 1799654.

Wasyl, D. (2014). Prevelance and characterisation of quinolone resistance mechanisms in commensal *Escherichia coli* isolated from slaughter animals in Poland, 2009-2012. *Microbial Drug Resistance, 20*(6), 544-549.

Werner, G., Coque, T. M., Franz, C. M., Grohmann, E., Hegstad, K., Jensen, L., van Schaik, W., & Weaver, K. (2013). Antibiotic resistant enterococci-tales of a drug resistance gene trafficker. *International Journal of Medical Microbiology, 303*(6-7), 360-379. doi:10.1016/j.ijmm.2013.03.001

Wiethoelter, A. K., Beltrán-Alcrudo, D., Kock, R., & Mor, S. M. (2015). Global trends in infectious diseases at the wildlife–livestock interface. *Proceedings of the National Academy of Sciences, 112*(31), 9662-9667.

Wille, M., Latorre-Margalef, N., & Waldenström, J. (2017). Of Ducks and Men: Ecology and Evolution of a Zoonotic Pathogen in a Wild Reservoir Host. In *Modeling the Transmission and Prevention of Infectious Disease* (pp. 247-286).ed C. J. Hurst. Springer International Publishing, Cham.

Wright, G. D. (2010). Antibiotic resistance in the environment: a link to the clinic? *Current Opinion in Microbiology, 13*(5), 589-594.

Yau, S., Liu, X. L., Djordjevic, S. P., & Hall, R. M. (2010). RSF1010-Like Plasmids in Australian *Salmonella enterica* Serovar Typhimurium and Origin of Their *sul2-strA-strB* Antibiotic Resistance Gene Cluster. *Microbial Drug Resistance, 16*(4), 249-252. doi:10.1089/mdr.2010.0033

Yong, D. L., Jain, A., Liu, Y., Iqbal, M., Choi, C. Y., Crockford, N. J., Milington, S., & Provencher, J. (2018). Challenges and opportunities for transboundary conservation of migratory birds in the East Asian-Australasian flyway. *Conservation Biology, 32*(3), 740-743. doi:10.1111/cobi.13041

Zhang, S., Sun, J., Liao, X., Q, H., Liu, B., Fang, L., Deng, H., Ma, J., Xiao, X., Zhu, H., & Liu, Y. (2013). Prevelence and plasmid characterization of the *qnrD* determinant in *Enterobacteriacae* isolated from animals, retail meat products, and humans. *Microbial Drug Resistance, 19*(4), 331-335.

Zhang, X., Paganelli, F. L., Bierschenk, D., Kuipers, A., Bonten, M. J., Willems, R. J., & van Schaik, W. (2012). Genome-wide identification of ampicillin resistance determinants in *Enterococcus faecium*. *PLoS Genetics, 8*(6), e1002804. doi:10.1371/journal.pgen.1002804

Zhao, H., Zhao, J., Shen, J., Fan, H., Guan, H., An, X., & Li, P. (2014). Prevelance and molecular characterization of fluoroquinolone resistance in *Escherichia coli* isolates from dairy cattle with endometritis in China. *Microbial Drug Resistance, 20*(2), 162-169.

Zurfluh, K., Power, K., Klumpp, J., Wang, J., Fanning, S., & Stephan, R. (2015). A novel *Tn3*-Like composite transposon harbouing bla_{VIM-1} in *Klebsiella pneumoniae* spp. *pneumoniae* isolated from river water. *Microbial Drug Resistance, 21*(1), 43-49.

Foreword to Chapter 2

Chapter 2 consists of one data chapter that seeks to validate the methodology utilised in this research project.

This manuscript investigated the potential impact of extended storage and fluctuating temperatures on the recovery of three bacterial target species – *E. coli*, *Enterococcus* sp., and *Salmonella* spp. Samples consisted of swabs spiked with target bacteria acting as controls, and swab samples taken from wild pest birds. In total, *E. coli* and *Salmonella* had survival rates of 100% and 87% respectively after storage for 6 weeks, while *Enterococcus* had a 20% survival rate. The effect of temperature on survival was much more variable, with 67% of *Enterococcus* surviving at 30°C compared to a 20% survival rate at room temperature (RT).

This study ensures that the results displayed in later chapters have been collected using sound methodology that can be easily replicated by future researchers.

2. Evaluation of the impact of holding period and temperature of swabs before conducting bacterial culture

2. 1 Introduction

When considering the role of wild birds in the transmission of bacterial species, it is important to understand that carriage rates of bacteria are impacted by a number of variables, such as species, location, and age of the bird sampled (Benskin et al., 2009). In the past decade, there has been a growing appreciation for how diseases and disease management is affected by the interplay between human, animal and environmental health (Gortazar et al., 2015; Smith et al., 2017). Infectious diseases do not occur in isolated systems, and risk management strategies for disease control increasingly take into account the potential for pathogenic bacteria to be transmitted into human communities from wild animal populations (Borges et al., 2017; Smith et al., 2017; Wilharm et al., 2017).

To determine the risk a certain population (such as migratory populations, or those residing in urban environments) pose in the dissemination of pathogenic bacteria, it is important to know the prevalence rate of that bacteria in the population. For example, a population that has a high carriage rate of bacteria of interest has a greater potential to transmit those pathogens than populations in which that pathogen occurs rarely (Boehm, Hutchings, & White, 2009; Dutta et al.,2012).

For microbiological studies where detailed data are sought for individual bacterial isolates, culture remains a necessity as it allows for extensive testing on individual isolates that remain difficult to perform using (culture independent) genetic approaches. To obtain bacteria for culture, it is often necessary to collect the sample with a swab (Rishmawi et al., 2007). However, the use of swabs can affect the recovery rates of different bacterial species (Rishmawi et al., 2007), especially when there is an extended lag-time between initial sample collection and culture (Gästrin, Kallings, & Marcetic, 1968).

The duration a swab is held prior to culture set-up, and the temperature the swab is stored, affect the viability of the bacteria on the swab. When plating up swabs it is generally regarded best practice to plate up the swabs within 24 hours, and depending on the organism/specimen, keep them stored below 5°C (Barry, Fay, & Sauer, 1972; Collee et al., 1974; Gnarpe, 1976; Hallander, Flodström, & Holmberg, 1975). Doing so minimises changes to the composition of the bacterial flora. However, some studies have found that extended storage and sub-optimal temperatures may have only a minor negative impact on bacterial flora (Bai et al., 2012; Lauber et al., 2010; Rubbo & Benjamin, 1951).

While a sub-24hr transport/storage period is desired, it is not always possible. Studies that sample in remote areas, or in resource-poor settings with broken chains of transport, can see transport times extended to days or weeks. Such settings may lack access to refrigeration for samples, leading to non-continuous cold-chain transportation.

When conducting microbiological studies on wild migratory birds (and similarly for many other species of wild animals) in remote locations, samples may need to be collected in batches and processed at the end of the sample expedition. Depending on the location of the field work, it might be 4 weeks or longer between collection of samples and culture, and access to refrigeration may be limited. It was therefore necessary to determine to what extent bacterial recovery was affected by variable storage conditions, and to what extent this might have impacted on the ability to measure bacterial prevalence rates in shorebirds. This chapter describes the potential impact of extended storage and fluctuating temperatures have on the recovery of three target bacterial species, namely *E. coli, Enterococcus*, and *Salmonella*.

A study was established using cloacal swabs from birds. The effects of storage at 5 °C, room temperature (RT) and 30°C for 1 week was investigated. Furthermore, the effects of storage at 5 °C for 1 hour, 3 weeks, and 6 weeks had on the recovery of bacteria were investigated. The results of this chapter were used to inform sampling procedures in subsequent chapters.

2.2 Materials and methods

To create control samples, three bacterial strains were used: *E. coli* ATCC 25922, *Enterococcus faecalis* ATCC 51299, and *Salmonella* Typhimurium ATCC 13311. Rayon-tipped aluminium wire swabs (Copan) were used for all experiments. Selective agars used were MacConkey agar (Oxoid) for the detection of *E. coli*, MacConkey II agar (Oxoid) for the detection of *Enterococcus*, and Xylose-Lysine-Dextrose (XLD) agar (Becton Dickinson) for the detection of *Salmonella*. Biochemical testing was performed on bacterial isolates (including catalase, oxidase, indole, and Gram staining). Bacterial speciation was performed using polymerase chain reaction (PCR) on a Veriti 96-Well Thermal Cycler (Thermofisher). Presumptive *E. coli* isolates were confirmed by a PCR assay targeting a 450bp fragment in the Internal Transcribed Spacer (ITS) region (Khan et al., 2007). Presumptive *Enterococcus* isolates were confirmed by a PCR assay targeting the 16S rRNA gene (Ryu et al., 2013). Presumptive *Salmonella* isolates were confirmed by a PCR assay targeting the *invA* gene (Malorny et al., 2003). Data analysis was conducted using Microsoft Excel and SPSS (IBM).

2.2.1 Creation and preparation of tested samples

The birds used in this study were two European Starlings (*Sturnus vulgaris*), two Spotted Doves (*Spilopelia chinensis*), one Indian Myna (*Acridotheres tristis*), and one European Blackbird (*Turdus merula*). Age was unknown for all birds. The starlings, doves and myna were euthanized as part of a pest control program in the Latrobe Valley, VIC, Australia, and the blackbird was from a car strike. As all animals involved were euthanised prior to and separate from this study, and were introduced and invasive pests, no ethical permissions were required.

Samples from wild birds (the 'wild swabs') were collected by dissecting the provided birds, and then rolling the swab in the contents of the cloaca and the lower intestines. These swabs were not spiked with bacteria: the only bacteria present on the swabs were directly from the gastrointestinal tract of the birds. These swabs were used to inform how prospective shorebird swabs might react under the field sampling conditions. In total, 36 swabs were collected from six individual birds ('wild' swabs).

A swab control group (the 'spiked' swabs) was created by adding the target bacteria (*E. coli, Enterococcus faecalis* and *Salmonella* Typhimurium) to sterile collection swabs. To create the control swabs (the 'spiked' group), swabs were placed in 500µl sterile saline, in 1.5ml Eppendorf tubes spiked with ~10 cells of *E. coli*, *Enterococcus faecalis* and *Salmonella* Typhimurium. For the following experiments (See Section 2.2.2 and 2.2.3), nine spiked swabs were used as a control to the cloacal swabs collected from wild birds. These spiked swabs were used to ensure that the results gained from this experiment were due to the effect of the trial conditions on the bacteria, and not due to any other confounding variables i.e. the effect of faecal matter on the swabs. Fifty-four 'spiked' swabs were created, resulting in a total of 90 swabs created for this experiment.

2.2.2 Bacterial culture

Six groups were created to test the effect of storage duration on bacterial viability. The groups and their associated variables are listed in Table 1 and 2. After exposure to the trial conditions, each swab was used to inoculate three separate plates. Each swab was swabbed onto the three agar plates in order of selectivity of the media (MacConkey, MacConkey II, and XLD). All plates were incubated at 35 °C for 24 – 48hr. Plates were then inspected for species-appropriate bacterial growth, and colonies underwent biochemical testing to presumptively identify isolates. Isolates were then subject to species-specific PCR assays (refer to Section 2.2.2 for details) to confirm the results of the biochemical testing. Results were recorded as either 'positive' (bacterial growth of the target species on the plate selecting for growth of that bacteria) or 'negative' (no growth, or growth of non-target bacteria).

2.2.3 Statistical analysis

Positive and negative counts of the target species were recorded for each variable, and these counts were tabulated with the percentages of positive recoveries for each group calculated. Chi Square testing was used to determine if the tested variables impacted the recovery of target species.

Table 1. The time trial conditions and the number of swabs used for each experiment. All swabs for the time trials were stored at 5°C for the duration of their storage time.

Time		
1 Hour	**3 Weeks**	**6 Weeks**
Spiked swab *(n=9)*	Spiked swab *(n=9)*	Spiked swab *(n=9)*
Wild bird swab *(n=6)*	Wild bird swab *(n=6)*	Wild bird swab *(n=6)*
1 hour total *(n=15)*	3 weeks total *(n=15)*	6 weeks total *(n=15)*
Total swabs (Time) ***(n=45)***		

Table 2. The temperature trial conditions and the number of swabs used for each experiment. All swabs in the temperature trials were stored for 1 week at their respective temperatures.

Temperature		
5°C	**Room Temperature**	**30°C**
Spiked swab *(n=9)*	Spiked swab *(n=9)*	Spiked swab *(n=9)*
Wild bird swab *(n=6)*	Wild bird swab *(n=6)*	Wild bird swab *(n=6)*
5°C total *(n=15)*	Room temperature total *(n=15)*	30°C total *(n=15)*
Total swabs (Temperature) ***(n=45)***		

2.3 Results

2.3.1 Effect of time spent in storage on bacterial viability

Recovery rates (the number of swabs that returned growth of bacterial target species on media plates) varied between bacterial species for differing storage times, with no consistent trends across the three species over the three durations (Table 3).

Table 3. The bacterial recovery rates of *E. coli*, *Enterococcus* and *Salmonella* for the Time trials. The number of swabs used for each swab category (Spiked vs Wild birds) is provided in parenthesis. The percentages provided are the percentage of swabs that returned positive growth of a bacterial target species.

Swabs used	**1 hour**	**3 weeks**	**6 weeks**
		E. coli	
Spiked	78% (7/9)	55% (5/9)	100% (9/9)
Wild birds	84% (5/6)	20% (2/6)	100% (6/6)
Total	80% (12/15)	47% (7/15)	100% (15/15)
		Enterococcus	
Spiked	55% (5/9)	33% (3/9)	0% (0/9)
Wild birds	20% (2/6)	50% (3/6)	50% (3/6)
Total	47% (7/15)	40% (6/15)	20% (3/15)
		Salmonella	
Spiked	100% (9/9)	100% (9/9)	100% (9/9)
Wild birds	84% (5/6)	50% (3/6)	66% (4/6)
Total	93% (14/15)	80% (12/15)	87% (13/15)

Overall, the length of time in storage had the greatest impact on the recovery of *Enterococcus* sp., with total recovery rates dropping from 47% after 1 hour storage to 20% after 6 weeks. The total recovery rates of *E. coli* were more variable, ranging from 47% (at 3 weeks) to 100% (at 6 weeks). *Salmonella* sp. recovery rates remained mostly stable, ranging from 80% to 93% over the different storage durations.

For the spiked samples, 1 hour of storage at 5°C had the greatest impact on the recovery of *Enterococcus* sp., with only 55% of the spiked swabs and 20% of the bird swabs returning a positive Enterococcal culture. *E. coli* recovery rates were also impacted, but not to the same extent, with an 80% total recovery rate. *Salmonella* sp. recovery was affected the least after 1 hour in storage, with a 93% recovery rate overall (100% recovery rate for spiked, 84% for experimental samples).

After 3 weeks of storage there was a detrimental impact on *Enterococcus* sp. in the spiked samples, with a 33% recovery rate; halved from the 1 hour storage. However, the recovery rate for *Enterococcus* in the wild bird samples was 50%; in contrast to the spiked samples. *E. coli* was also impacted by 3 weeks of storage, with the recovery rates for both the spiked and experimental samples dropping (55% and 20% respectively). *Salmonella* sp. remained unaffected by storage time in the spiked samples (100% recovery rate) but were significantly impacted in the experimental samples (50% recovery rate).

After 6 weeks of storage there was a large impact on *Enterococcus* sp., with there being no positive cultures recorded from the spiked samples. The experimental samples had the same recovery rate as at 3 weeks (50%), but in total for *Enterococcus* after 6 weeks in storage the recovery rate was only 20%. In contrast, the *E. coli* recovery rate was 100% after 6 weeks for both spiked and experimental samples. The *Salmonella* spiked samples, likewise, were not affected by 6 weeks storage, with a 100% recovery rate; however the experimental samples only had a 66% recovery rate (87% total).

2.3.2 Effect of temperature in storage on bacterial viability

As with time, the impact of storage in differing temperatures on the recovery rates of the different bacterial species varied (Table 4).

Table 4. The bacterial recovery rates of *E. coli*, *Enterococcus* and *Salmonella* after storage at three different temperatures after 1 week of storage. The number of swabs used for each swab category (Spiked vs Wild birds) is provided in parenthesis. The percentages provided are the percentage of swabs that returned positive growth of a bacterial target species.

Swabs used	**5°C**	**RT**	**30°C**
		E. coli	
Spiked	66% (6/9)	100% (9/9)	88% (8/9)
Wild birds	84% (5/6)	50% (3/6)	50% (3/6)
Total	73% (11/15)	80% (12/15)	73% (11/15)
		Enterococcus	
Spiked	33% (3/9)	22% (2/9)	66% (6/9)
Wild birds	16% (1/6)	16% (1/6)	50% (3/6)
Total	27% (4/15)	20% (3/15)	67% (10/15)
		Salmonella	
Spiked	100% (9/9)	78% (7/9)	66% 6(/9)
Wild birds	33% (2/6)	16% (1/6)	33% (2/6)
Total	73% (11/15)	53% (8/15)	53% (8/15)

Enterococcus sp. was adversely impacted by storage in different temperatures, though was more impacted after 1 week storage at 5 °C (27% total recovery rate) and RT (20%) than it was at 30 °C (67%). *E. coli* was tolerant of the differing temperatures, with recovery rates ranging from 73 – 80% in total. *Salmonella* sp. was negatively impacted by storage at any temperature, and more so at higher temperatures (73% recovery rate for 5 °C, compared to 53% for RT and 30 °C).

After 1 week storage at 5 °C the number of positive *Enterococcus* swabs decreased the greatest (relative to other temperatures), with the total recovery rate 27%. The spiked samples had a greater recovery rate than the experimental samples (33% to 16%), but still displayed a negative impact from the storage. *E. coli* fared better, with a total recovery rate of 73% (66% spiked, 84% experimental). The recovery rates between the spiked and experimental *Salmonella* samples varied greatly- all of the spiked swabs

yielded positive bacterial culture, while the experimental swabs only had a 33% recovery rate.

The recovery rate for *Enterococcus* was poor when kept at RT, with an overall recovery rate of 20%. Similar to the 5°C storage, *E. coli* was able to tolerate the variable RT to a greater extent than *Enterococcus*, with an 80% recovery rate in total; though there were large discrepancies between the spiked (100%) and experimental (50%) groups. A similar difference between groups was also seen in *Salmonella* recovery (78% spiked compared to 16% experimental).

In contrast to every other storage condition, under the 30°C storage conditions *Enterococcus* had a majority positive recovery rate (67% total). *E. coli* had a similar total recovery rate of 73%, with marked difference between the spiked (88%) and experimental (50%). *Salmonella* also exhibited a difference in recovery between experimental (33%) and spiked (66%) groups.

2.3.2 Relationship between time, temperature and bacterial recovery

The positive and negative recovery rates of the three targeted bacteria in each test condition were used to determine if there was a relationship between how the swabs were stored, and bacterial recovery. For this, only the results from the wild bird swabs were utilised (Table 5).

Table 5. The chi square results of the temperature and time trial conditions. Only the samples taken from wild birds were used. Positive percentages refer to the percentage of swabs that returned target bacterial growth. Negative percentages refer to the percentage of swabs that returned either non-target bacterial growth, or no growth.

Temperature									
	E. coli			*Enterococcus*			*Salmonella*		
	Positive %	Negative %	*p*	Positive %	Negative %	*p*	Positive %	Negative %	*p*
5°C	84	16	<0.05	16	84	<0.05	33	67	<0.05
RT	50	50		16	84		16	84	
30°C	50	50		50	50		33	67	
Time									
	E. coli			*Enterococcus*			*Salmonella*		
	Positive %	Negative %	*p*	Positive %	Negative %	*p*	Positive %	Negative %	*p*
1 hour	84	16	<0.05	20	80	<0.05	84	16	<0.05
3 weeks	20	80		50	50		50	50	
6 weeks	100	0		50	50		66	34	

2.4 Discussion

The results of this study demonstrate the variability of survival of bacteria over storage duration and temperature, with the different bacteria investigated in this study exhibiting different rates of survival.

Enterococcus was greatly affected by even short periods of storage, with bacteria on over 70% of the swabs losing viability, whereas *E. coli* and *Salmonella* were more stable over extended storage periods, with all *E. coli* swabs and ~90% of *Salmonella* swabs retaining viability. Similar findings were found for the temperature trials, with 50 – 70% of *E. coli* and *Salmonella* swabs retaining viability during each temperature testing condition, while *Enterococcus* was viable in ~20% of the swabs. The sole exception was seen in the 30°C trial, where *Enterococcus* was successfully recovered from 67% of the swabs after 1 week of storage.

When looking at the survival of each species in detail, it appears that *Enterococcus* is adversely affected by as little as 1 hour in storage. This may be due to the temperature rather than the time: *Enterococcus* was also negatively impacted by storage at 5°C. Recovery was halved in the spiked swabs after no more than 1 hour on a swab, and halved again after 6 weeks storage. At 6 weeks, *Enterococcus* could no longer be

recovered from any of the spiked samples, and only half of the wild bird samples. Despite 5°C being considered the 'optimum' temperature for swab storage, these results show that for species such as *Enterococcus* this temperature has a marked negative impact on bacterial recovery with only 27% of swabs returning a positive result. In contrast, enterococci fared much better when stored at 30°C, with a 67% recovery rate compared to 27%.

There are many potential reasons for why Enterococcus appeared to survive better in higher temperature conditions than in 'ideal' storage conditions of -5 – 5°C. It could be that enterococci are outcompeted by *E. coli* and *Salmonella* at higher temperatures, and are only able to begin to grow as other bacteria die off, though this seems unlikely as both *E. coli* and *Salmonella* were not overly affected by storage at higher temperatures. Another explanation may be that enterococci are more fastidious on selective and non-enriched agars such as MacConkey than are *E. coli* (Reuter, 1992), i.e. they may have failed to thrive on the media used in this study. Recovery rates may improve if grown on more enterococci-permissive media such as blood-based media (Isenberg, Goldberg, & Sampson, 1970) or enterococci-specific enrichment media (Slanetz & Bartley, 1957). Despite differing conditions, recovery for *Enterococcus* was poor in all scenarios; such findings will have to be acknowledged when analysing recovery rates for *Enterococcus* in subsequent chapters.

E. coli and *Salmonella* demonstrated higher recovery rates over long storage periods relative to *Enterococcus* sp., with a 100% total recovery rate after 6 weeks for *E. coli*, and an 87% recovery rate for *Salmonella*. Temperature had a greater impact on both bacterial species, with *E. coli* demonstrating a 73% total recovery rate after 1 week storage at 30°C, while *Salmonella* recovery dropped to 53% for both ambient and 30°C storage conditions. These results indicate that, in contrast to *Enterococcus*, *Salmonella* is more suited to the 'gold standard' storage temperature of 5°C. *E. coli* was affected little by any temperature differences, and (aside from a poor recovery rate in the 3 week sample) seemed to be affected less by differing storage conditions than either *Salmonella* or *Enterococcus.*

The results raise questions about how tolerant some bacterial species are of extended periods in transit on a swab, and whether longer storage time may introduce some unintentional bias due to the uneven recovery rates of different bacterial species. Species such as *Salmonella* and *E. coli* were able to tolerate storage on swabs for extended periods of time far better than *Enterococcus*, leading to the conclusion that, while theoretically possible to store bacterial swab samples for extended periods and still be able to recover viable bacteria, further steps would be needed to improve bacterial survivability between sample collection and culture, especially with regards to more potentially time-sensitive species such as *Enterococcus*.

An interesting finding was the variation seen in the time trials. Recovery rates did not correlate as may have been expected, with *E. coli* and *Salmonella* both having higher recovery rates after 6 weeks storage than 3 weeks storage. There are multiple potential reasons for this; as mentioned, there may have been less bacterial competition on the sample swabs after six weeks storage, due to the majority of the viable *Enterococcus* isolates on the swab becoming non-viable after 6 weeks storage. Another reason may be that, despite best efforts, not all the swabs received equal bacteria loads when inoculated or swabbed. As such, there may have been less bacteria present in the 3 week trial swabs than the 6 week and 1 hour swabs, unintentionally biasing the results. Lastly, variation seen in the wild bird swabs may be due to the sampling methodology; due to the small sizes of the birds utilised in this study, different parts of the gastrointestinal tract were swabbed to collect samples on all the swabs used in this experiment. This may have introduced different bacterial species onto the wild bid swabs, dependant on where they were sampling in the gastrointestinal tract; such variation in bacterial flora may have had an impact on the survival of the target species. While it is not possible to identify how many viable bacterial cells are collected on each swab, it is possible to ensure that collection methodologies used in future chapters are targeting the same collection site on the bird (the cloaca). As such, all swab samples collected in Chapters 3, 4 and 5 collected samples only from the cloaca of the bird, inserting the swabs at the same depth to reduce the chances of bacterial variation due to sampling site.

It is important to note the bacterial strains used in this study were laboratory derived ATCC strains; these strains were chosen to imitate potential pathogenic and AMR bacterial strains that may be present in the target shorebird species. However, they may not be representative of environmental strains of *E. coli, Enterococcus*, and *Salmonella*. There is the potential that environmental strains may be more tolerant of sub-optimal conditions than laboratory-derived strains. As such it may be prudent to repeat these trials with environmental strains to determine if they maintain viability on swabs to a greater degree than laboratory strains.

It is also important to note that the species of birds used in this study differs from the target shorebird species of this thesis. The choice of pest and non-native birds in this study was due to the reliability of obtaining these birds from pest spiked operations, and the proximity of the collected birds to the laboratory; it was possible to ensure a <1 hour transit time for bacterial swabs collected. This ensured that the swabs were not in storage for extended periods outside of the indented testing parameters. In addition, the difference in target species between this study and subsequent chapters is not hypothesised to affect the recovery of target bacterial species; a study on *E. coli* in differing species of wild Australian birds found that *E. coli* communities were similar between birds sampled in suburban and wilderness areas (Blyton et al., 2015). As such, the results of this study are expected to be applicable to subsequent bacterial studies in shorebirds.

While 30°C had the best recovery rates for enterococci, the negative effects such temperatures have on *Salmonella* recovery and the unfeasibility of storing samples at such a temperature (consistently without fluctuation) means it is an avenue that cannot be followed. However, this result does shows that variable temperatures may not have as large an impact on bacterial recovery as first feared. This is useful knowledge, as many expeditions to remote locations lack access to electricity to run temperature-regulated storage units, or such locations may be carry in, carry out sites only; in such locations, it may be physically impossible to bring such devices into the field when sampling.

The poor recovery of *Enterococcus* does not mean that the use of microbial culture is inappropriate for investigations into bacterial species present in wild animals. As previously stated, microbial culture is necessary for the majority of in-depth analyses that are conducted on bacterial specimens. Such analyses cannot be performed on the low amounts of fragmentary bacterial DNA obtained from cloacal swabs only (Kemp et al., 2013). Studies that intend to investigate both presence and prevalence would work best with a two-pronged approach of both culture and sequencing, with metagenomic sequencing identifying the prevalence of specific bacterial species within a given sample, and culture enabling in-depth investigations into the genetic determinants of individual isolates (Jost et al., 2013). Existing literature regarding the prevalence of common clinically important bacteria circulating in wild bird populations in Australia is scarce; specific studies investigating the prevalence rate of such bacteria in migratory birds are rarer. Currently, studies exist for *E. coli* and *Salmonella* (Blyton et al., 2015; Dolejska et al., 2016; Epstein et al., 2006), but these studies do not investigate migratory populations. While these findings mean that it will not be possible to determine prevalence rates for *Enterococcus* in shorebirds, it may still be possible to do so for *E. coli* and *Salmonella*, providing a broad understanding of the carriage of these two bacteria in Australian shorebirds.

As such, while it is possible to undertake studies into bacterial presence in wild birds, inferring prevalence data from swab cultures may not be possible for species such as *Enterococcus* and *Salmonella*. For these bacterial species, studies focusing instead on the antimicrobial resistance patterns, genetic relationships and virulence genes of the isolates may be better suited.

The results demonstrate that for this study, it is still possible to detect the presence of certain, clinically relevant bacterial species, even after long periods in transit. This is of importance when planning for expeditions to Western Australia, which have a 3 week duration between collection of some swabs and culture set-up.

2.5 Conclusion

The impact extended storage, or storage in adverse conditions has on the cultivability of bacteria seems to be dependent on the species of the bacteria in question, with *E. coli* and *Salmonella* tolerating long storage conditions far better than *Enterococcus*, which in turn tolerates higher storage temperatures to a greater extent than *Salmonella*. These results were used to inform research aims, culture methodology and data analysis for subsequent chapters. Based on results, it was decided to take extra steps to help increase bacterial recovery and survival. Swabs were transported in charcoal Aimes media (to reduce bacterial die-off and extend longevity), and swabs were subject to a 24hr pre-enrichment step in brain heart infusion broth before undergoing culture to help bacterial populations recover before being subject to growth on selective media.

While these results do show that it may not be possible to analyse prevalence rates in wild populations for bacteria such as *Enterococcus*, it may still be possible for *E. coli.* In addition, subsequent analysis for genetic traits, such as virulence factors and antimicrobial resistance genes, are still viable research options with such bacterial samples. Regardless, the most important finding is that bacteria can survive extremely extended durations in transit or storage, and still remain viable after even 6 weeks on the swab. Further enrichment steps, and using media designed to help support bacterial survival on the swab will most likely increase recovery rates further, enabling long-duration expeditions to be performed during this project without too deleterious an impact upon the samples collected.

2.6 References

Bai, G., Gajer, P., Nandy, M., Ma, B., Yang, H., Sakamoto, J., Blanchard, M. H., Ravel, J., & Brotman, R. M. (2012). Comparison of storage conditions for human vaginal microbiome studies. *PLoS ONE, 7*(5), e36934.

Barry, A., Fay, G., & Sauer, R. (1972). Efficiency of a transport medium for the recovery of aerobic and anaerobic bacteria from applicator swabs. *Applied microbiology, 24*(1), 31-33.

Benskin, C. M., Wilson, K., Jones, K., & Hartley, I. R. (2009). Bacterial pathogens in wild birds: a review of the frequency and effects of infection. *Biological Reviews of the Cambridge Philosophical Society, 84*(3), 349-373. doi:10.1111/j.1469-185X.2008.00076.x

Blyton, M. D., Pi, H., Vangchhia, B., Abraham, S., Trott, D. J., Johnson, J. R., & Gordon, D. M. (2015). Genetic Structure and Antimicrobial Resistance of *Escherichia coli* and Cryptic

Clades in Birds with Diverse Human Associations. *Applied and Environmental Microbiology, 81*(15), 5123-5133. doi:10.1128/AEM.00861-15

Boehm, M., Hutchings, M. R., & White, P. C. (2009). Contact networks in a wildlife-livestock host community: identifying high-risk individuals in the transmission of bovine TB among badgers and cattle. *PLoS ONE, 4*(4), e5016.

Borges, C. A., Cardozo, M. V., Beraldo, L. G., Oliveira, E. S., Maluta, R. P., Barboza, K. B., Werther, K., & Avila, F. A. (2017). Wild birds and urban pigeons as reservoirs for diarrheagenic *Escherichia coli* with zoonotic potential. *Journal of Microbiology, 55*(5), 344-348. doi:10.1007/s12275-017-6523-3

Collee, J., Watt, B., Brown, R., & Johnstone, S. (1974). The recovery of anaerobic bacteria from swabs. *Epidemiology & Infection, 72*(3), 339-347.

Dolejska, M., Masarikova, M., Dobiasova, H., Jamborova, I., Karpiskova, R., Havlicek, M., Carlile, N., Priddel, D., Cizek, A., & Literak, I. (2016). High prevalence of *Salmonella* and IMP-4-producing Enterobacteriaceae in the silver gull on Five Islands, Australia. *Journal of Antimicrobial Chemotherapy, 71*(1), 63-70. doi:10.1093/jac/dkv306

Dutta, B., Scherm, H., Gitaitis, R., & Walcott, R. (2012). *Acidovorax citrulli* seed inoculum load affects seedling transmission and spread of bacterial fruit blotch of watermelon under greenhouse conditions. *Plant disease, 96*(5), 705-711.

Epstein, J. H., McKee, J., Shaw, P., Hicks, V., Micalizzi, G., Daszak, P., Kilpatrick, A., & Kaufman, G. (2006). The Australian White Ibis (*Threskiornis molucca*) as a Reservoir of Zoonotic and Livestock Pathogens. *EcoHealth, 3*(4), 290-298. doi:10.1007/s10393-006-0064-2

Gästrin, B., Kallings, L., & Marcetic, A. (1968). The survival time for different bacteria in various transport media. *Acta pathologica et microbiologica Scandinavica, 74*(3), 371-380.

Gnarpe, H. (1976). Survival of bacteria in four different transportation systems. *Scandinavian Journal of Infectious Diseases, 8*(4), 279-280.

Gortazar, C., Diez-Delgado, I., Barasona, J. A., Vicente, J., De La Fuente, J., & Boadella, M. (2015). The wild side of disease control at the wildlife-livestock-human interface: a review. *Frontiers in Veterinary Science, 1*, 27.

Hallander, H., Flodström, A., & Holmberg, K. (1975). Influence of the collection and transport of specimens on the recovery of bacteria from peritonsillar abscesses. *Journal of Clinical Microbiology, 2*(6), 504-509.

Isenberg, H. D., Goldberg, D., & Sampson, J. (1970). Laboratory studies with a selective *Enterococcus* medium. *Applied microbiology, 20*(3), 433-436.

Jost, T., Lacroix, C., Braegger, C., & Chassard, C. (2013). Assessment of bacterial diversity in breast milk using culture-dependent and culture-independent approaches. *British Journal of Nutrition, 110*(7), 1253-1262.

Kemp, M., Bangsborg, J., Kjerulf, A., Schmidt, T. A., & Christensen, J. (2013). Advantages and limitations of ribosomal RNA PCR and DNA sequencing for identification of bacteria in cardiac valves of Danish patients. *The Open Microbiology Journal, 7*, 146.

Khan, I. U., Gannon, V., Kent, R., Koning, W., Lapen, D. R., Miller, J., Neumann, N., Phillips, R., Robertson, W., Topp, E., van Bochove, E., & Edge, T. A. (2007). Development of a rapid quantitative PCR assay for direct detection and quantification of culturable and non-culturable *Escherichia coli* from agriculture watersheds. *Journal of Microbiological Methods, 69*(3), 480-488. doi:10.1016/j.mimet.2007.02.016

Lauber, C. L., Zhou, N., Gordon, J. I., Knight, R., & Fierer, N. (2010). Effect of storage conditions on the assessment of bacterial community structure in soil and human-associated samples. *FEMS Microbiology Letters, 307*(1), 80-86. doi:10.1111/j.1574-6968.2010.01965.x

Malorny, B., Hoorfar, J., Bunge, C., & Helmuth, R. (2003). Multicenter validation of the analytical accuracy of *Salmonella* PCR: towards an international standard. *Applied and Environmental Microbiology, 69*(1), 290-296.

Reuter, G. (1992). Culture media for enterococci and group D-streptococci. *International Journal of Food Microbiology, 17*(2), 101-111.

Rishmawi, N., Ghneim, R., Kattan, R., Ghneim, R., Zoughbi, M., Abu-Diab, A., Turkuman, S., Dauodi, R., Shomali, I., & Issa, A. E.-R. (2007). Survival of fastidious and nonfastidious aerobic bacteria in three bacterial transport swab systems. *Journal of Clinical Microbiology, 45*(4), 1278-1283.

Rubbo, S. D., & Benjamin, M. (1951). Survival of Pathogenic Bacteria on Cotton-wool Swabs. *British Medical Journal, 1*(4713), 983.

Ryu, H., Henson, M., Elk, M., Toledo-Hernandez, C., Griffith, J., Blackwood, D., Noble, R., Gourmelon, M., Glassmeyer, S., & Santo Domingo, J. W. (2013). Development of quantitative PCR assays targeting the 16S rRNA genes of *Enterococcus* spp. and their application to the identification of enterococcus species in environmental samples. *Applied and Environmental Microbiology, 79*(1), 196-204. doi:10.1128/AEM.02802-12

Slanetz, L., & Bartley, C. H. (1957). Numbers of enterococci in water, sewage, and feces determined by the membrane filter technique with an improved medium. *Journal of Bacteriology, 74*(5), 591.

Smith, K. M., Machalaba, C. M., Jones, H., Caceres, P., Popovic, M., Olival, K. J., Ben Jebara, K., & Karesh, W. B. (2017). Wildlife hosts for OIE-Listed diseases: considerations regarding global wildlife trade and host-pathogen relationships. *The Journal of Veterinary Medical Science, 3*(2), 71-81. doi:10.1002/vms3.57

Wilharm, G., Skiebe, E., Higgins, P. G., Poppel, M. T., Blaschke, U., Leser, S., Heider, C., Heindorf, M., Brauner, P., Jackel, U., Bohland, K., Cuny, C., Lopinska, A., Kaminski, P., Kasprzak, M., Bochenski, M., Ciebiera, O., Tobolka, M., Zolnierowicz, K. M., Siekiera, J., Seifert, H., Gagne, S., Salcedo, S. P., Kaatz, M., Layer, F., Bender, J. K., Fuchs, S., Semmler, T., Pfeifer, Y., & Jerzak, L. (2017). Relatedness of wildlife and livestock avian isolates of the nosocomial pathogen *Acinetobacter baumannii* to lineages spread in hospitals worldwide. *Environmental Microbiology, 19*(10), 4349-4364. doi:10.1111/1462-2920.13931

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Foreword to Chapter 3

Chapter 3 consists of one pre-publication manuscript that investigates the presence of AMR in *Escherichia coli*, *Enterococcus*, and *Salmonella* collected from wild Australian shorebirds. The impact that species, migratory habit, age and sex of the bird sampled had on the carriage of AMR bacteria was also investigated.

This manuscript analyses the phenotypic resistance profiles of the target bacterial genera recovered from the shorebirds sampled in three Australian states (Victoria, South Australia, and Western Australia) over a 3 year period, from 2016-2019. Three bacterial species were targeted in this manuscript; *E. coli, Enterococcus* and *Salmonella*. Of 1022 birds sampled, 206 *E. coli*, 266 *Enterococcus*, and 20 *Salmonella* isolates were recovered, with AMR detected in 42% of the *E. coli*, 85% of the *Enterococcus*, and 10% of the *Salmonella*. Resistance was noted to clinically important antibiotics, such as colistin and ceftazidime (*E. coli*) and vancomycin (*Enterococcus*). Additionally, analysis of the results revealed that resident shorebirds were more likely to carry AMR *Enterococcus* than migratory shorebirds.

To date, there has been no comprehensive study investigating the presence of antibiotic resistance (AMR) in the bacteria of wild Australian shorebirds. Previous studies have either looked at small populations, at birds heavily impacted by human activity, or have utilised broad-scale metagenomic approaches to investigate genes present in whole populations. This study provides a broad view of what AMR phenotypes are present in common enteric bacteria in Australian shorebirds, and future research can investigate how AMR may differ by shorebird species or location.

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3. Presence and antimicrobial resistance profiles of *Escherichia coli*, *Enterococcus* spp. and *Salmonella* sp. in 12 species of wild Australian shorebirds.

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Presence and antimicrobial resistance profiles of *Escherichia coli*, *Enterococcus* spp. and *Salmonella* sp. in 12 species of wild Australian shorebirds.

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3.1 Summary

Antibiotic resistance is an ongoing threat to both human and animal health. Migratory birds are a potential vector for the spread of novel pathogens and antibiotic resistance genes. To date, there has been no comprehensive study investigating the presence of antibiotic resistance (AMR) in the bacteria of wild Australian shorebirds. In the current study, 1022 individual birds representing four species of non-migratory shorebird, six species of migratory shorebird and two coastal tern species were sampled across three states (Victoria, South Australia, and Western Australia) and tested for the presence of AMR strains of three bacteria that have the potential to be zoonotic pathogens; *Escherichia coli*, *Enterococcus* spp., and *Salmonella* sp. In total, 206 *E. coli*, 266 *Enterococcus* spp., and 20 *Salmonella* sp. isolates were recovered, with AMR detected in 42% of *E. coli*, 85% of *Enterococcus* spp., and 10% of *Salmonella* sp. Resistance was commonly detected to erythromycin (79% of *Enterococcus* spp. that were positive for AMR), ciprofloxacin (31% of *Enterococcus* spp.) and streptomycin (21% of *E. coli*). Investigations into the relationship between AMR carriage and the migratory habits of

the bird revealed that resident birds were more likely to carry AMR bacteria. This study acts as a broad baseline study into the carriage of AMR bacteria in wild Australian shorebirds. While it is common for bacteria isolated from shorebirds to have resistance to at least one antibiotic, this study did not detect emerging multi-drug resistant (MDR) bacteria in Australian shorebirds. With evidence that a suite of shorebird and tern species (some of which reside in remote locations during their breeding and non-breeding seasons) harbour AMR bacteria, wild bird populations clearly serve as a potential reservoir and vector for this emerging threat to human and animal health.
Keywords: AMR, enteric bacteria, migratory shorebirds, wildlife

3.3 Impacts

- The AMR profiles of zoonotic pathogens *E. coli*, *Enterococcus* spp. and *Salmonella* sp. isolated from wild Australian shorebirds were assessed over a three-year period (2016-2019). From 1066 individual bird samples, 266 *Enterococcus* spp., 206 *E. coli* and 20 *Salmonella* sp. isolates were recovered.

- AMR was detected in 61% of all recovered bacteria, with 42% of *E. coli*, 85% of *Enterococcus* spp., and 10% of *Salmonella* sp. demonstrating some level of AMR. Resistance to clinically important antibiotic classes, such as the quinolones and aminoglycosides, was detected.

- Resident species of shorebird were more likely to carry AMR *Enterococcus* spp. than migrant species, however this pattern of occurrence was not noted for AMR *E. coli* and *Salmonella* sp.

3.4 Introduction

Antimicrobial resistance (AMR) is recognised as a critical threat to human health (Nesme et al., 2014), increasing the length of stay and mortality risks in patients hospitalised with drug-resistant infections (Heffernan et al., 2018). Through decades of selection pressures exerted by the misuse of antibiotics in clinical, community, and agricultural settings, AMR bacteria have become increasingly common (McGettigan et al., 2019;

Wright, 2010). Some bacteria are more capable of acquiring AMR genes than others and, as such, pose a significant risk in clinical settings (Gao, Howden, & Stinear, 2018). An extra 700,000 deaths annually are reported worldwide due to antibiotic resistance, and this is expected to rise to 10 million extra deaths annually by 2050 (O'Neill, 2014).

Commensal organisms found in the gut have become an emerging clinical problem as they acquire AMR genes and become significant nosocomial pathogens (Gao et al., 2018). Increasing attention is being paid to the presence of AMR bacteria in wild animals and in environmental settings (Arnold, Williams, & Bennett, 2016; Torres et al., 2019). The spread of resistant bacteria into these areas has many potential ramifications; increasing the chance of exposure of bacteria to AMR genes, providing new vectors for further spread of AMR genes, and hampering conservation efforts for threatened wildlife populations. Wild birds in particular have been investigated for their potential to act as disease vectors, due to their high movement potentials and for some species, their close interactions with humans and livestock (Allen et al., 2010).

Wild birds are known vectors of zoonotic enteric pathogens that can potentially infect humans (Giacopello et al., 2016; Palmgren et al., 1997; Radhouani et al., 2012), and are reservoirs of enteric bacteria such as *E. coli*, *Enterococcus* spp., and *Salmonella* sp. (Blyton et al., 2015; Santos et al., 2013; Smith et al., 2020). Common commensal gut microbes play vital roles in digestion and host immune function, but some are capable of causing disease to the host and to other animals (Batt, Rutgers, & Sancak, 1996; Clemente et al., 2012). *Enterococcus* spp. have drawn considerable attention due to the impact species such as *E. faecalis* and *E. faecium* have had in clinical settings, being one of the most commonly isolated Gram-positive nosocomial pathogens in health settings globally (Gao et al., 2018). In addition, *Salmonella* is one of the most common causes of human morbidity and mortality associated with foodborne disease (Chlebicz & Slizewska, 2018). Increasing rates of AMR in enteric bacteria compound the problems they pose in clinical settings (Alvarez-Uria et al., 2018; Coombs et al., 2013; Gao et al., 2018).

Migratory shorebirds are potentially an important vector of emerging diseases and AMR due to their highly mobile behaviour. Many Australian shorebird species routinely migrate between the northern and southern hemisphere each year, stopping over in regions that support approximately one-third of the global human population on their migrations (Yong et al., 2018). Millions of migrant birds make regular movements between the Artic and Australia (Oldland et al., 2009), and come into close contact with human activity as they do so. Encroaching human development at major stopover sites such as the Yellow Sea and hunting of shorebirds for food increase the amount of contact these migrating birds have with humans (Piersma et al., 2017; Studds et al., 2017; Wauchope et al., 2017). This then increases the likelihood of the transfer of zoonotic diseases and AMR bacteria (Altizer, Bartel, & Han, 2011) between birds and humans.

