Can microbes be contributing to the decline of the North Island Brown Kiwi (*Apteryx mantelli*)?

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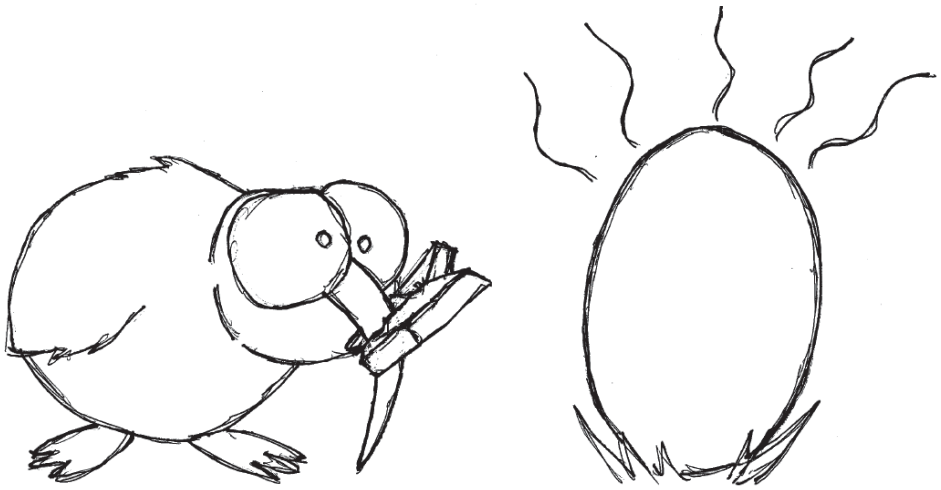
This thesis is dedicated to my daddy, who supports me through all my endeavours- even when they stink



Abstract

North Island Brown Kiwi (NIBK, *Apteryx mantelli*) are considered nationally vulnerable. Current conservation efforts concentrate on the predator vulnerable chicks, through both intensive predator control and Operation Nest Egg (ONE), a captive hatching and rearing scheme for wild eggs. While these methods are having a positive impact on some NIBK populations, they are expensive to maintain and many NIBK populations are dependent on this intensive management to maintain and increase numbers. Ideally, a point will be reached when less intensive management is needed to maintain NIBK populations. Therefore, ONE is not a permanent conservation strategy; the aim is to phase out intensive management when predator control is deemed sufficient to protect a majority of chicks.

However, even with intensive management, overall NIBK numbers are still declining. A potentially significant and previously overlooked factor in this decline could be that NIBK eggs experience high mortality. Indeed 60 per cent of NIBK eggs in the wild do not hatch. Both infertility and predators are unlikely to be major mortality factors in NIBK eggs. Consequently, predator control efforts do little to protect eggs. Research into why NIBK eggs experience such high hatching failure is needed and future conservation work needs to be adjusted in light of the results.

The overall objective of this project was to investigate if microbes could contribute to NIBK egg mortality. This project had two aims within this objective: 1. to determine if microbes that could impact hatching success are present on and in NIBK eggs; and 2. to use the results to direct future work and conservation efforts for NIBK. These aims were addressed using four studies, which together support each other in terms of conclusions and give an understanding of the microbes present at different stages in NIBK egg development, in locations throughout the population's range.

The first two studies used 16S rRNA sequencing and/or phenotypic identification methods to identify 1. the bacteria and 2. the fungi on the shells of wild NIBK eggs. Together these provided an understanding of the types of microbes that are present on living eggs during active incubation. In contrast, the third study used 16S rRNA sequencing to identify the bacteria present inside un-hatched infertile NIBK eggs,

collected from across the North Island. In the final study, a method was designed to determine if a target bacterium could penetrate through the NIBK egg's defensive shell. This method was not finalised because the NIBK eggshells could not be sterilised. However, this result showed that NIBK eggshells harbour bacteria that survive even through medical grade cleaning. The consequence of this may mean that bacteria can survive in the shell during adverse conditions, which may result in increased penetration when conditions become suitable.

Both the shell and the contents of NIBK eggs in this study had microbes present that could impact hatching success. Of these the most prevalent was *Staphylococcus,* and while no work has been done on the impact of *Staphylococcus* on NIBK, members of this genus have been shown to significantly impact the hatching of success of chickens and other birds. The prevalence of *Staphylococcus* in NIBK eggs indicates that it may be a significant factor in NIBK hatching success and warrants further, focused investigation.

That potentially pathogenic genera were isolated from NIBK eggs in this study has consequences for both fieldwork and NIBK conservation. NIBK are known to have dangerous and contagious pathogens in their blood and digestive tracts, such as *Cryptococcus* spp. Through this research, the potentially dangerous genera *Aspergillus, Staphylococcus*, *Streptococcus* and *Pseudomonas* are added to this list.

The Kiwi Best Practice Manual states that 'thin sterile latex gloves' should be worn when handing eggs, however, to use dry, bare hands 'rather than gloved' when collecting an ONE egg from the wild, to 'increase sensitivity to holding the egg ', as the eggs are cleaned upon arrival at the ONE facility. The eggshells in this project harboured bacteria that survive even through medical grade cleaning; therefore, the cleaning at ONE is unlikely to remove all bacteria. The conclusions of this project are that gloves should be worn at all stages of egg and bird handling, including collecting ONE eggs. This is because of the risk to the handler, as well as the egg. The results of this project also emphasise the need for all equipment used to be cleaned between individuals; this includes callipers, candling torches and weighing bags.

In regards to NIBK conservation, the results of this project suggest that predators are not the only factor in NIBK mortality. This project has shown that there are potentially

serious pathogens present on and in wild NIBK eggs that can kill avian embryos and could be contributing to NIBK egg mortality. We still do not know definitively what is causing the 60 per cent hatching failure in NIBK, but these results highlight the need for egg mortality and microbial factors to be factored in to NIBK conservation and recovery plans. Intensive management of NIBK should be phased out not only when predator control is deemed sufficient to protect the majority of chicks, but when researchers have a better understanding of what other factors contribute to NIBK mortality, at all stages of life. We need long-term, cost-effective ways to keep NIBK populations self-sustaining that protect the eggs as well as the chicks and adults. This means that phasing out of ONE needs to be considered in terms of egg mortality and not just chick survival.

More detailed studies are needed to both further identify the microbes present on wild NIBK eggs and to experimentally prove/disprove that NIBK embryos can be killed by these pathogens. This can be achieved by infecting eggs, or by cleaning them.

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Chapter one: Kiwi – an introduction

1.1 Kiwi biology

Kiwi (Apterygidae; five species) are nocturnal, flightless insectivores endemic to New Zealand (Marchant and Higgins, 1990). They are the smallest member of the Palaeognathae clade; a group of flightless birds including Tinamous (Tinamidae), Cassowaries (Casuariidae), Emus (Dromaiidae), Ostriches (Struthionidae), Rheas (Rheidae) (Johnston, 2011) and the extinct Moa (Dinornithidae) and Elephant Bird (Aepyornithidae). The Palaeognathae are characterised by their flat sternum (breastbone), poorly developed breast muscles, undeveloped tail-bone and palate resembling that of reptiles (Murray and Fowler, 1991).

All five species of Kiwi are endangered and have decreasing populations (Holzapfel et al., 2008; International Union for Conservation of Nature, 2012). The North Island Brown Kiwi (NIBK, *Apteryx mantelli*) is the most abundant and widespread species (Holzapfel et al., 2008) and it is the focus of my study. The NIBK population is estimated to be circa. 25,000 birds (Holzapfel et al., 2008) with an isolated and fragmented distribution (see figure 1.1). Ten year estimates of population numbers predicts a serious decline, and with some populations declining at a rate of almost six per cent per year, NIBK numbers are halved every decade (Perrins, 2003; Holzapfel et al., 2008) (see section 1.1.3 Kiwi Conservation).

1.2 Kiwi breeding

NIBK are predominantly monogamous but records of polyandry and polygynandry exist (McLennan, 1988; Potter, 1989; Taborsky and Taborsky, 1999; Ziesemann, 2011). Breeding occurs in austral winter, with peak laying from mid-winter to mid-spring (Marchant and Higgins, 1990). Environmental conditions at this time are usually considered negative to egg survival, with high average humidity and low temperatures (see chapter two, section 2.2.2). NIBK nest in excavated cavities or modified hollow logs; leaves and twigs are often taken into the nesting chamber and sometimes used to cover the entrance (McLennan, 1988; Folch, 1992). Nest reuse is common in some populations; 68 per cent of clutches observed on Ponui Island were laid in burrows that the male had used in a previous breeding season (Ziesemann et al., 2011). Clutches

typically consist of one or two eggs, with one or two clutches per season (McLennan, 1988). On average, the first and second eggs are laid 25-30 days apart with some records indicating there could be up to 76 days between eggs (McLennan, 1988; Folch, 1992).