Information regarding the presence of bacterial species of clinical importance and associated AMR in Australian shorebirds is scarce. Globally, studies concerning shorebirds are lacking, with most investigating the presence of pathogenic Enterobacteriaceae (Keeler & Huffman, 2009; Santos et al., 2012). As such, it is important to develop baseline measures of AMR in shorebird communities, covering both migrant and resident species. Due to the high number of shorebird species present (37 species), Australia presents an exemplary study site for this purpose (Weller & Lee, 2017).

Here we investigate the presence and AMR profiles of *E. coli*, *Enterococcus* spp. and *Salmonella* sp. as three common commensal gut bacteria that occur in wild shorebirds. Our aim is to quantify the occurrence rates of AMR bacteria in a shorebird community that contains both migrant and resident species, and to explore factors that may influence AMR carriage in wild shorebirds.

3.5 Materials and methods

Shorebirds were captured by the Victorian Wader Study Group (VWSG), Australasian Wader Study Group (AWSG), and Friends of Shorebirds South East (FOSSE) from the

20/12/2016-18/02/2019, as part of ongoing scientific and conservation efforts these focal species throughout the East-Asian Australasian flyway. Birds were captured with the aid of cannon nets, handheld nets, or captured by hand, as appropriate for species. Birds were sampled in 22 different geographic locations, across the Australian states of Victoria, South Australia and Western Australia. The locations sampled were sites that were part of the VWSG or AWSG's scientific research plan. Cloacal swabs were taken from each bird using Mini Tip Aimes with Charcoal specimen swabs (Copan). Transit time from sample collection to culture varied, ranging from 9-20 days due to the often-remote nature of the fieldwork. During transit, swab samples were stored at ~5°C in a portable refrigeration unit.

All research was conducted under animal ethics permits issued by Federation University Australia (permit no. 16-002), scientific research permits issued by the Department of Environment, Land, Water and Planning for Victoria (DELWP) (permit no. 10008032), the Department of Environment and Water (DEW) (permit no. 35/2016), and the Department of Parks and Water (DPaW) (permit no. 01-000179-1).

3.5.1 Bacterial culture, antimicrobial susceptibility testing and statistical analysis

In the laboratory, swabs were pre-enriched by removing tips aseptically and transferring to 5ml brain heart infusion broth (Oxoid) and incubated at 35°C for ~24 hours. Aliquots of 100µl were subsequently used to inoculate each of mannitol broth (Oxoid), azide dextrose broth (Oxoid) and selenite broth (Becton Dickinson [BD]). All selective enrichment broths were incubated at 35°C for 18-48 hours, and then plated onto MacConkey II agar (Oxoid) or xylose lysine deoxycholate (XLD) agar (BD) as appropriate.

All plates were incubated at 35°C for 24-48 hours. Colonies were selected by choosing a single isolate that had the appropriate phenotypic characterisations. Suspect *E. coli, Enterococcus* spp. or *Salmonella* sp. isolates were sub-cultured for purity on the respective selective plates and preliminary testing included Gram reaction, catalase and oxidase testing. Presumptive *E. coli* isolates were confirmed by the indole test. Presumptive *Salmonella* sp. were confirmed by a PCR assay targeting the *invA* gene

(Malorny et al., 2003), and presumptive *Enterococcus* spp. were confirmed by a PCR assay targeting the 16s rRNA gene (Ryu et al., 2013).

Confirmed isolates were tested for antimicrobial susceptibility using the Kirby-Bauer Disk Diffusion method (Clinical and Laboratory Standards Institute, 2012). The antimicrobials (Oxoid) tested were dependent upon the bacterial species in question (Table 5), and were chosen based on their importance to human and veterinary medicine. ATCC strains appropriate for each species as guided by the Clinical and Laboratory Standards were used as controls. Antimicrobial resistance (AMR) was defined as reduced susceptibility to at least one agent tested, while multi-drug resistance (MDR) was defined as reduced susceptibility to at least one agent in three or more antimicrobial classes (Magiorakos et al., 2012).

Table 6. The antimicrobial agents used in the antimicrobial susceptibility tests.

E. coli	*Enterococcus* spp.	*Salmonella* sp.
Ampicillin / AMP (10µg)	Ampicillin / AMP (10µg)	Ampicillin / AMP (10µg)
Amoxicillin-clavulanic acid / AMC (20/10µg)	-	Amoxicillin-clavulanic acid / AMC (20/10µg)
Amikacin / AK (30µg)	-	-
Chloramphenicol / C (30µg)	Chloramphenicol / C (30µg)	Chloramphenicol / C (30µg)
Ceftazidime / CAZ (30µg)	-	Ceftazidime / CAZ (30µg)
Cefotaxime / CTX (30µg)	-	Cefotaxime / CTX (30µg)
-	-	Cefalothin / KF (30µg)
-	-	Ceftiofur / EFT (30µg)
Ciprofloxacin / CIP (5µg)	Ciprofloxacin / CIP (5µg)	Ciprofloxacin / CIP (5µg)
Colistin / CT (10µg)	-	Colistin / CT (10µg)
-	Erythromycin / E (15µg)	-
Gentamicin / CN (10µg)	Gentamicin / CN (120µg)	Gentamicin / CN (10µg)
Imipenem / IMP (10µg)	-	Imipenem / IMP (10µg)
Nalidixic Acid / NA (30µg)	-	Nalidixic Acid / NA (30µg)
Sulfamethoxazole-trimethoprim / SXT (1.25/23.75µg)	-	Sulfamethoxazole-trimethoprim / SXT (1.25/23.75µg)
Streptomycin/ S (10µg)	Streptomycin / S (300µg)	Streptomycin / S (10µg)
Tetracycline / T (30µg)	Tetracycline / T (30µg)	Tetracycline / T (30µg)
-	Vancomycin / VA (30µg)	-

3.5.2 Data analysis

Chi-square analysis was used in tandem with observed vs expected counts to investigate potential relationships between variables. The proportion of antimicrobial resistant bacteria between the categories of each explanatory variable was tested to assess the relationship. For chi-square analysis, a p value of <0.05 was considered significant and indicates that the explanatory variable (e.g. the species of bird sampled, the migratory habits of the birds) correlated with the AMR profile of the bacteria recovered. All statistical analysis was performed using SPSS (IBM SPSS Statistics Version 25).

3.6 Results

3.6.1 Sample collection and bacterial recovery

Between 20/12/16 – 18/02/19, 1022 swabs were collected from twelve species of shorebird. From these swabs, *E. coli* was isolated from 20% (206 of 1022), *Salmonella* sp.

isolated from 2% (20 of 1022), and *Enterococcus* spp. from 26% (266 of 1022), with all 12 species of shorebird positive for one or more of the target bacteria species (Table 6).

Table 7. Recovery rates of bacteria (*E. coli*, *Salmonella* sp., and *Enterococcus* spp.) from the twelve species of shorebird sampled. The proportion of birds tested that were positive for each bacterial species is provided in parenthesis.

Species	*E. coli*	*Salmonella* sp.	*Enterococcus* spp.
Crested Tern *Thalasseus bergii (n=95)*	68 (72%)	2 (2%)	68 (72%)
Caspian Tern *Hydroprogne caspia (n=89)*	78 (88%)	0 (0%)	78 (88%)
Pied Oystercatcher *Haematopus longirostris (n=31)*	4 (13%)	1 (3%)	18 (58%)
Sooty Oystercatcher *Haematopus fuliginosus (n=12)*	5 (42%)	0 (0%)	10 (83%)
Sharp-tailed Sandpiper *Calidris acuminata* (n=131)	22 (16%)	0 (0%)	23 (18%)
Sanderling *Calidris alba (n=10)*	1 (10%)	0 (0%)	4 (40%)
Red-necked Stint *Calidris ruficollis (n=86)*	0 (0%)	0 (0%)	20 (23%)
Curlew Sandpiper *Calidris ferruginea (n=180)*	4 (2%)	0 (0%)	19 (11%)
Ruddy Turnstone *Arenaria interpres (n=37)*	1 (3%)	0 (0%)	3 (8%)
Bar-tailed Godwit *Limosa lapponica (n=264)*	4 (5%)	17 (6%)	17 (6%)
Double-banded Plover *Charadrius bicinctus (n=84)*	8 (10%)	0 (0%)	6 (7%)
Red-capped Plover *Charadrius ruficapillus (n=3)*	1 (33%)	0 (0%)	0 (0%)
Total *(n=1022)*	206 (20%)	20 (2%)	266 (26%)

The three species of bird from which *E. coli* was most commonly detected were Caspian Tern (88%), Crested Tern (72%) and Sooty Oystercatcher (42%). The same three bird species had the highest recovery rates of *Enterococcus* spp. (Caspian Terns 88%, Crested Terns 72%, and Sooty Oystercatcher 83%). By contrast, *Salmonella* sp. was only rarely detected in shorebirds. Of the *Salmonella* sp. isolates, 85% were identified as *Salmonella enterica* serovar Hvittingfoss (n=17, all from Bar-tailed Godwit, previously reported in Smith et al, 2020), 10% as *Salmonella enterica* serovar Typhimurium (n=2, one from a

Crested Tern and one from a Pied Oystercatcher), and 5% as *Salmonella enterica* serovar Bahrenfeld (n=1, from a Crested Tern).

3.6.2 Antibiotic resistance among bacterial isolates

Antibiotic susceptibility is shown for *E. coli* in Table 7. Because of lab restrictions due to the COVD-19 pandemic, not all enterococcal isolates had susceptibility testing performed: the data for 206/266 (77%) isolates are provided in Table 8.

Overall, AMR was observed in 88 of 210 (42%) *E. coli*, 175 of 206 (85%) *Enterococcus* spp., and 2 of 20 (10%) *Salmonella* sp. In total, 265 of 436 isolates (61%) demonstrated AMR. For *E. coli*, AMR bacteria were detected in all focal bird species. The species with the highest recovery of AMR bacteria was the Caspian Tern, with 45% (n=35) of all *E. coli* isolated from this species demonstrating resistance to at least one of the agents tested. Colistin resistance was noted in four species (Caspian Tern, Pied Oystercatcher, Sooty Oystercatcher, and Curlew Sandpiper), and ciprofloxacin resistance was noted in two species (Caspian Tern and Bar-tailed Godwit) (Table 7).

Two *Salmonella* sp. isolates demonstrated intermediate resistance against a single antibiotic (streptomycin). These isolates were recovered from a Bar-tailed Godwit and a Crested Tern. All other *Salmonella* sp. isolates were susceptible to all tested antibiotics as outlined in Table 5.

AMR *Enterococcus* spp. were recovered from all species sampled bar the Red-capped Plover. Vancomycin-resistant *Enterococcus* (VRE) was isolated from three species (Crested Tern, Caspian Tern, and Double-banded Plover). The most commonly observed resistance was to the macrolide class to which >70% of enterococcal isolates were resistant. One-third (32%) of *Enterococcus* spp. demonstrated resistance to quinolones (Table 8).

Table 8. Antibiotic susceptibility of *E. coli* isolates recovered from Australian Shorebird species. For species with <10 isolates recovered, the total isolates recovered were as such: Pied Oystercatcher (n=4), Sooty Oystercatcher (n=5), Red-capped Plover (n=1), Curlew Sandpiper (n=4), Ruddy Turnstone (n=1), Sanderling (n=1), and Double-banded Plover (n=8). † denotes colistin, which had 203 isolates tested. Explanations for abbreviations of the antibiotics used can be found in Table 5. ‡Bird species for which less than 10 samples were collected.

Bird species (No. birds positive)		AK	AMC	AMP	CTX	CAZ	C	CIP	†CT	CN	IPM	NA	S	SXT	TE
Crested Tern (n=68)	Resistant	3	-	3	-	-	-	-	-	-	-	1	5	1	5
	Intermediate	5	1	2	-	-	-	-	-	-	-	-	11	-	1
	Susceptible	60	67	63	68	68	68	68	68	68	68	67	52	67	62
Caspian Tern (n=78)	Resistant	2	1	14	-	-	-	1	2	-	-	-	6	3	13
	Intermediate	1	2	4	-	-	1	-	-	-	1	-	18	-	1
	Susceptible	75	75	60	77	78	77	77	76	78	77	78	54	75	64
Bar Tailed Godwit (n=14)	Resistant	-	1	-	-	-	-	-	-	-	-	-	-	-	-
	Intermediate	-	-	1	-	-	-	-	-	-	-	-	1	-	-
	Susceptible	14	13	13	14	14	14	14	14	14	14	14	13	14	14
Sharp-tailed Sandpiper (n=22)	Resistant	-	-	-	-	-	-	-	-	-	-	-	-	1	1
	Intermediate	-	-	4	-	-	-	-	-	-	-	-	-	-	-
	Susceptible	22	22	18	22	22	22	22	†19	22	22	22	22	21	21
Other‡ (n=24)	Resistant	2	1	2	-	2	-	-	3	-	-	2	2	-	-
	Intermediate	-	7	-	-	-	-	1	-	-	1	-	1	-	-
	Susceptible	22	16	22	24	22	24	23	21	24	23	22	21	24	24
Total (n= 206)	Resistant	7	3	19	-	2	-	1	†5	-	-	3	13	5	19
	Intermediate	6	10	11	-	-	1	1	-	-	2	-	31	-	2
	Susceptible	193	193	176	206	204	205	204	†198	206	204	203	162	201	185

Table 9. Antibiotic susceptibility of *Enterococcus* spp. isolates recovered from Australian shorebird species. For species with <10 isolates recovered, the total isolates recovered were as such: Ruddy Turnstone (n=3), Sanderling (n=4), and Double-banded Plover (n=7). Explanations for abbreviations of the antibiotics used can be found in Table 5. ‡Bird species for which less than 10 samples were collected.

Bird species (No. birds positive)		AMP	C	CIP	E	CN	S	TE	VA
Crested Tern (n=49)	Resistant	1	2	4	7	-	-	15	1
	Intermedia	-	1	24	36	1	-	-	12
	Susceptible	48	46	21	6	48	49	34	36
Caspian Tern (n=49)	Resistant	11	2	1	1	-	1	4	-
	Intermedia	-	3	10	44	-	-	-	1
	Susceptible	38	44	38	4	49	48	45	48
Pied Oystercatcher (n=16)	Resistant	-	-	-	1	-	-	-	-
	Intermedia	-	-	3	11	-	-	-	1
	Susceptible	16	16	13	4	16	16	16	15
Sooty oystercatcher (n=10)	Resistant	-	-	-	-	-	-	-	-
	Intermedia	-	-	-	7	-	-	-	-
	Susceptible	10	10	10	3	10	10	10	10
Bar Tailed Godwit (n=16)	Resistant	-	-	-	-	-	1	-	-
	Intermedia	-	-	5	6	-	1	-	-
	Susceptible	16	16	11	10	16	14	16	16
Red-necked Stint (n=20)	Resistant	5	-	-	1	-	-	1	-
	Intermedia	-	2	4	15	-	-	-	-
	Susceptible	15	18	16	4	20	20	19	20
Curlew Sandpiper (n=14)	Resistant	-	-	-	-	-	-	-	-
	Intermedia	-	-	7	11	-	-	-	-
	Susceptible	14	14	7	3	14	14	14	14
Sharp-tailed Sandpiper (n=18)	Resistant	-	-	-	1	-	-	-	-
	Intermedia	-	-	4	13	-	-	-	-
	Susceptible	18	18	14	4	18	18	18	18
Other‡ (n=14)	Resistant	-	-	-	-	-	-	-	-
	Intermedia	-	-	3	9	-	-	-	1
	Susceptible	14	14	11	5	14	14	14	13
Total (n=206)	Resistant	17	4	5	11	-	2	20	1
	Intermedia	-	6	60	152	1	1	-	15
	Susceptible	189	196	141	43	205	203	186	190

3.6.3 Bird species and antibiotic resistance

A correlation was observed between species of bird and decreased antibiotic susceptibility (χ^2=73.84, r=-0.291, df=22, p=≤0.001). Further analysis revealed that this relationship was only statistically significant for AMR *Enterococcus* spp. (χ^2=56, r=-0.336 df=20, p=≤0.001).

The relationship between habitat preference and the recovery of AMR bacteria was investigated, with birds assigned to one of two habitat types. Obligate 'Coastal' birds were defined as species that exclusively utilised coastal habitats, while 'Wetland' birds were defined as species that utilise both coastal habitats and freshwater wetlands. 'Coastal' birds included the Sanderling, the Bar-tailed Godwit, the Pied Oystercatcher, the Sooty Oystercatcher, the Ruddy Turnstone and the Crested Tern. 'Wetland' species included the Curlew Sandpiper, the Sharp-tailed Sandpiper, the Red-necked Stint, the Double-banded Plover, the Red-capped Plover and the Caspian Tern. . No statistically significant relationship was found between habitat preferenceand the recovery of AMR bacteria (of any species).

The relationship between the movement ecology of each shorebird species and the recovery of AMR bacteria was also investigated. Birds were defined as 'migratory' if they undertake trans-equatorial migration on an annual basis between high Arctic breeding grounds and non-breeding grounds in Australia, and 'resident' otherwise. Resident birds were more likely to carry AMR *Enterococcus* spp. than migratory birds. Results indicated resident species had significantly higher rates of AMR bacteria resistance than migrant species relationship (χ^2=19.723, df=2, p≤0.001) and closer investigation revealed this specifically applied to AMR *Enterococcus* spp. (χ^2=8.801, df=2, p=0.012). No such relationship was detected for AMR *E. coli* (χ^2=2.7, df=2, p=0.259) or *Salmonella* sp. Recovery (χ^2=2.135, df=1, p=0.144).

The number of multi-drug resistant (MDR) strains of bacteria was also investigated. No *Salmonella* sp. isolates were resistant to more than one class of antibiotic. Of *Enterococcus* spp., 17% (n=35) were resistant to ≥3 classes of antibiotics, 4% (n=8) to ≥4, and 1.5% (n=3) to ≥5 (Fig. 7). Of the *E. coli* isolates, 7% (n=15) were resistant to three or

more classes of antibiotics. One percent (n=2) of *E. coli* isolates were resistant to four or more classes of antibiotics (Fig. 7).

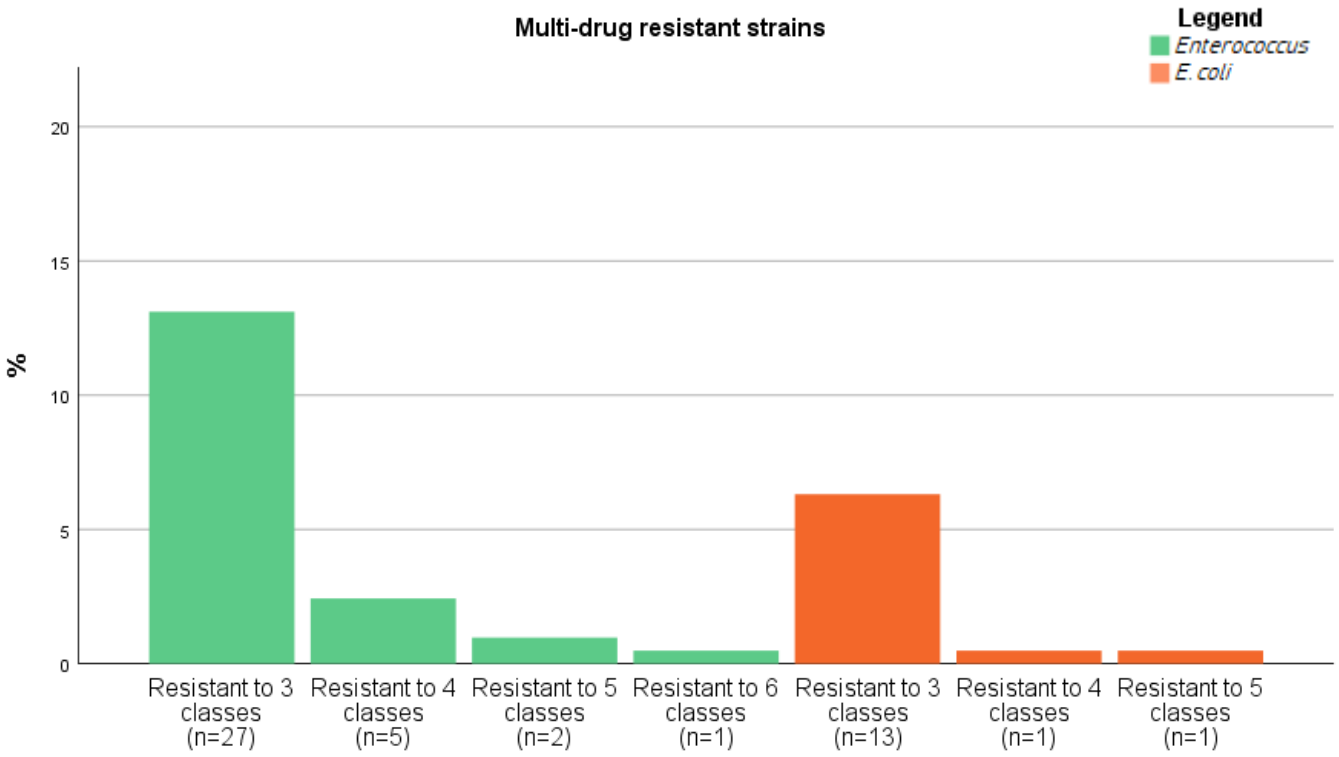

Figure 7. The percentage of multi-drug resistant strains of *E. coli* and *Enterococcus* spp. isolates recovered from Australian shorebirds. Scale is capped at 20%. N equals the number of isolates that demonstrated multi-drug resistance.

3.7 Discussion

This study demonstrates that while AMR is present in wild Australian shorebirds, resistance to clinically important antibiotics and MDR is currently uncommon in these populations. Resistance to clinically important classes of antibiotics such as the glycopeptides and carbapenems was rare: less than 8% (n=16) of all *Enterococcus* spp. isolated were resistant to glycopeptides, and less than 1% (n=2) of *all E. coli* isolates were resistant to carbapenems. Notably, all resistance to carbapenems and >90% of glycopeptide resistance was intermediate, rather than complete resistance. MIC testing would further clarify the phenotypic resistance profiles for these intermediate isolates. Multi-drug resistance was uncommon, with 17% of all *Enterococcus* spp. isolates resistant to three or more classes of antibiotics, and of those, 1.5% were resistant to five

or more classes. For *E. coli,* MDR was also uncommon, with 17% being resistant to three or more antibiotics and only 1% being resistant to four or more antibiotics. No MDR was detected in any of the *Salmonella* sp. recovered, and of those isolates, only 2 of 20 demonstrated intermediate resistance to a single antibiotic (streptomycin).

Resident birds were more likely to carry AMR *Enterococcus* spp. than migratory birds. This trend was not apparent in *E. coli* and *Salmonella* sp. There are two potential reasons for this finding; one may be due to the fact that *Enterococcus* spp. are able to acquire AMR genes with ease due to their malleable genomes, and as such may be picking up resistance genes from other gut bacteria or the environment to a higher degree than *E. coli* (Ramos et al., 2020). The second reason may be that *Enterococcus* spp. originating from faecal contamination (such as that from human sources) persist in environmental and aqueous environments to a greater degree than *E. coli* (Jin et al., 2004). Populations of both *E. coli* and *Enterococcus* are highly variable in wild bird populations (Fogarty et al., 2003) and further studies need to be conducted to confirm if this finding holds true across multiple species through different temporal periods.

One explanation for reduced rates of AMR bacterial infection of migratory shorebirds as observed here may be due to migratory culling and migratory separation (Altizer et al., 2011). These processes are theorised to curtail parasite and pathogen dispersal amongst migratory birds as infections negatively impact dispersal (migratory separation) and survival (migratory culling). Positive infection status in birds was associated with reduced movement and lowered survival rates (Risely, Klaassen, & Hoye, 2018). Previous studies have shown that enterococci cause disease in multiple bird species (Devriese et al., 1992; Devriese et al., 1990; Herdt et al., 2009). This may be the cause of the potential relationship seen here - migratory birds carrying *Enterococcus* could be less likely to thrive in the long term, while resident birds that do not undergo the same yearly movements are able to tolerate potential infections to a greater degree. However, it must be noted that there may be bias inherent in this study due to the non-randomized sampling methodology used.

All three target bacterial species (*E. coli*, *Enterococcus* spp., and *Salmonella* sp.) were present in Australian shorebirds. *Enterococcus* spp. and *E. coli* were present in the majority of species sampled, though *E. coli* was not isolated from the Red-necked Stint. *Salmonella* sp. was present in only three species (Bar-Tailed Godwit, Crested Tern, and Pied Oystercatcher). The overall isolation rates of *E. coli*, *Enterococcus* spp. and *Salmonella* sp. in the tested shorebirds was 20%, 26% and 2% respectively. Previous studies have shown that extended storage times only have a minor negative impact on bacteria present in swab samples; albeit in different experimental settings – lower enterococcal detection rates were also noted in Chapter 2 (Bai et al., 2012; Lauber et al., 2010). While it remains a possibility in our study that isolation rate was affected by transport times, it may not have had a major impact on detection rates. Other studies have also found low carriage rates of *E. coli*, *Enterococcus* spp., and *Salmonella* sp. in migratory birds, with *E. coli* having a carriage rate of 1-9% and *Salmonella* sp. having a 0-2% carriage rate (Brittingham, Temple, & Duncan, 1988; Najdenski et al., 2018), similar to this study. While there are no studies that examine the prevalence of *Enterococcus* spp. in migratory birds, studies investigating wild bird populations (consisting of wild raptors and Coraciiforms) detected carriage levels of 74-84% (Marrow et al., 2009; Splichalova et al., 2015). Despite the extended duration between sample collection and culture, the prevalence of target bacteria in this study was broadly comparable to those observed in other studies.

Contrary to the findings of other studies on Australian birds, AMR in Australian shorebirds was far less prevalent than previously reported for some species. For *Salmonella* sp., phenotypic resistance was only noted to streptomycin in two of the 20 (10%) isolates. Overall resistance rates were 42% in *E. coli*. Overall AMR rates were high in *Enterococcus* spp. at 85%, however resistance in *Enterococcus* spp. was confined mostly to older, broad-spectrum antibiotic classes such as the macrolides. Among *E. coli*, resistance was spread more evenly between both first-line and last-line antibiotic classes such as the penicillins and aminoglycosides, respectively. It must be noted that the colistin resistance detected was determined via disk-diffusion testing, a method that has known shortcomings when measuring values for colistin in the intermediate resistance range (Galani et al., 2008). Vancomycin resistance in *Enterococcus* spp. was found in

≤10% of all isolates, falling between previously reported rates of VRE in wild Australian birds; a study on Silver Gulls by Oravcova et al (2017) detected less than 1% of VRE in the sampled birds, while a study by Smith et al. detected VRE in 31% of recovered *Enterococcus* spp. (Oravcova, Svec, & Literak, 2017; Smith et al., 2019).

These findings suggest Australian shorebirds harbour bacteria that are sensitive to many clinically important antibiotics. This may be due to the ecology of shorebirds (both migratory and non-migratory) when compared to other species; they do not have significant interactions with anthropogenic or livestock populations, and may only interact with AMR bacteria through environmental sources such as wetland and coastal substrates and water. However it is difficult to state with any certainty whether shorebirds acquire AMR bacteria from the environment, as microbiome studies have demonstrated conflicting results. Shorebirds were considered to have a low intake of environmental bacteria (around 2% transfer between environmental bacteria and bird gut microbiota) by Risely et al. (2017), but Grond (2017) suggested that sampling site is the main driver in variation of shorebird gut microbiota. Further studies investigating the genetics and origins of AMR bacteria in shorebird populations are needed to determine where shorebirds acquire AMR bacteria and to provide a more comprehensive assessment of the scope of this problem. Investigations that determine whether migratory shorebirds are capable of transferring AMR bacteria between their own populations, and between human and livestock populations would also be of value.

3.8 Conclusion

This study shows that shorebirds are a potential reservoir of AMR pathogens, and are capable of carrying bacteria that are resistant to clinically important antibiotics. This study also shows that migratory status may affect carriage of AMR bacteria, with resident shorebirds demonstrating higher rates of AMR *Enterococcus* spp. than migrant shorebirds. These populations have the potential to act as both hosts and vectors of AMR enteric bacteria. It is anticipated that habitat degradation and loss will place further pressures on these populations, which may increase contact with human populations and further increase the potential for zoonotic transfer. Further studies are

required to track AMR in shorebird populations, and to begin to determine how wild populations are acquiring these bacteria.

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3.10 Conflict of Interest Statement

The authors declare they have no conflicts of interest.

3.11 References

Ahmad, M., & Khan, A. U. (2019). Global economic impact of antibiotic resistance: a review. *Journal of Global Antimicrobial Resistance, 19*, 313-316.

Allen, H. K., Donato, J., Wang, H. H., Cloud-Hansen, K. A., Davies, J., & Handelsman, J. (2010). Call of the wild: antibiotic resistance genes in natural environments. *Nature Reviews Microbiology, 8*(4), 251-259. doi:10.1038/nrmicro2312

Altizer, S., Bartel, R., & Han, B. A. (2011). Animal migration and infectious disease risk. *Science, 331*(6015), 296-302. doi:10.1126/science.1194694

Alvarez-Uria, G., Gandra, S., Mandal, S., & Laxminarayan, R. (2018). Global forecast of antimicrobial resistance in invasive isolates of *Escherichia coli* and *Klebsiella pneumoniae*. *International Journal of Infectious Diseases, 68*, 50-53. doi:10.1016/j.ijid.2018.01.011

Arnold, K. E., Williams, N. J., & Bennett, M. (2016). 'Disperse abroad in the land': the role of wildlife in the dissemination of antimicrobial resistance. *Biology Letters, 12*(8), 20160137.

Bai, G., Gajer, P., Nandy, M., Ma, B., Yang, H., Sakamoto, J., Blanchard, M. H., Ravel, J., & Brotman, R. M. (2012). Comparison of storage conditions for human vaginal microbiome studies. *PLoS ONE, 7*(5), e36934.

Batt, R., Rutgers, H., & Sancak, A. (1996). Enteric bacteria: friend or foe? *Journal of Small Animal Practice, 37*(6), 261-267.

Blyton, M. D., Pi, H., Vangchhia, B., Abraham, S., Trott, D. J., Johnson, J. R., & Gordon, D. M. (2015). Genetic Structure and Antimicrobial Resistance of *Escherichia coli* and Cryptic Clades in Birds with Diverse Human Associations. *Applied and Environmental Microbiology, 81*(15), 5123-5133. doi:10.1128/AEM.00861-15

Brittingham, M. C., Temple, S. A., & Duncan, R. M. (1988). A survey of the prevalence of selected bacteria in wild birds. *Journal of Wildlife Diseases, 24*(2), 299-307.

Chlebicz, A., & Slizewska, K. (2018). Campylobacteriosis, Salmonellosis, Yersiniosis, and Listeriosis as Zoonotic Foodborne Diseases: A Review. *International Journal of Environmental Research and Public Health, 15*(5). doi:10.3390/ijerph15050863

Clemente, J. C., Ursell, L. K., Parfrey, L. W., & Knight, R. (2012). The impact of the gut microbiota on human health: an integrative view. *Cell, 148*(6), 1258-1270. doi:10.1016/j.cell.2012.01.035

Clinical and Laboratory Standards Institute. (2012). Performance standards for antimicrobial susceptibility testing; Twenty-second informational supplement. In (Vol. 32, pp. 188). Wayne, Pennsylvania, U.S.A: Clinical and Laboratory Standards Institute.

Coombs, G. W., Daley, D. A., Lee, Y. T., & Pang, S. (2019). Australian Group on Antimicrobial Resistance (AGAR) Australian *Staphylococcus aureus* Sepsis Outcome Programme (ASSOP) Annual Report 2013. *Communicable Diseases Intelligence, 43*.

Devriese, L., Cruz Colque, J., Haesebrouck, F., Desmidt, M., Uyttebroek, E., & Ducatelle, R. (1992). *Enterococcus hirae* in septicaemia of psittacine birds. *Veterinary Record, 130*(25), 558-559.

Devriese, L. A., Ceyssens, K., Rodrigues, U. M., & Collins, M. D. (1990). *Enterococcus columbae*, a species from pigeon intestines. *FEMS Microbiol Lett, 59*(3), 247-251. doi:10.1016/0378-1097(90)90228-i

Fogarty, L., Haack, S., Wolcott, M., & Whitman, R. (2003). Abundance and characteristics of the recreational water quality indicator bacteria *Escherichia coli* and enterococci in gull faeces. *Journal of Applied Microbiology, 94*(5), 865-878.

Galani, I., Kontopidou, F., Souli, M., Rekatsina, P. D., Koratzanis, E., Deliolanis, J., & Giamarellou, H. (2008). Colistin susceptibility testing by Etest and disk diffusion methods. *International Journal of Antimicrobial Agents, 31*(5), 434-439.

Gao, W., Howden, B. P., & Stinear, T. P. (2018). Evolution of virulence in *Enterococcus faecium*, a hospital-adapted opportunistic pathogen. *Current Opinion in Microbiology, 41*, 76-82. doi:10.1016/j.mib.2017.11.030

Giacopello, C., Foti, M., Mascetti, A., Grosso, F., Ricciardi, D., Fisichella, V., & Lo Piccolo, F. (2016). Antimicrobial resistance patterns of Enterobacteriaceae in European wild bird species admitted in a wildlife rescue centre. *Veterinaria Italiana, 52*(2), 139-144. doi:10.12834/VetIt.327.1374.2

Grond, K. (2017). *Development and dynamics of gut microbial communities of migratory shorebirds in the Western Hemisphere.* Doctoral Dissertation, Kansas State University.

Heffernan, A. J., Sime, F. B., Lipman, J., & Roberts, J. A. (2018). Individualising Therapy to Minimize Bacterial Multidrug Resistance. *Drugs, 78*(6), 621-641. doi:10.1007/s40265-018-0891-9

Herdt, P. d., Defoort, P., Steelant, J. v., Swam, H., Tanghe, L., Goethem, S. v., & Vanrobaeys, M. (2009). *Enterococcus cecorum* osteomyelitis and arthritis in broiler chickens. *Vlaams Diergeneeskundig Tijdschrift, 78*(1), 44-48.

Jin, G., Englande, A., Bradford, H., & Jeng, H. W. (2004). Comparison of *E. coli*, enterococci, and fecal coliform as indicators for brackish water quality assessment. *Water Environment Research, 76*(3), 245-255.

Keeler, S., & Huffman, J. (2009). Identification of *Staphylococcus* spp. and aerobic gram-negative bacteria from the cloacae of migratory shorebirds (Family *Scolopacidae*) from Delware Bay, New Jersey. *Journal of the Pennsylvania Academy of Science*, 34-37.

Lauber, C. L., Zhou, N., Gordon, J. I., Knight, R., & Fierer, N. (2010). Effect of storage conditions on the assessment of bacterial community structure in soil and human-associated samples. *FEMS Microbiology Letters, 307*(1), 80-86. doi:10.1111/j.1574-6968.2010.01965.x

Magiorakos, A.-P., Srinivasan, A., Carey, R., Carmeli, Y., Falagas, M., Giske, C., Harbarth, S., Hindler, J., Kahlmeter, G., & Olsson-Liljequist, B. (2012). Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clinical Microbiology and Infection, 18*(3), 268-281.

Malorny, B., Hoorfar, J., Bunge, C., & Helmuth, R. (2003). Multicenter validation of the analytical accuracy of *Salmonella* PCR: towards an international standard. *Applied and Environmental Microbiology, 69*(1), 290-296.

Marrow, J., Whittington, J. K., Mitchell, M., Hoyer, L. L., & Maddox, C. (2009). Prevalence and antibiotic-resistance characteristics of *Enterococcus* spp. isolated from free-living and captive raptors in Central Illinois. *Journal of Wildlife Diseases, 45*(2), 302-313.

McGettigan, P., Roderick, P., Kadam, A., & Pollock, A. (2019). Threats to global antimicrobial resistance control: Centrally approved and unapproved antibiotic formulations sold in India. *British Journal of Clinical Pharmacology, 85*(1), 59-70. doi:10.1111/bcp.13503

Najdenski, H., Dimova, T., Zaharieva, M. M., Nikolov, B., Petrova-Dinkova, G., Dalakchieva, S., Popov, K., Hristova-Nikolova, I., Zehtindjiev, P., & Peev, S. (2018). Migratory birds along the Mediterranean–Black Sea Flyway as carriers of zoonotic pathogens. *Canadian Journal of Microbiology, 64*(12), 915-924.

Nesme, J., Cecillon, S., Delmont, T. O., Monier, J. M., Vogel, T. M., & Simonet, P. (2014). Large-scale metagenomic-based study of antibiotic resistance in the environment. *Current Biology, 24*(10), 1096-1100. doi:10.1016/j.cub.2014.03.036

Oldland, J., Rogers, D., Clemens, R., Berry, L., Maguire, G., & Gosbell, K. (2009). Shorebird conservation in Australia. *Birds Australia Conservation Statment, 14*(20).

Oravcova, V., Svec, P., & Literak, I. (2017). Vancomycin-resistant enterococci with *vanA* and *vanB* genes in Australian gulls. *Environmental Microbiology Reports, 9*(3), 316-318. doi:10.1111/1758-2229.12542

Palmgren, H., Sellin, M., Bergstrom, S., & Olsen, B. (1997). Enteropathogenic bacteria in migrating birds arriving in Sweden. *Scandinavian Journal of Infectious Disease, 29*(6), 565-568. doi:10.3109/00365549709035895

Piersma, T., Chan, Y.-C., Mu, T., Hassell, C. J., David S, D. S., Peng, H.-B., Ma, Z., Zhang, Z., & Wilcove, D. S. (2017). Loss of habitat leads to loss of birds: reflections on the Jiangsu, China, coastal development plans. *Wader Study, 124*(2). doi:10.18194/ws.00077

Radhouani, H., Poeta, P., Goncalves, A., Pacheco, R., Sargo, R., & Igrejas, G. (2012). Wild birds as biological indicators of environmental pollution: antimicrobial resistance patterns of *Escherichia coli* and enterococci isolated from common buzzards (*Buteo buteo*). *Journal of Medical Microbiology, 61*(Pt 6), 837-843. doi:10.1099/jmm.0.038364-0

Ramos, S., Silva, V., Dapkevicius, M. d. L. E., Igrejas, G., & Poeta, P. (2020). Enterococci, from Harmless Bacteria to a Pathogen. *Microorganisms, 8*(8), 1118.

Raymond, B. (2019). Five rules for resistance management in the antibiotic apocalypse, a road map for integrated microbial management. *Evolutionary Applications, 12*(6), 1079-1091. doi:10.1111/eva.12808

Risely, A., Klaassen, M., & Hoye, B. J. (2018). Migratory animals feel the cost of getting sick: A meta-analysis across species. *Journal of Animal Ecology, 87*(1), 301-314. doi:10.1111/1365-2656.12766

Risely, A., Waite, D., Ujvari, B., Klaassen, M., & Hoye, B. (2017). Gut microbiota of a long-distance migrant demonstrates resistance against environmental microbe incursions. *Molecular Ecology, 26*(20), 5842-5854.

Ryu, H., Henson, M., Elk, M., Toledo-Hernandez, C., Griffith, J., Blackwood, D., Noble, R., Gourmelon, M., Glassmeyer, S., & Santo Domingo, J. W. (2013). Development of quantitative PCR assays targeting the 16S rRNA genes of *Enterococcus* spp. and their application to the identification of *Enterococcus* species in environmental samples. *Applied and Environmental Microbiology, 79*(1), 196-204. doi:10.1128/AEM.02802-12

Santos, S., Pardal, S., Proença, D. N., Lopes, R. J., Ramos, J. A., Mendes, L., & Morais, P. V. (2012). Diversity of cloacal microbial community in migratory shorebirds that use the Tagus estuary as stopover habitat and their potential to harbor and disperse pathogenic microorganisms. *FEMS Microbiology Ecology, 82*(1), 63-74.

Santos, T., Silva, N., Igrejas, G., Rodrigues, P., Micael, J., Rodrigues, T., Resendes, R., Goncalves, A., Marinho, C., Goncalves, D., Cunha, R., & Poeta, P. (2013). Dissemination of antibiotic resistant *Enterococcus* spp. and *Escherichia coli* from wild birds of Azores Archipelago. *Anaerobe, 24*, 25-31. doi:10.1016/j.anaerobe.2013.09.004

Smith, H. G., Bean, D. C., Hawkey, J., Clarke, R. H., Loyn, R., Larkins, J.-A., Hassel, C., Valcanis, M., Pitchers, W., & Greenhill, A. R. (2020). Detection and characterisation of *Salmonella enterica* serovar Hvittingfoss in Bar-tailed Godwits (*Limosa lapponica*) from Roebuck Bay, north-western Australia. *Applied and Environmental Microbiology*.

Smith, H. G., Clarke, R. H., Larkins, J.-A., Bean, D. C., & Greenhill, A. R. (2019). Wild Australian birds and drug-resistant bacteria: characterisation of antibiotic-resistant *Escherichia coli* and *Enterococcus* spp. *Emu-Austral Ornithology, 119*(4), 384-390.

Splichalova, P., Svec, P., Ghosh, A., Zurek, L., Oravcova, V., Radimersky, T., Bohus, M., & Literak, I. (2015). Prevalence, diversity and characterization of enterococci from three coraciiform birds. *Antonie van Leeuwenhoek, 107*(5), 1281-1289.

Studds, C. E., Kendall, B. E., Murray, N. J., Wilson, H. B., Rogers, D. I., Clemens, R. S., Gosbell, K., Hassell, C. J., Jessop, R., Melville, D. S., Milton, D. A., Minton, C. D., Possingham, H. P., Riegen, A. C., Straw, P., Woehler, E. J., & Fuller, R. A. (2017). Rapid population decline in migratory shorebirds relying on Yellow Sea tidal mudflats as stopover sites. *Nature Communications, 8*, 14895. doi:10.1038/ncomms14895

Torres, R. T., Carvalho, J., Cunha, M. V., & Fonseca, C. (2019). Antimicrobial resistance and ecology: a dialog yet to begin. *EcoHealth, 16*(3), 402-403.

Wauchope, H. S., Shaw, J. D., Varpe, O., Lappo, E. G., Boertmann, D., Lanctot, R. B., & Fuller, R. A. (2017). Rapid climate-driven loss of breeding habitat for Arctic migratory birds. *Global Change Biology, 23*(3), 1085-1094. doi:10.1111/gcb.13404

Weller, D. R., & Lee, C. (2017). Migratory Shorebird Conservation Action Plan. *BirdLife Australia unpublished report. BirdLife Australia: Carlton*.

Wright, G. D. (2010). Antibiotic resistance in the environment: a link to the clinic? *Current Opinion in Microbiology, 13*(5), 589-594.

Yong, D. L., Jain, A., Liu, Y., Iqbal, M., Choi, C. Y., Crockford, N. J., Milington, S., & Provencher, J. (2018). Challenges and opportunities for transboundary conservation of migratory birds in the East Asian-Australasian flyway. *Conservation Biology, 32*(3), 740-743. doi:10.1111/cobi.13041

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Foreword to Chapter 4

With the results of Chapter 3 providing valuable information into the AMR present in *E. coli, Enterococcus* and *Salmonella* recovered from wild Australian shorebirds, a detailed investigation into the genetics of one of the target bacterial species was conducted. Chapter 4 consists of one pre-publication manuscript that investigates the genetic diversity of *E. coli* isolated from wild Australian shorebirds. This study investigated the pool of virulence and AMR genes present in 16 *E. coli* isolates that had undergone WGS. These isolates had been investigated previously in Chapter 3, and were selected based on their AMR profiles, with a secondary consideration to sequence isolates from a variety of bird species that covered both state sampled in and whether the birds were migratory or sedentary. The Sequence Types (STs) were investigated to gain insights into potential sites of origin, other host species and potential pathogenicity of the isolates.