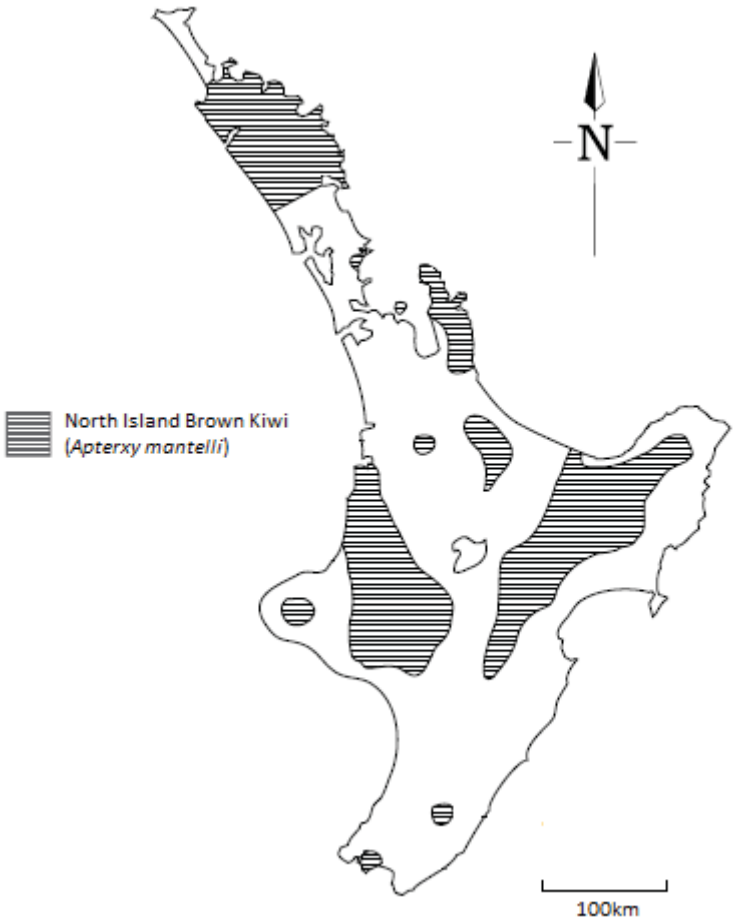

Figure 1.1: Map of the North Island of New Zealand showing the distribution of North Island Brown Kiwi populations (shaded areas). Please note that some offshore islands with NIBK populations present are missing. Modified from Department of Conservation (nd).

1.2.1 The Kiwi egg

The general structure of the NIBK egg is like that of most birds in that it consists of a shell, albumen and yolk, yet the NIBK egg is extreme in many ways. The egg of the NIBK is exceptionally large (Calder, 1979); the average egg is 128 (standard deviation (SD) ±10; n = 14) x 78mm (SD = ±8, n = 14) and weighs 434 grams (SD= ±20, n = 10) (Marchant and Higgins, 1990; Folch, 1992). The egg can weigh up to 20 per cent of the female's body weight (see figure 1.2) and is four times larger than expected from a bird the size of the NIBK (Calder, 1979; Andrews et al., 1990). The yolk of the NIBK egg is one of the

largest yolks of any known bird, making up 65 per cent of the total egg weight. At 280 grams on average, it is the same size as the yolk produced by a 35 kilogram emu (Calder et al., 1978 ; Andrews et al., 1990).

NIBK eggs have a very thin shell; this may reduce the weight the female has to carry while she is pregnant (Calder, 1979). However, a thin shell increases the risk of mechanical damage, cracking and hair line fractures which can impact embryo health and development (Silyn-Roberts, 1983; Stadelman, 1995). A thin shell also decreases the length of the pores and shorter pores have been shown to lead to increased water loss and microbial penetration (Board and Tranter, 1995). However, NIBK eggshells have sparsely distributed, funnel-shaped pores that range from 10 -100 µm in diameter (Silyn-Roberts, 1983). This sparse distribution of the pores may counter the negative effects of a thin shell and short pores, as they limit water loss and microbial penetration.

Figure 1.2: X-ray of a pregnant North Island Brown Kiwi showing size of egg in relation to female's body. Taken from (Kiwis For Kiwis, nd).

The levels of egg white proteins in NIBK eggs have been shown to be markedly different to the levels found in other birds (see table 1.1) (Osuga and Feeny, 1968; Prager and Wilson, 1974). Out of 14 bird species studied, the amount of ovoinhibitor and lysozyme in the egg contents were significantly higher in NIBK (Osuga and Feeny, 1968; Prager and Wilson, 1974). These two proteins have strong antimicrobial functions; ovoinhibitor

impacts microbial growth by inhibiting trypsin and chymotrypsin (proteases that are secreted by invading pathogens to aid in digestion) (Bourin et al., 2011) and lysozyme attacks the peptidoglycans that make up the cell wall of bacteria (Imoto, 2009) (see chapter two, section 2.4.2). NIBK lysozyme was found to be five to ten times more active than chicken (*Gallus gallus domesticus*) lysozyme in lysing (breaking down) bacterial cell walls. However, as enzymatic action progressed NIBK lysozyme appeared to cease lytic action whilst chicken lysozyme continued until bacterial cells experienced complete lysis (Osuga and Feeny, 1968).

Table 1.1: A comparison of the composition of egg white proteins from several avian species, modified from Osuga & Feeny, 1968. Chicken egg white was used as a control and standard, percentages are averaged from: 10 eggs from 2 cassowary; 10 eggs from 3 emu; 30 eggs from 6 rhea; 15 eggs from 3 ostrich; 50 eggs from 4 tinamou, and 1 egg from 1 kiwi. For more information see Osuga & Feeny (1968).

Avian Species	Dry weight of egg white (mg/ml)	Lysozyme (%)	Ovoinhibitor (%)
North Island Brown Kiwi (*Apteryx mantelli*)	123	5	2.3
Chicken (*Gallus gallus*)	121	3.4	1.5
Turkey (*Maleagris gallopavo*)	120	3.1	0.5
Cassowary (*Casuarius aruensis*)	114	0.5	0.6
Emu (*Dromiceius n. hollandiae*)	101	0.05	0.5
Ostrich (*Struthio camelus*)	113	0.45	0.6
Rhea (*Rhea americana*)	110	2	0.5
Tinamou (*Eudromia elegans*)	118	0.83	0.5

Most information on avian egg structure, function and composition is based on the domestic chicken. As can be seen by the above information; the NIBK egg is markedly

different in several characteristics, demonstrating the need for more research to fully understand this area.

1.2.2 North Island Brown Kiwi incubation

NIBK exhibit sex role reversal, the males incubate the egg after it is laid (Buller, 1888). The routine of an individual NIBK pair during early incubation varies. In most cases observed (39 of 47) the female will remain in the burrow for less than 24 hours after laying her first egg, and even less time when she returns to lay the second (Reid and Williams, 1975; McLennan, 1988; Potter, 1989; Marchant and Higgins, 1990; Taborsky and Taborsky, 1999).

The onset of full incubation differs between males; males can delay incubation anywhere from three to 15 days (n= 14) (McLennan, 1988; Potter, 1989; Colbourne, 2002). However, the first laid egg can be left up to 30 days before the male begins incubation (n= 1) (McLennan, 1988). One male was also observed intermittently incubating for 20-30 days before beginning full incubation (McLennan, 1988). Larger sample sizes are needed to draw solid conclusions about the incubation patterns during early incubation, but data thus far indicates that NIBK eggs are not incubated immediately after laying.

The incubation period of the NIBK is one of the longest known amongst birds, lasting up to 80 days (Calder et al., 1978 ; Folch, 1992). The males retain their nocturnal habits during incubation, leaving the nest every night to feed (McLennan, 1988). The egg is left un-incubated for four - five hours a night while the male forages (Marchant and Higgins, 1990).

NIBK eggs are large, relative to the male's body and the size of his brood patch (area on the belly that loses feathers during incubation). Thus, in a typical two egg clutch only a small area of an egg is actually in contact with the brood patch and receiving direct heating (Colbourne, 2002). The temperatures of the egg during incubation reflect this; as during incubation, the mean temperature on the top of the egg (in contact with the male's brood patch) can be as high as 37^{o}C, while the bottom of the egg can be up to ten degrees lower. Some males take up to ten days to develop a brood patch after the

first egg is laid; during this time, the temperature on the top of the egg is lower than when the male's brood patch is developed. When the male leaves the nest at night the core temperature of the egg can drop as low as 13^{o}C (Colbourne, 2002).

There is a significant difference between the laying interval of the eggs in a clutch (25-30 days) and the hatching interval (5-13 days) (Folch, 1992). Incubation patterns such as partial or intermittent incubation of the first laid egg, and the lower incubation temperature at the start of incubation may contribute to the longer incubation period of the first laid egg observed in NIBK (Colbourne, 2002).

1.3 North Island Brown Kiwi conservation

The NIBK is considered nationally vulnerable due to a combination of population decline, recruitment failure, habitat fragmentation and the fact that they are dependent on intensive conservation (Miskelly et al., 2008; Townsend et al., 2008). The Kiwi Recovery Programme was launched in 1991 with the aim of using coordinated, conservation management action to prevent the extinction of Kiwi (Butler and McLennan, 1991). Both community groups and the Department of Conservation (DOC) protect over 120,000 hectares of Kiwi habitat, and this number keeps growing (Holzapfel et al., 2008). Yet overall NIBK populations are still declining. Whilst adult NIBK have low annual mortality (5-16%), chick mortality in areas without predator control is high. Only five per cent of chicks reach adulthood, with stoats and feral cats being significant predators (McLennan et al., 1996; Basse et al., 1999).

The Kiwi Recovery Plan aims to restore and enhance the abundance, distribution and genetic diveristy of all Kiwi spp., mainly by reducing exposure to predators (Robertson and Colbourne, 2003; Holzapfel et al., 2008). Currently Kiwi spp. conservation is focused on two management strategies: intensive predator control at particular sites and Operation Nest Egg (ONE), a captive hatching and rearing scheme for wild eggs. ONE is a process where eggs are taken from monitored wild populations and hatched in captive breeding facilities. Chicks are raised to a predator-safe size (≥1000 g) then released in the wild (Colbourne et al., 2005).