A total of 33 virulence genes were identified, of which 22 were present in a single isolate. Two of the three most common virulence genes identified were associated with survival; *gad* conferred increased acid resistance, which is theorised to assist in survival in birds due to the high acidities of their stomachs, while *iss* conferred increased serum survival. *lpfA*, the third most common virulence gene, was an adhesive factor associated with enterohemorrhagic *E. coli*. There was a small pool of AMR genes present in the *E. coli* investigated, with one gene (*mdfA*) being found in all isolates. The resistance genes *qnrB65* and *dfrA5* were of interest- *qnrB65* encodes for resistance to quinolones, and *dfrA5* to trimethoprim, both of which are clinically important classes of antibiotics. The presence of these genes in shorebirds is of concern, as they descend from clinical settings; this indicates that shorebirds are coming into contact with anthropogenically associated bacteria in an unknown fashion. This was corroborated by the ST's present; in particular, ST155 has been identified to frequently occur in avian pathogenic *E. coli* (APEC) and human extraintestinal pathogenic *E. coli* (ExPEC) lineages. ST analysis indicated that *E. coli* populations in shorebirds are diverse, with multiple different geographic origins and host species identified for the different STs. In addition, a novel ST was identified, ST11348. These findings indicate a wide range of genetic diversity in *E.*

coli populations present in shorebirds, and that some of these *E. coli* are potentially zoonotic pathogens. As such, the continued monitoring of shorebird populations is necessary to track how pathogenic bacteria may be spreading in the environment.

4. Genetic characterisation of *Escherichia coli* isolated from migratory and sedentary wild Australian shorebirds

Genetic characterisation of *Escherichia coli* isolated from migratory and sedentary wild Australian shorebirds

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4.1 Abstract

While most strains of *Escherichia coli* are commensals, some strains are clinically important, causing disease in humans and other animals. It is genetic determinants that dictate whether *E. coli* is a commensal or a pathogen. The diversity of *E. coli* in wild Australian shorebirds is relatively unknown, but shorebirds present a potential reservoir for lineages and genes of clinical importance. This study sought to gain further knowledge about strains of *E. coli* isolated from Australian shorebirds, by conducting genotyping and investigating virulence and genetic mechanisms of antimicrobial resistance (AMR) in isolates from ten species of Australian shorebird. Of a total of 206 *E. coli* isolated from wild Australian shorebirds, 16 isolates underwent whole genome

sequencing (WGS). They were then analysed for serotype, sequence type (ST), AMR genes and virulence genes. There was a diversity of STs present, with each isolate having a unique ST. One isolate belonged to ST155 and another to ST141, both of which are associated with disease in poultry. A total of 33 recognised virulence genes were detected, with 22 of these genes being present in a single isolate. The virulence genes most commonly detected were *gad, lpfA* and *iss*. A total of eight AMR genes were detected, with *mdfA* occurring in all isolates. Of note was the detection of the trimethoprim resistance gene *dfrA5* and the quinolone resistance gene *qnrB65.* The diversity of *E. coli* present in wild shorebirds, and the presence of clinically important AMR genes indicates that wild populations are capable of harbouring *E. coli* that is potentially pathogenic and could play a role in the transmission of clinically important AMR and virulence genes. There is a need to monitor wild shorebirds to determine if these species play a role in the dissemination of *E. coli* of clinical importance.

4.2 Introduction

E. coli, a Gram negative enteric bacteria that colonises a wide range of hosts and persists in a multiplicity of environments, is an incredibly diverse species (Souza et al., 1999). While the majority of *E. coli* are commensal, some strains are pathogenic and are specialised to cause disease in specific hosts (Denton, 2007, Johnson, 1991). Characterisation of strains of *E. coli* in host species can help establish whether the bacteria belong to zoonotic or pathogenic lineages of concern, or are commensal organisms (Fratamico et al., 2016, Joensen et al., 2015). It is becoming more evident that the health of wildlife, the environment, and humans are all linked, with One Health approaches taking these intertwined factors into consideration when developing plans to address the increasing spread of AMR and disease in both the environment and the community (Marcelino et al., 2019). The lineages of *E. coli* present in wild animal populations in Australia and the broader Pacific region are not well known, and to date studies in Australia have focused on domestic animals or wild populations impacted by human activity (Gordon & Cowling 2003, Blyton et al., 2015). The potential virulence gene pool of *E. coli* in Australian wildlife has also been understudied (Blyton et al., 2015, Marcelino et al., 2019), leaving large gaps in our knowledge. It is important to

understand the distribution of potentially pathogenic or zoonotic strains in such populations, due to the risk these lineages may present to human populations, with consideration to human-associated *E. coli*. This risk also extends to livestock and wild populations, with some strains associated with specific hosts (Ewers et al., 2009). Along with the danger posed by pathogenic *E. coli*, the threat of antimicrobial resistance (AMR) is an increasing problem for both human and animal health. Infections caused by AMR bacteria are linked to increased mortality and economic burdens (Alvarez-Uria et al., 2018), and resistance continues to worsen despite improving management and surveillance (Kahn, 2017). There is increasing interest in the potential of wild birds, especially migratory birds, to disseminate antimicrobial resistance (AMR) through their travels. Global studies on migratory birds have shown that these populations can carry AMR bacteria (Foti et al., 2011, Baez et al., 2015, Raza et al., 2017). The bacteria of Australian migratory bird populations (especially those in remote areas) remain understudied (Marcelino et al., 2019, Smith et al., 2019, Blyton et al., 2015). Further research is required to assess if migratory birds coming to Australia are acting as reservoirs for AMR and virulence genes, and if so, how they may be disseminating these genes into the wider environment (Keseler et al., 2005).

The health of many wild animal species is under threat globally (Daszak, Cunningham, & Hyatt, 2000). Many shorebird species in Australia are negatively impacted by human activity through habitat loss, degradation (Studds et al., 2017) and climate change (Wauchope et al., 2017), and are exposed to pathogenic lineages and AMR genes that have arisen from anthropogenic activity (Marcelino et al., 2019). The potential for these populations to acquire and disseminate clinically important *E. coli* is a driving factor in our need to understand what potential pathogenic *E. coli* may be colonising Australian shorebird populations.

We isolated and studied enteric bacteria, including *E. coli,* for presence of AMR (Smith et al., in review). We then selected 16 *E. coli* isolates, guided by phenotypic AMR observations, and conducted WGS on those isolates to look for genetic determinants of AMR, and genes associated with virulence in either humans or birds. As an observational

study, the aim was to conduct characterisation of *E. coli* from shorebirds in Australia to determine whether this niche harboured bacteria that could be a health risk to animals and/or humans.

4.3 Materials and methods

E. coli were isolated from bird cloacal swabs. Sample collection was conducted as described in Smith et al, 2021. This research was conducted under animal ethics permits issued by Federation University Australia (permit no. 16-002), scientific research permits issued by the Department of Environment, Land, Water and Planning for Victoria (DELWP) (permit no. 10008032), the Department of Environment and Water (DEW) (permit no. 35/2016), and the Department of Parks and Water (DPaW) (permit no. 01-000179-1).

4.3.1 Bacterial culture and antimicrobial susceptibility testing

Swab tips were used to inoculate pre-enrichment media of Brain Heart Infusion broth (Oxoid) for 4 hr at 35°C. This pre-enrichment broth was used to inoculate Mannitol broth (Oxoid), incubated at 35°C for 24 hrs, for the isolation of *E. coli*. Broths were checked for turbidity and colour change, and those positive for colour change were plated onto MacConkey II (Oxoid) plates incubated at 35°C for 24-48 hrs. Suspected *E. coli* were confirmed by biochemical testing, consisting of Gram stain, catalase, oxidase and indole test.

4.3.2 Whole genome sequencing, gene identification and data analysis

DNA extraction was performed on 16 *E. coli* isolates. Isolates were chosen for diversity in host species, host sampling location, and phenotypic AMR results. DNA was collected from overnight colonies using a Qiagen DNeasy kit, and quantified using an Invitrogen Qubit 2. Sequencing was conducted at the Australian Genome Research Facility using the Illumina MiSeq, with library preparation being Illumina gDNA shotgun library preparation with bead size selection protocol, generating 150bp paired-end reads. Raw sequences were uploaded to the Galaxy web platform v 20.01 (Afgan et al., 2018). Genomes were assembled *de novo* using Unicycler (Wick et al., 2017), and genome

assembly quality was analysed using QUAST (Mikheenko et al., 2018). Online services hosted by the Centre for Genetic Epidemiology were then used to investigate the assembled genomes. The virulence genes (Joensen et al., 2014) and antimicrobial resistance genes (Bortolaia et al., 2020) present in the isolates were identified, as well as the MLSTs of each isolate (Larsen et al., 2012). Isolates with novel STs were uploaded to Enterobase (Zhou et al., 2020) to assign a new ST. The *E. coli* ST of each isolate were then investigated using Enterobase and literature searches to determine their most likely host niche, their potential distribution, and whether they were, or were associated with, pathogenic *E. coli* strains.

4.4 Results

4.4.1 MLST profiles and serotypes

All 16 *E. coli* isolates had unique STs, recorded in Table 9. All but one of the 16 STs have been found to be present in a wide range of niches (various animal hosts, food, and the environment), with global distributions. Specifically, 10 STs are commonly associated with birds, with seven previously detected in wild birds. Isolate 1239b was found to have a novel ST of 11348, as assigned by Enterobase. Isolate 626a belonged to ST86, a ST not recorded in Australia to date. Isolate 1158a belonged to ST155, which has been identified as frequently occurring in avian pathogenic *E. coli* (APEC) and human extraintestinal pathogenic *E. coli* (ExPEC) lineages (Maluta et al., 2014).

Serotype analysis based on genomic data (Table 9) revealed that three isolates were serotype H6, two isolates H8, two isolates H10 and a further two isolates H20. Fourteen isolates had unique O antigens, with the remaining two isolates (1060a and 1158a) having no O antigens detected. While none of the H serogroups identified were associated with poultry or wild birds specifically, isolates 932a and 986a were part of the O2 serogroup, a serogroup that is associated with disease in humans and animals (Delannoy et al., 2017, Fratamico et al., 2010). Serotype O28ab:H9 has previously been identified as enterotoxigenic *E. coli* (ETEC) (Begaud, Mondet, & Germani, 1993), while serotypes O185:H16 (Furlan et al. 2019) and O116:H49 have been identified as shiga-toxin-producing *E. coli* (STEC) (Furlan et al., 2019, Costa et al., 2020).

Table 10. The host species, sampling location, MLST, serotype and accession number for the *E. coli* isolates recovered. * indicates no antigen was identified.

Sample ID	Host Species	Latitude / Longitude	MLST	Accession Number	Predicted Serotype
516a	Crested Tern (*Thalasseus bergii*)	-38.277457, 144.768842	10	SAMN13884676	O28ab:H9
626a	Caspian Tern (*Hydroprogne caspia*)	-38.71166667, 46.70444444	86	SAMN13884677	O86:H10
709a	Sanderling (*Calidris alba*)	-19.212794, 121.434314	2520	SAMN13884678	O116:H49
932a	Pied Oystercatcher (*Haematopus longirostris*)	-38.669456, 146.697359	1159	SAMN13884680	O2/O50:H6
939b	Sooty Oystercatcher (*Haematopus fuliginosus*)	-38.656479, 146.727470	68	SAMN13884681	O1:H6
942a	Sooty Oystercatcher	-38.656479, 146.727470	1611	SAMN13884682	O100:H19
986a	Pied Oystercatcher	-38.675844, 146.595594	141	SAMN13884684	O2/O50:H6
1023a	Crested Tern	-38.277457, 144.768842	372	SAMN13884686	O21:H14
1060a	Caspian Tern	-38.277457, 144.768842	602	SAMN13884687	O-:H21
1110b	Sharp-tailed Sandpiper (*Calidris acuminata*)	-38.003826, 144.596880	2280	SAMN13884689	O185:H16
1113b	Sharp-tailed Sandpiper	-38.003826, 144.596880	1851	SAMN13884690	O19:H4
1119a	Curlew Sandpiper (*Calidris ferruginea*)	-38.003826, 144.596880	711	SAMN13884691	O120:H10
1156a	Red-capped Plover (*Charadrius ruficapillus*)	-38.276219, 145.313470	154	SAMN13884693	O142:H8
1158a	Bar-tailed Godwit (*Limosa lapponica*)	-19.212794, 121.434314	155	SAMN13884694	O-:H20
1232a	Pied Oystercatcher	-17.963887, 122.279828	191	SAMN13884695	O150:H20
1239b	Ruddy Turnstone (*Arenaria interpres*)	-17.979327, 122.336533	11348	SAMN13884696	O83:H8

4.4.2 Virulence genes

A total of 32 virulence genes were detected in the *E. coli* isolates. Table 10 displays all the genes that were present in more than one isolate. Twenty-four of these genes were present in individual isolates; these genes were *pic, terC, papC, celb, f17A, f17G, air, eilA, tsh, cea, chuA, cfn1, focCsfaE, focG, focl, fyuA, hra, ibeA, irp2, ompT, papA_F13, sitA, usp and yfcV.* One isolate, 1023a, contained 22 of 33 virulence genes detected. The isolates with the next highest number of virulence genes were 626a and 986a, both of which contained ten virulence genes. Of the virulence genes present in multiple isolates, three (*mchB*, *mchC, mcmA*) are associated with the synthesis of inhibitory toxins against bacterial growth. Two of the genes present in eleven separate isolates (*gad* and *iss*) are associated with increased persistence in hosts.

Table 11. The virulence genes present in *E. coli* isolates from wild birds.

Virulence gene	Name	Role / Function	No. isolates present in	Reference
gad	Glutamate decarboxylase	Confers increased acid resistance (up to pH 2.5)	11	(Castanie-Cornet et al., 1999)
iss	Increased serum survival	Increases complement resistance and virulence	11	(Johnson, Wannemuehler, & Nolan, 2008)
lpfA	Long polar fimbriae	Adhesive factor of enterohemorrhagic *E. coli*	11	(Dogan et al., 2012)
iroN	Enterobactin siderophore receptor protein	Contributes to invasion of urothelial cells	4	(Feldmann et al., 2007)
vat	Vacuolating autotransporter toxin	Synthesis of an autotransporter serine protease toxin	4	(Spurbeck et al., 2012)
mchC	Bacteriocin synthesis	Synthesis of toxins to inhibit bacterial growth	2	(Kurnick et al., 2019)
mcmA	Microcin M part of colicin H	Synthesis of toxins to inhibit bacterial growth	2	(Kurnick et al., 2019)
mchB	Microcin H47 part of colicin H	Synthesis of toxins to inhibit bacterial growth	2	(Kurnick et al., 2019)

4.4.3 AMR genes and resistance profiles

Eight antimicrobial resistance genes were identified in total. All 16 isolates contained at least one AMR gene, with the resistance gene *mdfA* present in all isolates (Table 11). All other AMR genes were present only in two isolates, 626a and 1060a. The phenotypical AMR profiles of the sequenced isolates can be found in Smith et al. (in review). Isolate 1060a demonstrated no phenotypic resistance patterns, while isolate 626a was phenotypically resistant to amoxycillin,

ampicillin, streptomycin, and sulfamethoxazole-trimethoprim. Eight of the 16 isolates displayed no phenotypic AMR patterns. Seven of the eight AMR genes identified were associated with antibiotic inactivation or target alteration, while the most commonly detected AMR gene *mdfA* is associated with drug efflux when upregulated.

Table 12. AMR genes present in the *E. coli* isolates recovered from wild Australian shorebirds.

AMR Gene	Resistance conferred	Phenotypic resistance	Resistance mechanism	Isolates present in
mdfA	Multidrug resistance	626a: AMC, AMP, S, SXT 1060a: None	Multidrug transporter (Edgar & Bibi 1997)	All isolates
aph(3")-lb	Aminoglycosides	626a: S 1060a: None	Aminoglycoside-modifying Enzyme (Zeng & Jin 2003)	626a
aph(6)-ld	Aminoglycosides	626a: S 1060a: None	Streptomycin phosphotransferase (Ashenafi et al., 2014)	626a
*bla*TEM-1B	Beta-lactams	626a: None 1060a: None	Cephalosporin hydrolysing enzyme (Peixe et al., 1997)	626a
sul2	Sulfanomides	626a: SXT 1060a: None	Targets the enzyme dihydropteroate synthase (DHPS) in the folic acid pathway (Sköld, 2000)	626a
dfrA5	Trimethoprim	626a: SXT 1060a: None	Antibiotic target replacement (McArthur et al., 2013)	626a
*bla*CMY-98	Beta-lactams	626a: None 1060a: None	Serine beta-lactamase, specificity for cephalosporins (Consortium, 2019)	1060a
qnrB65	Quinolones	626a: None 1060a: None	Antibiotic target protection (McArthur et al., 2013)	1060a

AMC=Amoxycillin, AMP=Ampicillin, S=Streptomycin, SXT=Sulfamethoxazole-trimethoprim.

4.5 Discussion

This study demonstrates that wild Australian shorebirds harbour a diversity of *E. coli* strains, including a newly identified serotype (ST 11348). Many of the STs detected colonize multiple host species and the environment, such as STs 1611, 68 and 154. However, some STs are associated with both domestic and wild birds, such as STs 155, 141 and 2520. While the majority of strains identified in this study have no specific links with disease, isolates belonging to STs 155, 602 and 141 have been linked to disease in humans and livestock (ST602) or disease in poultry and birds (STs 155 and 141). All STs bar ST11348 are found globally.

Each sequenced *E. coli* isolate was serotypically unique. This diversity is potentially indicative of a high degree of movement in the *E. coli* communities present in wild shorebirds, and for *E. coli* in wild populations in general. Shorebirds are renowned for their high movement potential, with millions of birds each year leaving Australia to migrate to breeding grounds scattered across India, Asia, and the high Artic (Yong et al., 2018). As they migrate, these birds encounter selection pressures along their migratory route through the EAAF. For shorebirds that breed in the Artic, they pass over one-third of the human population and travel through heavily developed and degraded environments (Studds et al., 2017). Land reclamation, habitat destruction and pollution are present in this flyway (Zhu et al., 2016). Such human activity has the potential to expose the birds to bacteria with AMR resistance genes and/or virulence genes, thus shorebirds could act as vectors for further dissemination. Fortunately, this study did not reveal a widespread problem in this regard and does not suggest a site of origin of the *E. coli* strains or genetic mechanisms of virulence or AMR.

Various genetic mechanisms of AMR were detected in *E. coli* isolates, and their presence did not always correlate with phenotypic resistance. While such observations are neither new nor surprising, it does demonstrate the potential for AMR genes to be much more widespread in wild populations than previously expected from traditional culture-based microbiology studies. Previous studies into the occurrence of AMR genes in non-shorebird Australian species have found that AMR genes are present, including

resistance to critically important antibiotics, in birds exposed to anthropogenic activity such as Silver Gulls (Vangchhia et al., 2016, Mukerji et al., 2019), and in birds living in extremely remote areas (Marcelino et al. 2019, Smith et al., 2019, Smith et al., 2020).

Some of the genes identified in this study, such as *blaTEM*, *sul2* and *dfrA5* have been previously detected in bacteria from Australian shorebirds (Marcelino et al., 2019), and *blaCMY* genes have been observed in bacteria from shorebirds in Europe (Veldman et al., 2013). Of interest is the presence of *mdfA* in all of the isolates sequenced. This gene was not present in any previous studies of wild Australian birds. While *mdfA* can contribute to antibiotic resistance, it must be upregulated to do so (Edgar & Bibi 1997). Considering this, when paired with the phenotypic AMR profiles of the isolates tested, it is possible that this gene is either inactive, or not upregulated to levels required for the efflux of antibiotics. As mentioned previously, this gene demonstrates the potential problem of spreading widely through different bird and animal populations.

Of note is the detection of *qnrB65* in one isolate; as a resistance gene to a chemically derived antibiotic, it is probable that this gene originated from use of quinolones in clinical settings rather than occurring naturally in the environment. The quinolone enrofloxacin has been used previously on injured wildlife in Australia (Blyton et al., 2015), and this may explain its origin- however, it is still worrying to find quinolone resistance in bacteria from wild birds that have little direct human association. This resistance gene has not been noted in Australian shorebirds previously (Marcelino et al., 2019), though it was identified in other Australian species (Blyton et al., 2015). These findings provide more evidence that AMR genes in *E. coli*- and potentially, all enteric bacteria- are persisting in animal populations in the absence of continued antibiotic pressure.

The virulence genes *gad*, *iss* and *lpfA* were found in 11 of the 16 isolates sequenced, though in addition to contributing to virulence these genes play roles in general survival. In particular, *gad* confers increased acid resistance; this may be an adaptation by enteric *E. coli* living in birds due to the high acidity of their stomachs (anywhere from pH 1.2-3.0, dependant on species (Beasley et al., 2015). The majority of the isolates recovered

contained only a small number of virulence genes, which is a positive finding for the general health of the wild populations studied. A number of the virulence genes identified (*ompT, sitA, iroN*) have been previously identified in wild Australian bird populations (Blyton et al., 2015, Mukerji et al., 2019).

One isolate, 1023a (isolated from a Crested Tern), contained 22 of all 33 virulence genes isolated. This isolate belonged to ST372, which is associated with infections in humans and domestic dogs (Melo et al., 2017, Valat et al., 2020), and its presence in wild birds could pose two threats. First, virulent, pathogenic *E. coli* strains of anthropogenic origin are entering wild bird populations. Secondly, shorebirds may act as vectors for the continued spread of such clinically important strains. Such strains could then potentially be re-introduced into livestock, domestic animals and community populations through interactions with shorebirds (Santos et al., 2012, Dusek et al., 2014).

An isolate (986a, isolated from a Pied Oystercatcher) belonging to ST141, is also associated with disease. This lineage is zoonotic, with a wide range of hosts including seals in Antarctica (Mora et al., 2018). Of note is the host species of these isolates, Pied Oystercatcher and Crested Tern, both species are sedentary and have spatially restricted home ranges when compared to migrants. Crested Terns typically have foraging ranges of <40km (McLeay et al., 2010) while Pied Oystercatchers will typically remain within 30km of their home territory with small movements between estuaries (Taylor et al., 2014). It has been hypothesised that migratory birds are negatively impacted by the carriage of infectious or virulent bacteria, with migratory culling and migratory separation impacting bird survival (Risely, Klaassen, & Hoye, 2018, Altizer, Bartel, & Han 2011). This leads to the act of migration itself lowering disease prevalence in migratory populations (Johns & Shaw, 2016); sedentary populations however, do not experience this reductive effect. This may be one reason for why these virulent strains of *E. coli* were found in sedentary birds, rather than migrants.

4.6 Conclusion

This study shows that wild Australian shorebirds are capable of carrying a diverse range of *E. coli*, some of which carry AMR genes of clinical importance. While there was a relatively low number of AMR genes detected in these isolates, the presence of resistance genes that code against clinically important antibiotics (*dfrA5, blaCMY-98, qnrB65*) shows that resistance is still a concern in wild shorebird populations. The diversity of virulence genes recovered also points to a potentially large virulence pool circulating among *E. coli* in these populations which may impact the health of, and future conservation efforts for, these populations. Routine targeted surveillance programs are needed for Australian shorebird populations to monitor for new potential pathogenic *E. coli* lineages emerging in these wild populations.

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4.8 Conflict of Interest Statement

The authors declare they have no conflicts of interest.

4.9 References

Afgan, E., Dannon B., Bérénice B., Marius B., Dave B., Martin Č., John C., Dave C., Nate C., & Björn A. (2018). The Galaxy platform for accessible, reproducible and collaborative biomedical analyses: 2018 update. *Nucleic acids research, 46*(W1):W537-W544.

Altizer, S., Bartel, R., & Han, B. A. (2011). Animal migration and infectious disease risk. *Science, 331*(6015), 296-302. doi:10.1126/science.1194694

Alvarez-Uria, G., Gandra, S., Mandal, S., & Laxminarayan, R. (2018). Global forecast of antimicrobial resistance in invasive isolates of *Escherichia coli* and *Klebsiella pneumoniae*. *International Journal of Infectious Diseases, 68*, 50-53. doi:10.1016/j.ijid.2018.01.011

Ashenafi, M., Ammosova, T., Nekhai, S., & Byrnes, W. M. (2014). Purification and characterization of aminoglycoside phosphotransferase APH (6)-Id, a streptomycin-inactivating enzyme. *Molecular and Cellular Biochemistry, 387*(1-2), 207-216.

Baez, J., Hernandez-Garcia, M., Guamparito, C., Diaz, S., Olave, A., Guerrero, K., Canton, R., Baquero, F., Gahona, J., Valenzuela, N., Del Campo, R., & Silva, J. (2015). Molecular characterization and genetic diversity of ESBL-producing *Escherichia coli* colonizing the migratory Franklin's gulls (*Leucophaeus pipixcan*) in Antofagasta, North of Chile. *Microbial Drug Resistance, 21*(1), 111-116. doi:10.1089/mdr.2014.0158

Beasley, D. E., Koltz, A. M., Lambert, J. E., Fierer, N., & Dunn, R. R. (2015). The evolution of stomach acidity and its relevance to the human microbiome. *PLoS ONE, 10*(7), e0134116.

Begaud, E., Mondet, D., & Germani, Y. (1993). Molecular characterization of enterotoxigenic *Escherichia coli* (ETEC) isolated in New Caledonia (value of potential protective antigens in oral vaccine candidates). *Research in Microbiology, 144*(9), 721-728.

Blyton, M. D., Pi, H., Vangchhia, B., Abraham, S., Trott, D. J., Johnson, J. R., & Gordon, D. M. (2015). Genetic Structure and Antimicrobial Resistance of *Escherichia coli* and Cryptic Clades in Birds with Diverse Human Associations. *Applied and Environmental Microbiology, 81*(15), 5123-5133. doi:10.1128/AEM.00861-15

Bortolaia, V., Kaas, R. S., Ruppe, E., Roberts, M. C., Schwarz, S., Cattoir, V., Philippon, A., Allesoe, R., Rebelo, A., & Florensa, A. F. (2020). ResFinder 4.0 for predictions of phenotypes from genotypes. *Journal of Antimicrobial Chemotherapy, 75*(12), 3491-3500.

Castanie-Cornet, M.-P., Penfound, T. A., Smith, D., Elliott, J. F., & Foster, J. W. (1999). Control of acid resistance in *Escherichia coli*. *Journal of Bacteriology, 181*(11), 3525-3535.

Costa, M., Pracca, G., Sucari, A., Galli, L., Ibargoyen, J., Gentiluomo, J., Brusa, V., Zugazua, M. M., Figueroa, Y., & Londero, A. (2020). Comprehensive evaluation and implementation of improvement actions in bovine abattoirs to reduce pathogens exposure. *Preventive Veterinary Medicine 176*, 104933.

Daszak, P., Cunningham, A. A., & Hyatt, A. D. (2000). Emerging infectious diseases of wildlife--threats to biodiversity and human health. *Science, 287*(5452), 443-449.

Delannoy, S., Beutin, L., Mariani-Kurkdjian, P., Fleiss, A., Bonacorsi, S., & Fach, P. (2017). The *Escherichia coli* serogroup O1 and O2 lipopolysaccharides are encoded by multiple O-antigen gene clusters. *Frontiers in Cellular and Infection Microbiology, 7*, 30.

Denton, M. (2007). *Enterobacteriaceae*. *International Journal of Antimicrobial Agents, 29 Suppl 3*(0), S9-S22. doi:10.1016/S0924-8579(07)72174-X

Dogan, B., Rishniw, M., Bruant, G., Harel, J., Schukken, Y. H., & Simpson, K. W. (2012). Phylogroup and *lpfA* influence epithelial invasion by mastitis associated *Escherichia coli*. *Veterinary Microbiology, 159*(1-2), 163-170.

Dusek, R. J., Hallgrimsson, G. T., Ip, H. S., Jonsson, J. E., Sreevatsan, S., Nashold, S. W., TeSlaa, J., Enomoto, S., Halpin, R., Lin, X., Fedorova, N., Stockwell, T., Dugan, V., Wentworth, D., & Hall, J. S. (2014). North Atlantic migratory bird flyways provide routes for

intercontinental movement of avian influenza viruses. *PLoS ONE, 9*(3), e92075. doi:10.1371/journal.pone.0092075

Edgar, R., & Bibi, E. (1997). m*dfA*, an *Escherichia coli* multidrug resistance protein with an extraordinarily broad spectrum of drug recognition. *Journal of Bacteriology, 179*(7), 2274-2280.

Ewers, C., Antao, E. M., Diehl, I., Philipp, H. C., & Wieler, L. H. (2009). Intestine and environment of the chicken as reservoirs for extraintestinal pathogenic *Escherichia coli* strains with zoonotic potential. *Applied and Environmental Microbiology, 75*(1), 184-192. doi:10.1128/AEM.01324-08

Feldmann, F., Sorsa, L. J., Hildinger, K., & Schubert, S. (2007). The salmochelin siderophore receptor IroN contributes to invasion of urothelial cells by extraintestinal pathogenic *Escherichia coli* in vitro. *Infection and Immunity, 75*(6), 3183-3187.

Foti, M., Rinaldo, D., Guercio, A., Giacopello, C., Aleo, A., De Leo, F., Fisichella, V., & Mammina, C. (2011). Pathogenic microorganisms carried by migratory birds passing through the territory of the island of Ustica, Sicily (Italy). *Avian Pathology, 40*(4), 405-409. doi:10.1080/03079457.2011.588940

Fratamico, P. M., DebRoy, C., Liu, Y., Needleman, D. S., Baranzoni, G. M., & Feng, P. (2016). Advances in molecular serotyping and subtyping of *Escherichia coli*. *Frontiers in Microbiology, 7*, 644.

Fratamico, P. M., Yan, X., Liu, Y., DebRoy, C., Byrne, B., Monaghan, A., Fanning, S., & Bolton, D. (2010). *Escherichia coli* serogroup O2 and O28ac O-antigen gene cluster sequences and detection of pathogenic *E. coli* O2 and O28ac by PCR. *Canadian Journal of Microbiology, 56*(4), 308-316.

Furlan, J., Gallo, I., de Campos, A., Navarro, A., Kobayashi, R., Nakazato, G., & Stehling, E. G. (2019). Characterization of non-O157 Shiga toxin-producing *Escherichia coli* (STEC) obtained from feces of sheep in Brazil. *World Journal of Microbiology and Biotechnology, 35*(9), 134.

Gordon, D. M., & Cowling, A. (2003). The distribution and genetic structure of *Escherichia coli* in Australian vertebrates: host and geographic effects. *Microbiology, 149*(Pt 12), 3575-3586. doi:10.1099/mic.0.26486-0

Joensen, K. G., Tetzschner, A. M., Iguchi, A., Aarestrup, F. M., & Scheutz, F. (2015). Rapid and easy in silico serotyping of *Escherichia coli* isolates by use of whole-genome sequencing data. *Journal of clinical microbiology*, *53*(8), 2410-2426.

Joensen, K. G., Scheutz, F., Lund, O., Hasman, H., Kaas, R. S., Nielsen, E. M., & Aarestrup, F. M. (2014). Real-time whole-genome sequencing for routine typing, surveillance, and outbreak detection of verotoxigenic *Escherichia coli*. *Journal of Clinical Microbiology, 52*(5), 1501-1510.

Johns, S., & Shaw, A. K. (2016). Theoretical insight into three disease-related benefits of migration. *Population Ecology, 58*(1), 213-221.

Johnson, J. R. (1991). Virulence factors in *Escherichia coli* urinary tract infection. *Clinical Microbiology Reviews, 4*(1), 80-128. doi:10.1128/cmr.4.1.80

Johnson, T. J., Wannemuehler, Y. M., & Nolan, L. K. (2008). Evolution of the *iss* gene in *Escherichia coli*. *Applied and Environmental Microbiology, 74*(8), 2360-2369.

Kahn, L. H. (2017). Antimicrobial resistance: a One Health perspective. *Transactions of the Royal Society of Tropical Medicine and Hygine, 111*(6), 255-260. doi:10.1093/trstmh/trx050

Keseler, I. M., Collado-Vides, J., Gama-Castro, S., Ingraham, J., Paley, S., Paulsen, I. T., Peralta-Gil, M., & Karp, P. D. (2005). EcoCyc: a comprehensive database resource for *Escherichia coli*. *Nucleic Acids Research, 33*(suppl_1), D334-D337.

Kurnick, S. A., Mannion, A. J., Feng, Y., Madden, C. M., Chamberlain, P., & Fox, J. G. (2019). Genotoxic *Escherichia coli* Strains Encoding Colibactin, Cytolethal Distending Toxin, and Cytotoxic Necrotizing Factor in Laboratory Rats. *Comparative Medicine, 69*(2), 103-113.

Larsen, M. V., Cosentino, S., Rasmussen, S., Friis, C., Hasman, H., Marvig, R. L., Jelsbak, L., Sicheritz-Ponten, T., Ussery, D., & Aarestrup, F. M. (2012). Multilocus sequence typing of total-genome-sequenced bacteria. *Journal of Clinical Microbiology, 50*(4), 1355-1361.

Maluta, R. P., Logue, C. M., Casas, M. R. T., Meng, T., Guastalli, E. A. L., Rojas, T. C. G., Montelli, A., Sadatsune, T., de Carvalho Ramos, M., & Nolan, L. K. (2014). Overlapped sequence types (STs) and serogroups of avian pathogenic (APEC) and human extra-intestinal pathogenic (ExPEC) *Escherichia coli* isolated in Brazil. *PLoS ONE, 9*(8), e105016.

Marcelino, V. R., Wille, M., Hurt, A. C., González-Acuña, D., Klaassen, M., Schlub, T. E., Eden, J-S., Shi, M., Iredell, J., & Sorrell, T. C. (2019). Meta-transcriptomics reveals a diverse antibiotic resistance gene pool in avian microbiomes. *BMC biology, 17*(1), 1-11.

McArthur, A. G., Waglechner, N., Nizam, F., Yan, A., Azad, M. A., Baylay, A. J., Bhullar, K., Canova, M., De Pascale, G., & Ejim, L. (2013). The comprehensive antibiotic resistance database. *Antimicrobial Agents and Chemotherapy, 57*(7), 3348-3357.

McLeay, L., Page, B., Goldsworthy, S., Paton, D., Teixeira, C., Burch, P., & Ward, T. (2010). Foraging behaviour and habitat use of a short-ranging seabird, the crested tern. *Marine Ecology Progress Series, 411*, 271-283.

Melo, L. C., Boisson, M. N., Saras, E., Médaille, C., Boulouis, H.-J., Madec, J.-Y., & Haenni, M. (2017). OXA-48-producing ST372 *Escherichia coli* in a French dog. *Journal of Antimicrobial Chemotherapy, 72*(4), 1256-1258.

Mikheenko, A., Prjibelski, A., Saveliev, V., Antipov, D., & Gurevich, A. (2018). Versatile genome assembly evaluation with QUAST-LG. *Bioinformatics, 34*(13), i142-i150.

Mora, A., García-Peña, F. J., Alonso, M. P., Pedraza-Diaz, S., Ortega-Mora, L. M., Garcia-Parraga, D., Lopez, C., Viso, S., Dahbi, G., & Marzoa, J. (2018). Impact of human-associated *Escherichia coli* clonal groups in Antarctic pinnipeds: presence of ST73, ST95, ST141 and ST131. *Scientific Reports, 8*(1), 1-11.

Mukerji, S., Stegger, M., Truswell, A. V., Laird, T., Jordan, D., Abraham, R. J., Harb, A., Barton, M., O'Dea, M., & Abraham, S. (2019). Resistance to critically important antimicrobials in Australian silver gulls (*Chroicocephalus novaehollandiae*) and evidence of anthropogenic origins. *Journal of Antimicrobial Chemotherapy, 74*(9), 2566-2574.

Peixe, L., Sousa, J., Perez-Diaz, J., & Baquero, F. (1997). A bla (TEM-1b)-derived TEM-6 beta-lactamase: a case of convergent evolution. *Antimicrobial Agents and Chemotherapy, 41*(5), 1206.

Raza, S., Mohsin, M., Madni, W. A., Sarwar, F., Saqib, M., & Aslam, B. (2017). First Report of bla CTX-M-15-Type ESBL-Producing *Klebsiella pneumoniae* in Wild Migratory Birds in Pakistan. *EcoHealth, 14*(1), 182-186. doi:10.1007/s10393-016-1204-y

Risely, A., Klaassen, M., & Hoye, B. J. (2018). Migratory animals feel the cost of getting sick: A meta-analysis across species. *Journal of Animal Ecology, 87*(1), 301-314. doi:10.1111/1365-2656.12766

Santos, S., Pardal, S., Proença, D. N., Lopes, R. J., Ramos, J. A., Mendes, L., & Morais, P. V. (2012). Diversity of cloacal microbial community in migratory shorebirds that use the Tagus estuary as stopover habitat and their potential to harbor and disperse pathogenic microorganisms. *FEMS Microbiology Ecology, 82*(1), 63-74.

Sköld, O. (2000). Sulfonamide resistance: mechanisms and trends. *Drug Resistance Updates, 3*(3), 155-160.

Smith, H. G., Bean, D. C., Hawkey, J., Clarke, R. H., Loyn, R., Larkins, J.-A., Hassel, C., Valcanis, M., Pitchers, W., & Greenhill, A. R. (2020). Detection and characterisation of *Salmonella enterica* serovar Hvittingfoss in Bar-tailed Godwits (*Limosa lapponica*) from Roebuck Bay, north-western Australia. *Applied and Environmental Microbiology*.

Smith, H. G., Clarke, R. H., Larkins, J.-A., Bean, D. C., & Greenhill, A. R. (2019). Wild Australian birds and drug-resistant bacteria: characterisation of antibiotic-resistant *Escherichia coli* and *Enterococcus* spp. *Emu-Austral Ornithology, 119*(4), 384-390.

Souza, V., Rocha, M., Valera, A., & Eguiarte, L. E. (1999). Genetic structure of natural populations of *Escherichia coli* in wild hosts on different continents. *Applied and Environmental Microbiology, 65*(8), 3373-3385.

Spurbeck, R. R., Dinh, P. C., Walk, S. T., Stapleton, A. E., Hooton, T. M., Nolan, L. K., Kim, K., Johnson, J., & Mobley, H. L. (2012). *Escherichia coli* isolates that carry *vat, fyuA, chuA,* and *yfcV* efficiently colonize the urinary tract. *Infect Immun, 80*(12), 4115-4122.

Studds, C. E., Kendall, B. E., Murray, N. J., Wilson, H. B., Rogers, D. I., Clemens, R. S., Gosbell, K., Hassell, C. J., Jessop, R., Melville, D. S., Milton, D. A., Minton, C. D., Possingham, H. P., Riegen, A. C., Straw, P., Woehler, E. J., & Fuller, R. A. (2017). Rapid population decline in migratory shorebirds relying on Yellow Sea tidal mudflats as stopover sites. *Nature Communications, 8*, 14895. doi:10.1038/ncomms14895

Taylor, I. R., Newman, O. M. G., Park, P., Hansen, B., Minton, C. D., Harrison, A., & Jessop, R. (2014). Conservation assessment of the Australian Pied Oystercatcher *Haematopus longirostris*. *International Wader Studies, 20*, 116-128.

UniProt Consortium, U. (2019). UniProt: a worldwide hub of protein knowledge. *Nucleic acids research, 47*(D1), D506-D515.

Valat, C., Drapeau, A., Beurlet, S., Bachy, V., Boulouis, H.-J., Pin, R., Cazeau, G., Madec, J-Y., & Haenni, M. (2020). Pathogenic *Escherichia coli* in Dogs Reveals the Predominance of ST372 and the Human-Associated ST73 Extra-Intestinal Lineages. *Frontiers in Microbiology, 11*, 580.

Vangchhia, B., Abraham, S., Bell, J. M., Collignon, P., Gibson, J. S., Ingram, P. R., Johnson, J., Kennedy, K., Trott, D., & Turnidge, J. D. (2016). Phylogenetic diversity, antimicrobial susceptibility and virulence characteristics of phylogroup F *Escherichia coli* in Australia. *Microbiology, 162*(11), 1904-1912.

Veldman, K., van Tulden, P., Kant, A., Testerink, J., & Mevius, D. (2013). Characteristics of cefotaxime-resistant *Escherichia coli* from wild birds in the Netherlands. *Applied and Environmental Microbiology, 79*(24), 7556-7561. doi:10.1128/AEM.01880-13

Wauchope, H. S., Shaw, J. D., Varpe, O., Lappo, E. G., Boertmann, D., Lanctot, R. B., & Fuller, R. A. (2017). Rapid climate-driven loss of breeding habitat for Arctic migratory birds. *Global Change Biology, 23*(3), 1085-1094. doi:10.1111/gcb.13404

Wick, R. R., Judd, L. M., Gorrie, C. L., & Holt, K. E. (2017). Unicycler: resolving bacterial genome assemblies from short and long sequencing reads. *PLoS Computational Biology, 13*(6), e1005595.

Yong, D. L., Jain, A., Liu, Y., Iqbal, M., Choi, C. Y., Crockford, N. J., Milington, S., & Provencher, J. (2018). Challenges and opportunities for transboundary conservation of migratory birds in the East Asian-Australasian flyway. *Conservation Biology, 32*(3), 740-743. doi:10.1111/cobi.13041

Zeng, L., & Jin, S. (2003). aph (3')-IIb, a gene encoding an aminoglycoside-modifying enzyme, is under the positive control of surrogate regulator HpaA. *Antimicrobial Agents and Chemotherapy, 47*(12), 3867-3876.

Zhou, Z., Alikhan, N.-F., Mohamed, K., Fan, Y., Achtman, M., Brown, D., Chattaway, M., Dallman, T., Delahay, R., & Kornschober, C. (2020). The EnteroBase user's guide, with case studies on *Salmonella* transmissions, *Yersinia pestis* phylogeny, and *Escherichia* core genomic diversity. *Genome Research, 30*(1), 138-152.

Zhu, G. R., Xie, Z. L., Xu, X. G., Ma, Z. W., & Wu, Y. L. (2016). The landscape change and theory of orderly reclamation sea based on coastal management in rapid industrialization area in Bohai Bay, China. *Ocean & Coastal Management, 133*, 128-137. doi:10.1016/j.ocecoaman.2016.09.016

Foreword to Chapter 5

Chapter 5 consists of the final accepted manuscript of a published article that investigates the finding of a strain of *Salmonella enterica* serovar Hvittingfoss in a flock of Bar-tailed Godwits sampled in Roebuck Bay, Western Australia. The details of the publication are:

Smith, H. G., Bean, D. C., Hawkey, J., Clarke, R. H., Loyn, R., Larkins, J. A., Hassell, C., Valcanis, M., Pitchers W. & Greenhill, A. R. (2020). *Salmonella enterica* serovar Hvittingfoss in Bar-Tailed Godwits (*Limosa lapponica*) from Roebuck Bay, Northwestern Australia. *Applied and Environmental Microbiology*, *86*(19): e01312-20. doi: 10.1128/AEM.01312-20.

These isolates were collected from a single flock of Bar-tailed Godwits that were sampled on the 01/03/19.

WGS was performed on the isolated *S*. Hvittingfoss, and phylogenetic analysis revealed that these isolates were closely related to *S.* Hvittingfoss isolates that had previously caused an outbreak of salmonellosis in 2016. The *S.* Hvittingfoss from the Bar-tailed Godwits differed from the 2016 outbreak isolates by 68 Single Nucleotide Polymorpisms (SNP's), and all 17 of the *S.* Hvittingfoss isolates recovered from the Bar-tailed Godwits were clonal, with 1 SNP difference at maximum.

The 2016 salmonellosis outbreak occurred in all Australian states, and originated from a single farm in the Northern Territory. The original vector of *S.* Hvittingfoss onto the farm was never identified. While the current study was not designed to investigate that transmission event, it does demonstrate that wild birds do carry this important food-borne strain. An outcome of this study is that wild birds cannot be discounted as a vector for *Salmonella*, an extremely important foodborne pathogen. The role of wild birds as a mechanism of transmission of *Salmonella* in foodborne outbreaks should be considered in the future if ecological parameters support such a route of transmission (i.e. for wild birds have access to the produce).

It is unknown where the Bar-tailed Godwits acquired the isolates; however, there was a large sewerage spill that occurred at the same time as the sampling event. This may have been responsible, but it is not possible to positively identify if this was indeed where the birds acquired *S.* Hvittingfoss.

This paper shows that shorebirds can carry clinically important pathogens, and potentially can act as reservoirs for such bacteria. Further studies at a later time point would be required to assess whether Godwits are capable or persistent carriage of *S.* Hvittingfoss. The referencing in this text has been modified from the published manuscript due to the difference in citation practices between the journal and Federation University standards.

1

5. Detection and characterisation of *Salmonella enterica* serovar Hvittingfoss in Bar-tailed Godwits (*Limosa lapponica*)

Detection and characterisation of *Salmonella enterica* serovar Hvittingfoss in Bar-tailed Godwits (*Limosa lapponica*) from Roebuck Bay, north-western Australia.

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Keywords: *Salmonella* Hvittingfoss, shorebirds, zoonotic potential, enteric pathogens, wildlife

5.1 Abstract

Salmonella enterica serovar Hvittingfoss is an important foodborne serotype of *Salmonella*, being detected in many countries where surveillance is conducted. Outbreaks can occur, including a recent multi-state foodborne outbreak in Australia. *S.*

Hvittingfoss can be found in animal populations, though a definitive animal host has not been established. Six species of birds were sampled at Roebuck Bay, a designated Ramsar site in north-western Australia, resulting in 326 cloacal swabs for bacterial culture. A single flock of 63 Bar-tailed Godwit (*Limosa lapponica menzbieri*) caught at Wader Spit, Roebuck Bay in 2018, resulted in 17 (27%) culture-positive for *Salmonella* spp. All other birds were negative for *Salmonella.* The isolates were identified as *Salmonella enterica* serovar Hvittingfoss. Phylogenetic analysis revealed a close relationship between isolates collected from godwits and the *S.* Hvittingfoss responsible for a 2016 multi-state foodborne outbreak originating from tainted cantaloupes (rock melons) in Australia. While it is not possible to determine how this strain of *S.* Hvittingfoss was introduced into the Bar-tailed Godwits, these findings show that wild Australian birds are capable of carrying *Salmonella* strains of public health importance.

5.2 Importance

Salmonella is a zoonotic pathogen that causes gastroenteritis and other disease presentations in both humans and animals. Serovars of *S. enterica* commonly cause foodborne disease in Australia, and globally. In 2016-17, *S.* Hvittingfoss was responsible for an outbreak in that resulted in 110 clinically confirmed human cases throughout Australia. The origins of the contamination that led to the outbreak was never definitively established. Here, we identify a migratory shorebird, the Bar-tailed Godwit, as an animal reservoir of *S*. Hvittingfoss. These birds were sampled in north-western Australia, during their non-breeding period. The presence of a genetically similar *S.* Hvittingfoss strain circulating in a wild bird population, two years after the 2016-17 outbreak and ~1,500 km from the suspected source of the outbreak, demonstrates a potentially unidentified environmental reservoir of *S*. Hvittingfoss. While the birds cannot be implicated in the outbreak 2 years prior, this study does demonstrate the potential role for wild birds in the transmission of this important foodborne pathogen.

5.3 Introduction

Salmonellosis is a disease caused by bacteria of the genus *Salmonella*, and is a major public health concern worldwide (1). *Salmonella* are important zoonotic pathogens that

causes disease in humans, and both domestic and wild animal populations (2, 3). Globally, *Salmonella* spp. are estimated to cause 93.8 million cases of gastroenteritis each year (4), and are one of the most common causes of human mortality associated with foodborne disease (3). The largest burden of salmonellosis is in young children in low- and middle-income countries, with the greatest impacts in Africa, South-eastern Asia, and the Eastern Mediterranean (3). In Australia, an estimated 40,000 salmonellosis cases per year are attributed to contaminated food (5).

The majority of foodborne disease outbreaks are associated with *Salmonella enterica* subsp. *enterica*; serovars of this subspecies are found predominantly in humans and animals and account for ~99% of *Salmonella* infections in humans (2). Globally, the three serovars most commonly isolated from infections in humans are *S.* Enteriditis, *S.* Typhimurium and *S*. Newport, which contribute to 65%, 12% and 4% of clinical isolates, respectively (6).

Salmonella enterica serovar Hvittingfoss has caused outbreaks of salmonellosis in the USA (7), and over the past two decades has been responsible for several outbreaks in Australia, most recently an outbreak in 2016 caused by contaminated cantaloupes (8, 9). In Australia *S.* Hvittingfoss is isolated from human, animal and environmental sources in northern Australia (10, 5); but during outbreaks the organism can be disseminated through the distribution of fresh produce to all states, including as far south as Tasmania (9).

Here we report a high incidence of *S.* Hvittingfoss recovered from pre-migratory Bar-tailed Godwits (*Limosa lapponica menzbieri*) captured in Roebuck Bay, Broome, Western Australia, in March, 2018. The study was a part of broader research investigating pathogenic and antimicrobial-resistant bacteria in Australian shorebirds, and the high prevalence of *Salmonella* led to the further characterization of these isolates, and the determination of their genetic relationship to other isolates.

5.4 Methods

5.4.1 Sample collection

Shorebirds were captured by the Australasian Wader Study Group in two separate expeditions: 24 - 28 February 2017, and 23 February - 3 March 2018. These expeditions were conducted in a designated Ramsar site approximately 15 km from Broome township in the northern region of Western Australia, as part of ongoing scientific and conservation efforts involving shorebirds throughout the East-Asian Australasian flyway. Birds were captured with the aid of cannon nets and biometrics such as age and weight were recorded. Age was recorded according to moult. Focal species were subject to cloacal swabs before release. Cloacal swabs were taken from these birds on Mini Tip Aimes with Charcoal specimen swabs (Copan). Transit time from sample collection to culture varied, ranging from 9-20 days, due to the remote nature of the fieldwork. During transit, samples were stored at ~5°C in a portable refrigeration unit. This work was conducted under animal ethics permits issued by Federation University Australia (permit no. 16-002) and the Department of Parks and Water (permit no. 01-000179-1).

5.4.1 Bacterial culture, antimicrobial susceptibility testing and statistical analysis

In the laboratory, swab tips were aseptically transferred to 5ml of brain heart infusion broth and incubated at 35°C for 24 hours for pre-enrichment. Aliquots of 100µl were subsequently used to inoculate mannitol broth (Oxoid), azide dextrose broth (Oxoid) and selenite broth (Becton Dickinson [BD]). All selective enrichment broths were incubated at 35°C for 18-48 hours, then plated onto MacConkey II agar (Oxoid) or xylose lysine deoxycholate (XLD) agar (BD) as appropriate. All plates were incubated at 35°C for 24-48 hours. Suspect *Salmonella* isolates were sub-cultured for purity on XLD and preliminary testing included Gram reaction, catalase and oxidase testing. Presumptive *Salmonella* were confirmed by a PCR assay targeting the *invA* gene (25).

Confirmed *Salmonella* were tested for antimicrobial susceptibility using the Kirby-Bauer Disk Diffusion method (26). The antimicrobials (Oxoid) tested included: ampicillin (10µg), amoxicillin-clavulanic acid (20/10µg), ceftazidime (30µg), cephalothin (30µg), cefotaxime

(30µ), imipenem (10µg), chloramphenicol (30µg), tetracycline (30µg), gentamicin (10µg), streptomycin (10µg), sulphamethoxazole-trimethoprim (1.25/23.75µg), nalidixic acid (30µg), ciprofloxacin (5µg), and colistin (10µg). Statistical analysis was performed using SPSS (IBM SPSS Statistics Version 25).

5.4.2 Serotyping, MLST and phylogenetics

DNA extraction was performed on the seventeen positive *Salmonella* isolates, with DNA being collected from overnight colonies using a Qiagen DNeasy kit and quantified using an Invitrogen Qubit 2. The *in silico* serotype was inferred from WGS by the Microbiological Diagnostic Unit Public Health Laboratory, Doherty Institute at the University of Melbourne. Multi-locus sequence typing (MLST) was also conducted for the other *Salmonella* isolates via this service. Sequencing was conducted at the Australian Genome Research Facility using the Illumina MiSeq, with library preparation being Illumina gDNA shotgun library preparation with bead size selection protocol, generating 150bp paired-end reads.

Raw sequences were uploaded to the Galaxy web platform, and data were analyzed via the public server at usegalaxy.org (27). Genomes were assembled using Unicycler (28), and genome assembly quality was analyzed using QUAST (29). Online services hosted by the Centre for Genetic Epidemiology were used to determine antimicrobial resistance genes (30), pathogenicity islands (https://cge.cbs.dtu.dk/services/SPIFinder/), and sequence type for Multi-locus sequencing typing (31). Serotyping for the three sequenced isolates was determined using SeqSero (32). The newly determined genomes were uploaded to NCBI and made publically available under Bioproject PRJNA602163.

Raw reads were also uploaded to Enterobase (33) for purposes of phylogenetic analysis. Phylogenetic trees were constructed in Enterobase, using their SNP Projects option. This method uses the maximum likelihood estimation to create phylogenies. The uploaded *S.* Hvittingfoss isolates were compared to a dataset of 183 additional *S.* Hvittingfoss publicly-available whole genome sequences in the database. Using the inbuilt dendrogram option, a phylogenetic tree of the genetic relationship between the 17 Bar-tailed Godwits isolates, and all other global *S.* Hvittingfoss isolates was created (34).

Trees were then visualized and edited in FigTree 1.4.2 (35), Microreact (36) and iTOL (37). Tree colour schemes were created by Color Brewer 2.0 (38).

SNPs in the collected isolates were identified via ParSNP and visualized in Gingr (https://harvest.readthedocs.io/en/latest/content/harvest-tools.html). SNP analysis of the ST 2062 subgroup was performed using the alignment matrix generated by EnteroBase's SNP Projects in IQtree v1.6.12 (39), using a GTR substitution model with 100 bootstrap replicates. SNPpar (https://github.com/d-j-e/SNPPar) was used to map SNPs back onto the ST2062 tree.

5.5 Results

A total of 326 birds were sampled (190 in 2017, 136 in 2018), representing six species of wild Australian shorebirds: Ruddy Turnstone (*Arenaria interpres*) (n=23); Curlew Sandpiper (*Calidris ferruginea*, n=114); Pied Oystercatcher (*Haematopus longirostris*, n=9); Bar-tailed Godwit (n=130); Red-necked Stint (*Calidris ruficollis*, n=17) and Greater Sand Plover (*Charadrius leschenaultia*, n=33). All were screened for *Salmonella* spp. No individual from the 2017 expedition returned a positive *Salmonella* spp. culture.

All but one of the Bar-tailed Godwits caught in 2018 (n=63) were caught from a single flock at Wader Spit, Roebuck Bay (-17.979327, 122.336533) on 1st March, 2018. Seventeen (27%) of these birds were positive for *Salmonella* as determined by culture, with isolates confirmed by PCR. None of the other sampled shorebirds (n=73) were positive for *Salmonella*. All 17 isolates were susceptible to the tested antibiotics. All isolates carried the aminoglycoside resistance gene *aac(6')-Iaa*, which is conserved in all *Salmonella* but not normally expressed (11). There was no significant difference in *Salmonella* carriage rates in the godwits based on the sex (χ^2=1.104, df=2, p=0.576), weight (χ^2=8.456, df=8, p=0.390), or age (χ^2=1.036, df=2, p=0.596) of the bird. Serotype prediction using Whole Genome Sequencing analysis revealed all isolates to be *S.* Hvittingfoss, and MLST showed all isolates belonged to the same sequence type (ST), ST2062.

The 17 sequenced isolates were determined to be closely related via SNP comparisons, with 0-1 SNPs between each of the 16 isolates, bar one. Isolate 1293a was noted to have 23 SNPs different between it and all other godwit isolates, however these SNPs were likely to be due to recombination. The isolates obtained in this study were compared to 198 publicly available *S.* Hvittingfoss genomes, which were from a variety of different sources of global origin (Fig. 8).

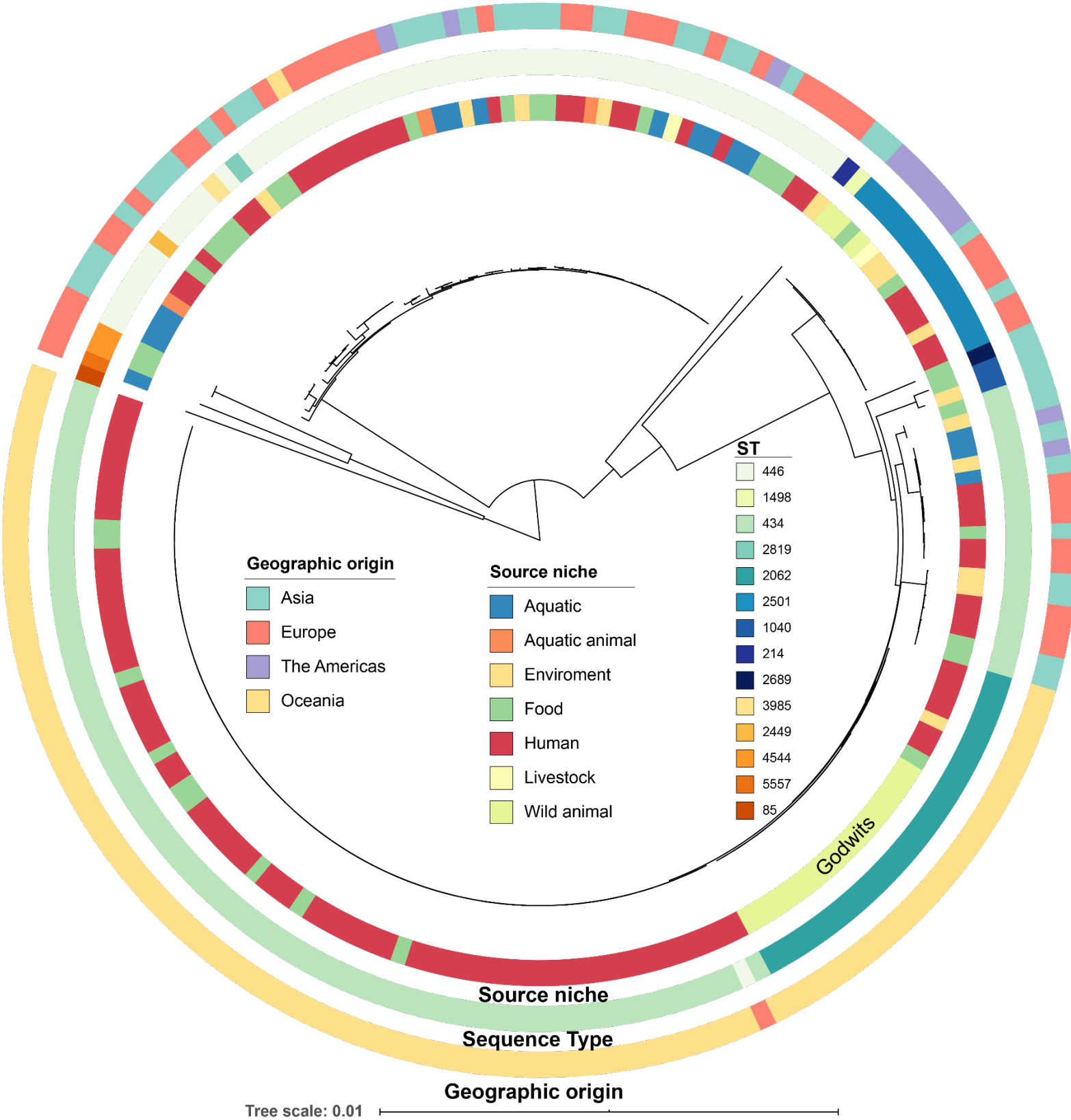

Figure 8. Maximum-likelihood phylogenetic tree showing the relationship between isolates collected from Bar-tailed Godwits (this study) and 198 globally-sourced and publicly-available S. Hvittingfoss genomes on Enterobase (11). Tree was outgroup rooted with *S*. Paratyphi ATCC 9150 strain. Colour-coding of rings is based on geographic origin, source niche and sequence type of the isolates. Australian isolates are indicated within the wild animal section marked 'Godwits'. Tree scale represents the number of substitutions per site. The raw data for this tree can be viewed in Microreact at https://microreact.org/project/PB7XPUPsv/.

Of the 198 *S.* Hvittingfoss genomes that were available, 186 were uploaded to Enterobase by the Centre for Infectious Diseases and Microbiology Public Health, University of Sydney. These publicly available Australian isolates were collected during the 2016 *S.* Hvittingfoss outbreak and formed a separate clade to all other global isolates. The genomes of isolates from the Bar-tailed Godwits collected in March 2018 (this study) clustered with these outbreak genomes. One non-Australian *S.* Hvittingfoss isolate also occupied this same genetic clade. Nevertheless, diversity was noted among the Australian *S*. Hvittingfoss isolates, with three different ST's identified (ST434, ST446 and ST2062). No other genome sequence from an Australian *S.* Hvittingfoss isolate from any other year, nor from any other animal, was available for comparison.

As all the Bar-tailed Godwit samples belonged to ST2062, a phylogenetic tree was constructed of all available ST2062 genomes. When examining only these isolates, SNP analysis showed two distinct linages; one associated with the 2016 *S*. Hvittingfoss outbreak in humans, and the other encompassing the 2018 godwit isolates. Sixty-eight SNPs separated the godwit isolates from the 2016 outbreaks at the nearest common ancestor (Fig. 9).

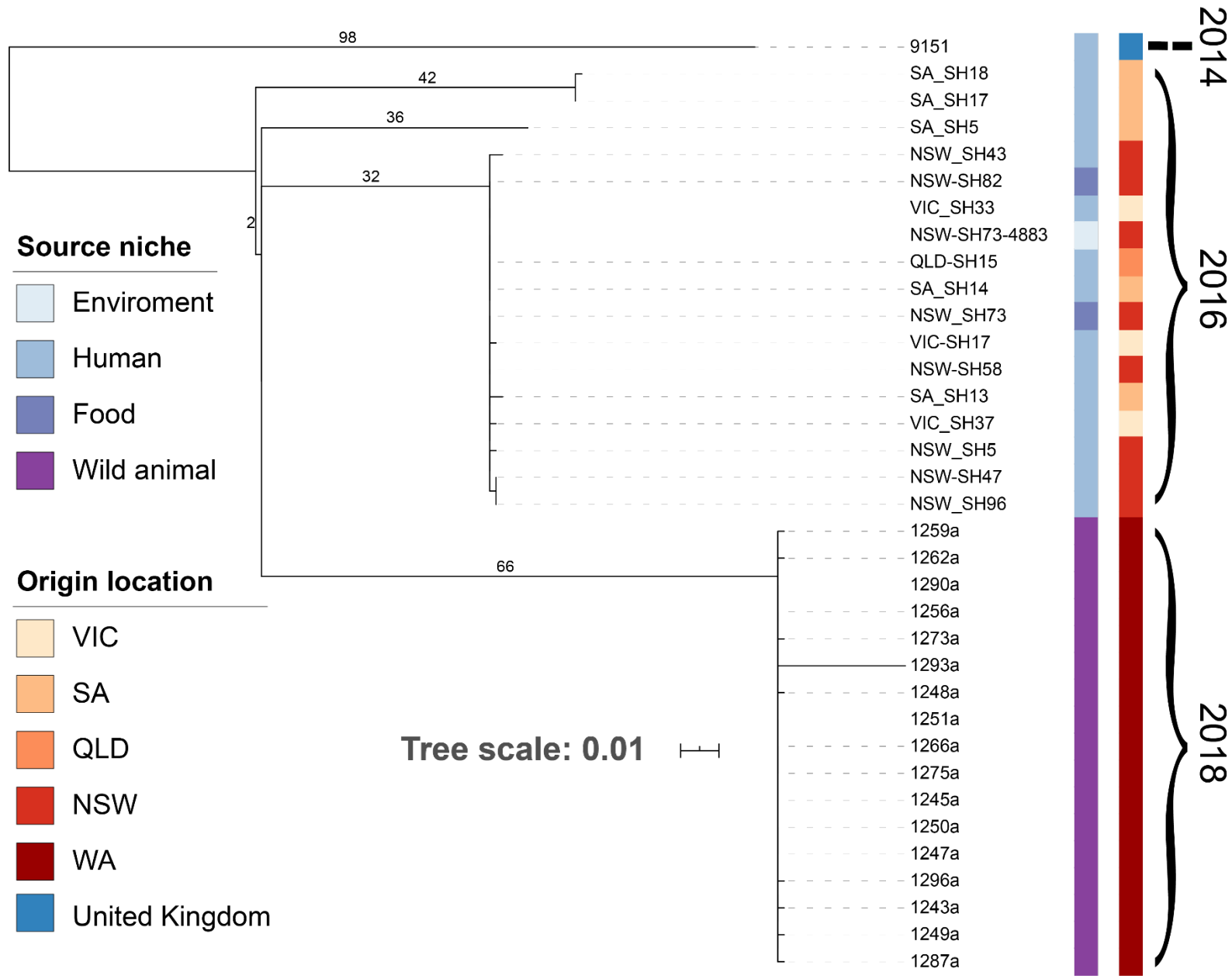

Figure 9. Maximum-likelihood phylogenetic tree of ST2062 *S.* Hvittingfoss isolates. The SNPs are noted on major branches of the tree. Colour coding is based on the source niche and geographical location of each isolate. Tree scale represents number of substitutions per site.

5.6 Discussion

This study demonstrates that *S.* Hvittingfoss can be present in shorebirds. We detected a high prevalence of *S.* Hvittingfoss in Bar-tailed Godwits during a sampling event at a single location in north-western Australia. These isolates were genetically similar to those causing a foodborne outbreak in humans who consumed tainted cantaloupes in 2016. While no direct link between shorebirds and foodborne outbreaks has been

established in this study, the relatedness of the respective strains suggests there may be an as-yet unknown avenue of introduction of this pathogen into the wider environment.

When the genomes of S. Hvittingfoss isolates collected from the godwits were compared pairwise, all except one genome were separated by at most a single SNP. A small amount of recombination was noted in a single isolate, resulting in a total of 23 SNPs; however, all isolates are considered clonal. This was based upon the findings of Octavia et al. (12), who determined a cutoff of four SNP's for an outbreak caused by a single strain of *Salmonella* spp. The close relatedness of these isolates suggests a recent colonization event in the godwits, likely from a single source.

Genetic analysis of the *S.* Hvittingfoss isolated from the Bar-tailed Godwits sampled in north-western Australia demonstrated that the isolates were related to isolates of *S*. Hvittingfoss responsible for the 2016-17 outbreak linked to contaminated cantaloupes in Australia. There were 68 SNPs to the most recent common ancestor. The foodborne outbreak of 2016-17 was traced back to a single farm in the Northern Territory, however no vector was identified that introduced *S*. Hvittingfoss onto the farm initially (13, 14). It is noteworthy that within the cantaloupe outbreak lineage there is some diversity (Fig. 9). This may be due to other *S*. Hvittingfoss strains circulating independent of the outbreak strains during the 2016-17 timeframe, as is commonly the case in Australia and various other countries where surveillance is conducted. Nonetheless, there is a group of highly related *S*. Hvittingfoss linked to the cantaloupe outbreak, and those isolates are closely related to the godwit isolates detected in this study.

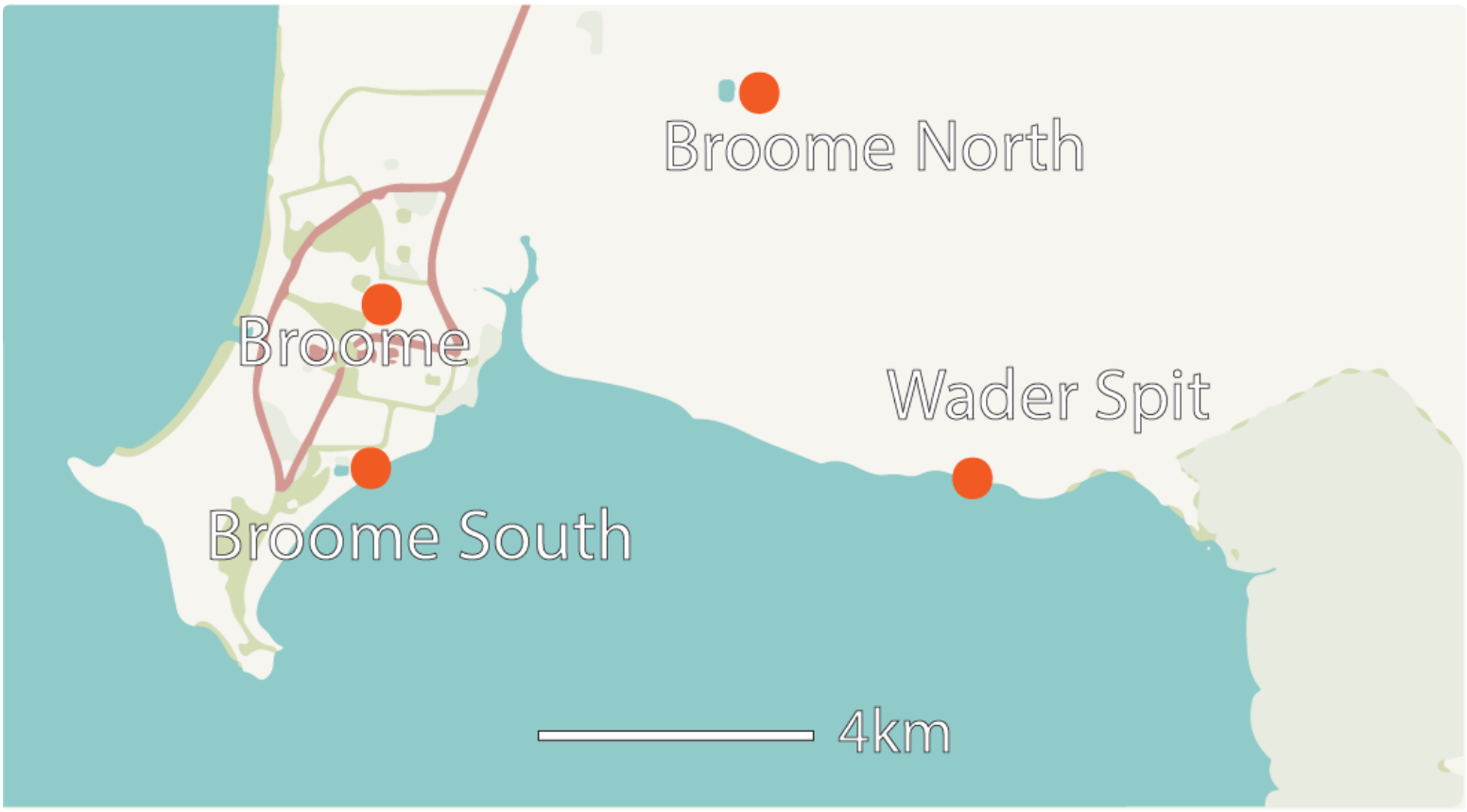

Figure 10. The location of the Broome North and South sewerage plants, and the location of the beach Wader Spit, where the flock of Bar-tailed Godwits was caught originally. Map adapted from OpenStreetMap.

The godwit isolates clearly have a shared ancestry with the cantaloupe outbreak strains, but we are not suggesting a direct epidemiological link between wild birds and foodborne outbreaks. Rather, there is likely an unidentified common ancestor from which the 2016 and 2018 isolates descended. The *S*. Hvittingfoss from godwits were isolated after the 2016 outbreak, thus there is sufficient temporal and genetic difference to rule out a direct link. Despite the importance of the outbreak, the original source of the outbreak strain remains undetermined (i.e. how *Salmonella* got onto or into the cantaloupes) (8, 15). The presence of closely related *S.* Hvittingfoss in birds suggests there may be an as of yet unknown animal and/or environmental reservoir of this serotype.

Outside of this study, the recovery of *S*. Hvittingfoss from wild animals has been rare. There have been only three previous reports of *S*. Hvittingfoss recovered from wild birds: a single species of waterfowl (two Plumed Whistling-duck, *Dendrocygna eytoni,*

out of 42 birds in total) in Australia (16) and in two migrating crane species (Hooded Crane, *Grus monacha* and White-naped Crane, *Antigone vipio,* nine birds of 359) in Japan (17). Within Australia, *S*. Hvittingfoss has also been recovered from reptiles (two positive of 97 sampled) and feral pigs (five of 139) (16, 18, 19).

As previously mentioned, the close genetic relatedness of all isolates present in the godwits is suggestive of recent colonization from a single source. One possible source of contamination is wastewater spills that occurred in Roebuck Bay in January-February 2018. During two separate events, both the Broome South and Broome North wastewater treatment plants overflowed. While the spills at Broome North were contained on-site, the Broome South wastewater treatment plant released 39.8 million litres of treated wastewater into Roebuck Bay (20). However, there is insufficient data to state with any certainty that these wastewater spills had an impact on the *Salmonella* carriage in Bar-tailed Godwits. A direct anthropogenic transmission is unlikely, as Bar-tailed Godwits do not have regular human contact. They feed mainly on marine invertebrates from broad expanses of tidal mudflats in areas with low human populations (e.g. Roebuck Bay) (21).

Salmonella infections can affect the health of avian populations, with higher mortality reported in infected birds relative to uninfected birds (21). Mass mortality events have been recorded due to *Salmonella* infections; one such event in New Zealand saw elevated mortality in many passerine (perching bird) species, from an outbreak of *Salmonella* Typhimurium DT160 (22). The *menzbieri* subspecies of the Bar-tailed Godwit is listed as nationally critically endangered (24) with a downward population trend. Although the birds in this study were considered healthy, there are potential negative impacts of *Salmonella* infection including the possibility of salmonellosis, which could hamper conservation and population recovery.

S. Hvittingfoss is present in Australian Bar-tailed Godwits, suggesting godwits, and possibly other wild birds, are able to acquire infectious agents associated with human illness. It is possible that *S*. Hvittingfoss was present in other shorebird species sampled in the area, but it was not detected; with a contributing factor being that this study was

restricted to two short sampling events across two years. Long-term surveillance of shorebird populations, and their environment as a whole, is needed to better understand the ecology of *S*. Hvittingfoss and wild bird populations (particularly godwits), and determine the potential health threat for both wild bird populations and humans.

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5.8 Data availability

All sequences have been uploaded to NCBI. All isolates have been deposited in SRA under Bioproject PRJNA602163. Individual *S.* Hvittingfoss isolate data were deposited under accession numbers; SAMN13884698 (1243a), SAMN13884699 (1245b), SAMN13884700 (1247a), SAMN13884701 (1248a), SAMN13884702 (1249a), SAMN13884703 (1250a), SAMN13884704 (1251a), SAMN13884705 (1256a), SAMN13884706 (1259a), SAMN13884707 (1262a), SAMN13884708 (1266a), SAMN13884709 (1273a), SAMN13884711 (1275a), SAMN13884712 (1287a), SAMN13884713 (1290a), SAMN13884714 (1293a), and SAMN13884715 (1296a).

5.9 References

1. Yan SS, Pendrak ML, Abela-Ridder B, Punderson JW, Fedorko DP, & Foley SL. (2004). An overview of *Salmonella* typing: public health perspectives. *Clinical and Applied Immunology, 4*(3), 189-204.
2. Ashton P, Nair, S Peters T, Bale J, Powell D, Painset A, Tewolde R, Schaefer U, Jenkins C, Dallman T. 2016. Identification of *Salmonella* for public health surveillance using whole genome sequencing. *PeerJ*, *4*. doi: 10.7717/peerj.1752
3. Havelaar AH, Kirk MD, Torgerson PR, Gibb HJ, Hald T, Lake RJ, Praet N, Bellinger DC, De Silva NR, Gargouri N, Speybroeck N. 2015. World Health Organization global estimates and regional comparisons of the burden of foodborne disease in 2010. *PLoS Medicine, 12*(12), e1001923.
4. Majowicz SE, Musto J, Scallan E, Angulo FJ, Kirk M, O'Brien SJ, Jones TF, Fazil A, Hoekstra RM 2010. The global burden of nontyphoidal *Salmonella* gastroenteritis. *Clinical Infectious Diseases, 50*(6), 882-889.
5. Ford L, Glass K, Veitch M, Wardell R, Polkinghorne B, Dobbins T, Lal A, Kirk MD. 2016. Increasing incidence of *Salmonella* in Australia, 2000-2013. *PLoS ONE, 11*(10), e0163989.
6. Eng SK, Pusparajah P, Ab Mutalib NS, Ser HL, Chan KG, & Lee, LH. 2015. *Salmonella*: a review on pathogenesis, epidemiology and antibiotic resistance. *Frontiers in Life Science, 8*(3), 284-293.
7. Greenhalgh M. 2010. *Salmonella* lawsuit filed against Subway. Retrieved from https://www.foodsafetynews.com/2010/06/salmonella-lawsuit-filed-against-subway/
8. Flynn D. 2016. How did *Salmonella* Hvittingfoss get on Aussie rockmelons? Retrieved from https://www.foodsafetynews.com/2016/08/130219/
9. Munnoch S, Irwin M, Oxenford C, Hanson R, Owen R, Black A, & Bell R. 2005. Investigation of a multi-state outbreak of *Salmonella* Hvittingfoss using a web-based case reporting form. *Communicable Diseases Intelligence Quaterly Report, 29*(4), 379.
10. Fearnley E J, Lal A, Bates J, Stafford R, Kirk M D, & Glass K. 2018. *Salmonella* source attribution in a subtropical state of Australia: capturing environmental reservoirs of infection. *Epidemiol Infect, 146*(15), 1903-1908. doi:10.1017/S0950268818002224
11. Magnet S, Courvalin P, & Lambert, T. 1999. Activation of the Cryptic *aac(6')-Iy* Aminoglycoside Resistance Gene of *Salmonella* by a Chromosomal Deletion Generating a Transcriptional Fusion. J Bacteriol, 181 (21), 6650-6655.
12. Octavia S, Wang Q, Tanaka M, Kaur S, Sintchenko V, Lan R. 2015. Delineating Community Outbreaks of *Salmonella enterica* Serovar Typhimurium by Use of Whole-Genome Sequencing: Insights into Genomic Variability within an Outbreak. J. Clin. Microbiol. *53*(4), 1063-1071. doi:10.1128/JCM.03235-14.
13. Australian Associated Press. 2016. Rockmelon industry devastated by S*almonella* outbreak linked to NT farm. *The Guardian*. Accessed 15 August, 2019.
14. Keen B. 2017. *Stories from the Frontline: Investigating causes of salmonellosis in Australia.* New South Wales Government
15. Claughton D, Kontominas B, & Logan T. 2018. Rockmelon listeria: Rombola Family Farms named as source of outbreak. *ABC Rural.* Accessed 18 August, 2019.
16. Hoque M A, Burgess G W, Greenhill A R, Hedlefs R, & Skerratt L F. 2012. Causes of morbidity and mortality of wild aquatic birds at Billabong Sanctuary, Townsville, North Queensland, Australia. *Avian Dis, 56*(1), 249-256. doi:10.1637/9863-072611-Case.1
17. Kitadai N, Ninomiya N, Murase T, Obi T, & Takase K J. 2010. *Salmonella* isolated from the feces of migrating cranes at the Izumi Plain (2002-2008): serotype, antibiotic sensitivity and PFGE type. *Journal of Veterinary Medical Science, 72*(7), 939-942.
18. Iveson J B, Mackay-Scollay E M, & Bamford V. 1969. *Salmonella* and Arizona in reptiles and man in Western Australia. *J Hyg (Lond), 67*(1), 135-145. doi:10.1017/s0022172400041516

18. Ward MP, Cowled BD, Galea F, Garner MG, Laffan SW, Marsh I, Negus K, Sarre SD, Woolnough AP. 2013. *Salmonella* infection in a remote, isolated wild pig population. *Vet Microbiol, 162*(2-4), 921-929. doi:10.1016/j.vetmic.2012.11.036
19. Water Corporation. 2018. Broome high rainfall events January-February 2018: Water quality sampling results report. (1-65).
20. Higgins, P.J. and Davies, S.J.J.F. (eds.) 1996. Hanbook of Australian, New Zealand & Antarctic Birds. Volume 3 Snipe to Pigeons. Oxford University Press, Melbourne.
21. Risely A, Klaassen M, & Hoye B J. 2018. Migratory animals feel the cost of getting sick: A meta-analysis across species. *J Anim Ecol, 87*(1), 301-314. doi:10.1111/1365-2656.12766
22. Alley MR, Connolly JH, Fenwick SG, Mackereth GF, Leyland MJ, Rogers LE, Haycock M, Nicol C, Reed CE. (2002). An epidemic of salmonellosis caused by *Salmonella Typhimurium* DT160 in wild birds and humans in New Zealand. *N Z Vet J, 50*(5), 170-176. doi:10.1080/00480169.2002.36306
23. Birdlife Australia. 2019. Bird profile: Bar-tailed Godwit. Retrieved from http://birdlife.org.au/bird-profile/bar-tailed-godwit
24. Malorny B, Hoorfar J, Bunge C, & Helmuth R. 2003. Multicenter validation of the analytical accuracy of *Salmonella* PCR: towards an international standard. *Applied and Enviromental Microbiology, 69*(1), 290-296. doi:10.1128/AEM.69.1.290–296.2003
25. Clinical and Laboratory Standards Institute. 2012. Performance standards for antimicrobial susceptibility testing; Twenty-second informational supplement. In (Vol. 32, pp. 188). Wayne, Pennsylvania, U.S.A: Clinical and Laboratory Standards Institute.
26. Afgan E, Baker D, Batut B, Van Den Beek M, Bouvier D, Čech M, Chilton J, Clements D, Coraor N, Grüning BA, Guerler A. 2018. The Galaxy platform for accessible, reproducible and collaborative biomedical analyses: 2018 update. *Nucleic acids research 46*(W1), W537-W544.
27. Wick R R, Judd L M, Gorrie C L, & Holt K E. 2017. Unicycler: resolving bacterial genome assemblies from short and long sequencing reads. *13*(6), e1005595.
28. Gurevich A, Saveliev V, Vyahhi N, & Tesler G J B. 2013. QUAST: quality assessment tool for genome assemblies.*Bioinformatics*, *29*(8), 1072-1075.
29. Zankari E, Hasman H, Cosentino S, Vestergaard M, Rasmussen S, Lund O, Aarestrup FM, Larsen MV. 2012. Identification of acquired antimicrobial resistance genes. *67*(11), 2640-2644.
30. Larsen MV, Cosentino S, Rasmussen S, Friis C, Hasman H, Marvig RL, Jelsbak L, Sicheritz-Pontén T, Ussery DW, Aarestrup FM, Lund O. 2012. Multilocus sequence typing of total-genome-sequenced bacteria. *50*(4), 1355-1361.
31. Zhang S, Yin Y, Jones MB, Zhang Z, Kaiser BL, Dinsmore BA, Fitzgerald C, Fields PI, Deng X. 2015. *Salmonella* serotype determination utilizing high-throughput genome sequencing data. *Journal of Clinical Microbiology, 53*(5), 1685-1692.
32. Zhou Z, Alikhan NF, Mohamed K, & Achtman M. 2019. The user's guide to comparative genomics with EnteroBase. Three case studies: micro-clades within *Salmonella enterica* serovar Agama, ancient and modern populations of *Yersinia pestis*, and core genomic diversity of all *Escherichia.* 613554
33. Stamatakis A. 2014. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics, 30*(9), 1312-1313. doi:10.1093/bioinformatics/btu033
34. Rambaut A. FigTree 1.4. 2 software. Institute of Evolutionary Biology, Univ. Edinburgh. 2014.
35. Argimón S, Abudahab K, Goater R, Fedosejev A, Bhai J, Glasner C, Feil E, Holden M, Yeats C, Grundmann H, Spratt B. 2016. Microreact: visualizing and sharing data for genomic epidemiology and phylogeography. *Microbial Genomics*. 2(11).
36. Letunic, I., & Bork, P. 2019. Interactive Tree Of Life (iTOL) v4: recent updates and new developments. *Nucleic acids research*, *47*(1).

37. Brewer, C. 1994. Color use guidelines for mapping and visualization. Chapter 7 (pp. 123-147) in Visualization in Modern Cartography
38. Nguyen L, Schmidt, H, von Haeseler A, Minh, B. (2015) IQ-TREE: A fast and effective stochastic algorithm for estimating maximum likelihood phylogenies. *Molecular Biology and Evolution*, 32, 268-274. https://doi.org/10.1093/molbev/msu300

6. General Discussion

The rapid increase in AMR bacteria is presenting a crisis for human health, and a multifaceted approach is required to combat the problem. While the most important factors are arguably the development of new antimicrobial agents and the prudent use of existing ones, there is also a need to monitor the spread of AMR bacteria, including in environments and hosts with little direct interactions with humans. AMR bacteria have been detected in remote habitats and isolated populations (Sjölund et al. 2008, Power et al. 2016, Guenther et al. 2012, Ramey et al. 2018). Mobile species such as birds can move disease between and across entire continents, and present a unique problem in the transmission and dissemination of both pathogenic bacteria and AMR.

With the aforementioned risks in mind, this thesis was developed to address current gaps in our awareness and understanding of pathogenic enteric bacteria in wild Australian shorebirds, with a focus on identifying AMR bacteria present in these populations. This thesis aimed to investigate if wild shorebirds are importing pathogens and AMR into Australia, and to determine if shorebirds were a natural reservoir for pathogens and AMR bacteria. Specifically, this thesis investigated the presence, genetic diversity and AMR profiles of *E. coli, Enterococcus* and *Salmonella*. While some recent studies have looked at AMR in enteric bacteria in Australian birds, these studies have either focused on urban associated birds (Oravcova, Svec, and Literak 2017, Dolejska et al. 2016, Mukerji et al. 2019), or on small samples of wild birds (Blyton et al. 2015, Smith et al. 2019). This study draws on a sample size of 1022 birds to provide a more detailed insight into the dissemination of AMR into these wild bird populations, and while doing so explore the zoonotic and animal health risk of selected bacteria associated with the birds.

In brief, the key findings of this study are: 1) shorebirds carry low levels of AMR bacteria, but clinically important AMR phenotypes and genes are present in Australian shorebird populations; 2) resident birds are more likely to carry AMR bacteria than migratory birds; 3) Australian shorebirds can carry (and potentially act as a reservoir for) pathogenic bacteria of human populations; and 4) *Escherichia* in Australian shorebirds

are diverse, and harbour a variety of *E. coli* associated with other animal species and geographic locations**.** These findings and their implications to future research are further explored below.

6.1 Shorebirds carry low levels of AMR, but clinically important AMR is present in the populations studied.

The main finding of this thesis has been that while AMR in the bacteria of Australian shorebirds is uncommon, resistance to clinically important antibiotics (such as ceftazidime, colistin and ciprofloxacin) is present and potentially circulating in these populations. Compared to numerous other studies (both Australian and overseas) used to compare against Australian shorebirds, AMR in Australian shorebirds is relatively uncommon, and resistance to clinically important last-line antibiotics such as glycopeptides and carbapenems was rare. However, these finding are not at complete odds with other published data. The AMR profiles obtained in this study are similar to studies that have also investigated birds that either have little contact with humans, or inhabit remote areas (Blyton et al., 2015, Oravcova, Svec & Literak, 2017).

The apparent correlation between interaction with anthropogenic activity and carriage of AMR bacteria is one that has been noted before (Thaller et al. 2010, Navarro-Gonzalez et al. 2012). Understanding that AMR, and the 'severity' of the AMR genes present is amplified by increased levels of human interactions, suggests that there is a growing risk for Australian shorebirds to become significant reservoirs of AMR bacteria- and potentially as vectors of dissemination for AMR bacteria back into human populations. As discussed in Chapter 1, shorebird habitat is under increasing pressure from human development and encroachment. This is particularly the case for habitat along the EAAF, which Australian shorebirds use, and is occurring at all stages along the flyway including in Australia. Even when habitat changes seem minor at the local scale, there is the risk of 'death by a thousand cuts' as loss of habitat continues to push shorebirds closer and closer to human activity (Milton & Harding 2011). If the trends reported in other studies of wild birds (Cizek et al. 2007, Ramey et al. 2018, Smith et al. 2019) apply to Australian shorebirds (assuming continued habitat destruction and closer

wildlife-human interactions), migratory bird populations that visit Australia will likely continue to acquire AMR bacteria and associated genes. Wild animals are a potentially important, but often overlooked, component of the One Health concept. The flow-on effects could be significant, with the potential to be detrimental to other wildlife, agriculture and human health.

6. 2 Resident birds are more likely to carry AMR than migratory birds

An important finding from this study was that migratory birds were less likely to carry AMR bacteria than resident birds. Interestingly, this finding differs to at least one other study looking at non-shorebird migrants. Migratory Rooks (*Corvus frugilegus*) in the Czech Republic, for example, were more likely to carry AMR bacteria than resident Rooks (Loncaric et al. 2013). However, direct comparisons are difficult due to differing migration strategies, feeding ecologies and habitat preferences of shorebirds and corvids. Rooks generally have a closer association with anthropogenically impacted habitats than shorebirds.

The higher likelihood of bacterial AMR carriage in resident shorebirds may be due to the much higher fitness requirements that migratory birds have for their annual migrations; a meta-analysis by Risely, Klaassen, and Hoye (2018) found that infected migrants were both less likely to migrate, and were less likely to survive migration, than non-infected migrants. This may also extend to the carriage of AMR bacteria, especially if such bacteria are pathogenic to the bird; as resident birds have much lower movement requirements and defined home ranges, they may be able to tolerate the carriage of potential pathogens and AMR bacteria to a greater degree than migrants. However, resistance does not necessarily correlate with pathogenicity, as discussed in Chapter 1, and so the presence of AMR bacteria alone may not have negative effects on the host. The finding that sedentary birds are more likely to harbour AMR bacteria than migratory birds was obtained using a sample size of 1022 birds (708 migratory and 314 non-migratory), and achieved statistical significance. However, there may have been confounding factors that contributed to the finding. The finding may have been

influenced by the target bacteria and/or sampling approach. It is important to note that the difference between migratory and non-migratory birds hinged upon the detection of AMR *Enterococcus* spp. (Section 3.6.3). As addressed in Chapter 2, *Enterococcus* fared poorly in long-term storage trials; in this study, the majority of the samples from sedentary birds were collected on short-term, overnight expeditions that allowed for more immediate culture of the sample swabs . This fact may act as a confounding variable, and may make it appear that sedentary birds are more likely to harbour AMR bacteria than migrants.