Since 1995 ONE has been used every breeding season and has been successful in increasing NIBK numbers. Hatching success of a ONE egg is above 90 per cent and compared with wild hatched birds, a ONE bird has a 60 per cent increase in the chance of surviving to maturity (Bassett, 2012). However, ONE is also expensive; at one facility, the cost to fund this program for one year is estimated to be over $400,000 and with only 1000 chicks being hatched since 1995 this is as much as $7,600 per egg (Kiwi Encounter, 2013). Also being so heavily conservation dependent is contributing to the NIBK vulnerable status (Miskelly et al., 2008). Therefore, ONE is not a permanent conservation strategy; the end goal is to phase out intensive management when predator control is deemed sufficient to protect the majority of chicks (Robertson, 2003; Holzapfel et al., 2008).

Even with intensive conservation NIBK recovery in the wild is slow, and overall populations are still declining. An important contributing factor is egg mortality. NIBK egg mortality in the wild is extremely high, on average less than 36 per cent of eggs hatch (McLennan, 1988; Potter, 1989; McLennan et al., 1996; Ziesemann et al., 2011) (see section 1.4 below). ONE is protecting the egg stage as well as the chick stage. Successful survival of eggs could improve NIBK population recovery greatly, but before this can happen, information is needed about what is causing the average 64 per cent hatching failure in wild eggs.

1.4 Threats to wild North Island Brown Kiwi hatching success

Infertility has been considered the main cause of hatching failure in several wild bird species (Potti and Merino, 1996; Cordero et al., 1999; Cabezas-Diaz and Virgos, 2007). For example, infertility rates were as high as 70 per cent in wild Red-Legged Partridge (*Alectoris rufa)* (Cabezas-Diaz and Virgos, 2007). A study of a NIBK population in Northland revealed the NIBK had an infertility rate of around 15 per cent (Potter, 1989); this is significantly less than some other endangered New Zealand birds such as the Kakapo (*Strigops habroptila*) (Jamieson and Ryan, 2000) (see table 1.2). The high hatching rate of NIBK eggs from most populations in ONE also shows that infertility is unlikely to be a major factor explaining the high hatching failure of wild eggs.

Table 1.2: Comparison of egg infertility for several endangered bird species in New Zealand. Modified from Jamieson & Ryan, 2000.

Species	Total no. of eggs laid	Infertile eggs (% of total eggs laid)	Failed eggs (% of total eggs laid)	Infertile eggs (% of failed eggs)
North Island Brown Kiwi (*Apteryx mantelli*)	26	15	77	20
Kakapo (*Strigops habroptila*)	45	42	66	64
Yellow eyed penguin (*Megadyptes antipodes)*	104	14	15	89
Takahe (*Porphyrio hochstetteri*)	61	19	31	60

Few studies exist on the fate of NIBK eggs in the wild (McLennan, 1988; Potter, 1989; McLennan et al., 1996; Ziesemann et al., 2011). Hatching success varies across the populations, but on average is less than 36 per cent per year (see table 1.3). Predation accounts for less than two per cent of known egg failures. Visible microbial infection was noted in at least 19 per cent of eggs examined. The remaining 43 per cent of eggs were abandoned, broken, or disappeared.

The focus of these studies (McLennan, 1988; Potter, 1989; McLennan et al., 1996; Ziesemann et al., 2011) was not to record the fate of the eggs; therefore impacts of both predation and microbial infection could be higher than recorded. Four factors make NIBK eggs extremely resistant to predators: nest site selection and camouflage; parental attentiveness; parental defence against would-be egg predators; and the large size and weight of the NIBK eggs to the size of available predators (McLennan et al., 1996). Therefore, the low level of predation is likely to be accurate.

In all studies, microbial contamination was recorded as being present or absent based on visual observation. The eggs that were abandoned or broken were not analysed for microbial contamination and no analysis was conducted on the identification of microbial species. It is not known if the microbes seen were the cause of embryo death or secondary invaders. Therefore, microbial presence could be far higher than recorded

and, as suggested by McLennan et al. (1996), if this is the case then microbes are the main cause of NIBK egg mortality.

Table 1.3: The fate of North Island Brown Kiwi eggs from three separate populations (McLennan et al., (1996) has information on two populations). Hatched = eggs hatched, Eaten = known predation, Microbial presence = visual observation of microbial presence in egg contents, Abandoned = male deserted egg, other/unknown = egg disappeared; male was disturbed due to the sampling process. Modified from McLennan et al 1996, Ziesemann et al 2011 and Ziesemann pers. comm..