There are various potential approaches to overcome potential experimental bias speculated above. A future study could focus on a bacterial species less prone to loss of viability in storage. Alternatively, a culture independent approach could be taken, where metagenomic analysis seeks to detect AMR genes rather than AMR bacteria. The potential benefits and current impediments to metagenomic approaches are discussed below (Section 6.7).

Due to the highly mobile nature of many long-distance migrants, designing and conducting studies to sample birds at multiple points along their migratory pathways is difficult due to the logistics involved. Performing detailed investigations on whether migratory bird populations are acquiring and disseminating AMR bacteria, and where acquisition and shedding occurs would be highly beneficial, but difficult to coordinate. Until such time that coordinated international studies can be conducted, studies such as the work presented in this thesis and other research that compares migratory and resident populations provide valuable information, and should continue to be conducted to further investigate how AMR carriage differs between migratory and resident populations.

6. 3 Shorebirds can carry (and potentially act as a reservoir for) pathogenic bacteria of human populations

While it is not possible to determine prevalence rates of target bacteria in wild shorebirds (due to the limitations investigated in Chapter 2), it is still possible to

determine presence of bacterial species in the wild birds. From this study, we have been able to confirm the presence of *E. coli*, *Enterococcus*, and *Salmonella* in wild shorebirds. The finding of a strain of *S.* Hvittingfoss in Bar-tailed Godwits that is genetically related to the isolates that caused a nationwide outbreak in 2016 illustrates beyond doubt the capacity of wild birds to harbour pathogens of zoonotic potential. By extension, these birds (and shorebirds and other wild birds in general) may be able to introduce foodborne and pathogenic bacteria into agricultural crops, irrigation water and/or the wider environment.

Alongside finding diverse, AMR bacteria, finding a clinically important bacteria that had very recently caused a major outbreak of salmonellosis in a single flock of wild shorebirds highlights how important regular and systematic surveillance is of wild shorebirds. Wild birds have been responsible for outbreaks of salmonellosis before, including outbreaks from food (Alley et al., 2002, Lawson et al., 2014), and shorebirds have been identified as reservoirs of other clinically important diseases such as *Staphylococcus* (Keeler & Huffman, 2009) and *Klebsiella* (Raza et al., 2017). The finding that shorebirds can carry foodborne pathogens is of particular concern as this means that wild birds can be part of transmission routes to humans with no direct contact. The finding of *S.* Hvittingfoss in shorebirds also highlights how many diseases are potentially slipping under the radar with shorebirds due to a lack of consistent surveillance. Biosecurity is a major consideration for both the agricultural industry and the wider community, and Australia has some of the strictest biosecurity laws in the world to safeguard our various disease-free statuses. Yet despite the considerable effort put into biosecurity, the failure to establish consistent, effective wildlife testing for animals such as shorebirds (that regularly travel into and out of the country in great numbers) is a gap in our defences against disease introduction.

During the sampling period, there was a large sewage spill into the nearby waterways that the Bar-tailed Godwits used to forage (Water Corporation, 2018). While it is not possible to directly link this sewage spill to the findings of *S.* Hvittingfoss in the birds, it is important to be aware that the only nearby township of Broome does not have access to rapid pathology testing. Potentially, *S.* Hvittingfoss could have been circulating in the

local population undetected and was therefore present in the sewerage during the spill. If this is the case, then it raises concerns for both the community in the area (the waters around Broome are often used for fishing) and for the fauna in the area (as Roebuck Bay is listed as a RAMSAR site).

6. 4 *Escherichia* in shorebirds are diverse and harbour *E. coli* from many other organisms and locations.

The results of Chapter 4 provide a greater understanding of the genetic diversity of *E. coli* in wild shorebirds. Birds carried a genetically diverse range of *E. coli* lineages including both zoonotic and avian-specific *strains.* Birds were also found to carry bacteria that either had, or were closely related to, *E. coli* lineages that have caused disease in humans, livestock and wild animals. The diversity present in these shorebirds suggest they are able to acquire and maintain bacteria from a wide range of sources.

These findings have the potential to be extrapolated to other bacterial species and help provide insights into how shorebirds may act as reservoirs for a wide range of bacterial lineages of both pathogenic, zoonotic, and commensal organisms. As discussed earlier, the finding of AMR genes in some isolates shows there is the potential for previously susceptible bacterial strains to acquire AMR genes without ever being exposed directly to antibiotics. There is much evidence that the gut acts as a 'melting pot' of genetic determinants (Robinson, Bohannan, & Britton 2019), which poses the threat that wild birds may be able to introduce AMR genes into a wide variety of bacterial strains and then disseminate these further.

In addition to identifying the genetic diversity in *E. coli*, other enteric bacteria were identified. A newly recognised *Escherichia* species, *E. marmotae*, was isolated and identified via analysis of WGS data. This species was first identified in 2016, and has only been detected in a limited number of species and environments, namely Himalayan Marmots (*Marmota himalayana*), cattle and wastewater. *E. marmotae* is closely related to Clade V *E. coli*, and further phylogenetic analysis is required to better understand what niches *E. marmotae* occupies and clarify the relationship between clade V *E. coli*

and *E. marmotae*. In addition, three *Citrobacter* species were isolated from shorebirds; *C. freundii*, *C. braakii*, and *C. amalonaticus* (Appendix 8.1). These species can play important roles in the dissemination of AMR genes, and can act as indicators of environmental contamination with AMR. Future surveillance of wild shorebird populations for AMR genes might consider targeting *Citrobacter* species as indicator organisms of AMR spread.

6. 5 Extended transport may impact organism recovery, which impacts prevalence studies

Traditional culture-based methodology was used in this study over newer, DNA-based approaches (that do not require viable bacteria). There was considerable deliberation over the most suitable approach to use during the design of this study; however, it was decided that culture-based methods remain the gold standard for AMR detection (see below for discussion on culture-independent genetic approaches). The decision to use culture led to a preliminary study to determine the impact of long-term storage of swabs prior to the setup of culture on viability of bacteria (Chapter 2). The rate of bacterial survival in transit differed according to species of the bacteria; however, there was survival of target bacteria over periods of up to 6 weeks. This finding meant that a culture-based study could be used to isolate bacteria from wild birds despite long field trips meaning culture setup was delayed for up to 1-month post sample collection. However, the variability in survival of bacterial means that determining the prevalence of a given bacterial species is not ideal in the current study.

Research in remote wild populations is likely to remain important in the coming decades, as humans have an obligation to monitor the health of wild populations while there remains increasing anthropogenic pressures on wild animal populations and their habitat. In this study it has been demonstrated that if there is a need to gain a detailed understanding of bacteria colonising wild animals, culture can be used even if there is an extended period between sample collection and culture set up.

The ability to successfully culture target bacteria up to 6 weeks post 'collection' (as demonstrated in Chapter 2 in the preliminary experiment to determine viability of target organisms) enabled this study to conduct culture and thus determine AMR and conduct detailed genetic analysis on selected isolates. However, it is worth noting that it is not possible to appreciate what was 'lost' in that process. There is no evidence-based reasoning to my knowledge that suggests long-term survival of target organism would bias AMR results. However, it is likely to impact on the diversity of bacteria present, with some strains of bacteria likely to survive long-term storage than other strains. The heterogeneity of *E. coli* strains detected in this study (Chapter 4) could be interpreted as successfully capturing much of the genetic diversity present; however there is no way of knowing if indeed there could have been dominant strains present at the time of sample collection that were not detected due to limitations in experimental design.

6. 6 Further consideration of AMR bacteria in wild birds

AMR bacteria is present in populations with seemingly low selection pressures warrants further consideration. As discussed in Chapter 4, some AMR genes detected in this study had more functions than just antibiotic resistance. The gene *mdfA* is an efflux pump that can confer AMR when upregulated, but this may not be the main function of this gene. Many genes that confer antibiotic resistance do so as a side effect of their regular function (Beaber, Hochhut, & Waldor 2004, Gullberg et al. 2014). Resistance in wild populations may not be solely due to overconsumption of antibiotics in agricultural and human populations, and as such it may be found in future research that even with increased restrictions in human antibiotic use, AMR in wild populations is still widespread and persisting. This may also tie into how resistance genes seem to persist in populations, even after antibiotic usage stops (Hsu et al. 2014); there is no extra energetic 'cost' for essential genes in bacterial genomes, and so they are not lost upon the cessation of the selection pressure (in this case, antibiotic exposure).

Nonetheless, a number of the genes detected in the shorebirds in this study specifically functioned as resistance mechanisms. The *qnrB65* gene is an example of such a resistance gene (Jacoby et al. 2006), and was detected in one of the shorebirds tested.

The presence of this gene raises multiple questions regarding how it entered the shorebird population; the birds could have acquired this gene from environments contaminated with antibiotics, or by becoming colonised with a clinically derived isolate. The diversity of *E. coli* detected in shorebirds (Chapter 4), many with associations to other animal hosts, suggest it is possible that a clinically-associated isolates carrying this resistance gene has come into contact with the isolate detected with the *qnrB65* gene.

Many antibiotics are either wholly or partially derived from compounds produced by environmental bacteria and fungi. As such, even the bacteria of populations that are completely untouched by human activity are still likely to come into contact with antibiotic-like compounds, and so have an ecological need for AMR genes. The difference between antibiotic exposure under natural settings and antibiotic exposure from anthropogenic sources is often a matter of scale; clinical concentrations of antibiotics are many times higher than what is present in natural environments, and may result in AMR genes either being expressed far more than they would under natural circumstances. In addition, some of the antibiotics utilised in clinical and agricultural settings are entirely synthetic and have no environmental analogues- yet resistance genes to them are still present in the environment and in wild populations (Cizek et al. 2007, Literak et al. 2010, Foti et al. 2011, Wilson et al. 2019). Resistance to synthetic antibiotics such as nalidixic acid was detected in the shorebirds in this study, indicating that Australian shorebirds are indeed either coming into contact with human-associated bacteria, or with AMR genes descended from human activity.

6. 7 Genetic approaches to pathogen surveillance

While this study intentionally used culture to isolate bacterial strain for further characterisation, much can be gained from a genomic-only research approach, and future studies are likely to be increasingly dependent on genomic detection and characterisation. Genomic approaches have been employed to investigate pathogens in Australian and other shorebirds to great effect (Power et al. 2016, Risely et al. 2018, Risely et al. 2017). To date approaches such as 16S RNA gene sequencing and

metagenomic approaches provide methods for investigating community composition, and in doing so can readily detect microbes of genus level.

In the current study, DNA isolation was attempted in tandem with bacterial culture. In addition to a swab collected in transport media for subsequent culture, a dry swab was collected for potential application of genomic detection methods. However, preliminary attempts revealed challenges in obtaining sufficient DNA to enable meaningful genomic detection such as PCR to be conducted (data not shown). The collection of suitable volumes of DNA from these swabs proved to be unreliable, for which there may have been a multitude of reasons. The DNA swabs were all collected from shorebirds after swabbing for bacterial isolates, which may have reduced the available material for DNA isolation. In addition, the sizes of the shorebird species targeted varied greatly; the smallest species targeted (the Red-necked Stint) had an average weight of 20-50g. This impacted the ability to swab small birds twice due to ethical concerns. The process of DNA isolation itself also presented problems, despite multiple extraction methods being trialled; further research investigating how to collect appropriate DNA samples from such birds would be of great use in shorebird research, and indeed in wild bird research in general. Efficiency of DNA extraction continues to improve, with pursuits such as molecular forensics driving the need to be able to detect minute amounts of DNA. Thus, although direct genetic analysis was conducted in this study, there is no suggestion that it could not, or should not be applied to research investigating avian microbiomes, including bacterial zoonotic pathogens in wild birds in the future.

PCR and other nucleic amplification techniques are rightfully considered highly sensitive and are likely to be the main approach used in most future studies like this one. However, one should not overlook the importance of obtaining adequate DNA for the amplification technique to be conducted, and if insufficient DNA is obtained from samples the results will be impacted upon to the detriment of the study. It is also worth considering the potential sensitivity of culture: one viable bacterial cell can result in successful culture of a target organisms. The chance of obtaining a specific organism can be further enhanced using enrichment media. Such an approach is commonplace in detection of foodborne pathogens and to a lesser extent clinically relevant pathogens.

Where initial numbers may be low an enrichment can enable viable bacteria to multiple to a point where they can be readily detected using molecular approaches. For future research, it would be prudent for traditional culture methodologies to be combined further with genomic and sequencing methodologies.

While methods currently exist for direct genetic detection of bacteria (and other microorganisms) the widescale application of such approaches to studies with a large sample size remains expensive. In this study, conducting culture and then selecting bacteria for further genetic characterisation enabled the development of knowledge in a cost-efficient manner. This is often a limitation when conducting research on wild animal populations where the health and economic benefits to humans are often considered peripheral or not immediately threatening. The cost of conducting WGS has decreased considerable over the past ~10 years, and may continue to decrease as technologies develop. Thus it is anticipated that cost will become less of a critical consideration in experimental design in the future.

Combining genetic and culture techniques to investigate whether birds can directly transmit clinically important or pathogenic bacteria into another bird, other animal or human population could help overcome some of the shortcomings of this study. Ideally, prevalence would be determined by direct genetic detection (notwithstanding the limitations outlined above), either through a metagenomic approach or by targeting specific species through PCR or other nucleic amplification technique. There may even be the potential to conduct some such analyses in the field in the future, with the development of portable approaches such as LAMP, field-PCR and MinION sequencing technology. Where further characterisation including AMR profiles are required, culture could later be conducted in the laboratory.

6. 8 Implications of findings for wild bird conservation and human public health

When considering migratory birds as potential disease vectors, it should be recognised that humans should not attempt to directly control the movements and behaviours of

the birds themselves. Birds have been migrating for millennia, and humans have no way of changing how the birds behave without massive impacts on bird populations and broader ecological webs. While monitoring the health of and tracking the movements of livestock or caged birds is possible (and often, is undertaken as part of routine biosecurity regulations in many countries) tracking the movement and health of wild birds is more challenging. This is especially the case for migratory birds; as mentioned in Chapter 1, migratory flyways such as the EAAF span two hemispheres and multiple countries. Coordinating long term monitoring programs between the many countries involved is difficult; though it remains a worthy goal and there have been successes in the past for such programs. For example, the East Asian-Australasian Flyway Partnership is a voluntary initiative to protect migratory shorebirds and their habitat, and has been adopted by 18 countries and 6 intergovernmental agencies (Szabo et al. 2016). Such a long term, interconnected operation is possible for monitoring shorebird populations for disease emergence, and would provide a deep understanding of disease transmission and AMR presence in the flyway as a whole, rather than in just individual countries.

Effective control of AMR spread into the wild is not something that can be done by controlling the populations affected directly. What we can- and should- control, is how our antibiotic usage affects and enters the environment. We are aware of the main avenues of how antibiotic compounds and AMR bacteria pass from anthropogenic sources and out into the wider environment, but there is little done to curb this transmission. When designing sewerage treatment plants and planning where effluent will be expelled to, we need to take into concern what bacteria we are releasing as well as looking at what pollutants are being let out. When feeding livestock, we need to weigh up what is more important: the short-term economic gain produced through slightly higher product yields, or the long-term disadvantages that will face the entire population when AMR bacteria inevitably spreads out from the farm and into the wider community. While such measures may not remove all traces of AMR from wild populations, they will ensure that resistance is not widespread in wild populations, and that resistance to clinically important or synthetic antibiotics does not enter such populations at all.

With regards to public health, these findings show that although resistance to some clinically important antibiotics was detected, they were not common, and therefore Australian shorebirds do not appear to currently be significant reservoirs of AMR bacteria of clinical importance. This finding is promising for the continued protection of both wild populations and communities against the development and proliferation of AMR bacteria, and is something that needs to be protected against further intrusion. As demonstrated in multiple other studies, remoteness and lack of human interaction alone does not prevent AMR bacteria from colonising wild populations; it merely delays colonisation. The continued degradation and human encroachment into shorebird habitat increases the chances of these populations becoming reservoirs for AMR bacteria, to the detriment of both the community and the wild populations themselves.

6. 9 Conclusions

In summary, the results from this thesis show that Australian shorebirds are capable of carrying (and potentially transmitting) enteric pathogens of clinical importance. While prevalence data cannot be reliably calculated from sample swabs in extended transit, culturing these swabs for further characterisation of individual isolates can be conducted, and has provided insights into bacteria present in a difficult to sample group of animals. Shorebirds are capable of harbouring bacteria of human health importance, as demonstrated by Bar-tailed Godwits being shown for the first time to be a potential reservoir for the foodborne pathogen *S.* Hvittingfoss. Further surveillance is needed for these populations, to both determine bacterial prevalence and to understand what role shorebirds may play in the transmission of other pathogenic and AMR bacteria.

6.10 References

Beaber, J. W., Hochhut, B., & Waldor, M. K. (2004). SOS response promotes horizontal dissemination of antibiotic resistance genes. *Nature, 427*(6969), 72-74. doi:10.1038/nature02241

Blyton, M. D., Pi, H., Vangchhia, B., Abraham, S., Trott, D. J., Johnson, J. R., & Gordon, D. M. (2015). Genetic Structure and Antimicrobial Resistance of *Escherichia coli* and Cryptic Clades in Birds with Diverse Human Associations. *Applied and Environmental Microbiology, 81*(15), 5123-5133. doi:10.1128/AEM.00861-15

Cizek, A., Dolejska, M., Karpiskova, R., Dedicova, D., & Literak, I. (2007). Wild black-headed gulls (*Larus ridibundus*) as an environmental reservoir of *Salmonella* strains resistant to

antimicrobial drugs. *European Journal of Wildlife Research, 53*(1), 55-60. doi:10.1007/s10344-006-0054-2

Dolejska, M., Masarikova, M., Dobiasova, H., Jamborova, I., Karpiskova, R., Havlicek, M., Carlile, N., Priddel, D., Cizek, A., & Literak, I. (2016). High prevalence of *Salmonella* and IMP-4-producing Enterobacteriaceae in the silver gull on Five Islands, Australia. *Journal of Antimicrobial Chemotherapy, 71*(1), 63-70. doi:10.1093/jac/dkv306

Epstein, J. H., McKee, J., Shaw, P., Hicks, V., Micalizzi, G., Daszak, P., Kilpatrick, A., & Kaufman, G. (2006). The Australian White Ibis (*Threskiornis molucca*) as a Reservoir of Zoonotic and Livestock Pathogens. *EcoHealth, 3*(4), 290-298. doi:10.1007/s10393-006-0064-2

Fearnley, E., Lal, A., Bates, J., Stafford, R., Kirk, M., & Glass, K. (2018). *Salmonella* source attribution in a subtropical state of Australia: capturing environmental reservoirs of infection. *Epidemiology & Infection, 146*(15), 1903-1908.

Foti, M., Rinaldo, D., Guercio, A., Giacopello, C., Aleo, A., De Leo, F., Fisichella, V., & Mammina, C. (2011). Pathogenic microorganisms carried by migratory birds passing through the territory of the island of Ustica, Sicily (Italy). *Avian Pathology, 40*(4), 405-409. doi:10.1080/03079457.2011.588940

Guenther, S., Aschenbrenner, K., Stamm, I., Bethe, A., Semmler, T., Stubbe, A., Stubbe, M., Batsajkhan, N., Glupszynski, Y., Wieler, L, & Ewers, C. (2012). Comparable high rates of extended-spectrum-beta-lactamase-producing *Escherichia coli* in birds of prey from Germany and Mongolia. *PLoS ONE, 7*(12), e53039. doi:10.1371/journal.pone.0053039

Gullberg, E., Albrecht, L. M., Karlsson, C., Sandegren, L., & Andersson, D. I. (2014). Selection of a multidrug resistance plasmid by sublethal levels of antibiotics and heavy metals. *mBio, 5*(5), e01918-01914. doi:10.1128/mBio.01918-14

Hsu, J. T., Chen, C. Y., Young, C. W., Chao, W. L., Li, M. H., Liu, Y. H., Lin, C. M., & Ying, C. (2014). Prevalence of sulfonamide-resistant bacteria, resistance genes and integron-associated horizontal gene transfer in natural water bodies and soils adjacent to a swine feedlot in northern Taiwan. *Journal of Hazardous Materials, 277*, 34-43. doi:10.1016/j.jhazmat.2014.02.016

Jacoby, G. A., Walsh, K. E., Mills, D. M., Walker, V. J., Oh, H., Robicsek, A., & Hooper, D. C. (2006). *qnrB*, another plasmid-mediated gene for quinolone resistance. *Antimicrobial Agents and Chemotherapy, 50*(4), 1178-1182.

Literak, I., Dolejska, M., Janoszowska, D., Hrusakova, J., Meissner, W., Rzyska, H., Bzoma, S., & Cizek, A. (2010). Antibiotic-resistant *Escherichia coli* bacteria, including strains with genes encoding the extended-spectrum beta-lactamase and *QnrS*, in waterbirds on the Baltic Sea Coast of Poland. *Applied and Environmental Microbiology, 76*(24), 8126-8134. doi:10.1128/AEM.01446-10

Loncaric, I., Stalder, G. L., Mehinagic, K., Rosengarten, R., Hoelzl, F., Knauer, F., & Walzer, C. (2013). Comparison of ESBL--and AmpC producing Enterobacteriaceae and methicillin-resistant *Staphylococcus aureus* (MRSA) isolated from migratory and resident population of rooks (*Corvus frugilegus*) in Austria. *PLoS ONE, 8*(12), e84048. doi:10.1371/journal.pone.0084048

Milton, D. A., & Harding, S. B. (2011). Death by a thousand cuts–the incremental loss of coastal high tide roosts for Shorebirds in Australia: Sandfly Creek Environmental Reserve, Central Queensland. *Stilt, 60*, 22-33.

Mukerji, S., Stegger, M., Truswell, A. V., Laird, T., Jordan, D., Abraham, R. J., Harb, A., Barton, M., O'Dea, M., & Abraham, S. (2019). Resistance to critically important antimicrobials in Australian silver gulls (*Chroicocephalus novaehollandiae*) and evidence of anthropogenic origins. *Journal of Antimicrobial Chemotherapy, 74*(9), 2566-2574.

Navarro-Gonzalez, N., Mentaberre, G., Porrero, C. M., Serrano, E., Mateos, A., Lopez-Martin, J. M., Lavin, S., & Dominguez, L. (2012). Effect of cattle on *Salmonella* carriage, diversity

and antimicrobial resistance in free-ranging wild boar (*Sus scrofa*) in northeastern Spain. *PLoS ONE, 7*(12), e51614. doi:10.1371/journal.pone.0051614

Oravcova, V., Svec, P., & Literak, I. (2017). Vancomycin-resistant enterococci with *vanA* and *vanB* genes in Australian gulls. *Environmental Microbiology Reports, 9*(3), 316-318. doi:10.1111/1758-2229.12542

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

Ramey, A. M., Hernandez, J., Tyrlov, V., Uher-Koch, B. D., Schmutz, J. A., Atterby, C., Jarhult, J. D., & Bonnedahl, J. (2018). Antibiotic-Resistant *Escherichia coli* in Migratory Birds Inhabiting Remote Alaska. *EcoHealth, 15*(1), 72-81. doi:10.1007/s10393-017-1302-5

Risely, A., Waite, D., Ujvari, B., Klaassen, M., & Hoye, B. (2017). Gut microbiota of a long-distance migrant demonstrates resistance against environmental microbe incursions. *Molecular Ecology, 26*(20), 5842-5854.

Risely, A., Klaassen, M., & Hoye, B. J. (2018). Migratory animals feel the cost of getting sick: A meta-analysis across species. *Journal of Animal Ecology, 87*(1), 301-314. doi:10.1111/1365-2656.12766

Risely, A., Waite, D. W., Ujvari, B., Hoye, B. J., & Klaassen, M. (2018). Active migration is associated with specific and consistent changes to gut microbiota in *Calidris* shorebirds. *Journal of Animal Ecology, 87*(2), 428-437. doi:10.1111/1365-2656.12784

Robinson, C. D., Bohannan, B. J., & Britton, R. A. (2019). Scales of persistence: transmission and the microbiome. *Current Opinion in Microbiology, 50*, 42-49.

Sjolund, M., Bonnedahl, J., Hernandez, J., Bengtsson, S., Cederbrant, G., Pinhassi, J., Kahlmeter, G., & Olsen, B. (2008). Dissemination of multidrug-resistant bacteria into the Arctic. *Emerging Infectious Diseases, 14*(1), 70-72. doi:10.3201/eid1401.070704

Smith, G. C., & Carlile, N. (1993). Food and Feeding Ecology of Breeding Silver Gulls (*Larus Novaehollandiae*) in Urban Australia. *Colonial waterbirds, 16*(1), 9-17. doi: 10.2307/1521551

Smith, H. G., Clarke, R. H., Larkins, J.-A., Bean, D. C., & Greenhill, A. R. (2019). Wild Australian birds and drug-resistant bacteria: characterisation of antibiotic-resistant *Escherichia coli* and *Enterococcus* spp. *Emu-Austral Ornithology, 119*(4), 384-390.

Szabo, J. K., Choi, C.-Y., Clemens, R. S., & Hansen, B. (2016). Conservation without borders–solutions to declines of migratory shorebirds in the East Asian–Australasian Flyway. *Emu, 116*(2), 215-221.

Thaller, M. C., Migliore, L., Marquez, C., Tapia, W., Cedeno, V., Rossolini, G. M., & Gentile, G. (2010). Tracking acquired antibiotic resistance in commensal bacteria of Galapagos land iguanas: no man, no resistance. *PLoS ONE, 5*(2), e8989. doi:10.1371/journal.pone.0008989

Vangchhia, B., Abraham, S., Bell, J. M., Collignon, P., Gibson, J. S., Ingram, P. R., Johnson, J., Kennedy, K., Trott, D., & Turnidge, J. D. (2016). Phylogenetic diversity, antimicrobial susceptibility and virulence characteristics of phylogroup F *Escherichia coli* in Australia. *Microbiology, 162*(11), 1904-1912.

Water Corporation. (2018). *Broome high rainfall events January-February 2018: Water quality sampling results report*. (1-65).

Wilson, A., Fox, E. M., Fegan, N., & Kurtboke, D. I. (2019). Comparative Genomics and Phenotypic Investigations Into Antibiotic, Heavy Metal, and Disinfectant Susceptibilities of *Salmonella enterica* Strains Isolated in Australia. *Frontier Microbiology, 10*, 1620. doi:10.3389/fmicb.2019.01620

1

7. References

Abo-Amer, A. E., & Shobrak, M. Y. (2015). Isolation and Molecular Characterization of Multidrug-Resistant *Salmonella, Shigella* and *Proteus* from Domestic Birds. *Thai Journal of Veterinary Medicine, 45*(1), 23-34.

Abt, M. C., & Artis, D. (2013). The dynamic influence of commensal bacteria on the immune response to pathogens. *Current Opinion in Microbiology, 16*(1), 4-9.

Abulreesh, H., Goulder, R., & Scott, G. (2007). Wild birds and human pathogens in the context of ringing and migration. *Ringing & Migration, 23*(4), 193-200.

Afgan, E., Dannon B., Bérénice B., Marius B., Dave B., Martin Č., John C., Dave C., Nate C., & Björn A. (2018). The Galaxy platform for accessible, reproducible and collaborative biomedical analyses: 2018 update. *Nucleic acids research, 46*(W1):W537-W544.

Aharon-Rotman, Y. (2015). Challenges within the annual cycle of long-distance migratory waders along the East Asian-Australasian flyway. PhD thesis, Deakin University.

Ahmad, M., & Khan, A. U. (2019). Global economic impact of antibiotic resistance: a review. *Journal of Global Antimicrobial Resistance, 19*, 313-316.

Ahoyo, T., Bankole, H., Adeoti, F., Gbohoun, A., Assavedo, S., Amoussou-Guenou, M., Kinde-Gazard, D., Pittet, D. (2014). Prevelance of nosocomial infections and anti-infective therapy in Benin: results of the first nationwide survery in 2012. *Antimicrobial Resistance and Infection Control, 3*(17), 1-16.

Albers, H. J., Lee, K. D., Rushlow, J. R., & Zambrana-Torrselio, C. (2020). Disease risk from human-environment interactions: environment and development economics for joint conservation-health policy. *Environmental and Resource Economics, 76*(4), 929-944.

Albrechtova, K., Kubelova, M., Mazancova, J., Dolejska, M., Literak, I., & Cizek, A. (2014). High prevelence and variability of CTX-M-15-Producing and Fluoroquinolone-Resistant *Escherichia coli* observed in stray dogs in rural Angola. *Microbial Drug Resistance, 20*(4), 372-5.

Alkasir, R., Liu, X., Zahra, M., Ferreri, M., Su, J., & Han, B. (2013). Characteristics of *Staphylococcus aureus* Small Colony Variant and its parent strain isolated from chrinic mastitis at a dairy farm in Beijin, China. *Microbial Drug Resistance, 19*(2), 138-145.

Allen, H. K., Donato, J., Wang, H. H., Cloud-Hansen, K. A., Davies, J., & Handelsman, J. (2010). Call of the wild: antibiotic resistance genes in natural environments. *Nature Reviews Microbiology, 8*(4), 251-259. doi:10.1038/nrmicro2312

Alley, M. R., Connolly, J. H., Fenwick, S. G., Mackereth, G. F., Leyland, M. J., Rogers, L. E., Haycock, M., Nicol, C., & Reed, C. E. (2002). An epidemic of salmonellosis caused by *Salmonella* Typhimurium DT160 in wild birds and humans in New Zealand. *New Zealand Veterinary Journal, 50*(5), 170-176. doi:10.1080/00480169.2002.36306

Alroy, K., & Ellis, J. C. (2011). Pilot study of antimicrobial-resistant *Escherichia coli* in herring gulls (*Larus argentatus*) and wastewater in the northeastern United States. *Journal of Zoo and Wildlife Medicine, 42*(1), 160-163. doi:10.1638/2010-0130.1

Altizer, S., Bartel, R., & Han, B. A. (2011). Animal migration and infectious disease risk. *Science, 331*(6015), 296-302. doi:10.1126/science.1194694

Alvarez-Uria, G., Gandra, S., Mandal, S., & Laxminarayan, R. (2018). Global forecast of antimicrobial resistance in invasive isolates of *Escherichia coli* and *Klebsiella pneumoniae*. *International Journal of Infectious Diseases, 68*, 50-53. doi:10.1016/j.ijid.2018.01.011

Amabile-Cuevs, C. (2013). Antibiotic resistance: From Darwin to Lederberg to Keynes. *Microbial Drug Resistance, 19*(2), 73-87.

Andersson, D. I., & Hughes, D. (2012). Evolution of antibiotic resistance at non-lethal drug concentrations. *Drug Resistance Updates, 15*(3), 162-172. doi:10.1016/j.drup.2012.03.005

Ao, T. T., Feasey, N. A., Gordon, M. A., Keddy, K. H., Angulo, F. J., & Crump, J. A. (2015). Global burden of invasive nontyphoidal *Salmonella* disease, 2010. *Emerging Infectious Diseases, 21*(6), 941.

Argimón, S., Abudahab, K., Goater, R. J., Fedosejev, A., Bhai, J., Glasner, C., Feil, E., Holden, M., Yeats, C., & Grundmann, H. (2016). Microreact: visualizing and sharing data for genomic epidemiology and phylogeography. *Microbial genomics, 2*(11).

Armand-Lefevre, L., Ruimy, R., & Andremont, A. (2005). Clonal comparison of *Staphylococcus aureus* isolates from healthy pig farmers, human controls, and pigs. *Emerging Infectious Diseases, 11*(5), 711-714. doi:10.3201/eid1105.040866

Arnold, K. E., Williams, N. J., & Bennett, M. (2016). 'Disperse abroad in the land': the role of wildlife in the dissemination of antimicrobial resistance. *Biology Letters, 12*(8), 20160137.

Aryee, A., & Price, N. (2015). Antimicrobial stewardship - can we afford to do without it? *British Journal of Clincal Pharmacology, 79*(2), 173-181. doi:10.1111/bcp.12417

Ashenafi, M., Ammosova, T., Nekhai, S., & Byrnes, W. M. (2014). Purification and characterization of aminoglycoside phosphotransferase APH (6)-Id, a streptomycin-inactivating enzyme. *Molecular and Cellular Biochemistry, 387*(1-2), 207-216.

Ashton, P. M., Nair, S., Peters, T. M., Bale, J. A., Powell, D. G., Painset, A., Tewolde, R., Schaefer, U., Kenkins, C., & Dallman, T. (2016). Identification of *Salmonella* for public health surveillance using whole genome sequencing. *PeerJ, 4*, e1752.

Australian Associated Press. (2016). Rockmelon industry devastated by *Salmonella* outbreak linked to NT farm. Retrieved from https://www.theguardian.com/australia-news/2016/aug/05/rockmelon-industry-devastated-by-salmonella-outbreak-linked-to-nt-farm

Azevedo, J., Araujo, S., Oliveira, C., Correia, A., & Henriques, I. (2013). Analysis of antibiotic resistance in bacteria isolated from the surface microlayer and underlying water of an estuarine enviroment. *Microbial Drug Resistance, 19*(1), 64-71.

Baba, T., Takeuchi, F., Kuroda, M., Yuzawa, H., Aoki, K., Oguchi, A., Nagai, Y., Iwama, N., Asano, K., Naimi, T., Kuroda, H., Cui, L., Yamamoto, K., & Hiramatsu, K. (2002). Genome and virulence determinants of high virulence community-acquired MRSA. *Lancet, 359*(9320), 1819-1827. doi:10.1016/s0140-6736(02)08713-5

Babic, M., Hujer, A. M., & Bonomo, R. A. (2006). What's new in antibiotic resistance? Focus on beta-lactamases. *Drug Resistance Updates, 9*(3), 142-156. doi:10.1016/j.drup.2006.05.005

Baez, J., Hernandez-Garcia, M., Guamparito, C., Diaz, S., Olave, A., Guerrero, K., Canton, R., Baquero, F., Gahona, J., Valenzuela, N., Del Campo, R., & Silva, J. (2015). Molecular characterization and genetic diversity of ESBL-producing *Escherichia coli* colonizing the migratory Franklin's gulls (*Leucophaeus pipixcan*) in Antofagasta, North of Chile. *Microbial Drug Resistance, 21*(1), 111-116. doi:10.1089/mdr.2014.0158

Bai, G., Gajer, P., Nandy, M., Ma, B., Yang, H., Sakamoto, J., Blanchard, M. H., Ravel, J., & Brotman, R. M. (2012). Comparison of storage conditions for human vaginal microbiome studies. *PLoS ONE, 7*(5), e36934.

Bailie, R. S., Carson, B. E., & McDonald, E. L. (2004). Water supply and sanitation in remote Indigenous communities-priorities for health development. *Australian and New Zealand journal of public health, 28*(5), 409-414.

Barry, A., Fay, G., & Sauer, R. (1972). Efficiency of a transport medium for the recovery of aerobic and anaerobic bacteria from applicator swabs. *Applied microbiology, 24*(1), 31-33.

Bartoloni, A., Pallecchi, L., Rodríguez, H., Fernandez, C., Mantella, A., Bartalesi, F., Strohmeyer, M., Kristiansson, C., Gotuzzo, E., & Paradisi, F. (2009). Antibiotic resistance in a very remote Amazonas community. *International Journal of Antimicrobial Agents, 33*(2), 125-129.

Barton, M. D. (2010). *Antibiotic resistance in Australian animals in 2010–what lies ahead?* Paper presented at the APVMA Science Fellows Symposium.

Batt, R., Rutgers, H., & Sancak, A. (1996). Enteric bacteria: friend or foe? *Journal of Small Animal Practice, 37*(6), 261-267.

Bauer, S., & Hoye, B. J. (2014). Migratory animals couple biodiversity and ecosystem functioning worldwide. *Science, 344*(6179).

Beaber, J. W., Hochhut, B., & Waldor, M. K. (2004). SOS response promotes horizontal dissemination of antibiotic resistance genes. *Nature, 427*(6969), 72-74. doi:10.1038/nature02241

Beasley, D. E., Koltz, A. M., Lambert, J. E., Fierer, N., & Dunn, R. R. (2015). The evolution of stomach acidity and its relevance to the human microbiome. *PLoS ONE, 10*(7), e0134116.

Beceiro, A., Tomas, M., & Bou, G. (2013). Antimicrobial resistance and virulence: a successful or deleterious association in the bacterial world? *Clinical Microbiology Reviews, 26*(2), 185-230. doi:10.1128/CMR.00059-12

Begaud, E., Mondet, D., & Germani, Y. (1993). Molecular characterization of enterotoxigenic *Escherichia coli* (ETEC) isolated in New Caledonia (value of potential protective antigens in oral vaccine candidates). *Research in Microbiology, 144*(9), 721-728.

Beiring, M. (2013). Determination of valuable areas for migratory songbirds along the East-Asian Australasian Flyway (EEAF), and an approach for strategic conservation planning. Masters Thesis, University of Natural Resources and Life Sciences Vienna.

Belkaid, Y., & Hand, T. W. (2014). Role of the microbiota in immunity and inflammation. *Cell, 157*(1), 121-141. doi:10.1016/j.cell.2014.03.011

Bennun, L. (2006). Migratory birds as scapegoats. *Multinational Monitor, 27*(2), 21.

Benskin, C. M., Wilson, K., Jones, K., & Hartley, I. R. (2009). Bacterial pathogens in wild birds: a review of the frequency and effects of infection. *Biological Reviews of the Cambridge Philosophical Society, 84*(3), 349-373. doi:10.1111/j.1469-185X.2008.00076.x

Berglund, B. (2015). Environmental dissemination of antibiotic resistance genes and correlation to anthropogenic contamination with antibiotics. *Infection Ecology & Epidemiology, 5*(1), 28564. doi:10.3402/iee.v5.28564

Birdlife Australia. (2019). Bar-tailed Godwit. Retrieved from http://birdlife.org.au/bird-profile/bar-tailed-godwit

Biswas, M., Roy, D. N., Tajmim, A., Rajib, S. S., Hossain, M., Farzana, F., & Yasmen, N. (2014). Prescription antibiotics for outpatients in Bangladesh: a cross-sectional health survey conducted in three cities. *Annals of Clinical Microbiology and Antimicrobials, 13*(15), 15. doi:10.1186/1476-0711-13-15

Blanco, G., Lemus, J. A., Grande, J., Gangoso, L., Grande, J. M., Donazar, J. A., Arroyo, B., Frias, O., & Hiraldo, F. (2007). Geographical variation in cloacal microflora and bacterial antibiotic resistance in a threatened avian scavenger in relation to diet and livestock farming practices. *Environmental Microbiology, 9*(7), 1738-1749. doi:10.1111/j.1462-2920.2007.01291.x

Blyton, M. D., Pi, H., Vangchhia, B., Abraham, S., Trott, D. J., Johnson, J. R., & Gordon, D. M. (2015). Genetic Structure and Antimicrobial Resistance of *Escherichia coli* and Cryptic Clades in Birds with Diverse Human Associations. *Applied and Environmental Microbiology, 81*(15), 5123-5133. doi:10.1128/AEM.00861-15

Boehm, M., Hutchings, M. R., & White, P. C. (2009). Contact networks in a wildlife-livestock host community: identifying high-risk individuals in the transmission of bovine TB among badgers and cattle. *PLoS ONE, 4*(4), e5016.

Boere, G., & Stroud, D. (2006). *Waterbirds around the world: A global overview of the conservation, management and research of the world's waterbird flyways*. The Stationery Office, 71 Lothian Road, Edinburgh, UK: Scottish Natural Heritage.

Bonnedahl, J. (2011). Antibiotic resistance in *Enterobacteriacea* isolated from wild birds. *Digital Comprehensive Summaries of Uppsala Dissertations, 641*, 1-62.

Bonnedahl, J., Drobni, M., Gauthier-Clerc, M., Hernandez, J., Granholm, S., Kayser, Y., Melhus, A., Kahlmeter, G., Waldenstrom, J., Johansson, A., & Olsen, B. (2009). Dissemination of *Escherichia coli* with CTX-M type ESBL between humans and yellow-legged gulls in the south of France. *PLoS ONE, 4*(6), e5958. doi:10.1371/journal.pone.0005958

Bonnedahl, J., Drobni, P., Johansson, A., Hernandez, J., Melhus, A., Stedt, J., Olsen, B., & Drobni, M. (2010). Characterization, and comparison, of human clinical and black-headed gull (*Larus ridibundus*) extended-spectrum beta-lactamase-producing bacterial isolates from Kalmar, on the southeast coast of Sweden. *Journal of Antimicrobial Chemotherapy, 65*(9), 1939-1944. doi:10.1093/jac/dkq222

Bonnedahl, J., & Jarhult, J. D. (2014). Antibiotic resistance in wild birds. *Upsala Journal of Medical Sciences, 119*(2), 113-116. doi:10.3109/03009734.2014.905663

Borges, C. A., Cardozo, M. V., Beraldo, L. G., Oliveira, E. S., Maluta, R. P., Barboza, K. B., Werther, K., & Avila, F. A. (2017). Wild birds and urban pigeons as reservoirs for diarrheagenic *Escherichia coli* with zoonotic potential. *Journal of Microbiology, 55*(5), 344-348. doi:10.1007/s12275-017-6523-3

Bortolaia, V., Kaas, R. S., Ruppe, E., Roberts, M. C., Schwarz, S., Cattoir, V., Philippon, A., Allesoe, R., Rebelo, A., & Florensa, A. F. (2020). ResFinder 4.0 for predictions of phenotypes from genotypes. *Journal of Antimicrobial Chemotherapy, 75*(12), 3491-3500.

Boucher, H. W., & Corey, G. R. (2008). Epidemiology of methicillin-resistant *Staphylococcus aureus*. *Clinical Infectious Diseases, 46 Suppl 5*(5), S344-349. doi:10.1086/533590

Brewer, C. A. (1994). Color use guidelines for mapping. *Visualization in modern cartography, 1994*, 123-148.

Brittingham, M. C., Temple, S. A., & Duncan, R. M. (1988). A survey of the prevalence of selected bacteria in wild birds. *Journal of Wildlife Diseases, 24*(2), 299-307.

Brown, C. (2004). Emerging zoonoses and pathogens of public health significance - an overview. *Revue Scientifique Et Technique De L Office International Des Epizooties, 23*(2), 435-442. doi:DOI 10.20506/rst.23.2.1495

Brown, C. R., Brown, M. B., Moore, A. T., & Komar, N. (2007). Bird movement predicts Buggy Creek virus infection in insect vectors. *Vector Borne Zoonotic Diseases, 7*(3), 304-314. doi:10.1089/vbz.2006.0646

Burroughs, T., Knobler, S., & Lederberg, J. (2002). *The emergence of zoonotic diseases: Understanding the impact on animal and human health: workshop summary*. National Academy Press, Washington.

Buzby, J. C., & Roberts, T. (2009). The economics of enteric infections: human foodborne disease costs. *Gastroenterology, 136*(6), 1851-1862.

Camarda, A., Circella, E., Pennelli, D., Madio, A., Bruni, G., Lagrasta, V., Marzano, G., Mallia, E., & Campagnari, E. (2006). Wild birds as biological indicators of environmental pollution: biotyping and antimicrobial resistance patterns of *Escherichia coli* isolated from Audouin's gulls (*Larus audouinii*) living in the Bay of Gallipoli (Italy). *Italian Journal of Animal Science, 5*(3), 287-290.

Casadevall, A., & Pirofski, L. (2001). Host-pathogen interactions: the attributes of virulence. *Journal of Infectious Disease, 184*(3), 337-344. doi:10.1086/322044

Castanie-Cornet, M.-P., Penfound, T. A., Smith, D., Elliott, J. F., & Foster, J. W. (1999). Control of acid resistance in *Escherichia coli*. *Journal of Bacteriology, 181*(11), 3525-3535.

Cecchini, M., Langer, J., & Slawomirski, L. (2015). Antimicrobial resistance in G7 countries and beyond: economic issues, policies and options for action. *Paris: Organization for Economic Co-operation and Development*, 1-75.

Chan, J. F., To, K. K., Chen, H., & Yuen, K. Y. (2015). Cross-species transmission and emergence of novel viruses from birds. *Current Opinion in Virology, 10*, 63-69. doi:10.1016/j.coviro.2015.01.006

Chatterjee, S., Ghosh, A. K., & Chattopadhyay, J. (2006). Controlling disease in migratory bird population: a probable solution through mathematical study. *Dynamical Systems: An International Journal, 21*(3), 265-288. doi:10.1080/14689360500456279

Chlebicz, A., & Slizewska, K. (2018). Campylobacteriosis, Salmonellosis, Yersiniosis, and Listeriosis as Zoonotic Foodborne Diseases: A Review. *International Journal of Environmental Research and Public Health, 15*(5). doi:10.3390/ijerph15050863

Choi, J. M., & Woo, G. J. (2013). Molecular characterization of high-level gentamicin-resistant *Enterococcus faecalis* from chicken meat in Korea. *International Journal of Food Microbiology, 165*(1), 1-6. doi:10.1016/j.ijfoodmicro.2013.02.016

Chomel, B. B., Belotto, A., & Meslin, F. X. (2007). Wildlife, exotic pets, and emerging zoonoses. *Emerging Infectious Diseases, 13*(1), 6-11. doi:10.3201/eid1301.060480

Christou, L. (2011). The global burden of bacterial and viral zoonotic infections. *Clinical Microbiology and Infection, 17*(3), 326-330. doi:10.1111/j.1469-0691.2010.03441.x

Chua, K. B., Chua, B. H., & Wang, C. W. (2002). Anthropogenic deforestation, El Niño and the emergence of Nipah virus in Malaysia. *Malaysian Journal of Pathology, 24*(1), 15-21.

Chua, K. Y., Grayson, M. L., Burgess, A. N., Lee, J. Y., & Howden, B. P. (2014). The growing burden of multidrug-resistant infections among returned Australian travellers. *Medical Journal of Australia, 200*(2), 116-118. doi:10.5694/mja13.10592

Churria, C. D. G., Arias, N., Origlia, J., Netri, C., Marcantoni, H., Píscopo, M., Loyola, M., & Petruccelli, M. (2014). *Citrobacter freundii* infection in two captive Australian king parrots (*Alisterus scapularis*). *Journal of Zoo and Aquarium Research, 2*(2), 52-53.

Cizek, A., Dolejska, M., Karpiskova, R., Dedicova, D., & Literak, I. (2007). Wild black-headed gulls (*Larus ridibundus*) as an environmental reservoir of *Salmonella* strains resistant to antimicrobial drugs. *European Journal of Wildlife Research, 53*(1), 55-60. doi:10.1007/s10344-006-0054-2

Cizek, A., Literak, I., Hejlicek, K., Treml, F., & Smola, J. (1994). *Salmonella* contamination of the environment and its incidence in wild birds. *Journal of Veterinary Medicine Series B-Zentralblatt Fur Veterinarmedizin Reihe B-Infectious Diseases and Veterinary Public Health, 41*(5), 320-327.

Clarke, R. H. (2004). The avifauna of northern Torres Strait: Notes on a wet-season visit. *Australian Field Ornithology, 21*(2), 49.

Claughton, D., Kontominas, B., & Logan, T. (2019). Rockmelon listeria: Rombola Family Farms named as source of outbreak. ABC Rural. In.

Clemente, J. C., Ursell, L. K., Parfrey, L. W., & Knight, R. (2012). The impact of the gut microbiota on human health: an integrative view. *Cell, 148*(6), 1258-1270. doi:10.1016/j.cell.2012.01.035

Clermont, O., Olier, M., Hoede, C., Diancourt, L., Brisse, S., Keroudean, M., Glodt, J., Picard, B., Oswald, E., Denamur, E. (2011). Animal and human pathogenic *Escherichia coli* strains share common genetic backgrounds. *Infection, genetics and evolution, 11*(3), 654-662.

Clinical and Laboratory Standards Institute. (2012). Performance standards for antimicrobial susceptibility testing; Twenty-second informational supplement. In (Vol. 32, pp. 188). Wayne, Pennsylvania, U.S.A: Clinical and Laboratory Standards Institute.

CLSI. 2018. Performance standards for antimicrobial susceptibility testing: twenty-second informational supplement. 28th edition CLSI supplement M100. Wayne, PA. Clinical and Laboratory Standards Institute.

Cole, D., Drum, D. J., Stalknecht, D. E., White, D. G., Lee, M. D., Ayers, S., Sobsey, M., & Maurer, J. J. (2005). Free-living Canada geese and antimicrobial resistance. *Emerging Infectious Diseases, 11*(6), 935-938. doi:10.3201/eid1106.040717

Collee, J., Watt, B., Brown, R., & Johnstone, S. (1974). The recovery of anaerobic bacteria from swabs. *Epidemiology & Infection, 72*(3), 339-347.

Conly, J., & Johnston, M. (2005). Where are all the new antibiotcs? The new antibiotic paradox. *Infectious Diseases and Medical Microbiology, 16*(3), 159-160.

Coombs, G. W., Daley, D. A., Lee, Y. T., & Pang, S. (2019). Australian Group on Antimicrobial Resistance (AGAR) Australian *Staphylococcus aureus* Sepsis Outcome Programme (ASSOP) Annual Report 2013. *Communicable Diseases Intelligence, 43*.

Costa, M., Pracca, G., Sucari, A., Galli, L., Ibargoyen, J., Gentiluomo, J., Brusa, V., Zugazua, M. M., Figueroa, Y., & Londero, A. (2020). Comprehensive evaluation and implementation of improvement actions in bovine abattoirs to reduce pathogens exposure. *Preventive Veterinary Medicine 176*, 104933.

Cox, G., Koteva, K., & Wright, G. D. (2014). An unusual class of anthracyclines potentiate Gram-positive antibiotics in intrinsically resistant Gram-negative bacteria. *Journal of Antimicrobial Chemotherapy, 69*(7), 1844-1855. doi:10.1093/jac/dku057

Crombe, F., Willems, G., Dispas, M., Hallin, M., Denis, O., Suetens, C., Gordts, B., Struelens, M., & Butaye, P. (2012). Prevelance and antimicrobial susceptibility of methicillin-resistant *Staphylococcus aureus* among pigs in Belgium. *Microbial Drug Resistance, 18*(2), 125-131.

da Costa, P., Loureiro, L., & Matos, A. (2013). Transfer of Multidrug-Resistant Bacteria Between Intermingled Ecological Niches: The Interface Between Humans, Animals and the Environment. *International Journal of Environmental Research and Public Health, 10*(1), 278-294. doi:10.3390/ijerph10010278

Daszak, P., Cunningham, A. A., & Hyatt, A. D. (2000). Emerging infectious diseases of wildlife--threats to biodiversity and human health. *Science, 287*(5452), 443-449. doi:10.1126/science.287.5452.443

David, R. (2012). Pathogens and commensals fight it out. *Nature Reviews Microbiology, 10*(7), 445-445.

Davies, J., & Davies, D. (2010). Origins and evolution of antibiotic resistance. *Microbiology and Molecular Biology Reviews, 74*(3), 417-433. doi:10.1128/MMBR.00016-10

de Kraker, M. E., Stewardson, A. J., & Harbarth, S. (2016). Will 10 million people die a year due to antimicrobial resistance by 2050? *PLoS Medicine, 13*(11), e1002184.

Delannoy, S., Beutin, L., Mariani-Kurkdjian, P., Fleiss, A., Bonacorsi, S., & Fach, P. (2017). The *Escherichia coli* serogroup O1 and O2 lipopolysaccharides are encoded by multiple O-antigen gene clusters. *Frontiers in Cellular and Infection Microbiology, 7*, 30.

Denton, M. (2007). *Enterobacteriaceae*. *International Journal of Antimicrobial Agents, 29 Suppl 3*(0), S9-S22. doi:10.1016/S0924-8579(07)72174-X

Depardieu, F., Perichon, B., & Courvalin, P. (2004). Detection of the *van* alphabet and identification of enterococci and staphylococci at the species level by multiplex PCR. *Journal of Clinical Microbiology, 42*(12), 5857-5860. doi:10.1128/JCM.42.12.5857-5860.2004

Devriese, L., Cruz Colque, J., Haesebrouck, F., Desmidt, M., Uyttebroek, E., & Ducatelle, R. (1992). *Enterococcus hirae* in septicaemia of psittacine birds. *Veterinary Record, 130*(25), 558-559.

Dixon, B. (2013). Microbial ornithology? *Microbe, 8*(5), 196-197.

Dogan, B., Rishniw, M., Bruant, G., Harel, J., Schukken, Y. H., & Simpson, K. W. (2012). Phylogroup and *lpfA* influence epithelial invasion by mastitis associated *Escherichia coli*. *Veterinary Microbiology, 159*(1-2), 163-170.

Dolejska, M., Masarikova, M., Dobiasova, H., Jamborova, I., Karpiskova, R., Havlicek, M., Carlile, N., Priddel, D., Cizek, A., & Literak, I. (2016). High prevalence of *Salmonella* and IMP-4-producing Enterobacteriaceae in the silver gull on Five Islands, Australia. *Journal of Antimicrobial Chemotherapy, 71*(1), 63-70. doi:10.1093/jac/dkv306

Donnenberg, M. S. (2000). Pathogenic strategies of enteric bacteria. *Nature, 406*(6797), 768-774.
Dotto, G., Giacomelli, M., Grilli, G., Ferazzi, V., Carattoli, A., Fortini, D., & Piccirillo, A. (2014). High prevelance of *oqx*AB in *Escherichia coli* isolates from domestic and wild lagomorphs in Italy. *Microbial Drug Resistance, 29*(2), 118-123.
Draffan, R., Garnett, S., & Malone, G. (1983). Birds of the Torres Strait: an annotated list and biogeographical analysis. *Emu, 83*(4), 207-234.
Dusek, R. J., Hallgrimsson, G. T., Ip, H. S., Jonsson, J. E., Sreevatsan, S., Nashold, S. W., TeSlaa, J., Enomoto, S., Halpin, R., Lin, X., Fedorova, N., Stockwell, T., Dugan, V., Wentworth, D., & Hall, J. S. (2014). North Atlantic migratory bird flyways provide routes for intercontinental movement of avian influenza viruses. *PLoS ONE, 9*(3), e92075. doi:10.1371/journal.pone.0092075
Dutta, B., Scherm, H., Gitaitis, R., & Walcott, R. (2012). *Acidovorax citrulli* seed inoculum load affects seedling transmission and spread of bacterial fruit blotch of watermelon under greenhouse conditions. *Plant disease, 96*(5), 705-711.
Edgar, R., & Bibi, E. (1997). *mdfA*, an *Escherichia coli* multidrug resistance protein with an extraordinarily broad spectrum of drug recognition. *Journal of Bacteriology, 179*(7), 2274-2280.
Eng, S.-K., Pusparajah, P., Ab Mutalib, N.-S., Ser, H.-L., Chan, K.-G., & Lee, L.-H. (2015). *Salmonella*: a review on pathogenesis, epidemiology and antibiotic resistance. *Frontiers in Life Science, 8*(3), 284-293.
Epstein, J. H., McKee, J., Shaw, P., Hicks, V., Micalizzi, G., Daszak, P., Kilpatrick, A., & Kaufman, G. (2006). The Australian White Ibis (*Threskiornis molucca*) as a Reservoir of Zoonotic and Livestock Pathogens. *EcoHealth, 3*(4), 290-298. doi:10.1007/s10393-006-0064-2
Ewers, C., Antao, E. M., Diehl, I., Philipp, H. C., & Wieler, L. H. (2009). Intestine and environment of the chicken as reservoirs for extraintestinal pathogenic *Escherichia coli* strains with zoonotic potential. *Applied and Environmental Microbiology, 75*(1), 184-192. doi:10.1128/AEM.01324-08
Fauci, A. S. (2005). Emerging and reemerging infectious diseases: the perpetual challenge. *Annals of the Academy of Medicine, 80*(12), 1079-1085. doi:10.1097/00001888-200512000-00002
Fearnley, E., Lal, A., Bates, J., Stafford, R., Kirk, M., & Glass, K. (2018). *Salmonella* source attribution in a subtropical state of Australia: capturing environmental reservoirs of infection. *Epidemiology & Infection, 146*(15), 1903-1908.
Feldmann, F., Sorsa, L. J., Hildinger, K., & Schubert, S. (2007). The salmochelin siderophore receptor IroN contributes to invasion of urothelial cells by extraintestinal pathogenic *Escherichia coli* in vitro. *Infection and Immunity, 75*(6), 3183-3187.
Fernández-Delgado, M., & Suárez, P. (2009). Multiple antibiotic resistances of enteric bacteria isolated from recreational coastal waters and oysters of the Caribbean Sea. *Annals of Microbiology, 59*(3), 409.
Fick, J., Soderstrom, H., Lindberg, R. H., Phan, C., Tysklind, M., & Larsson, D. G. (2009). Contamination of surface, ground, and drinking water from pharmaceutical production. *Environmental Toxicology and Chemistry, 28*(12), 2522-2527. doi:10.1897/09-073.1
Field, H., Young, P., Yob, J. M., Mills, J., Hall, L., & Mackenzie, J. (2001). The natural history of Hendra and Nipah viruses. *Microbes and Infection, 3*(4), 307-314.
Flynn, D. (2016). How did *Salmonella* Hvittingfoss get on Aussie rockmelons. *Food Safety News, 9*.
Fogarty, L., Haack, S., Wolcott, M., & Whitman, R. (2003). Abundance and characteristics of the recreational water quality indicator bacteria *Escherichia coli* and enterococci in gull faeces. *Journal of Applied Microbiology, 94*(5), 865-878.
Ford, L., Glass, K., Veitch, M., Wardell, R., Polkinghorne, B., Dobbins, T., Lal, A., & Kirk, M. (2016). Increasing incidence of *Salmonella* in Australia, 2000-2013. *PLoS ONE, 11*(10), e0163989.

Forshell, L. P., & Wierup, M. (2006). *Salmonella* contamination: a significant challenge to the global marketing of animal food products. *Revue Scientifique et Technique-Office International des Epizooties, 25*(2), 541-554.

Foti, M., Rinaldo, D., Guercio, A., Giacopello, C., Aleo, A., De Leo, F., Fisichella, V., & Mammina, C. (2011). Pathogenic microorganisms carried by migratory birds passing through the territory of the island of Ustica, Sicily (Italy). *Avian Pathology, 40*(4), 405-409. doi:10.1080/03079457.2011.588940

Fratamico, P. M., DebRoy, C., Liu, Y., Needleman, D. S., Baranzoni, G. M., & Feng, P. (2016). Advances in molecular serotyping and subtyping of *Escherichia coli*. *Frontiers in Microbiology, 7*, 644.

Fratamico, P. M., Yan, X., Liu, Y., DebRoy, C., Byrne, B., Monaghan, A., Fanning, S., & Bolton, D. (2010). *Escherichia coli* serogroup O2 and O28ac O-antigen gene cluster sequences and detection of pathogenic *E. coli* O2 and O28ac by PCR. *Canadian Journal of Microbiology, 56*(4), 308-316.

French, G. L. (2005). Clinical impact and relevance of antibiotic resistance. *Advanced Drug Delivery Reviews, 57*(10), 1514-1527. doi:10.1016/j.addr.2005.04.005

French, G. L. (2010). The continuing crisis in antibiotic resistance. *International Journal of Antimicrobial Agents, 36 Suppl 3*(0), S3-7. doi:10.1016/S0924-8579(10)70003-0

Furlan, J., Gallo, I., de Campos, A., Navarro, A., Kobayashi, R., Nakazato, G., & Stehling, E. G. (2019). Characterization of non-O157 Shiga toxin-producing *Escherichia coli* (STEC) obtained from feces of sheep in Brazil. *World Journal of Microbiology and Biotechnology, 35*(9), 134.

Galani, I., Kontopidou, F., Souli, M., Rekatsina, P. D., Koratzanis, E., Deliolanis, J., & Giamarellou, H. (2008). Colistin susceptibility testing by Etest and disk diffusion methods. *International Journal of Antimicrobial Agents, 31*(5), 434-439.

Gallo-Cajiao, E., Morrison, T. H., Woodworth, B. K., Lees, A. C., Naves, L. C., Yong, D. L., Choi, C., Mundkur, T., Bird, J., & Jain, A. (2020). Extent and potential impact of hunting on migratory shorebirds in the Asia-Pacific. *Biological Conservation, 246*, 108582.

Gao, W., Howden, B. P., & Stinear, T. P. (2018). Evolution of virulence in *Enterococcus faecium*, a hospital-adapted opportunistic pathogen. *Current Opinion in Microbiology, 41*, 76-82. doi:10.1016/j.mib.2017.11.030

Gargiulo, A., Russo, T. P., Schettini, R., Mallardo, K., Calabria, M., Menna, L. F., Raia, P., Pagnini, U., Caputo, V., Fioretti, A, & Dipineto, L. (2014). Occurrence of enteropathogenic bacteria in urban pigeons (*Columba livia*) in Italy. *Vector Borne Zoonotic Diseases, 14*(4), 251-255. doi:10.1089/vbz.2011.0943

Gästrin, B., Kallings, L., & Marcetic, A. (1968). The survival time for different bacteria in various transport media. *Acta pathologica et microbiologica Scandinavica, 74*(3), 371-380.

Gaukler, S. M., Linz, G. M., Sherwood, J. S., Dyer, N. W., Bleier, W. J., Wannemuehler, Y. M., Nolan, L. K., & Logue, C. M. (2009). *Escherichia coli, Salmonella*, and *Mycobacterium avium* subsp *paratuberculosis* in Wild European Starlings at a Kansas Cattle Feedlot. *Avian Diseases, 53*(4), 544-551. doi:DOI 10.1637/8920-050809-Reg.1

Gearin, C. (2016). Detective gulls sniff out illegally dumped trash. *New Scientist,* (3085), 22.

Giacopello, C., Foti, M., Mascetti, A., Grosso, F., Ricciardi, D., Fisichella, V., & Lo Piccolo, F. (2016). Antimicrobial resistance patterns of Enterobacteriaceae in European wild bird species admitted in a wildlife rescue centre. *Veterinaria Italiana, 52*(2), 139-144. doi:10.12834/VetIt.327.1374.2

Gilchrist, M. J., Greko, C., Wallinga, D. B., Beran, G. W., Riley, D. G., & Thorne, P. S. (2007). The potential role of concentrated animal feeding operations in infectious disease epidemics and antibiotic resistance. *Environmental Health Perspective, 115*(2), 313-316. doi:10.1289/ehp.8837

Gilmore, M. S., Lebreton, F., & van Schaik, W. (2013). Genomic transition of enterococci from gut commensals to leading causes of multidrug-resistant hospital infection in the antibiotic era. *Current Opinions in Microbiology, 16*(1), 10-16. doi:10.1016/j.mib.2013.01.006

Glenn, L., Lindsey, R., Folster, J., Pecic, G., Boerlin, P., Gilmour, M., Harbottle, H., Zhao, S., McDermott, P., Fedorka-Cray, P., & Fyre, J. (2013). Antimicrobial resistance genes in multidrug-resistant *Salmonella enterica* isolated from animals, retail meats, and humans in the United States and Cnada. *Microbial Drug Resistance, 19*(3), 175-184.

Gnarpe, H. (1976). Survival of bacteria in four different transportation systems. *Scandinavian Journal of Infectious Diseases, 8*(4), 279-280.

Gordon, D. M., & Cowling, A. (2003). The distribution and genetic structure of *Escherichia coli* in Australian vertebrates: host and geographic effects. *Microbiology, 149*(Pt 12), 3575-3586. doi:10.1099/mic.0.26486-0

Gortazar, C., Diez-Delgado, I., Barasona, J. A., Vicente, J., De La Fuente, J., & Boadella, M. (2015). The wild side of disease control at the wildlife-livestock-human interface: a review. *Frontiers in Veterinary Science, 1*, 27.

Gortazar, C., Reperant, L. A., Kuiken, T., de la Fuente, J., Boadella, M., Martinez-Lopez, B., Ruiz-Fons, F., Estrada-Pena, A., Drosten, C., Medley, G., Ostfeld, R., Peterson, T., VerCauteren, K. C., Menge, C., Artois, M., Schultsz, C., Delahay, R., Serra-Cobo, J., Poulin, R., Keck, F., Aguirre, A. A., Henttonen, H., Dobson, A., Kutz, S., Lubroth, J., & Mysterud, A. (2014). Crossing the interspecies barrier: opening the door to zoonotic pathogens. *PLoS Pathogens, 10*(6), e1004129. doi:10.1371/journal.ppat.1004129

Gould, I. M. (2009). Antibiotic resistance: the perfect storm. *International Journal of Antimicrobial Agents, 34 Suppl 3*(0), S2-5. doi:10.1016/S0924-8579(09)70549-7

Greenhalgh, M. (2010). *Salmonella* lawsuit filed against Subway. *Food Safety News https://www. foodsafetynews. com/2010/06/salmonella-lawsuit-filed-against-subway*.

Greenhill, A. R., Guwada, C., Siba, V., Michael, A., Yoannes, M., Wawarie, Y., Ford, R., Siba, P., & Horwood, P. F. (2014). Antibiotic resistant *Shigella* is a major cause of diarrhoea in the Highlands of Papua New Guinea. *Journal of Infection in Developing Countries, 8*(11), 1391-1397. doi:10.3855/jidc.4396

Greger, M. (2007). The long haul: risks associated with livestock transport. *Biosecurity and Bioterrorism, 5*(4), 301-311. doi:10.1089/bsp.2007.0028

Greig, J., Rajic, A., Young, I., Mascarenhas, M., Waddell, L., & LeJeune, J. (2015). A scoping review of the role of wildlife in the transmission of bacterial pathogens and antimicrobial resistance to the food Chain. *Zoonoses Public Health, 62*(4), 269-284. doi:10.1111/zph.12147

Grond, K. (2017). *Development and dynamics of gut microbial communities of migratory shorebirds in the Western Hemisphere.* Doctoral Dissertation, Kansas State University.

Guenther, S., Aschenbrenner, K., Stamm, I., Bethe, A., Semmler, T., Stubbe, A., Stubbe, M., Batsajkhan, N., Glupszynski, Y., Wieler, L, & Ewers, C. (2012). Comparable high rates of extended-spectrum-beta-lactamase-producing *Escherichia coli* in birds of prey from Germany and Mongolia. *PLoS ONE, 7*(12), e53039. doi:10.1371/journal.pone.0053039

Gullberg, E., Albrecht, L. M., Karlsson, C., Sandegren, L., & Andersson, D. I. (2014). Selection of a multidrug resistance plasmid by sublethal levels of antibiotics and heavy metals. *mBio, 5*(5), e01918-01914. doi:10.1128/mBio.01918-14

Gurevich, A., Saveliev, V., Vyahhi, N., & Tesler, G. (2013). QUAST: quality assessment tool for genome assemblies. *Bioinformatics, 29*(8), 1072-1075.

Haft, D. H., DiCuccio, M., Badretdin, A., Brover, V., Chetvernin, V., O'Neill, K., Li, W., Chitsaz, F., Derbyshire, M., & Gonzales, N. R. (2018). RefSeq: an update on prokaryotic genome annotation and curation. *Nucleic Acids Research, 46*(D1), D851-D860.

Hallander, H., Flodström, A., & Holmberg, K. (1975). Influence of the collection and transport of specimens on the recovery of bacteria from peritonsillar abscesses. *Journal of Clinical Microbiology, 2*(6), 504-509.

Handeland, K., Refsum, T., Johansen, B. S., Holstad, G., Knutsen, G., Solberg, I., Schulze, J., & Kapperud, G. (2002). Prevalence of *Salmonella typhimurium* infection in Norwegian hedgehog populations associated with two human disease outbreaks. *Epidemiology and Infection, 128*(3), 523-527. doi:10.1017/s0950268802007021

Hartemink, N. A., Davis, S. A., Reiter, P., Hubalek, Z., & Heesterbeek, J. A. (2007). Importance of bird-to-bird transmission for the establishment of West Nile virus. *Vector Borne Zoonotic Diseases, 7*(4), 575-584. doi:10.1089/vbz.2006.0613

Hassell, J. M., Begon, M., Ward, M. J., & Fèvre, E. M. (2017). Urbanization and Disease Emergence: Dynamics at the Wildlife–Livestock–Human Interface. *Trends in Ecology & Evolution, 32*(1), 55-67.

Havelaar, A. H., Kirk, M. D., Torgerson, P. R., Gibb, H. J., Hald, T., Lake, R. J., Praet, N., Bellinger, D., De Silva, N., & Gargouri, N. (2015). World Health Organization global estimates and regional comparisons of the burden of foodborne disease in 2010. *PLoS medicine, 12*(12), e1001923.

Haydon, D., Cleaveland, S., Taylor, L., & Laurenson, M. (2002). Identifying reservious of infection: A conceptual and practical challenge. *Emerging Infectious Diseases, 8*(12), 1468-1473. doi:10.3201/eid0812.010317

Heffernan, A. J., Sime, F. B., Lipman, J., & Roberts, J. A. (2018). Individualising Therapy to Minimize Bacterial Multidrug Resistance. *Drugs, 78*(6), 621-641. doi:10.1007/s40265-018-0891-9

Herdt, P. d., Defoort, P., Steelant, J. v., Swam, H., Tanghe, L., Goethem, S. v., & Vanrobaeys, M. (2009). *Enterococcus cecorum* osteomyelitis and arthritis in broiler chickens. *Vlaams Diergeneeskundig Tijdschrift, 78*(1), 44-48.

Hernandez, J., Stedt, J., Bonnedahl, J., Molin, Y., Drobni, M., Calisto-Ulloa, N., Gomez-Fuentes, C., Astorga-Espana, M., Gonzalez-Acuna, D., Waldenstrom, J., Blomqvist, M., & Olsen, B. (2012). Human-associated extended-spectrum beta-lactamase in the Antarctic. *Applied and Environmental Microbiology, 78*(6), 2056-2058. doi:10.1128/AEM.07320-11

Hernandez-Divers, S.M., Villegas, P., Jimenez, C., Hernandez-Divers, S.J., Garcia, M., Riblet, S., Sanchez, S *et al*. (2008). Backyard chicken flocks pose a disease risk for neotropic birds in Costa Rica. *Avian Diseases, 52*(4), 558-566.

Higgins, P. J., & Davies, S. J. (1996). *Handbook of Australian, New Zealand & Antarctic birds. Vol. 3, Snipe to pigeons*: Oxford University Press.

Hiltunen, T., Virta, M., & Laine, A. L. (2017). Antibiotic resistance in the wild: an eco-evolutionary perspective. *Philosphical Transactions of the Royal Society of London B Biological Science, 372*(1712). doi:10.1098/rstb.2016.0039

Hoque, M., Burgess, G., Greenhil, A., Hedlefs, R., & Skerratt, L. F. (2012). Causes of morbidity and mortality of wild aquatic birds at Billabong Sanctuary, Townsville, North Queensland, Australia. *Avian Diseases, 56*(1), 249-256.

Horwood, P., & Greenhill, A. (2012). Cholera in Papua New Guinea and the importance of safe water sources and sanitation. *Western Pacific surveillance and response journal: WPSAR, 3*(1), 3.

Hsu, J. T., Chen, C. Y., Young, C. W., Chao, W. L., Li, M. H., Liu, Y. H., Lin, C. M., & Ying, C. (2014). Prevalence of sulfonamide-resistant bacteria, resistance genes and integron-associated horizontal gene transfer in natural water bodies and soils adjacent to a swine feedlot in northern Taiwan. *Journal of Hazardous Materials, 277*, 34-43. doi:10.1016/j.jhazmat.2014.02.016

Hutchings, M. I., Truman, A. W., & Wilkinson, B. (2019). Antibiotics: Past, present and future. *Current Opinion in Microbiology, 51*, 72-80.

Igbinosa, E. O. (2016). Detection and Antimicrobial Resistance of *Vibrio* Isolates in Aquaculture Environments: Implications for Public Health. *Microbial Drug Resistance, 22*(3), 238-245. doi:10.1089/mdr.2015.0169

Isenberg, H. D., Goldberg, D., & Sampson, J. (1970). Laboratory studies with a selective *Enterococcus* medium. *Applied microbiology, 20*(3), 433-436.

Iveson, J., Mackay-Scollay, E., & Bamford, V. (1969). *Salmonella* and Arizona in reptiles and man in Western Australia. *Epidemiology & Infection, 67*(1), 135-145.

Jackson, J., & Elgar, M. A. (1993). The foraging behaviour of the willie wagtail *Rhipidura leucophrys*: Why does it wag its tail? *Emu-Austral Ornithology, 93*(4), 284-286.

Jacoby, G. A., Walsh, K. E., Mills, D. M., Walker, V. J., Oh, H., Robicsek, A., & Hooper, D. C. (2006). *qnrB*, another plasmid-mediated gene for quinolone resistance. *Antimicrobial Agents and Chemotherapy, 50*(4), 1178-1182.

Jarnheimer, P. A., Ottoson, J., Lindberg, R., Stenstrom, T. A., Johansson, M., Tysklind, M., Winner, M., & Olsen, B. (2004). Fluoroquinolone antibiotics in a hospital sewage line; occurrence, distribution and impact on bacterial resistance. *Scandanavian Journal of Infectious Diseases, 36*(10), 752-755. doi:10.1080/00365540410021027

Jin, G., Englande, A., Bradford, H., & Jeng, H. W. (2004). Comparison of *E. coli*, enterococci, and fecal coliform as indicators for brackish water quality assessment. *Water Environment Research, 76*(3), 245-255.

Joensen, K. G., Scheutz, F., Lund, O., Hasman, H., Kaas, R. S., Nielsen, E. M., & Aarestrup, F. M. (2014). Real-time whole-genome sequencing for routine typing, surveillance, and outbreak detection of verotoxigenic *Escherichia coli*. *Journal of Clinical Microbiology, 52*(5), 1501-1510.

Joensen, K. G., Tetzschner, A. M., Iguchi, A., Aarestrup, F. M., & Scheutz, F. (2015). Rapid and easy in silico serotyping of *Escherichia coli* isolates by use of whole-genome sequencing data. *Journal of clinical microbiology*, *53*(8), 2410-2426.

Johns, S., & Shaw, A. K. (2016). Theoretical insight into three disease-related benefits of migration. *Population Ecology, 58*(1), 213-221.

Johnson, J. R. (1991). Virulence factors in *Escherichia coli* urinary tract infection. *Clinical Microbiology Reviews, 4*(1), 80-128. doi:10.1128/cmr.4.1.80

Johnson, T. J., Wannemuehler, Y. M., & Nolan, L. K. (2008). Evolution of the *iss* gene in *Escherichia coli*. *Applied and Environmental Microbiology, 74*(8), 2360-2369.

Jones, K. E., Patel, N. G., Levy, M. A., Storeygard, A., Balk, D., Gittleman, J. L., & Daszak, P. (2008). Global trends in emerging infectious diseases. *Nature, 451*(7181), 990-993. doi:10.1038/nature06536

Jorgensen, J. H., & Pfaller, M. A. (2015). Introduction to the 11th Edition of the Manual of Clinical Microbiology. In *Manual of Clinical Microbiology, Eleventh Edition* (pp. 3-4): American Society of Microbiology.

Jost, T., Lacroix, C., Braegger, C., & Chassard, C. (2013). Assessment of bacterial diversity in breast milk using culture-dependent and culture-independent approaches. *British Journal of Nutrition, 110*(7), 1253-1262.

Kahn, L. H. (2017). Antimicrobial resistance: a One Health perspective. *Transactions of the Royal Society of Tropical Medicine and Hygine, 111*(6), 255-260. doi:10.1093/trstmh/trx050

Kapperud, G., Gustavsen, S., Hellesnes, I., Hansen, A. H., Lassen, J., Hirn, J., Jahkola, M., Montenegro, M., & Helmuth, R. (1990). Outbreak of *Salmonella typhimurium* infection traced to contaminated chocolate and caused by a strain lacking the 60-megadalton virulence plasmid. *Journal of Clinical Microbiology, 28*(12), 2597-2601. doi:0095-1137/90/122597-05$02.00/0

Keeler, S., & Huffman, J. (2009). Identification of *Staphylococcus* spp. and aerobic gram-negative bacteria from the cloacae of migratory shorebirds (Family *Scolopacidae*) from Delware Bay, New Jersey. *Journal of the Pennsylvania Academy of Science*, 34-37.

Keen, B. (2017). *Stories from the Frontline: Investigating causes of salmonellosis in Australia.* New South Wales Government, Sydney, Australia.

Kemp, M., Bangsborg, J., Kjerulf, A., Schmidt, T. A., & Christensen, J. (2013). Advantages and limitations of ribosomal RNA PCR and DNA sequencing for identification of bacteria in cardiac valves of Danish patients. *The Open Microbiology Journal, 7*, 146.

Kennedy, K., & Collignon, P. (2010). Colonisation with *Escherichia coli* resistant to "critically important" antibiotics: a high risk for international travellers. *European Journal of Clinical Microbiology and Infectious Diseases, 29*(12), 1501-1506.

Keseler, I. M., Collado-Vides, J., Gama-Castro, S., Ingraham, J., Paley, S., Paulsen, I. T., Peralta-Gil, M., & Karp, P. D. (2005). EcoCyc: a comprehensive database resource for *Escherichia coli*. *Nucleic Acids Research, 33*(suppl_1), D334-D337.

Khalil, I. A., Troeger, C., Blacker, B. F., Rao, P. C., Brown, A., Atherly, D. E., Brewer, T., Engmann, C., Houpt, E., & Kang, G. (2018). Morbidity and mortality due to *Shigella* and enterotoxigenic *Escherichia coli* diarrhoea: the Global Burden of Disease Study 1990–2016. *The Lancet Infectious Diseases, 18*(11), 1229-1240.

Khan, I. U., Gannon, V., Kent, R., Koning, W., Lapen, D. R., Miller, J., Neumann, N., Phillips, R., Robertson, W., Topp, E., van Bochove, E., & Edge, T. A. (2007). Development of a rapid quantitative PCR assay for direct detection and quantification of culturable and non-culturable *Escherichia coli* from agriculture watersheds. *Journal of Microbiological Methods, 69*(3), 480-488. doi:10.1016/j.mimet.2007.02.016

Kirby, J. S., Stattersfield, A. J., Butchart, S. H. M., Evans, M. I., Grimmett, R. F. A., Jones, V. R., O'Sullivan, J., Tucker, G., & Newton, I. (2008). Key conservation issues for migratory land- and waterbird species on the world's major flyways. *Bird Conservation International, 18*(S1), S49-S73. doi:10.1017/s0959270908000439

Kitadai, N., Ninomiya, N., Murase, T., Obi, T., & Takase, K. (2010). *Salmonella* isolated from the feces of migrating cranes at the Izumi Plain (2002-2008): serotype, antibiotic sensitivity and PFGE type. *Journal of Veterinary Medical Science, 72*(7), 939-942.

Kurnick, S. A., Mannion, A. J., Feng, Y., Madden, C. M., Chamberlain, P., & Fox, J. G. (2019). Genotoxic *Escherichia coli* Strains Encoding Colibactin, Cytolethal Distending Toxin, and Cytotoxic Necrotizing Factor in Laboratory Rats. *Comparative Medicine, 69*(2), 103-113.

Lapierre, L., Gatica, M. A., Riquelme, V., Vergara, C., Yanez, J. M., San Martin, B., Saenz, L., Vidal, M., Martinez, M., Araya, P., Flores, R., Duery, O., & Vidal, R. (2016). Characterization of Antimicrobial Susceptibility and Its Association with Virulence Genes Related to Adherence, Invasion, and Cytotoxicity in *Campylobacter jejuni* and *Campylobacter coli* Isolates from Animals, Meat, and Humans. *Microbial Drug Resistance, 22*(5), 432-444. doi:10.1089/mdr.2015.0055

Larsen, M. V., Cosentino, S., Rasmussen, S., Friis, C., Hasman, H., Marvig, R. L., Jelsbak, L., Sicheritz-Ponten, T., Ussery, D., & Aarestrup, F. M. (2012). Multilocus sequence typing of total-genome-sequenced bacteria. *Journal of Clinical Microbiology, 50*(4), 1355-1361.

Lauber, C. L., Zhou, N., Gordon, J. I., Knight, R., & Fierer, N. (2010). Effect of storage conditions on the assessment of bacterial community structure in soil and human-associated samples. *FEMS Microbiology Letters, 307*(1), 80-86. doi:10.1111/j.1574-6968.2010.01965.x

Lavigne, J.-P., Defez, C., Bouziges, N., Mahamat, A., & Sotto, A. (2007). Clinical and molecular epidemiology of multidrug-resistant *Citrobacter* spp. infections in a French university hospital. *European Journal of Clinical Microbiology & Infectious Diseases, 26*(6), 439-441.

Lawson, B., de Pinna, E., Horton, R. A., Macgregor, S. K., John, S. K., Chantrey, J., Duff, J., Kirkwood, J., Simpson, V., Robinson, R., Wain, J., & Cunningham, A. A. (2014). Epidemiological evidence that garden birds are a source of human salmonellosis in England and Wales. *PLoS ONE, 9*(2), e88968. doi:10.1371/journal.pone.0088968

Lee, J. K., Jin-Young, P., Hwa-Jung, K., Hur, W.-H., Sung-Hyun, K., & Jin-Han, K. (2018). Effects of the Saemangeum Reclamation Project on migratory shorebird staging in the Saemangeum and Geum Estuaries, South Korea. *Bird Conservation International, 28*(2), 238-250.

Lee, R. S., Gonçalves da Silva, A., Baines, S. L., Strachan, J., Ballard, S., Carter, G. P., Kwong, J., Schultz, M., Bulach, D., & Seemann, T. (2018). The changing landscape of vancomycin-resistant *Enterococcus faecium* in Australia: a population-level genomic study. *Journal of Antimicrobial Chemotherapy, 73*(12), 3268-3278.

LeJeune, J., Homan, J., Linz, G., & Pearl, D. (2008). Role of the European Starling in the transmission of *E. coli* O157 on dairy farms. *Vertebrate Pest Conference, 23*, 31-34.

Lessa, F. C., Gould, C. V., & McDonald, L. C. (2012). Current status of *Clostridium difficile* infection epidemiology. *Clinical Infectious Diseases, 55*(Suppl 2), S65-S70.

Letunic, I., & Bork, P. (2019). Interactive Tree Of Life (iTOL) v4: recent updates and new developments. *Nucleic acids research, 47*(W1), W256-W259.

Levy, S., Fitzgerald, G., & Macone, A. (1976). Changes in intestinal flora of farm personnel after introduction of a tetracycline-supplement feed on a farm. *The New England Journal of Medicine, 295*(11), 583-588.

Lin, J., Nishino, K., Roberts, M. C., Tolmasky, M., Aminov, R. I., & Zhang, L. (2015). Mechanisms of antibiotic resistance. *Frontiers in Microbiology, 6*(34), 34. doi:10.3389/fmicb.2015.00034

Literak, I., Dolejska, M., Janoszowska, D., Hrusakova, J., Meissner, W., Rzyska, H., Bzoma, S., & Cizek, A. (2010). Antibiotic-resistant *Escherichia coli* bacteria, including strains with genes encoding the extended-spectrum beta-lactamase and *QnrS*, in waterbirds on the Baltic Sea Coast of Poland. *Applied and Environmental Microbiology, 76*(24), 8126-8134. doi:10.1128/AEM.01446-10

Loaiza, J. R., Dutari, L. C., Rovira, J. R., Sanjur, O. I., Laporta, G. Z., Pecor, J., Foley, D., Eastwood, G., Kramer, L., Radtke, M., & Pongsiri, M. (2017). Disturbance and mosquito diversity in the lowland tropical rainforest of central Panama. *Scientific Reports, 7*(1), 7248. doi:10.1038/s41598-017-07476-2

Loncaric, I., Stalder, G. L., Mehinagic, K., Rosengarten, R., Hoelzl, F., Knauer, F., & Walzer, C. (2013). Comparison of ESBL--and AmpC producing Enterobacteriaceae and methicillin-resistant *Staphylococcus aureus* (MRSA) isolated from migratory and resident population of rooks (*Corvus frugilegus*) in Austria. *PLoS ONE, 8*(12), e84048. doi:10.1371/journal.pone.0084048

Lowen, J. (2016). Reclamation of Yellow Sea causing serious declines in migratory shorebirds. Retrieved from https://www.birdlife.org/asia/news/reclamation-yellow-sea-causing-serious-declines-migratory-shorebirds

Lubik, N. (2011). Tools for tracking antibiotic resistance. *Environmental Health Perspectives, 119*(5), A214-A217.

Ma, L., Zhang, X., Zhao, F., Wu, B., Cheng, S., & Yang, L. (2013). Sewage treatment plant serves as a hot-spot reserviour of intergrons and gene cassettes. *Journal of Enviromental Biololgy, 34*(2 Suppl), 391-399.

Magiorakos, A.-P., Srinivasan, A., Carey, R., Carmeli, Y., Falagas, M., Giske, C., Harbarth, S., Hindler, J., Kahlmeter, G., & Olsson-Liljequist, B. (2012). Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clinical Microbiology and Infection, 18*(3), 268-281.

Magnet, S., Courvalin, P., & Lambert, T. (1999). Activation of the Cryptic aac (6')-IyAminoglycoside Resistance Gene of *Salmonella* by a Chromosomal Deletion Generating a Transcriptional Fusion. *Journal of Bacteriology, 181*(21), 6650-6655.

Majowicz, S. E., Musto, J., Scallan, E., Angulo, F. J., Kirk, M., O'Brien, S. J., Jones, T., Fazil, A., & Hoekstra, R. (2010). The global burden of nontyphoidal *Salmonella* gastroenteritis. *Clinical Infectious Diseases, 50*(6), 882-889.

Malorny, B., Hoorfar, J., Bunge, C., & Helmuth, R. (2003). Multicenter validation of the analytical accuracy of *Salmonella* PCR: towards an international standard. *Applied and Environmental Microbiology, 69*(1), 290-296.

Maluta, R. P., Logue, C. M., Casas, M. R. T., Meng, T., Guastalli, E. A. L., Rojas, T. C. G., Montelli, A., Sadatsune, T., de Carvalho Ramos, M., & Nolan, L. K. (2014). Overlapped sequence types (STs) and serogroups of avian pathogenic (APEC) and human extra-intestinal pathogenic (ExPEC) *Escherichia coli* isolated in Brazil. *PLoS ONE, 9*(8), e105016.

Marcelino, V. R., Wille, M., Hurt, A. C., González-Acuña, D., Klaassen, M., Schlub, T. E., Eden, J-S., Shi, M., Iredell, J., & Sorrell, T. C. (2019). Meta-transcriptomics reveals a diverse antibiotic resistance gene pool in avian microbiomes. *BMC Biology, 17*(1), 1-11.

Marrow, J., Whittington, J. K., Mitchell, M., Hoyer, L. L., & Maddox, C. (2009). Prevalence and antibiotic-resistance characteristics of *Enterococcus* spp. isolated from free-living and captive raptors in Central Illinois. *Journal of Wildlife Diseases, 45*(2), 302-313.

Martínez, I., Stegen, J. C., Maldonado-Gómez, M. X., Eren, A. M., Siba, P. M., Greenhill, A. R., & Walter, J. (2015). The gut microbiota of rural Papua New Guineans: composition, diversity patterns, and ecological processes. *Cell Reports, 11*(4), 527-538.

McArthur, A. G., Waglechner, N., Nizam, F., Yan, A., Azad, M. A., Baylay, A. J., Bhullar, K., Canova, M., De Pascale, G., & Ejim, L. (2013). The comprehensive antibiotic resistance database. *Antimicrobial Agents and Chemotherapy, 57*(7), 3348-3357.

McCullough, A. R., Rathbone, J., Parekh, S., Hoffmann, T. C., & Del Mar, C. B. (2015). Not in my backyard: a systematic review of clinicians' knowledge and beliefs about antibiotic resistance. *Journal of Antimicrobial Chemotherapy, 70*(9), 2465-2473. doi:10.1093/jac/dkv164

McEwen, S. A., & Fedorka-Cray, P. J. (2002). Antimicrobial use and resistance in animals. *Clinical Infectious Diseases, 34*(3), S93-S106. doi:10.1086/340246

McGettigan, P., Roderick, P., Kadam, A., & Pollock, A. (2019). Threats to global antimicrobial resistance control: Centrally approved and unapproved antibiotic formulations sold in India. *British Journal of Clinical Pharmacology, 85*(1), 59-70. doi:10.1111/bcp.13503

McLeay, L., Page, B., Goldsworthy, S., Paton, D., Teixeira, C., Burch, P., & Ward, T. (2010). Foraging behaviour and habitat use of a short-ranging seabird, the crested tern. *Marine Ecology Progress Series, 411*, 271-283.

Melo, L. C., Boisson, M. N., Saras, E., Médaille, C., Boulouis, H.-J., Madec, J.-Y., & Haenni, M. (2017). OXA-48-producing ST372 *Escherichia coli* in a French dog. *Journal of Antimicrobial Chemotherapy, 72*(4), 1256-1258.