Fate of Eggs	(McLennan et al., 1996)	(Ziesemann et al., 2011)	Total (%)
Hatched	26	17	36
Eaten	2	0	2
Microbial presence	13	9*	19
Abandoned	24	0	20
Other/unknown	18	9	23
Total eggs	83	35	100

*Note: the aim of Ziesemann study was not an investigation into the causes of egg mortality therefore direct recording of causes of egg failure was not done. No eggs were abandoned or eaten during this study, however, most eggs found dead had microbial infection (Ziesemann per. comm.). Therefore I used a conservative estimate of microbial presence in nine eggs (50% of failed eggs).

1.5 Thesis outline and objectives

The presence of microbes in a large number of un-hatched NIBK eggs has been noticed (see table 1.3). However, what these microbes are, and if they can impact the hatching of NIBK is yet to be investigated.

Several factors of NIBK eggs and their incubation make them susceptible to microbial infection. It has been shown that the longer an egg is left exposed and un-incubated, the

greater the chance of content contamination (Cook et al., 2003). The incubation period of the NIBK can be up to 80 days; which is one of the longest incubation periods of any bird (Calder et al., 1978 ; Folch, 1992). NIBK often lay two eggs in a clutch and the eggs can be laid up to 15 days apart; the males do not start full incubation until the last egg is laid (McLennan, 1988). Like most birds, NIBK incubation is intermittent; the male will leave the egg un-incubated during the night to forage (Colbourne, 2002). Periods of warming and cooling, like those the egg experiences when the male leaves to feed, creates a pressure change which can suck bacteria into the egg contents (Bruce and Drysdale, 1994; Berrang et al., 1999).

The shell is the primary layer of defence for avian eggs (Solomon et al., 1994), yet it is not impenetrable to bacteria. The shell of the NIBK is large, thin and has many pores (see section 1.2.1), each of these factors has been shown to increase bacterial penetration into avian eggs (see chapter two, section 2.4.2).

The nesting environment of birds is a reservoir of microbes and contamination of the egg contents is positively correlated with the microbial load of the nesting environment (Bruce and Drysdale, 1994). In our study population (see 1.6.2), perhaps due to the high density of birds, nests are used as roosts outside the breeding season (Ziesemann et al., 2011). NIBK often defecates inside roosts as well as nests (I. Castro pers. comm.). Because of these behaviours NIBK eggs are exposed to faecal material and while the bacteria present in the faeces of NIBK have not yet been investigated, faecal contamination is a significant factor in the infection of eggs by bacteria in domestic chickens (Graves and MacLaury, 1962; Drysdale, 1985). A large number of bacteria are also present in the soil, air and water and thus would be in contact with the NIBK egg during incubation. In addition, NIBK have incubation behaviours which may contribute to bacteria contamination; for example they tend to bury their eggs in soil and litter in the nest (McLennan, 1988; Colbourne, 2002).

The nesting environment also can increase bacterial penetration. For example, the infection of the egg contents is also influenced by the presence of water on the eggshell, as moisture supports microbial growth and assists bacterial penetration (Board and Halls, 1973; Board et al., 1979; Cook et al., 2003, 2005b). Under cool, humid conditions

infection of Pearly-Eyed Thrasher eggs increased (Cook et al., 2005a; Cook et al., 2005b). NIBK eggs are laid in burrows during winter, where rainfall and humidity is high; this high water content could facilitate microbial penetration in the NIBK eggs.

Information on NIBK eggs is poor and apart from the high visual presence of microbes in un-hatched NIBK eggs, and the factors above that make them vulnerable, no information exists on the threat microbes pose to NIBK hatching success. Information about the causes of hatching failure is needed so that conservation efforts can be optimised and the NIBK population size can increase.

In order to know if specific microbes can cause hatching failure in NIBK eggs or embryos, we would ultimately need to infect NIBK eggs with potential pathogens and monitor the resulting hatching success. However, this method raises several issues; one such concern is the ethical issues involved with infecting embryos with potential pathogens (New Zealand Government, 2014, January 1). Animal ethics approval is a major factor when conducting any research on an animals and there are strict rules around research on native New Zealand birds (New Zealand Government, 2014, January 1). In addition, placing potential pathogens on the shells of fertile NIBK eggs to research hatching success is unlikely to be logistically feasible. NIBK are endangered, every egg and every chick is important to the population. Thus, there are restrictions on the type of work that can be carried out. As well as this, difficulties arise with limited sample size, variable egg condition, variable age when eggs are obtained and unknown time of egg death. All these factors mean that a creative solution is needed to investigate microbial impacts on NIBK eggs.