Mennerat, A., Nilsen, F., Ebert, D., & Skorping, A. (2010). Intensive farming: evolutionary implications for parasites and pathogens. *Evolutionary Biology, 37*(2-3), 59-67.

Mikheenko, A., Prjibelski, A., Saveliev, V., Antipov, D., & Gurevich, A. (2018). Versatile genome assembly evaluation with QUAST-LG. *Bioinformatics, 34*(13), i142-i150.

Milton, D. A., & Harding, S. B. (2011). Death by a thousand cuts–the incremental loss of coastal high tide roosts for Shorebirds in Australia: Sandfly Creek Environmental Reserve, Central Queensland. *Stilt, 60*, 22-33.

Molton, J. S., Tambyah, P. A., Ang, B. S., Ling, M. L., & Fisher, D. A. (2013). The global spread of healthcare-associated multidrug-resistant bacteria: a perspective from Asia. *Clinical Infectious Diseases, 56*(9), 1310-1318.

Moores, N., Rogers, D. I., Rogers, K., & Hansbro, P. M. (2016). Reclamation of tidal flats and shorebird declines in Saemangeum and elsewhere in the Republic of Korea. *Emu-Austral Ornithology, 116*(2), 136-146.

Mora, A., García-Peña, F. J., Alonso, M. P., Pedraza-Diaz, S., Ortega-Mora, L. M., Garcia-Parraga, D., Lopez, C., Viso, S., Dahbi, G., & Marzoa, J. (2018). Impact of human-associated *Escherichia coli* clonal groups in Antarctic pinnipeds: presence of ST73, ST95, ST141 and ST131. *Scientific Reports, 8*(1), 1-11.

Morales, A. S., Fragoso de Araujo, J., de Moura Gomes, V. T., Reis Costa, A. T., dos Prazeres Rodrigues, D., Porfida Ferreira, T. S., de Lima, F., Felizardo, M., & Micke Moreno, A. (2012). Colistin resistance in *Escherichia coli* and *Salmonella enterica* strains isolated from swine in Brazil. *Scientific World Journal, 2012*, 109795. doi:10.1100/2012/109795

Mott, R., Herrod, A., & Clarke, R. H. (2017). Post-breeding dispersal of frigatebirds increases their exposure to mercury. *Marine Pollution Bulletin, 119*(1), 204-210. doi:10.1016/j.marpolbul.2017.03.050

Mott, R., Herrod, A., & Clarke, R. H. (2017). Resource partitioning between species and sexes in Great Frigatebirds and Lesser Frigatebirds. *The Auk: Ornithological Advances, 134*(1), 153-167.

Mourkas, E., Florez-Cuadrado, D., Pascoe, B., Calland, J. K., Bayliss, S. C., Mageiros, L., Meric, G., Hitchings, M., Quesada, A., Porrero, C., Ugarte-Ruiz, M., Guiterrez-Fernandez, J., Dominguez, L., & Sheppard, S. K. (2019). Gene pool transmission of multidrug resistance among *Campylobacter* from livestock, sewage and human disease. *Environmental Microbiology, 21*(12), 4597-4613. doi:10.1111/1462-2920.14760.

Mourkas, E., Taylor, A. J., Méric, G., Bayliss, S. C., Pascoe, B., Mageiros, L., Calland, J., Hitchings, M., Ridley, A., & Vidal, A. (2020). Agricultural intensification and the evolution of host specialism in the enteric pathogen *Campylobacter jejuni*. *Proceedings of the National Academy of Sciences, 117*(20), 11018-11028.

Mukerji, S., Stegger, M., Truswell, A. V., Laird, T., Jordan, D., Abraham, R. J., Harb, A., Barton, M., O'Dea, M., & Abraham, S. (2019). Resistance to critically important antimicrobials in Australian silver gulls (*Chroicocephalus novaehollandiae*) and evidence of anthropogenic origins. *Journal of Antimicrobial Chemotherapy, 74*(9), 2566-2574.

Munnoch, S., Irwin, M., Oxenford, C., Hanson, R., Owen, R., Black, A., & Bell, R. (2005). Investigation of a multi-state outbreak of *Salmonella* Hvittingfoss using a web-based case reporting form. *Communicable Diseases Intelligence Quaterly Report 29*(4), 379.

Najdenski, H., Dimova, T., Zaharieva, M. M., Nikolov, B., Petrova-Dinkova, G., Dalakchieva, S., Popov, K., Hristova-Nikolova, I., Zehtindjiev, P., & Peev, S. (2018). Migratory birds along the Mediterranean–Black Sea Flyway as carriers of zoonotic pathogens. *Canadian Journal of Microbiology, 64*(12), 915-924.

Navarro-Gonzalez, N., Mentaberre, G., Porrero, C. M., Serrano, E., Mateos, A., Lopez-Martin, J. M., Lavin, S., & Dominguez, L. (2012). Effect of cattle on *Salmonella* carriage, diversity and antimicrobial resistance in free-ranging wild boar (*Sus scrofa*) in northeastern Spain. *PLoS ONE, 7*(12), e51614. doi:10.1371/journal.pone.0051614

Nawaz, M., Khan, A. A., Khan, S., Sung, K., & Steele, R. (2008). Isolation and characterization of tetracycline-resistant *Citrobacter* spp. from catfish. *Food microbiology, 25*(1), 85-91.

Nesme, J., Cecillon, S., Delmont, T. O., Monier, J. M., Vogel, T. M., & Simonet, P. (2014). Large-scale metagenomic-based study of antibiotic resistance in the environment. *Current Biology, 24*(10), 1096-1100. doi:10.1016/j.cub.2014.03.036

Nguyen, L.-T., Schmidt, H. A., Von Haeseler, A., & Minh, B. Q. (2015). IQ-TREE: a fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. *Molecular Biology and Evolution, 32*(1), 268-274.

Nwankwo, C., Ayogu, T., Iroha, I., Ejikeugwu, C., Nwakaeze, E., Oji, A., & Ilang, D. (2014). Cloacal feacal carriage and occurrence of antibiotic resistant *Escherichia coli* in chicken grown with and without antibiotic supplemented feed. *Journal of Veterinary Medicine and Animal Health, 6*(3), 91-94. doi:10.5897/jvmah2013.0267

O'Niell, J. (2014). Antimicrobial resistance: Tackling a crisis for the health and wealth of nations. *Review on Antimicrobial Resistance*, 1-16.

Ochman, H., & Moran, N. A. (2001). Genes lost and genes found: evolution of bacterial pathogenesis and symbiosis. *Science, 292*(5519), 1096-1099.

Octavia, S., Wang, Q., Tanaka, M. M., Kaur, S., Sintchenko, V., & Lan, R. (2015). Delineating community outbreaks of *Salmonella enterica* serovar Typhimurium by use of whole-genome sequencing: insights into genomic variability within an outbreak. *Journal of Clinical Microbiology, 53*(4), 1063-1071.

Oldland, J., Rogers, D., Clemens, R., Berry, L., Maguire, G., & Gosbell, K. (2009). Shorebird conservation in Australia. *Birds Australia Conservation Statment,* (14).

Oravcova, V., Ghosh, A., Zurek, L., Bardon, J., Guenther, S., Cizek, A., & Literak, I. (2013). Vancomycin-resistant enterococci in rooks (*Corvus frugilegus*) wintering throughout Europe. *Environmental Microbiology, 15*(2), 548-556. doi:10.1111/1462-2920.12002

Oravcova, V., Svec, P., & Literak, I. (2017). Vancomycin-resistant enterococci with *vanA* and *vanB* genes in Australian gulls. *Environmental Microbiology Reports, 9*(3), 316-318. doi:10.1111/1758-2229.12542

Pallecchi, L., Bartoloni, A., Paradisi, F., & Rossolini, G. M. (2008). Antibiotic resistance in the absence of antimicrobial use: mechanisms and implications. *Expert Review of Anti-Infective Therapy, 6*(5), 725-732.

Palmgren, H., Sellin, M., Bergstrom, S., & Olsen, B. (1997). Enteropathogenic bacteria in migrating birds arriving in Sweden. *Scandinavian Journal of Infectious Disease, 29*(6), 565-568. doi:10.3109/00365549709035895

Peixe, L., Sousa, J., Perez-Diaz, J., & Baquero, F. (1997). A bla (TEM-1b)-derived TEM-6 beta-lactamase: a case of convergent evolution. *Antimicrobial Agents and Chemotherapy, 41*(5), 1206.

Pesapane, R., Ponder, M., & Alexander, K. (2013). Tracking pathogen transmission at the human–wildlife interface: banded mongoose and *Escherichia coli*. *EcoHealth, 10*(2), 115-128.

Piersma, T., Chan, Y.-C., Mu, T., Hassell, C. J., David S, D. S., Peng, H.-B., Ma, Z., Zhang, Z., & Wilcove, D. S. (2017). Loss of habitat leads to loss of birds: reflections on the Jiangsu, China, coastal development plans. *Wader Study, 124*(2). doi:10.18194/ws.00077

Piersma, T., Gudmundsson, G. A., & Lilliendahl, K. (1999). Rapid changes in the size of different functional organ and muscle groups during refueling in a long-distance migrating shorebird. *Physiological and Biochemical Zoology, 72*(4), 405-415. doi:10.1086/316680

Power, M. L., Emery, S., & Gillings, M. R. (2013). Into the wild: dissemination of antibiotic resistance determinants via a species recovery program. *PLoS ONE, 8*(5), e63017. doi:10.1371/journal.pone.0063017

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

Radhouani, H., Poeta, P., Goncalves, A., Pacheco, R., Sargo, R., & Igrejas, G. (2012). Wild birds as biological indicators of environmental pollution: antimicrobial resistance patterns of *Escherichia coli* and enterococci isolated from common buzzards (*Buteo buteo*). *Journal of Medical Microbiology, 61*(Pt 6), 837-843. doi:10.1099/jmm.0.038364-0

Radimersky, T., Frolkova, P., Janoszowska, D., Dolejska, M., Svec, P., Roubalova, E., Cikova, P., Cizek, A., & Literak, I. (2010). Antibiotic resistance in faecal bacteria (*Escherichia coli, Enterococcus* spp.) in feral pigeons. *Journal of Applied Microbiology, 109*(5), 1687-1695. doi:10.1111/j.1365-2672.2010.04797.x

Rambaut, A. (2014). FigTree 1.4. 2 software. *Institute of Evolutionary Biology, Univ. Edinburgh*.

Ramey, A. M., Hernandez, J., Tyrlov, V., Uher-Koch, B. D., Schmutz, J. A., Atterby, C., Jarhult, J. D., & Bonnedahl, J. (2018). Antibiotic-Resistant *Escherichia coli* in Migratory Birds Inhabiting Remote Alaska. *EcoHealth, 15*(1), 72-81. doi:10.1007/s10393-017-1302-5

Ramos, S., Silva, V., Dapkevicius, M. d. L. E., Igrejas, G., & Poeta, P. (2020). Enterococci, from Harmless Bacteria to a Pathogen. *Microorganisms, 8*(8), 1118.

Rappole, J. H., Derrickson, S. R., & Hubalek, Z. (2000). Migratory birds and spread of West Nile virus in the Western Hemisphere. *Emerging Infectious Diseases, 6*(4), 319-328. doi:10.3201/eid0604.000401

Raymond, B. (2019). Five rules for resistance management in the antibiotic apocalypse, a road map for integrated microbial management. *Evolutionary Applications, 12*(6), 1079-1091. doi:10.1111/eva.12808

Raza, S., Mohsin, M., Madni, W. A., Sarwar, F., Saqib, M., & Aslam, B. (2017). First Report of bla CTX-M-15-Type ESBL-Producing *Klebsiella pneumoniae* in Wild Migratory Birds in Pakistan. *EcoHealth, 14*(1), 182-186. doi:10.1007/s10393-016-1204-y

Reed, K. D., Meece, J. K., Henkel, J. S., & Shukla, S. K. (2003). Birds, migration and emerging zoonoses: west nile virus, lyme disease, influenza A and enteropathogens. *Clinical Medicine & Research, 1*(1), 5-12.

Reuter, G. (1992). Culture media for enterococci and group D-streptococci. *International Journal of Food Microbiology, 17*(2), 101-111.

Risely, A., Klaassen, M., & Hoye, B. J. (2018). Migratory animals feel the cost of getting sick: A meta-analysis across species. *Journal of Animal Ecology, 87*(1), 301-314. doi:10.1111/1365-2656.12766

Risely, A., Waite, D., Ujvari, B., Klaassen, M., & Hoye, B. (2017). Gut microbiota of a long-distance migrant demonstrates resistance against environmental microbe incursions. *Molecular Ecology, 26*(20), 5842-5854.

Risely, A., Waite, D. W., Ujvari, B., Hoye, B. J., & Klaassen, M. (2018). Active migration is associated with specific and consistent changes to gut microbiota in *Calidris* shorebirds. *Journal of Animal Ecology, 87*(2), 428-437. doi:10.1111/1365-2656.12784

Rishmawi, N., Ghneim, R., Kattan, R., Ghneim, R., Zoughbi, M., Abu-Diab, A., Turkuman, S., Dauodi, R., Shomali, I., & Issa, A. E.-R. (2007). Survival of fastidious and nonfastidious aerobic bacteria in three bacterial transport swab systems. *Journal of Clinical Microbiology, 45*(4), 1278-1283.

Robinson, C. D., Bohannan, B. J., & Britton, R. A. (2019). Scales of persistence: transmission and the microbiome. *Current Opinion in Microbiology, 50*, 42-49.

Robinson, H. L., Robinson, P. C., & Whitby, M. (2014). Poor compliance with community-acquired pneumonia antibiotic guidelines in a large Australian private hospital emergency department. *Microbial Drug Resistance, 20*(6), 561-567. doi:10.1089/mdr.2014.0064

Rogers, D. I., Yang, H.-Y., Hassell, C. J., Boyle, A. N., Rogers, K. G., Chen, B., Zhang, Z., & Piersma, T. (2010). Red Knots (*Calidris canutus piersmai* and *C. c. rogersi*) depend on a small threatened staging area in Bohai Bay, China. *Emu-Austral Ornithology, 110*(4), 307-315.

Rubbo, S. D., & Benjamin, M. (1951). Survival of Pathogenic Bacteria on Cotton-wool Swabs. *British Medical Journal, 1*(4713), 983.

Ryu, H., Henson, M., Elk, M., Toledo-Hernandez, C., Griffith, J., Blackwood, D., Noble, R., Gourmelon, M., Glassmeyer, S., & Santo Domingo, J. W. (2013). Development of quantitative PCR assays targeting the 16S rRNA genes of *Enterococcus* spp. and their application to the identification of *Enterococcus* species in environmental samples. *Applied and Environmental Microbiology, 79*(1), 196-204. doi:10.1128/AEM.02802-12

Samonis, G., Karageorgopoulos, D., Kofteridis, D., Matthaiou, D., Sidiropoulou, V., Maraki, S., & Falagas, M. (2009). *Citrobacter* infections in a general hospital: characteristics and outcomes. *European Journal of Clinical Microbiology & Infectious Diseases, 28*(1), 61.

Santos, S., Pardal, S., Proença, D. N., Lopes, R. J., Ramos, J. A., Mendes, L., & Morais, P. V. (2012). Diversity of cloacal microbial community in migratory shorebirds that use the Tagus estuary as stopover habitat and their potential to harbor and disperse pathogenic microorganisms. *FEMS Microbiology Ecology, 82*(1), 63-74.

Santos, T., Silva, N., Igrejas, G., Rodrigues, P., Micael, J., Rodrigues, T., Resendes, R., Goncalves, A., Marinho, C., Goncalves, D., Cunha, R., & Poeta, P. (2013). Dissemination of antibiotic resistant *Enterococcus* spp. and *Escherichia coli* from wild birds of Azores Archipelago. *Anaerobe, 24*, 25-31. doi:10.1016/j.anaerobe.2013.09.004

Sawant, A. A., Hegde, N. V., Straley, B. A., Donaldson, S. C., Love, B. C., Knabel, S. J., & Jayarao, B. M. (2007). Antimicrobial-resistant enteric bacteria from dairy cattle. *Applied and Environmental Microbiology, 73*(1), 156-163.

Sellin, M., Palmgren, H., Broman, T., Bergstrom, S., & Olsen, B. (2000). Involving ornithologists in the surveillance of vancomycin-resistant enterococci. *Emerging Infectious Diseases, 6*(1), 87-88. doi:10.3201/eid0601.000119

Sender, R., Fuchs, S., & Milo, R. (2016). Revised estimates for the number of human and bacteria cells in the body. *PLoS Biology, 14*(8), e1002533.

Shahcheraghi, F., Nobari, S., Rahmati Ghezelgeh, F., Nasiri, S., Owlia, P., Nikbin, V. S., & Imani Fooladi, A. A. (2013). First report of New Delhi metallo-beta-lactamase-1-producing *Klebsiella pneumoniae* in Iran. *Microbial Drug Resistance, 19*(1), 30-36. doi:10.1089/mdr.2012.0078

Sjolund, M., Bonnedahl, J., Hernandez, J., Bengtsson, S., Cederbrant, G., Pinhassi, J., Kahlmeter, G., & Olsen, B. (2008). Dissemination of multidrug-resistant bacteria into the Arctic. *Emerging Infectious Diseases, 14*(1), 70-72. doi:10.3201/eid1401.070704

Sköld, O. (2000). Sulfonamide resistance: mechanisms and trends. *Drug Resistance Updates, 3*(3), 155-160.

Slanetz, L., & Bartley, C. H. (1957). Numbers of enterococci in water, sewage, and feces determined by the membrane filter technique with an improved medium. *Journal of Bacteriology, 74*(5), 591.

Smith, G. C., & Carlile, N. (1993). Food and Feeding Ecology of Breeding Silver Gulls (*Larus Novaehollandiae*) in Urban Australia. *Colonial waterbirds, 16*(1), 9-17. doi: 10.2307/1521551

Smith, H. G., Bean, D. C., Hawkey, J., Clarke, R. H., Loyn, R., Larkins, J.-A., Hassel, C., Valcanis, M., Pitchers, W., & Greenhill, A. R. (2020). Detection and characterisation of *Salmonella enterica* serovar Hvittingfoss in Bar-tailed Godwits (*Limosa lapponica*) from Roebuck Bay, north-western Australia. *Applied and Environmental Microbiology*.

Smith, H. G., Clarke, R. H., Larkins, J.-A., Bean, D. C., & Greenhill, A. R. (2019). Wild Australian birds and drug-resistant bacteria: characterisation of antibiotic-resistant *Escherichia coli* and *Enterococcus* spp. *Emu-Austral Ornithology, 119*(4), 384-390.

Smith, K. M., Machalaba, C. M., Jones, H., Caceres, P., Popovic, M., Olival, K. J., Ben Jebara, K., & Karesh, W. B. (2017). Wildlife hosts for OIE-Listed diseases: considerations regarding global wildlife trade and host-pathogen relationships. *The Journal of Veterinary Medical Science, 3*(2), 71-81. doi:10.1002/vms3.57

Souza, V., Rocha, M., Valera, A., & Eguiarte, L. E. (1999). Genetic structure of natural populations of *Escherichia coli* in wild hosts on different continents. *Applied and Environmental Microbiology, 65*(8), 3373-3385.

Spellberg, B. (2009). *Rising Plague: The global threat from deadly bacteria and our dwindling arsenal to fight them*. Amherst, New York, USA: Spellberg Prometheus Books.

Splichalova, P., Svec, P., Ghosh, A., Zurek, L., Oravcova, V., Radimersky, T., Bohus, M., & Literak, I. (2015). Prevalence, diversity and characterization of enterococci from three coraciiform birds. *Antonie van Leeuwenhoek, 107*(5), 1281-1289.

Spurbeck, R. R., Dinh, P. C., Walk, S. T., Stapleton, A. E., Hooton, T. M., Nolan, L. K., Kim, K., Johnson, J., & Mobley, H. L. (2012). *Escherichia coli* isolates that carry *vat, fyuA, chuA,* and *yfcV* efficiently colonize the urinary tract. *Infect Immun, 80*(12), 4115-4122.

Stamatakis, A. (2014). RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics, 30*(9), 1312-1313.

Studds, C. E., Kendall, B. E., Murray, N. J., Wilson, H. B., Rogers, D. I., Clemens, R. S., Gosbell, K., Hassell, C. J., Jessop, R., Melville, D. S., Milton, D. A., Minton, C. D., Possingham, H. P., Riegen, A. C., Straw, P., Woehler, E. J., & Fuller, R. A. (2017). Rapid population decline in migratory shorebirds relying on Yellow Sea tidal mudflats as stopover sites. *Nature Communications, 8*, 14895. doi:10.1038/ncomms14895

Szabo, J. K., Choi, C.-Y., Clemens, R. S., & Hansen, B. (2016). Conservation without borders–solutions to declines of migratory shorebirds in the East Asian–Australasian Flyway. *Emu, 116*(2), 215-221.

Taylor, I. R., Newman, O. M. G., Park, P., Hansen, B., Minton, C. D., Harrison, A., & Jessop, R. (2014). Conservation assessment of the Australian Pied Oystercatcher *Haematopus longirostris*. *International Wader Studies, 20*, 116-128.

Thaller, M. C., Migliore, L., Marquez, C., Tapia, W., Cedeno, V., Rossolini, G. M., & Gentile, G. (2010). Tracking acquired antibiotic resistance in commensal bacteria of Galapagos land iguanas: no man, no resistance. *PLoS ONE, 5*(2), e8989. doi:10.1371/journal.pone.0008989

Tien, Y. C., Li, B., Zhang, T., Scott, A., Murray, R., Sabourin, L., Marti, R., & Topp, E. (2017). Impact of dairy manure pre-application treatment on manure composition, soil dynamics of antibiotic resistance genes, and abundance of antibiotic-resistance genes on vegetables at harvest. *The Science of the Total Environment, 581*, 32-39. doi:10.1016/j.scitotenv.2016.12.138

Topp, E., Larsson, D. J., Miller, D. N., Van den Eede, C., & Virta, M. P. (2018). Antimicrobial resistance and the environment: assessment of advances, gaps and recommendations for agriculture, aquaculture and pharmaceutical manufacturing. *FEMS Microbiology Ecology, 94*(3).

Torres, R. T., Carvalho, J., Cunha, M. V., & Fonseca, C. (2019). Antimicrobial resistance and ecology: a dialog yet to begin. *EcoHealth, 16*(3), 402-403.

UniProt Consortium, U. (2019). UniProt: a worldwide hub of protein knowledge. *Nucleic acids research, 47*(D1), D506-D515.

Ussui, M., Iwasa, T., Fukuda, A., Sato, T., Okubo, T., & Tamura, Y. (2013). The role of flies in spreading the extended-Spectrum β-lactamase gene from cattle *Microbial Drug Resistance, 19*(5), 415-420.

Valat, C., Drapeau, A., Beurlet, S., Bachy, V., Boulouis, H.-J., Pin, R., Cazeau, G., Madec, J-Y., & Haenni, M. (2020). Pathogenic *Escherichia coli* in Dogs Reveals the Predominance of ST372 and the Human-Associated ST73 Extra-Intestinal Lineages. *Frontiers in Microbiology, 11*, 580.

Van Schaik, G., Dijkhuizen, A., Benedictus, G., Barkema, H., & Koole, J. (1998). Exploratory study on the economic value of a closed farming system on Dutch dairy farms. *Veterinary Record, 142*(10), 240-242.

Vangchhia, B., Abraham, S., Bell, J. M., Collignon, P., Gibson, J. S., Ingram, P. R., Johnson, J., Kennedy, K., Trott, D., & Turnidge, J. D. (2016). Phylogenetic diversity, antimicrobial susceptibility and virulence characteristics of phylogroup F *Escherichia coli* in Australia. *Microbiology, 162*(11), 1904-1912.

Veldman, K., van Tulden, P., Kant, A., Testerink, J., & Mevius, D. (2013). Characteristics of cefotaxime-resistant *Escherichia coli* from wild birds in the Netherlands. *Applied and Environmental Microbiology, 79*(24), 7556-7561. doi:10.1128/AEM.01880-13

Verdet, C., Gautier, V., Chachaty, E., Ronco, E., Hidri, N., Decré, D., & Arlet, G. (2009). Genetic context of plasmid-carried blaCMY-2-like genes in Enterobacteriaceae. *Antimicrobial Agents and Chemotherapy, 53*(9), 4002-4006.

Wallace, M., Fishbein, S., & Dantas, G. (2020). Antimicrobial resistance in enteric bacteria: current state and next-generation solutions. *Gut Microbes, 12*(1), 1799654.

Ward, M. P., Cowled, B. D., Galea, F., Garner, M. G., Laffan, S. W., Marsh, I., Negus, K., Sarre, S., & Woolnough, A. P. (2013). *Salmonella* infection in a remote, isolated wild pig population. *Veterinary Microbiology, 162*(2-4), 921-929.

Wasyl, D. (2014). Prevelance and characterisation of quinolone resistance mechanisms in commensal *Escherichia coli* isolated from slaughter animals in Poland, 2009-2012. *Microbial Drug Resistance, 20*(6), 544-549.

Water Corporation. (2018). *Broome high rainfall events January-February 2018: Water quality sampling results report*. (1-65).

Wauchope, H. S., Shaw, J. D., Varpe, O., Lappo, E. G., Boertmann, D., Lanctot, R. B., & Fuller, R. A. (2017). Rapid climate-driven loss of breeding habitat for Arctic migratory birds. *Global Change Biology, 23*(3), 1085-1094. doi:10.1111/gcb.13404

Weller, D. R., & Lee, C. (2017). Migratory Shorebird Conservation Action Plan. *BirdLife Australia unpublished report. BirdLife Australia: Carlton*.

Werner, G., Coque, T. M., Franz, C. M., Grohmann, E., Hegstad, K., Jensen, L., van Schaik, W., & Weaver, K. (2013). Antibiotic resistant enterococci-tales of a drug resistance gene trafficker. *International Journal of Medical Microbiology, 303*(6-7), 360-379. doi:10.1016/j.ijmm.2013.03.001

Wick, R. R., Judd, L. M., Gorrie, C. L., & Holt, K. E. (2017). Unicycler: resolving bacterial genome assemblies from short and long sequencing reads. *PLoS Computational Biology, 13*(6), e1005595.

Wiethoelter, A. K., Beltrán-Alcrudo, D., Kock, R., & Mor, S. M. (2015). Global trends in infectious diseases at the wildlife–livestock interface. *Proceedings of the National Academy of Sciences, 112*(31), 9662-9667.

Wikelski, M., & Tertitski, G. (2016). Living sentinels for climate change effects. *Science, 352*(6287), 775-776.

Wilharm, G., Skiebe, E., Higgins, P. G., Poppel, M. T., Blaschke, U., Leser, S., Heider, C., Heindorf, M., Brauner, P., Jackel, U., Bohland, K., Cuny, C., Lopinska, A., Kaminski, P., Kasprzak, M., Bochenski, M., Ciebiera, O., Tobolka, M., Zolnierowicz, K. M., Siekiera, J., Seifert, H., Gagne, S., Salcedo, S. P., Kaatz, M., Layer, F., Bender, J. K., Fuchs, S., Semmler, T., Pfeifer, Y., & Jerzak, L. (2017). Relatedness of wildlife and livestock avian isolates of the nosocomial pathogen *Acinetobacter baumannii* to lineages spread in hospitals worldwide. *Environmental Microbiology, 19*(10), 4349-4364. doi:10.1111/1462-2920.13931

Wille, M., Latorre-Margalef, N., & Waldenström, J. (2017). Of Ducks and Men: Ecology and Evolution of a Zoonotic Pathogen in a Wild Reservoir Host. In *Modeling the Transmission and Prevention of Infectious Disease* (pp. 247-286).ed C. J. Hurst. Springer International Publishing, Cham.

Wilson, A., Fox, E. M., Fegan, N., & Kurtboke, D. I. (2019). Comparative Genomics and Phenotypic Investigations Into Antibiotic, Heavy Metal, and Disinfectant Susceptibilities of *Salmonella enterica* Strains Isolated in Australia. *Frontier Microbiology, 10*, 1620. doi:10.3389/fmicb.2019.01620

Wright, G. D. (2010). Antibiotic resistance in the environment: a link to the clinic? *Current Opinion in Microbiology, 13*(5), 589-594.

Yan, S. S., Pendrak, M. L., Abela-Ridder, B., Punderson, J. W., Fedorko, D. P., & Foley, S. L. (2004). An overview of *Salmonella* typing: public health perspectives. *Clinical and Applied Immunology Reviews, 4*(3), 189-204.

Yau, S., Liu, X. L., Djordjevic, S. P., & Hall, R. M. (2010). RSF1010-Like Plasmids in Australian *Salmonella enterica* Serovar Typhimurium and Origin of Their *sul2-strA-strB* Antibiotic Resistance Gene Cluster. *Microbial Drug Resistance, 16*(4), 249-252. doi:10.1089/mdr.2010.0033

Yong, D. L., Jain, A., Liu, Y., Iqbal, M., Choi, C. Y., Crockford, N. J., Milington, S., & Provencher, J. (2018). Challenges and opportunities for transboundary conservation of migratory birds in the East Asian-Australasian flyway. *Conservation Biology, 32*(3), 740-743. doi:10.1111/cobi.13041

Zankari, E., Hasman, H., Cosentino, S., Vestergaard, M., Rasmussen, S., Lund, O., Aarestrup, F., & Larsen, M. V. (2012). Identification of acquired antimicrobial resistance genes. *Journal of Antimicrobial Chemotherapy, 67*(11), 2640-2644.

Zeng, L., & Jin, S. (2003). aph (3')-IIb, a gene encoding an aminoglycoside-modifying enzyme, is under the positive control of surrogate regulator HpaA. *Antimicrobial Agents and Chemotherapy, 47*(12), 3867-3876.

Zhang, S., Sun, J., Liao, X., Q, H., Liu, B., Fang, L., Deng, H., Ma, J., Xiao, X., Zhu, H., & Liu, Y. (2013). Prevelence and plasmid characterization of the *qnrD* determinant in *Enterobacteriacae* isolated from animals, retail meat products, and humans. *Microbial Drug Resistance, 19*(4), 331-335.

Zhang, S., Yin, Y., Jones, M. B., Zhang, Z., Deatherage Kaiser, B. L., Dinsmore, B. A., Fitzgerald, C., Fields, P., & Deng, X. (2015). *Salmonella* serotype determination utilizing high-throughput genome sequencing data. *Journal of Clinical Microbiology, 53*(5), 1685-1692. doi:10.1128/JCM.00323-15

Zhang, X., Paganelli, F. L., Bierschenk, D., Kuipers, A., Bonten, M. J., Willems, R. J., & van Schaik, W. (2012). Genome-wide identification of ampicillin resistance determinants in *Enterococcus faecium*. *PLoS Genetics, 8*(6), e1002804. doi:10.1371/journal.pgen.1002804

Zhao, C., Sun, H., Wang, H., Liu, Y. H., B, Yu, Y., Sun, Z., Chu, Y., Cao, B., Liao, K., Lei, J., Hu, Z., Zhang, L., Zhang, X., Xu, Y., Wang, Z., & Chen, M. (2012). Antimicrobial resistance trends among 5608 clinical Gram-positive isolates in China: results from the Gram-Positive Cocci Resistance Surveillance program (2005–2010). *Diagnostic Microbiology and Infectious Disease, 73*, 174-181.

Zhao, H., Zhao, J., Shen, J., Fan, H., Guan, H., An, X., & Li, P. (2014). Prevelance and molecular characterization of fluoroquinolone resistance in *Escherichia coli* isolates from dairy cattle with endometritis in China. *Microbial Drug Resistance, 20*(2), 162-169.

Zhou, Z., Alikhan, N.-F., Mohamed, K., Fan, Y., Achtman, M., Brown, D., Chattaway, M., Dallman, T., Delahay, R., & Kornschober, C. (2020). The EnteroBase user's guide, with case studies on *Salmonella* transmissions, *Yersinia pestis* phylogeny, and *Escherichia* core genomic diversity. *Genome Research, 30*(1), 138-152.

Zhu, G. R., Xie, Z. L., Xu, X. G., Ma, Z. W., & Wu, Y. L. (2016). The landscape change and theory of orderly reclamation sea based on coastal management in rapid industrialization area in Bohai Bay, China. *Ocean & Coastal Management, 133*, 128-137. doi:10.1016/j.ocecoaman.2016.09.016

Zurfluh, K., Power, K., Klumpp, J., Wang, J., Fanning, S., & Stephan, R. (2015). A novel *Tn3*-Like composite transposon harbouing bla_{VIM-1} in *Klebsiella pneumoniae* spp. *pneumoniae* isolated from river water. *Microbial Drug Resistance, 21*(1), 43-49.

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8. Appendices

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Foreword to Appendix 8.1

During the analysis of *E. coli* WGS data in Chapter 4, four of the sequenced isolates were identified to be *Citrobacter* spp. These isolates were investigated for AMR genes and speciated, and genome assembly quality for all four isolates were investigated. These isolates were collected from three species of shorebird; the Curlew Sandpiper, Double-banded Plover and Bar-tailed Godwit.

This accepted manuscript shows that the carriage of AMR genes in bacteria from shorebirds is not restricted only to potential pathogens such as *E. coli, Enterococcus* and *Salmonella*. *Citrobacter* are known commensal organisms, and have potential as indicator organisms of AMR contamination in environmental settings. Future studies may consider targeting *Citrobacter* species if undertaking surveillance studies in wild bird populations.

Provided below is a copy of the accepted manuscript (re-formatted slightly to be consistent with the rest of this thesis). The details of the publication are as follows:

Smith, H.G.P., Bean, D.C, Pitchers, W., Valcanis, W., Clarke, R.H., Loyn, R., Hassell, C.J., Greenhill, A.R. (In Press). Draft genome sequences of four *Citrobacter* isolates recovered from wild Australian shorebirds. *Microbiology Resource Announcements*. 9:e01113-20. doi.org/10.1128/MRA.01113-20.

8. 1 Draft genome sequences of four *Citrobacter* isolates recovered from wild Australian shorebirds

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Running Head: *Citrobacter* from wild shorebirds

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8.1.1 Abstract

Citrobacter spp. are a ubiquitous bacterial genus that inhabit a variety of niches. Some species are clinically important for both antimicrobial resistance (AMR) carriage and as the cause of nosocomial infections. Surveillance of *Citrobacter* species in the environment can provide indicators of the spread of AMR genes outside clinical spaces. In this study we present draft-genome sequences of four *Citrobacter* isolates obtained from three species of wild Australian shorebirds.

8.1.2 Announcement

The genus *Citrobacter* comprises 11 species. They occupy a broad range of habitats, play a key role in the nitrogen cycle, and are frequently found in food and in the gut of animals including humans (1). *Citrobacter* is an opportunistic pathogen of humans, most commonly associated with infant meningitis, urinary tract infections, sepsis and pneumonia (2). Species most commonly isolated from clinical specimens are *C. koseri*, *C. freundii*, *C. youngae*, *C. braakii* and *C. amalonaticus* (3). *Citrobacter* species can act as reservoirs for AMR genes, and can transfer these genes to other pathogenic bacteria (4, 5). *Citrobacter* spp. have been isolated from both healthy (6) and diseased birds (7).
We present here draft genomes of four *Citrobacter* isolates collected from Australian shorebirds through 2017-2018.

Cloacal swabs collected from healthy Australian shorebirds recovered four *Citrobacter* isolates (Table 12). Cloacal swabs were pre-enriched by incubating overnight in Brain Heart Infusion broth at 35°C, followed by a secondary enrichment by transferring 100µl into Mannitol broth and again incubating overnight at 35°C. The broths were subsequently subcultured onto MacConkey II agar (Oxoid) and incubated overnight at 35°C.

Phenotypic testing of antimicrobial resistance was conducted using the disk diffusion method (8). For DNA extraction organisms were grown overnight on Nutrient Agar. Genomic DNA was extracted using a Qiagen DNeasy kit and quantified using an Invitrogen Qubit 2. Sequencing was conducted at the Australian Genome Research Facility using the Illumina MiSeq, with Illumina gDNA shotgun library preparation with bead size selection protocol generating 150bp paired-end reads.
Raw reads were uploaded to the Galaxy web platform, and data were analyzed via the public server at usegalaxy.org, version 20.01 (9). Genomes were assembled *de novo* and read quality control performed using Unicycler Ver. 0.4.8.0 (10), and genome assembly quality was analyzed using QUAST Ver. 5.0.2+galaxy1 (11). Genomes were uploaded to NCBI and annotated by PGAP, version 4.12 (12). Further information on genome parameters is given in Table 12.

Table 13. Genotypic and phenotypic features of the Citrobacter sp. isolates.

	Isolate data			
Strain	966a	1120a	1241a	1273b
Species	*Citrobacter amalonaticus*	*Citrobacter braakii*	*Citrobacter freundii*	*Citrobacter freundii*
Sampling location	38.324105 S 145.517553 E	38.003826 S 144.596880 E	17.979327 S 122.336533 E	17.979327 S 122.336533 E
Host	Double-banded Plover (*Charadrius bicinctus*)	Curlew Sandpiper (*Calidris ferruginea*)	Bar-tailed Godwit (*Limosa lapponica*)	Bar-tailed Godwit (*Limosa lapponica*)
Year of isolation	2017	2017	2018	2018
Phenotypic resistance	Amoxicillin, Ampicillin	Amoxicillin, Ampicillin	Amoxicillin	Amoxicillin, Ampicillin
AMR[1] genes	None	*blaCMY-48*	*blaCMY-48*	*blaCMY-48*
No. of total raw paired reads	753,330	617,160	728,982	493,188
No. of contigs	91	34	48	54
Total length (bp)	4,903,911	5,098,689	5,280,444	5,279,325
N^{50} length	225,898	851,789	571,851	571,851
Average depth	71.7x	71.7	71.7x	68,5x
GC content (%)	53.35	51.49	51.35	54
BioSample no.	SAMN13884683	SAMN13884692	SAMN13884697	SAMN13884710
Assembly no.	ASM1433303v1	ASM1433294v1	ASM1433285v1	ASM1433283v1

[1]Antimicrobial Resistance (AMR)

ResFinder 3.2 (13) hosted by the Centre for Genetic Epidemiology (http://www.genomicepidemiology.org/) was used to identify AMR genes. The AMR gene identified can be seen in Table 12. This gene is thought to have originated in *C. freundii* (14).

8.1.3 Data availability

Whole genome sequences, assemblies and raw reads for this project have been deposited in GenBank under the BioProject PRJNA602163. Raw reads are available under the SRA as SRR11613026 (*C. braakii*), SRR11613020 (*C. freundii*), SRR11613006 (*C. freundii*) and SRR11612996 (*C. amalonaticus*).

8.1.4 Acknowledgments

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8.1.5 References

Lavigne JP, Defez C, Bouziges N, Mahamat A, Sotto A. 2007. Clinical and molecular epidemiology of multidrug-resistant *Citrobacter* spp. infections in a French university hospital. *Eur J Clin Microbiol* 26(6): 439-441.

Jorgensen J, Pfaller M, Carroll K, Funke G, Landry ML, Richter S, Warnock D. 2015. Manual of Clinical Microbiology. 11th Edition.

Samonis G, Karageorgopoulos DE, Kofteridis DP, Matthaiou DK, Sidiropoulou V, Maraki S, Falagas ME. 2009. *Citrobacter* infections in a general hospital: characteristics and outcomes. *Eur J Clin Microbiol* 28(1): 61.

Nawaz M, Khan A, Khan S, Sung K, Steele R. 2008. Isolation and characterization of tetracycline-resistant *Citrobacter* spp. from catfish. *Food Microbiol* 25(1): 85-91.

Jacoby GA, Griffin C, Hooper D. 2011. *Citrobacter* spp. as a source of *qnrB* alleles. *Antimicrob. Agents Chemother* 55(11): 4979-4984.

Giacopello C, Foti M, Mascetti A, Grosso F, Ricciardi D, Fisichella V, Piccolo FL. 2016. Antimicrobial resistance patterns of *Enterobacteriaceae* in European wild bird species admitted in a wildlife rescue centre. *Veterinaria Italiana* 52(2): 139-144.

Churria C, Arias N, Origlia J, Netri C, Marcantoni H, Píscopo M, Loyola M, Petruccelli M. 2014. *Citrobacter freundii* infection in two captive Australian king parrots (*Alisterus scapularis*). *J Zoo Aquar Res* 2(2).

CLSI. 2018. Performance standards for antimicrobial susceptibility testing: twenty-second informational supplement. 28th edition CLSI supplement M100. Wayne, PA. Clinical and Laboratory Standards Institute.

Afgan E, Baker D, Batut B, Van Den Beek M, Bouvier D, Cech M, Chilton J, Clements D, Coraor N, Gruning B. 2018. The Galaxy platform for accessible, reproducible and collaborative biomedical analyses: 2018 update. *Nucleic Acids Res* 46(W1): W537-W544.

Wick R, Judd L, Gorrie C, Holt K. 2017. Unicycler: resolving bacterial genome assemblies from short and long sequencing reads. *PLoS Comput. Biol.* 13(6): e1005595.

Mikheenko A, Prjibelski A, Saveliev V, Antipov D, Gurevich A. 2018. Versatile genome assembly evaluation with QUAST-LG. *Bioinformatics* 34(13): i142-i150.

Haft DH, DiCuccio M, Badretdin A, Brover V, Chetvernin V, O'Neill K, Li W, Chitsaz F, Derbyshire, MK, Gonzales NR, Gwadz M. 2018. RefSeq: an update on prokaryotic genome annotation and curation. *Nucleic Acids Res* 46(D1): D851-D860.

Zankari E, Hasman H, Cosentino S, Vestergaard M, Rasmussen S, Lund O, Aarestrup F, Larsen M. 2012. Identification of acquired antimicrobial resistance genes. *J Antimicrob Chemother* 67(11): 2640-2644.

Verdet C, Gautier V, Chachaty E, Ronco E, Hidri N, Decre D, Arlet G. 2009. Genetic context of plasmid-carried *blaCMY*-2-like genes in Enterobacteriaceae. *Antimicrob. Agents Chemother* 53(9): 4002-4006.

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Foreword to Appendix 8.2

The initial question behind this thesis- do wild Australian birds carry AMR bacteria- was conceived as an undergraduate student research project. This question was expanded further as an Honours research project, and provided the preliminary results that confirmed the validity of expanding research to surveying Australian shorebirds, looking at both migratory and non-migratory species.

Most of the laboratory work for this publication was completed as part of the aforementioned Honours project, prior to commencement of this PhD. However, some laboratory analysis (such as confirmation of some AMR data and screening isolates for *van* genes), re-analysis of data and manuscript preparation was conducted during the early stages of this PhD. The findings of presented in this publication informed the aims of this research project, and the results guided the experimental design of this thesis.

Approximately 33% of the work required to complete this work to the stage of publication was conducted as part of this PhD. Provided below is a copy of the accepted manuscript (re-formatted slightly to be consistent with the rest of this thesis). The details of the publication are as follows:

Smith, H.G., Clarke, R.H., Larkins, J-A., Bean, D.C. & Greenhill, A.R. (2019). Wild Australian birds and drug-resistant bacteria: characterisation of antibiotic-resistant *Escherichia coli* and *Enterococcus spp*. *Emu – Austral Ornithology*, 119(4): 384-390. doi.org/10.1080/01584197.2019.1591162

8.2 Wild Australian birds and drug resistant bacteria: Characterisation of antibiotic resistant *Escherichia coli* and *Enterococcus* spp.

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8.2.1 Abstract

Birds can spread microorganisms through their movement; however it is still not fully understood how wild birds acquire and disperse antimicrobial resistant bacteria. We sampled wild Australian birds from three geographically distinct locations for the presence of AMR strains of two clinically important species of bacteria, *Escherichia coli* and *Enterococcus* spp. A total of 121 birds were sampled, representing 15 different species. Thirty *E. coli* and 54 *Enterococcus* isolates were tested for resistance to 12 and 8 different antibiotics, respectively. Resistance to at least one antibiotic was common, being detected in 96% of *Enterococcus* and 60% of *E. coli* isolates. The vancomycin resistance genes *vanA* and *vanD* were detected in 22% of enterococci (13% *vanA*, 9% *vanD*), while 9% displayed phenotypic resistance with no associated gene. Wild birds are a carrier of AMR bacteria in Australia, and are capable of harbouring a more diverse range of vancomycin resistance genes than is typically seen among Australian clinical isolates.

Keywords: Zoonotic, AMR, antimicrobial, avian, van genes.