1.5.1 Project objectives

Given the restrictions raised in the previous section, the overall objective of this project was to investigate if microbes could contribute to NIBK egg mortality. Originally, the project aimed to include studies dealing with: 1. the microbes present inside and outside the egg; 2. the development of a non-destructive technique to examine the ability of potential pathogens found in study one to penetrate the eggshell; and 3. the use of the method in 2 to examine the penetrability of NIBK eggshells under a variety of conditions. However, I was unable to complete the development of a method to

examine microbial penetration through shells because of the high presence of microbes in un-hatched NIBK eggs. Therefore, the thesis concentrates on the first two studies above, and uses the results to offer suggestions for future work and conservation efforts for NIBK. The overall hypothesis of this project is that microbes are contributing to NIBK hatching failure.

1.5.2 Outlines and aims of chapters

Chapter two: Literature review; the avian egg and the impact of microorganisms

This chapter provides background knowledge about avian eggs, potential causes of mortality and microbial impacts. Most of the work done in this field has been carried out on domestic chicken eggs, but where wild bird studies have occurred this is noted. This knowledge is necessary to understand the defences of the egg, how microbes can penetrate these defences, and the impacts when they do.

Chapter three: Identification of bacteria on the shells of live, wild North Island Brown Kiwi eggs and potential impacts on hatching success

Although the presence of microbes has been noted inside un-hatched Kiwi eggs (by visual examination during candling), the presence on living eggs is unknown. The first aim of this study was then to determine what bacteria are present on the shells of living NIBK eggs and investigate if they have the potential to impact hatching success (by comparing to studies in chickens and other birds' eggs). The eggs used in this study were alive at the time of sampling and were actively being incubated by wild birds. The second aim was to use information about the bacterial genera found to support and guide the direction of future work on NIBK egg hatching success.

Chapter four: Identification of fungi on the shells of live, wild North Island Brown Kiwi eggs and potential impacts on hatching success

Fungi also pose a threat to the avian egg and few studies to date have identified what fungi are present on wild bird eggs. The primary aim of this chapter was

then to determine what fungi are present on living wild NIBK eggs and to investigate if they have the potential to impact hatching success (by comparing to studies in chickens and other birds' eggs). The second aim of this study was to use the knowledge gained to make suggestions towards Kiwi egg conservation.

Chapter five: Identification of bacteria in the contents of un-hatched, wild North Island Brown Kiwi eggs and potential impacts on hatching success

The presence of bacteria inside un-hatched NIBK eggs has been noted in previous studies (McLennan, 1988; Potter, 1989; McLennan et al., 1996; Ziesemann et al., 2011); however identification of the bacteria and if they contributed to the death of the embryo is unknown. The first aim of this study was to identify the bacteria present inside un-hatched wild NIBK eggs and also investigate if the bacteria found have the potential to impact hatching success (by comparing to studies in chickens and other birds' eggs). The third aim of this chapter was to support and guide the direction of future work on NIBK egg hatching success.

As sampling of the contents of eggs would lead to the death of the embryo, for this study infertile or early embryo death ONE eggs were used.

Chapter six: Initial steps in the development of a non-destructive method to test the penetration of bacteria through the North Island Brown Kiwi eggshell

The avian eggshell has layers of defences to restrict the entry of microorganisms (see chapter two, section 2.4); but the main barrier is the shell itself. This chapter describes a method designed to test if the bacteria found on the shell of the wild NIBK eggs can grow through it and thus into the egg contents. This chapter's structure does not follow that of a manuscript for publication as the previous three, but rather it has been written as the series of steps followed to develop the method.

Chapter seven: Discussion and conclusions

The aim of all of the chapters above was to gain insight into the potential microbes have to impact on the hatching success in NIBK. Separately they aim to highlight the microbes that have the biggest potential. This chapter combines the results of all four studies to examine the overall hypothesis of this project, and to provide overall direction for future research into causes of hatching failure in NIBK.

1.6 General methods

Chapters three, four and five of this thesis have been written as individual manuscripts ready for publication and thus some repetition exists between the chapters (i.e. scientific names are given in each chapter the first time a species is mentioned). Other information is shared between chapters three, four, five and six therefore to avoid excessive repetition I have removed the information below from each of the chapters and written it instead in this section.

1.6.1 Laboratory site

All laboratory work was carried out in the mEpiLab, Hopkirk Institute, Massey University, Palmerston North (Massey University: 40^{o}38’ S, 175^{o}60’ E; see figure 1.3). Samples were analysed in a laboratory with a Physical Containment level 2 (PC2). As such, this laboratory complies with the operational and structural requirements of the Ministry of Agriculture and Forestry (MAF) Biosecurity and Environmental Risk Management Authority (ERMA) under the ‘Facilities for Microorganisms and Cell Cultures: 2007a’.