8.2.2 Introduction

Due to their highly mobile nature, birds have the potential to rapidly spread microorganisms between geographically distinct locations (Reed et al. 2003, Bauer & Hoye 2014). In addition

to microorganisms, wild birds are also capable of carrying antimicrobial resistant (AMR) bacteria, which are recognised as a growing threat to global health (da Costa et al. 2013, Reed et al. 2003). At least 700,000 deaths are associated with AMR each year, and this is predicted to reach 10 million deaths by 2050 (Kahn 2017). AMR bacteria have been found in environments and animal populations not directly exposed to antibiotics (Allen et al. 2010), including wild animal populations (da Costa et al. 2013). The transmission of bacteria between wild animals and humans has been noted in areas of high human-wildlife contact, such as urban fringes (Blyton et al. 2015, Pesapane et al. 2013). Moreover, genetically similar bacteria have been detected both animal and human hosts (Clermont 2011).

Enterococcus spp. and *Escherichia coli* are common gastrointestinal bacteria, and can be passed between wild birds and humans (Abulreesh et al. 2007, da Costa et al. 2013, Bonnedahl and Jarhult et al. 2014). Both are potential pathogens of humans and other animals, and are effective indicators of AMR in the environment due to the ease with which they acquire resistance genes (Radhouani et al. 2012). Enterococci are a serious concern in clinical settings, being ranked as the second most abundant healthcare-associated infection globally (Coombs et al. 2013). Of particular concern is resistance to the antibiotic vancomycin, a drug traditionally reserved for the treatment of severe *Enterococcus* and *Staphylococcus* infections in humans (Courvalin 2006).

The first AMR bacteria isolated from wildlife was recovered from pigeons (Columba livia) in 1975 (Bonnedahl and Jarhult 2014). Since then, AMR bacteria have been isolated in birds from every continent (Guenther et al. 2012, Bonnedahl and Jarhult, 2014, Blyton et al. 2015). AMR bacteria have been isolated from a diversity of avian taxa including representatives in the families Laridae (Cizek et al. 2007), Passerines (Hernandez-Divers et al. 2008, LeJeune et al. 2008), Columbidae (Radimersky et al. 2010) and Accipitriformes (Radhouani et al. 2012). Three recent studies have investigated AMR in Australian birds: one investigated *E. coli* in wild and domestic birds (Blyton et al. 2015); while two looked at AMR Enterobacteriaceae and vancomycin-resistant *Enterococcus* (VRE) in Silver Gulls from New South Wales (*Chroicocephalus novaehollandiae*; Dolejska et al. 2015, Oravcova et al. 2017). Whilst these studies provide much-needed insight into how wild birds are affected by anthropogenic activity and location, there remains a gap in knowledge for how AMR and VRE carriage may differ at sites with little to no anthropogenic impact.

In the current study, we demonstrate that resistance to clinically important antibiotics is pervasive in wild avian populations, regardless of location or human impact. Furthermore, the study provides clarification on the impact anthropogenic contact has on AMR carriage in wild Australian birds, by investigating habitats with little to no anthropogenic impact.

8.2.3 Methods

8.2.3.1 Sample sites and species investigated

Cloacal swabs were collected from three geographically distinct locations: Adele Island (15 31' 28" S, 123 09' 20" E), NW Australia, Boigu Island (9 13' 59"S, 142 13' 00"E), Torres Strait, Queensland and the Gippsland Lakes (37 57' 52"S 147 46' 15" E), Victoria, where capture of wild free-flying birds was being conducted for ecological research (see Mott et al. 2017a). Birds were captured using canon nets, mist nets or hand nets, as appropriate to the target species. In all instances sampled birds appeared healthy, displaying no signs of illness or injury. Cloacal swabs were collected using routine bird handling and sample collection methods. Swabs were placed in Aimes transport media and stored at 4°C until bacterial culture could be conducted. Transit time between sample collection and culture varied, with a 5 day transit time for the Gippsland Lakes samples, 2-12 days for the Torres Strait samples, and 4-8 days for the Adele Island samples. This work was conducted under wildlife permits issued by Parks Australia, Victorian Department of Environment, Land, Water and Planning (DELWP) and Queensland Environment Protection Agency and animal ethics approvals administered through Monash University BSCI/2012/08 (Adele Island), BSCI/2012/15 (Gippsland Lakes) and BSCI/2014/03 (Torres Strait).

8.2.3.2 Bacterial culture and identification

We selected two bacteria for our analysis: one Gram positive (*Enterococcus* spp.) and one Gram negative organism (*E. coli*). Each swab was used to inoculate duplicate Oxoid MacConkey II agar. Plates were incubated at 37°C and checked for growth at 24, 48 and 72 hours.

Suspected *E. coli* or *Enterococcus* were subcultured: up to six colonies were selected from each plate. Identification was conducted by biochemical tests (Catalase, Oxidase and Gram Stain) and PCR. *Enterococcus* isolates were identified to genus and species level by using established protocols that target the 16S rRNA gene (Ryu et al. 2013). Molecular confirmation of *E. coli* was conducted following protocols of Khan et al. (2007) that targeted a 450bp fragment in the Internal Transcribed Spacer (ITS) region.

Isolates were tested for AMR using the Kirby-Bauer disk diffusion test following CLSI guidelines. Antibiotics appropriate for the clinical treatment of each organism were chosen (Clinical and Laboratory Standards Institute 2012).

8.2.3.3 Antimicrobial resistance testing and *van* gene detection

E. coli isolates were tested for susceptibility to amikacin (10 µg), co-trimoxazole (1.25/23.75 µg), streptomycin (10 µg), nalidixic acid (30 µg), imipenem (10 µg), ceftazidime (30 µg), ampicillin (10 µg), tetracycline (30 µg), gentamicin (10 µg), chloramphenicol (30 µg), amoxicillin-clavulanic acid (30 µg) and ciprofloxacin (5 µg). *Enterococcus* isolates were tested against erythromycin (15 µg), tetracycline (30 µg), chloramphenicol (30 µg), ampicillin (10 µg), streptomycin (300 µg), gentamicin (120 µg), ciprofloxacin (5 µg) and vancomycin (30 µg). If multiple isolates were obtained from a single swab, susceptibility testing was conducted on all isolates. The presence of the vancomycin resistance genes *vanA*, *vanB*, *vanC* and *vanD* in the *Enterococcus* isolates was determined by PCR (Depardieu et al., 2004).

8.2.4 Results

8.2.4.1 Bird species

Samples were collected from 121 birds. In the Gippsland Lakes region of Victoria (a major human population centre) 26 Greater Crested Terns (*Thalasseus bergii*) were sampled. In the Torres Strait (a human population of 300-400 on the island), 46 individual birds were sampled, representing 10 species (two Coraciiformes, one Cuculiforme and seven Passeriformes; Table 13). From Adele Island (no permanent human population) 49 birds were sampled, representing four species of seabird (all Suliformes; Table 13).

8.2.4.2 Bacterial isolates

From 121 sampled birds, 15 (12%) were positive for *E. coli* (12 Greater Crested Tern, two Varied Honeyeater (*Gavicalis versicolor*), one Rainbow Bee-eater (*Merops ornatus*) and 14 (11%) for *Enterococcus* (5 Greater Crested Tern, two Varied Honeyeater, two Sacred Kingfisher (*Todiramphus sanctus*), and one each of; Willie Wagtail (*Rhipidura leucophrys*), Horsfields Bronze-Cuckoo (*Chrysococcyx basalis*), Rainbow Bee-eater, Masked Booby (*Sula dactylatra*), and Lesser Frigatebird (*Fregata ariel*), Table 13). A total of 71 presumptive *Enterococcus* isolates and 73 presumptive *E. coli* isolates were obtained, all of which were confirmed by PCR.

Table 14. Australian bird species sampled, and the proportions of birds positive for bacterial carriage, and the proportions of birds positive for AMR carriage.

Sampling location	Bird species (common name)	No. of birds sampled	Number (%) birds positive*		Number of birds yielding bacteria resistant to a specific antibiotic (number; %#)	
			E. coli	*Enterococcus* spp.	*E. coli*	*Enterococcus* spp.
Gippsland Lakes	*Thalasseus bergii* (Greater Crested Tern)	26	12 (46%)	5 (19%)	Amikacin (1; 8%) Ceftazidime (1; 8%) Chloramphenicol (1; 8%) Streptomycin (4; 33%)	Ciprofloxacin (3; 60%) Erythromycin (5; 100%) Gentamicin (5; 100%) Streptomycin (5; 100%) Vancomycin (2; 40%)
Boigu Island	*Rhipidura leucophrys* (Willie Wagtail)	1	0	1 (100%)	-	Ampicillin (1; 100%) Ciprofloxacin (1; 100%) Erythromycin (1; 100%) Gentamicin (1; 100%) Streptomycin (1; 100%) Vancomycin (1; 100%)
	Chrysococcyx basalis (Horsfields Bronze-Cuckoo)	1	0	1 (100%)	-	Ampicillin (1; 100%)
	Gavicalis versicolour (Varied Honeyeater)	23	2 (9%)	2 (9%)	Amikacin (1; 50%) Amoxicillin (1; 50%) Ampicillin (2; 100%) Streptomycin (2; 100%)	Ampicillin (1; 50%) Ciprofloxacin (1; 50%) Chloramphenicol (1; 50%) Erythromycin (1; 50%) Gentamicin (1; 50%) Streptomycin (1; 50%) Vancomycin (1; 50%)
	Todiramphus sanctus (Sacred Kingfisher)	2	0	2 (100%)	-	Ampicillin (2; 100%) Ciprofloxacin (2; 100%) Chloramphenicol (1; 50%) Erythromycin (2; 100%) Gentamicin (2; 100%) Streptomycin (2; 100%) Tetracycline (1; 50%) Vancomycin (2; 100%)
	Merops ornatu (Rainbow Bee-eater)	2	1 (50%)	1 (50%)	Ampicillin (1; 100%) Amoxicillin (1; 100%) Gentamicin (1; 100%) Streptomycin (1; 100%) Tetracycline (1; 100%)	Ampicillin (1; 100%) Amoxicillin (1; 100%) Gentamicin (1; 100%) Streptomycin (1; 100%) Tetracycline (1; 100%)
	Myiagra Alecto (Shining Flycatcher)	1	0	0	-	-
	Ramsayornis modestus (Brown-backed Honeyeater)	1	0	0	-	-
	Conopophila albogularis (Rufous Banded Honeyeater)	1	0	0	-	-
	Xanthotis flaviventer (Tawny-breasted Honeyeater)	12	0	0	-	-
	Dicrurus bracteatus (Spangled Drongo)	2	0	0	-	-
Adele Island	*Sula dactylatra* (Masked Booby)	9	0	1 (11%)	-	Ciprofloxacin (1; 100%) Erythromycin (1; 100%) Gentamicin (1; 100%) Streptomycin (1; 100%) Vancomycin (1; 100%)
	Fregata ariel (Lesser Frigatebird)	20	0	1 (5%)	-	Erythromycin (1; 100%) Streptomycin (1; 100%) Vancomycin (1; 100%)
	Sula leucogaster (Brown Booby)	16	0	0	-	-
	Fregata minor (Great Frigatebird)	4	0	0	-	-

For both *E. coli* and *Enterococcus* spp., multiple bacterial isolates from a single bird with identical AMR phenotypes were excluded, on the assumption they were clonal. This resulted in 30 distinct *E. coli* isolates and 54 distinct isolates of *Enterococcus* spp. *Enterococcus* spp. were isolated from birds in all three study sites, while *E. coli* was recovered from birds in two study sites (Gippsland Lakes and Boigu Island). Of the *Enterococcus* isolates, 45 (83%) were identified as *E. faecalis*, 2 (4%) as *E. faecium*, and 7 (13%) could not be identified to species.

8.2.4.3 Antimicrobial resistance and *van* genes

Of the 15 birds from which *E. coli* was isolated, AMR bacteria were detected in nine (60%) birds (seven Greater Crested Terns, two Varied Honeyeaters and one Rainbow Bee-Eater, Table 13). Of the *E. coli* isolates, 34% demonstrated resistance to streptomycin, 34% to ampicillin, 7% to amikacin, 7% to amoxicillin, 7% to gentamicin, 4% to chloramphenicol, 4% to ceftazidime, and 4% to tetracycline. All isolates were sensitive to imipenem, nalidixic acid, co-trimoxazole and ciprofloxacin. Multi-drug resistance (resistance to >1 drug) was recorded in 23% of *E. coli* isolates, in both sites where *E. coli* was detected.

Of the 14 individual birds from which *Enterococcus* spp. were isolated, AMR bacteria were detected in 13 (five Greater Crested Terns, two Sacred Kingfishers, and one each of Varied Honeyeater, Rainbow Bee-Eater, Willie Wagtail, Horsfields Bronze-Cuckoo, Lesser Frigatebird, and Masked Booby, Table 13). Ninety-six percent of *Enterococcus* isolates displayed AMR. Of these isolates, 83% demonstrated resistance to streptomycin, 50% to erythromycin, 48% to gentamicin, 31% to vancomycin, 26% to ciprofloxacin, 13% to ampicillin, 6% to chloramphenicol, and 2% to tetracycline. Resistance was observed to every antibiotic tested. Multi-drug resistance was recorded in 72% of *Enterococcus* isolates, and in all three sites.

Seventeen (31%) VRE isolates in total were discovered. Van genes were detected in 22% of enterococci; 13% (n=7) isolates were *vanA*, 9% (n=5) were *vanD*, while three carried both *vanA* and *vanD*. Five isolates were VRE, but did not carry either *vanA, B, C* or *D*.
No *vanB* or *vanC* genes were detected. VRE was isolated from six bird species, with *vanA* carried by two Sacred Kingfishers, one Willie Wagtail, one Masked Booby, and *vanD* carried

by one Willie Wagtail, one Sacred Kingfisher and one Greater Crested Tern (all of which also carried *vanA*). Vancomycin resistance was also present in one Greater Crested Tern, Varied Honeyeater and Lesser Frigatebird, but a *van* gene was not identified. Vancomycin resistance was detected in all three study sites.

8.2.5 Discussion

This study demonstrates that wild birds in three geographically distinct locations around the periphery of the Australian continent harbour AMR bacteria. Moreover, resistance was widespread across bird taxa, and was present in remote and isolated habitats.
The proportion of AMR *E. coli* isolates was generally higher in this study (60% of isolates, with 7% of birds carrying AMR bacteria) than in similar studies conducted in countries such as the US (49% of isolates; though 23% of birds carried AMR bacteria, Gaukler et al. 2009) and the Czech Republic (1.5% of isolates, with 1% of birds carrying AMR bacteria; Radimersky et al. 2010). However, a study of Common Buzzards (*Buteo buteo*) in Portugal found 97% of *E. coli* isolates displayed AMR, with 83% of birds carrying AMR bacteria. (Radhouani et al. 2012).

As was the case with *E. coli*, the prevalence of AMR enterococci was generally higher (96% of isolates carried drug resistance), though the prevalence of AMR enterococci in the overall population was lower (12% of birds carried AMR *Enterococcus* spp.) than in similar studies. For example, 31% of *Enterococcus* isolates in feral pigeons in the Czech Republic demonstrated AMR, with 18% of birds carrying AMR bacteria (Radimersky et al. 2010), and 46% of enterococci from wild birds in the Azores Archipelago demonstrated AMR, with 29% of birds carrying AMR enterococci (Santos et al. 2013). However, direct comparisons to previous studies are limited due to the small sample size in the current study. Moreover, it is important to note that bacterial recovery may have been adversely affected by time in transit between swab collection and culture.

Vancomycin resistance was investigated further due to its importance in human medicine. While vancomycin has never been available for use in livestock, a chemically similar antibiotic (Avoparcin) was used in Australian agriculture until 2007 (Barton 2010). VRE has only been described once in Australian birds, from Silver Gulls in both Sydney and Five

Islands, NSW (Oravcova et al. 2017). Our study isolated 17 VRE isolates, in six species, from all study sites. It is important to note that the culture methods used differed between this study and the Oravcova et al. study, which cultured for VRE isolates. Elsewhere, VRE have been reported in a number of avian species, including Rooks (*Corvus frugilegus*), and feral pigeons in Europe (Radimersky et al. 2010, Oravcova et al. 2013). Our findings compliment those of Oravcova et al. (2017), and demonstrate that VRE occurs in a range of taxonomically distinct Australian bird species that are geographically dispersed, and in association with both high and low human population densities. In addition to the *vanA* and *vanB* genotypes discovered by Oravcova et al., this study shows that *vanD* genotypes also are present in Australian birds. This genotype is not currently seen in the clinical population (Lee et al. 2018, Coombs et al. 2013). While Silver Gulls often live in close proximity to humans and may be exposed to clinically important bacteria through behaviour such as feeding on refuse (Smith et al. 1993), many of the identified carriers of VRE in this study are unlikely to have the same exposure due to habitat, foraging requirements or behavioural responses (Jackson et al. 1993, Mott et al. 2017a).

It is possible that the birds acquired AMR bacteria from environmental contamination (Berglund et al. 2015), or through interactions with other wildlife that may interact with anthropogenic environments (Smith et al. 1993). In addition, some of the species sampled (such as the Passerines) may be acquiring AMR bacteria indirectly from anthropogenic sources through diet, by ingesting insects that have fed on refuse. This is of importance in areas of high anthropogenic activity, such as the Gippsland Lakes, or in areas with poorer access to adequate sanitation, such as Boigu Island (Bailie et al. 2004, Horwood and Greenhill 2012).

This study clearly demonstrates that AMR bacteria are present in a diversity of wild bird species at distant points around Australia. Whilst acknowledging limited replication across just three sites, given that one of these sites (Adele Island) is remote from human population centres, and another (Boigu Island) is sparsely populated with a near complete absence of livestock, our results suggest that direct anthropogenic contact may only be one of a number of drivers of AMR and VRE at regional scales. It is possible that proximity to the Asia-Pacific is a contributing factor to this trend (Molton et al. 2013), especially given that

Torres Strait is recognised as an important migratory flyway (Draffan et al. 1986, Clarke 2004) and that some seabirds from Adele Island are known to spend the non-breeding season in Indonesian waters where individuals also accumulate high loads of heavy metals (Mott et al. 2017b).

Of particular interest is that *vanA* was detected in our isolates. In Australia *vanB* is most commonly associated with clinical VRE (Coombs et al 2013); whereas in China *vanA* has been shown to be common (Zhao et al. 2010). Four of the six *vanA* isolates were obtained from Boigu Island, Torres Strait. Although these particular species do not migrate to Asia, there is potential for crossover from migratory landbirds that breed as far afield as eastern Asia and travel through, or remain in, the Torres Strait during the non-breeding season (e.g Oriental Cuckoo, *Cuculus optatus*: Clarke 2004). Given the absence of direct AMR exposure pathways for the bird species assessed here, more work needs to be done to determine where and how such AMR bacteria are colonizing Australian birds.

8.2.6 Acknowledgments

This work was conducted under wildlife permits issued by Parks Australia, Victorian Department of Environment, Land, Water and Planning (DELWP) and Queensland Environment Protection Agency and animal ethics approvals administered through Monash University. The authors thank the Victorian Wader Study Group (VWSG) for the sampling opportunities, and Rowan Mott, Lee Peacock and Ashley Herrod for their assistance with sample collection. We also thank Dr Susan Ballard for the donation of control strains. This research was supported by the Holsworth Wildlife Research Endowment.

8.2.7 References

Allen, H., Donato, J., Wang, H., Cloud-Hansen, K., Davies, J., and Handelsman, J. (2010). Call of the wild: antibiotic resistance genes in natural environments. *Nature Reviews Microbiology,* **1**, 1-9. doi: 10.1038/nrmicro2312

Barton, D. (2010). Antibiotic resistance in Australian animals in 2010 – what lies ahead?. APVMA Science Fellows Symposium.

Bailie, R., Carson, B., McDonald, E. (2004). Water supply and sanitation in remote Indigenous communities – priorities for health development. *Australian and New Zealand Journal of Public Health,* **28**(5), 409-414.

Bauer, S., & Hoye, B. J. (2014). Migratory animals couple biodiversity and ecosystem functioning worldwide. *Science*, **344**(6179), 1242552.

Blyton, M., Pi, H., Vangchhia, B., Abraham, S., Trott, D., Johnson, J., and Gordon, D. (2015). The genetic structure and antimicrobial resistance of *Escherichia coli* and cryptic clades in birds with diverse human associations. *Applied and Enviromental Microbiology*, **81**(15), 5123-5133. doi: doi:10.1128/AEM.00861-15

Bonnedahl, J., and Jarhult, J. (2014). Antibiotic resistance in wild birds. *Upsala Journal of Medical Sciences*, **119**(2), 1-4. doi: 10.3109/03009734.2014.905663

Cizek, A., Dolejska, M., Karpiskova, R., Dedicova, D., and Literak, I. (2007). Wild black-headed gulls (*Larus ridibundus*) as an environmental reservoir of *Salmonella* strains resistant to antimicrobial drugs. *European Journal of Wildlife Research,* **53**, 55-60. doi: 10.1007/s10344-006-0054-2

Clarke, Rohan. (2004). The Avifauna of Northern Torres Strait: Notes on a Wet-season Visit. *Australian Field Ornithology,* **21**, 49-66.

Clermont, O., Olier, M., Hoede, C., Diancourt, L., Brisse, S., Keroudean, M., Glodt, J, *et al*. (2011). Animal and human pathogenic *Escherichia coli* strains share common genetic backgrounds. *Infection, Genetics and Evolution*, **11**(3), 654-662. doi: 10.1016/j.meegid.2011.02.005

Clinical and Laboratory Standards Institute. (2012). Performance Standards for Antimicrobial Susceptibility Testing. Twenty-Second Informational Supplement. *CLSI Document M100-S22*.

Coombs, G., Pearson, J., Christiansen, K., Gottleib, T., Bell, J., Geroge, N., and Turnidge, J. (2013). "Australian group on antimicrobial resistance *Enterococcus* surveillance programme annual report, 2010." *Communicable Diseases Intelligence*, **37**(3),199-209

da Costa, P., Loureiro, L., and Matos, A. (2013). Transfer of multidrug-resistant bacteria between intermingled ecological niches: The interface between humans, animals and the environment. *International Journal of Enviromental Research and Public Health,* **10**, 278-294. doi: 10.3390/ijerph10010278

Depardieu, F., Perichon, B., and Courvalin, P. (2004). Detection of the van alphabet and identification of enterococci and staphylococci at the species level by multiplex PCR. *Journal of Clinical Microbiology,* **42**(12), 5857-5860. doi: 10.1128/JCM.42.12.5857-5860.2004

Dolejska, M., Masarikova, M., Dobiasova, H., Jamborova, I., Karpiskova, R., Havlicek, M., Literak, I *et al*. (2015). High prevalence of *Salmonella* and IMP-4-producing Enterobacteriaceae in the Silver Gull on Five Islands, Australia. *Journal of Antimicrobial Chemotherapy,* **71**(1), 63-70.

Draffan, R.D.W., Garnett, S.T. and Malone, G.J. (1983). Birds of the Torres Strait: An Annotated List and Biogeographical Analysis. *Emu - Austral Ornithology,* **83**, 207-234.

Gaukler, S., Linz, G., Sherwood, J., Dyer, N., Bleier, W., Wannemuehler, Y., Louge, C *et al*. (2009). *Escherichia coli, Salmonella*, and *Mycobacterium avium subsp. paratuberculosis* in Wild European Starlings at a Kansas cattle feedlot. *Avian Diseases,* **53**(4), 544-551.

Guenther, S., Aschenbrenner, K., Stamm, I., Bethe, A., Semmler, T., Stubbe, A., Ewers, C *et al*. (2012). Comparable High Rates of Extended-Spectrum-Beta- Lactamase-Producing *Escherichia coli* in Birds of Prey from Germany and Mongolia. *PLoS ONE,* **7**(12), 1-6.

Hernandez-Divers, S.M., Villegas, P., Jimenez, C., Hernandez-Divers, S.J., Garcia, M., Riblet, S., Sanchez, S *et al*. (2008). Backyard chicken flocks pose a disease risk for neotropic birds in Costa Rica. *Avian Diseases,* **52**(4), 558-566.

Horwood, P., and Greenhill, A. (2012). Cholera in Papua New Guinea and the importance of safe water sources and sanitation. *Western Pacific Surveillance and Response,* **3**(1), 1-3.

Jackson, J., and Elgar, M. A. (1993). The foraging behaviour of the willie wagtail Rhipidura leucophrys: Why does it wag its tail? *Emu-Austral Ornithology,* **93**(4), 284-286.

Kahn, L. H. (2017). Antimicrobial resistance: a One Health perspective. *Transactions of the Royal Society of Tropical Medicine and Hygiene,* **111**(6), 255-260. doi: 10.1093/trstmh/trx050

Khan, I. U., Gannon, V., Kent, R., Koning, W., Lapen, D. R., Miller, J., Edge, T *et al*. (2007). Development of a rapid quantitative PCR assay for direct detection and quantification of culturable and non-culturable *Escherichia coli* from agriculture watersheds. *J Microbiol Methods,* **69**(3), 480-488. doi: 10.1016/j.mimet.2007.02.016

Lee, R., Goncalves, A., Baines, S., Strachan, J., Ballard, S., Carter, P., Kwong, J., Schultz, M., Bulach, D., Seemann, T., Stinear, T., and Howden, B. (2018). The changing landscape of vancomycin-resistant *Enterococcus faecium* in Australia: a population-level genomic study. *Journal of Antimicrobial Chemotherapy*. doi: 10.1093/jac/dky331.

LeJeune, J., Homan, J., Linz, G., and Pearl, D. (2008). Role of the European Starling in the transmission of *E. coli* O157 on dairy farms. *Vertebrate Pest Conference,* **23**, 31-34.

Molton, J., Tambyah, P., Ang, B., Ling, M., and Fisher, D. (2013). The global spread of healthcare-associated multidrug-resistant bacteria: a perspective from Asia. *Clinical infectious diseases,* **56**(9), 1310-1318.

Mott, R., Herrod, A., and Clarke, R. H. (2017a). Resource partitioning between species and sexes in Great Frigatebirds and Lesser Frigatebirds. *The Auk* **134**, 153–167.

Mott, R., Herrod, A. and Clarke, R.H. (2017b) Post-breeding dispersal of frigatebirds increases their exposure to mercury. *Marine Pollution Bulletin* **119**, 204-210.

Oravcova, V., Anuradha G., Ludek Z., Bardon, J., Guenther, S., Cizek, A., and Literak, I. (2013) Vancomycin-resistant enterococci in rooks (*Corvus frugilegus*) wintering throughout Europe. *Environmental microbiology* **15**(2), 548-556.

Oravcova, V., Svec, P., and Literak, I. (2017). Vancomycin-resistant enterococci with *vanA* and *vanB* genes in Australian gulls. *Enviromental Microbiology Reports,* **9**(3), 316-318. doi: 10.1111/1758-2229.12542

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

Radhouani, H., Poeta, P., Goncalves, A., Pacheco, R., Sargo, R., and Igrejas, G. (2012). Wild birds as biological indicators of environmental pollution: antimicrobial resistance patterns of *Escherichia coli* and enterococci isolated from Common Buzzards (*Buteo buteo*). *Journal of Medical Microbiology,* **61**, 837-843. doi: 10.1099/jmm.0.038364-0

Radimersky, T., Frolkova, P., Janoszowska, D., Dolejska, M., Svec, P., Roubalova, E., Literak, I *et al*. (2010). Antibiotic resistance in faecal bacteria (*Escherichia coli, Enterococcus* spp.) in feral pigeons. *Journal of Applied Microbiology,* **109**, 1687-1695. doi: 10.1111/j.1365-2672.2010.04797.x

Reed, K., Meece, J., Henkel, J., and Shukla, S. (2003). Birds, migration and emerging zoonoses: West Nile Virus, Lyme Disease, Influenza A and Enteropathogens. *Clinical Medicine & Research,* **1**(1), 5-12.

Ryu, H., Henson, M., Elk, M., Toledo-Hernandez, C., Griffith, J., Blackwood, D., Santo Domingo, J *et al*. (2013). Development of quantitative PCR assays targeting the 16S rRNA genes of *Enterococcus* spp. and their application to the identification of *Enterococcus* species in environmental samples. *Applied and Enviromental Microbiology,* **79**(1), 196-204. doi: 10.1128/AEM.02802-12

Santos, T., Silva, N., Igrejas, G., Rodrigues, P., Micael, J., Rodrigues, T., Poeta, P *et al*. (2013). Dissemination of antibiotic resistant *Enterococcus* spp. and *Escherichia coli* from wild birds of Azores Archipelago. *Anaerobe,* **24**, 25-31. doi: 10.1016/j.anaerobe.2013.09.004

Smith, G. C., and Carlile, N. (1993). Food and feeding ecology of breeding Silver Gulls (*Larus novaehollandiae*) in urban Australia. *Colonial waterbirds*, **16**(1), 9-16.

Zhao, C., Sun, H., Wang, H., Liu, Y. H., B, Yu, Y., Sun, Z., Chen, M *et al*. (2012). Antimicrobial resistance trends among 5608 clinical Gram-positive isolates in China: results from the Gram-Positive Cocci Resistance Surveillance program (2005–2010). *Diagnostic Microbiology and Infectious Disease,* **73**, 174-181.

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8.3 Final Report for Federation University Animal Ethics Committee

Complete all sections of this template pertaining to your project requirements. The information provided allows the AEC to determine whether animals used in this project have been justified; that it adheres to the principals of the 3R's and that the research has been conducted as approved.

1. PROJECT DETAILS:

Indicate the type of report:	☐ **Annual Report (Omit 9.2 & 11.2)** ☒ **Final Report**
Project No:	**16-002**
Project Name:	Zoonotic pathogens and the potential for antimicrobial resistance in the gut microbiota of wild Australian birds: the impact of migration on potential emerging diseases.
State:	▬ **VIC** ■ **OTHER** _____

2. RESEARCHER DETAILS:

Principal Researcher:	**Andrew Greenhill**
School/Section:	**School of Health and Life Sciences**
Phone:	**5122 8504**
Email:	**andrew.greenhill@federation.edu.au**
Other Researchers:	**Hannah Smith** **David Bean** **Maureen Christie** **Jeff Campbell**

3. PROJECT STATUS:

Please indicate the current status of the project:
☐ **Continuing** **Expected completion date:**

☒ Completed Completion date: **01 / 03 / 2019**	
☐ **Abandoned for the following reason:**	

4. SPECIAL CONDITIONS:

If this project was originally approved subject to certain conditions, have these been met?		
☐ N/A	☒ Yes	☐ **No** * NB: If 'no', please provide an explanation:

5. CHANGES TO PROJECT:

5.1 Have any amendments been made to the originally approved project?	
☐ **No**	☒ **Yes** * NB: List all Amendment details: **Provided further clarification of what capture methods would be used by the Victorian Wader Study Group to initially capture animals.** **Removed two species (Silver Gull, *Chroicocephalus novaehollandiae*, and Australian White Ibis, *Threskiornis moluccus*) from the list of species to be targeted. Added three more species (Double-banded Plover, *Charadrius bicinctus*, Red-Capped Plover, *Charadrius ruficapillus*, Ruddy Turnstone, *Arenaria interpres*) to the list of species to be targeted.** **Removed a species to be targeted- Banded Stilt, *Cladorhynchus leucocephalus*.** **Addition of locations in Western Australia (80 mile beach and Roebuck Bay)** Addition of locations in South Australia (Adelaide and Mount Lofty Ranges, South Australian Murray-Darling Basin, Eyre Peninsula, Northern and Yorke, South East, Piccannie Ponds Conservation Park, Little Dip Conservation Park, Beachport Conservation Park, Canunda National Park, Coorong National Park, Venus Bay Conservation Park, Bernouilli Conservation Reserve, Nene Valley Conservation Park, Guichen Bay Conservation Park, Baudin Rocks Conservation Park) **Addition of locations in Tasmania (King Island)** Addition of researchers Maureen Christie and Jeff Campbell

5.2 Do you propose to make further changes?	
☒ No	☐ **Yes** * NB: Submit an amendment request to the AEC for approval before implementing any changes. Note: Extensions cannot be granted retrospectively - submit them prior to expiry of approval

6. TRAINING:

Provide details of all training undertaken by members of the research team:		
Researcher Name:	**Andrew Greenhill**	
Technique/Procedure undertaken:	N/A – Did not take part in sample collection	
Trainer's Name:	**N/A**	
Trainer's level of experience:	**N/A**	
Advise when and how competency in the technique was tested:	**N/A**	Date of Training: **N/A**
Researcher Name:	**David Bean**	
Technique/Procedure undertaken:	**N/A – Did not take part in sample collection**	
Trainer's Name:	**N/A**	
Trainer's level of experience:	**N/A**	
	N/A	Date of

Advise when and how competency in the technique was tested:		Training: **N/A**
Researcher Name:	**Hannah Smith**	
Technique/Procedure undertaken:	**Cloacal swabbing of birds**	
Trainer's Name:	**Rohan Clarke**	
Trainer's level of experience:	**Written training provided by Rohan Clarke in the method of cloacal swabbing** **2+ years' experience handling, catching and banding shorebirds**	
Advise when and how competency in the technique was tested:	**N/A**	Date of Training: **N/A**
Researcher Name:	**Maureen Christie**	
Technique/Procedure undertaken:	**Cloacal swabbing**	
Trainer's Name:	**Hannah Smith**	
Trainer's level of experience:	A Class banding license 20+ years' experience handling, catching and banding shorebirds **Training in sampling techniques provided by Hannah Smith**	
Advise when and how competency in the technique was tested:	**N/A**	Date of Training: **N/A**
Researcher Name:	**Jeff Campbell**	
Technique/Procedure undertaken:	**Cloacal swabbing**	

Trainer's Name:	**Maureen Christie**	
Trainer's level of experience:	A Class banding license **20+ years experience handling, catching and banding shorebirds**	
Advise when and how competency in the technique was tested:	**N/A**	Date of Training: **N/A**

7. STORAGE OF DATA:

Please indicate where you are storing the data collected during the course of this project:
☒ **At my place of work.** **Details: Physical swabs, bacterial cultures, and DNA extractions are all kept in -80C storage. Using this storage method, samples can be preserved for a decade or more.** **Results gathered from this experiment are kept on paper and in an electronic format, which is backed up regularly onto external cloud-based servers hosted by Federation University (OneDrive) in case of computer malfunctions, as well as being held on personal external hard drives kept at Federation University.**
☐ **Other location:** **Arrangements for material held in other locations should be documented (Australian Code for the Responsib Conduct of Research: 2.2.2)**

8. RESEARCH SUBJECTS:

Have there been any events that might have had an adverse effect on the animal subjects OR unforeseen events tha might affect continued ethical acceptability of the project? Please clearly differentiate between speculation and fact. (Please note it is AEC policy that all unexpected deaths or adverse events are reported)	
☒ No	☐ **Yes: Provide details:**

Did the number of animals used vary from the number approved? (If reporting fauna surveys, please attach a list of all species and numbers surveyed)	
☐ No	☒ **Yes: Provide details: For some species, fewer birds than approved were sampled. This was due to fewer birds than expected being available for sample collection.**

9. PROJECT SUMMARY AND FINDINGS:

9.1. Provide a complete summary of work done and findings (in lay terms) for the duration of this project.
Sample collection began in December 2016, coinciding with the approval of DELWP permits and the resumption of the Victorian Wader Study Group (VWSG) expeditions. Since then, sampling expeditions have taken place at a number of sites in Victoria, South Australia, and Western Australia through the VWSG (with Australasian Wader Study Group partnership in Western Australia). Appropriate ethical approval and permits have been granted for all of these expeditions, and are all up to date. **Data collection has processed to plan, and preliminary analyses have been conducted. To date, 1085 birds have been processed, providing roughly 1,280 samples for culture and DNA extraction. Of the 1087 birds sampled, 33 were collected by another researcher on a separate project and permit.** **Along with the preliminary analyses of the data, Whole Genome Sequencing has been performed on a select population of isolates. This process will provide large amounts of high-quality, in-depth data that will be used in both the final thesis and journal articles.**

9.2 FINAL REPORT: Provide details about how the aims of the project, as stated in the application for approval, were achieved (or not achieved).
The stated aims of this project were as such: Determine the prevalence of pathogenic bacteria in wild Australian birds **This aim has been achieved, with samples collected from 1087 birds in total. Bacteria have been cultured from all of these swabs, identified, and the prevalence rates recorded for both the overall prevalence rates, and the prevalence rates for each species.** Determine the prevalence of AMR bacteria in wild Australian birds **This aim has been achieved, with all bacteria isolated being subject to testing to determine AMR. In addition, Whole Genome Sequencing on a select number of isolates has provided detailed information on what AMR genes are present in bacteria carried by wild Australian birds.** Determine what virulence genes are present in bacteria carried by wild Australian birds

This aim has been achieved. Similar to how AMR genes were identified, virulence genes were determined by Whole Genome Sequencing on a select number of isolates.

Determine if migration has an affect on bacterial transmission

This aim is in the process of being completed, with Whole Genome Sequencing data being analyzed to determine the origins of bacterial isolates, and to see if this differed between migratory and non-migratory birds.

Determine if AMR carriage has an effect on bird gut microbiota

This aim was discarded, due to failures in laboratory experiments.

9.3 For Laboratory projects: Has the wellbeing of the animals been consistent with that anticipated in the project? (Then go to 8.6)

☐ **Yes**	☐ **No: Provide details:**

9.4 For field studies list details of all animals involved (eg. handled/trapped) in the study this year.

State where handled/ trapped eg. Vic	Species name	Common name	Annual Number (handled/trapped during year)	Cumulative Totals for project
WA	**Haematopus longirostris**	**Pied Oystercatcher**	**9**	**31**
VIC	**Charadrius ruficapillus**	**Red-Capped Plover**	**2**	**3**
WA, VIC	**Limosa lapponica**	**Bar-Tailed Godwit**	**191**	**275**
WA, VIC	**Calidris ferruginea**	**Curlew Sandpiper**	**40**	**183**
WA	**Arenaria interpres**	**Ruddy Turnstone**	**2**	**38**
WA	**Calidris alba**	**Sanderling**	**5**	**12**
VIC	**Calidris acuminata**	**Sharp-Tailed Sandpiper**	**117**	**131**
VIC	**Charadrius bicinctus**	**Double-Banded Plover**	**51**	**84**
VIC	**Thalasseus bergii**	**Greater Crested Tern**	**0**	**95**
VIC	**Hydroprogne caspia**	**Caspian Tern**	**0**	**89**
VIC	**Haematopus fuliginosus**	**Sooty Oystercatcher**	**0**	**12**
VIC, WA	**Calidris ruficola**	**Red-Necked Stint**	**0**	**101**

9.5. Are any of the animals listed in 8.4 endangered species?

State where handled/ trapped eg. Vic	Species name	Common name	Annual Number (handled/trapped during year)	Cumulative Totals for project
WA, VIC	**Calidris ferruginea**	**Curlew Sandpiper**	**40**	**183**

10. INTENTIONAL / UNINTENTIONAL DEATH OF ANIMALS:

10.1 Have any animals died as a result of experimental procedures during the project?

☒ **No** ☐ **Yes** * NB: Provide details:

State eg.Vic	Species name	Common name	Annual Number during year	Cumulative Totals for project

10.2 Have any animals been euthanised during the project?

☒ **No** ☐ **Yes** * NB: Provide details of the process

State eg. Vic	Species name	Common name	Annual Number during year	Cumulative Totals for project

10.3 Have any animals been unintentionally killed during the project?

☒ **No** ☐ **Yes** * NB: Provide details:

State eg. Vic	Species name	Common name	Annual Number during year	Cumulative Totals for project

10.4 If unintentional deaths have occurred, what steps have you taken to ensure further deaths do not occur?

N/A

11. DISSEMINATION OF RESEARCH (Australian Code for the Responsible Conduct of Research 4.4-4.12)

11.1 Provide details of research dissemination outcomes for the previous year resulting from this project: eg: Community seminars; Conference attendance; Government reports and/or research publications

A research publication is currently in the final stages of editing for re-submission to the journal Emu: Austral Ornithology. In addition, the researcher Hannah Smith has attended three conferences held by Federation University- the HDR Research Conference, held annually. Ms Smith will also be attending the Australian Ornithological Conference in July, 2019.

Ms Smith has also provided a number of informal presentations with members of the public on voluntary expeditions with the Victorian Wader Study Group (VWSG).

11.2 FINAL REPORT: How will research project findings be disseminated to peers, colleagues and the wider community?

☒ Conference papers	☒ Journal article(s)
☒ Thesis	☐ Book
☒ Other – Ms Smith will provide talks on her project with other volunteers of the Victorian Wader Study Group and Australasian Wader Study Group.	☐ None - *Please explain*

12. FEEDBACK:

The AEC requires feedback on:

- Difficulties experienced with carrying out the teaching/research project; and/ or

• Appropriate suggestions which would lead to improvements in ethical clearance and monitoring of projects involving animals.
No difficulties were experienced with carrying out the sample collection of this project, and as such no suggestions or recommendations can be provided.

13. SIGNATURE/S: By signing the report you confirm that the research has been conducted in compliance with the Act; the Code; FedUni Guidelines and Standard Conditions of AEC approval

Principal Researcher:	**Print name: Andrew Greenhill**	Date:	**28/02/19**
Other/Student Researchers:	Hannah Smith……. **Print name:**	Date:	**28/02/19**
	……. **Print name:**	Date:	

8.4 Approved Animal Ethics Permit

Principal Researcher:	Ms Hannah Smith
Student/Other Researcher/s:	Dr Andrew Greenhill David Bean
School/Section:	**School of Applied and Biomedical Sciences** **Faculty of Science & Technology**
Project Number:	**16-002**
Project Title:	Zoonotic pathogens and the potential for antimicrobial resistance in the gut microbiota of wild Australian birds: the impact of migration on potential emerging diseases.
For the period:	11/05/2016 **to** 01/03/2019
Approved Location:	Victoria
Project Summary:	There has been very little work done on antibiotic resistance in bacteria in wild bird populations in Australia, and determining the prevalence rate of many bacterial species in Australian birds. No work has been done on the risk migrating birds may play in the role of bringing new bacterial strains or diseases to Australia. This project aims to fill these gaps in our knowledge by determining: The prevalence of pathogenic (disease-causing) and zoonotic (transmits between species) diseases in wild Australian birds. The prevalence of antibiotic resistance and their associated genes in pathogenic bacteria from wild Australian birds. The presence and prevalence of virulence genes in bacteria from wild Australian birds. Determining the risk migrating birds play in transmitting new diseases to Australia. Determining if the carriage of antibiotic resistant bacteria, pathogenic bacteria or virulence genes has an effect on the composition of bird gut microbiota (the ecological community of bacteria in the gut).

Please quote the Project No. 16-002 in all correspondence regarding this application.

Amendment Approved with Comment: P9 which still refers to Ms Smith as the Principal applicant, despite Dr Andrew Greenhill being the Principal Researcher on this project.

REPORTS TO AEC:

An Annual Report must be submitted to the Ethics Officer prior to 15 January each year until the conclusion of the project.

A final report for this project must be submitted to the Ethics Officer prior to:
01/04/2019
These forms can be found at:
http://federation.edu.au/research-and-innovation/research-support/ethics/animal-ethics/downloadforms

Irene Hall
Ethics Officer
11 May 2016
Please see attached 'Conditions of Approval'.

CONDITIONS OF APPROVAL

1. The project must be conducted in accordance with the approved application, including any conditions and amendments that have been approved. You must comply with all of the conditions imposed by the AEC, and any subsequent conditions that the AEC may require.

2. You must report **IMMEDIATELY** anything which might affect ethical acceptance of your project, including:

    - Adverse effects on the welfare of animals;
    - Unforeseen events/incidents;
    - Other matters that might affect continued ethical acceptability of the project.

3. Proposed changes or amendments to the research must be applied for, using a '**Request for Amendments**' form, and approved by the AEC before these may be implemented.

4. If an extension is required beyond the approved end date of the project, a '**Request for Extension**' should be submitted, allowing sufficient time for its consideration by the committee. Extensions cannot be granted retrospectively, and will not be approved beyond four years, to ensure all projects meet current Code guidelines

5. If changes are to be made to the project's personnel, a '**Changes to Personnel**' form should be submitted for approval.

6. An '**Annual Report**' must be provided by the due date specified each year for the project to have continuing approval.

7. A '**Final Report**' must be provided at the conclusion of the project.

8. If, for any reason, the project does not proceed or is discontinued, you must advise the committee in writing, using a '**Final Report**' form.

9. You must advise the AEC immediately, in writing, if any complaint is made about the conduct of the project.

10. You must notify the Ethics Office of any changes in contact details including address, phone number and email address.

11. The AEC may conduct random audits and / or require additional reports concerning the research project.

Failure to comply with the *Australian code for the care and use of animals for scientific purposes (2013)* and with the conditions of approval will result in suspension or withdrawal of approval.