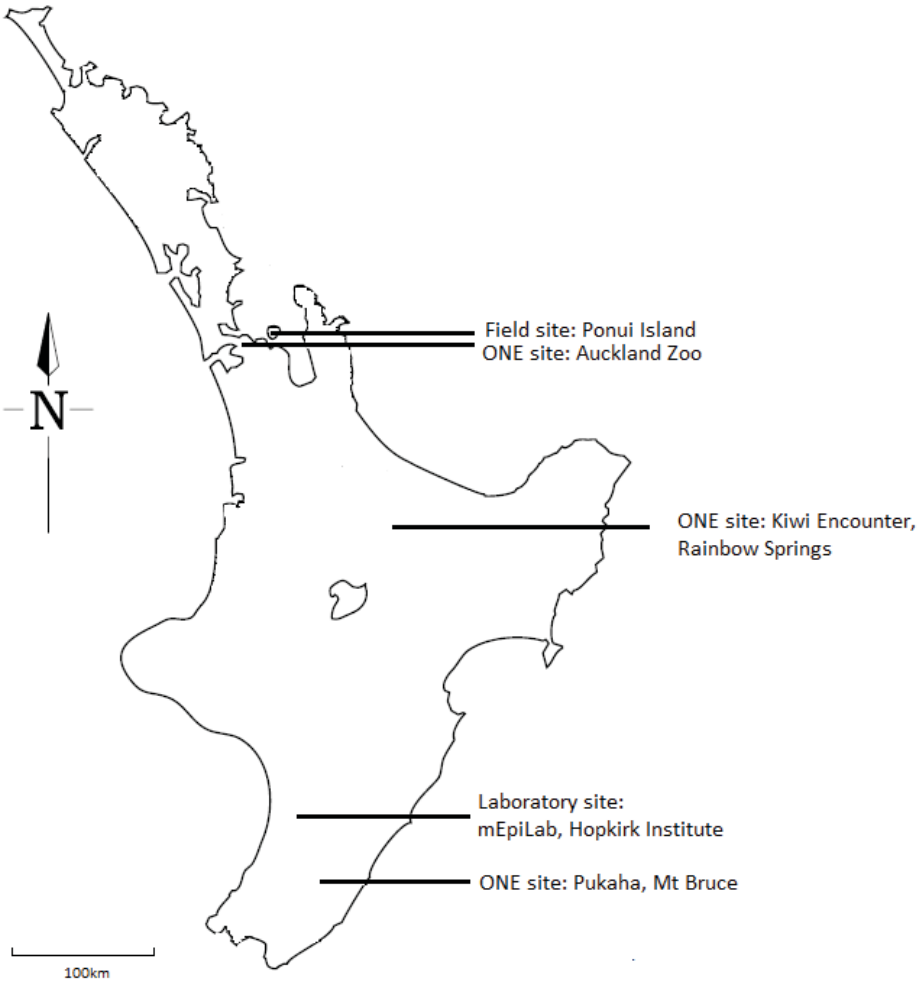

Figure 1.3: Map of the North Island of New Zealand showing field site, laboratory site and the three Operation Nest Egg sites used in this study. Modified from Department of Conservation (nd).

1.6.2 North Island Brown Kiwi study population and study site

Wild eggs were swabbed on Ponui Island (36°55' S, 175°11' E; see figure 1.3) during the 2011 breeding season. The Ponui Island population is a high density population, with estimates of 100 NIBK per km^2 (Cunningham et al., 2007). Introduced NIBK predators include feral Cats (*Felis catus*) and farm Dogs (*Canis lupus familiaris*). Both nesting success (47%) and fledging success (89%) in this population are high compared with other NIBK populations (Ziesemann et al., 2011). On Ponui, male NIBK provided sole parental care of the egg and the incubation period was typically between 74 and 84 days. The breeding season of the population has been shown to be variable across years, but typically occurs in the austral winter, with the peak laying period from mid-winter to mid-spring (June-October) (Ziesemann et al., 2011). High rainfall and low temperatures are typical of this time of year, on average temperatures are under 12°C

and over 120 mm of rain fall (this is based on the weather station in Auckland, the nearest large city to Ponui island) (National Institute of Water and Atmospheric Research, 2012).

One egg clutches were dominant on the island (69%) during the period of this study, however two and even three egg clutches have been recorded in this population (I. Castro pers. comm.). The frequency of nest reuse is high (68%) and nests are used as roosting sites outside the breeding season (Ziesemann, 2011). This reuse of nests can often lead to faecal build up within the burrow. However, observations of nest cleaning before the egg is laid have been made (I. Castro pers. comm.).

1.6.3 Sources of un-hatched North Island Brown Kiwi eggs

NIBK eggs were obtained from the three main ONE facilities in the North Island; Pukaha Mt Bruce (40°43' S, 175°37' E), Auckland Zoo (36°51' S, 174°43' E) and Kiwi Encounter at Rainbow Springs (38°06' S, 176°13' E; see table 1.4 and figure 1.3). These facilities obtain eggs from reserves across the North Island, therefore the eggs obtained in this study are representative sample of the New Zealand mainland population.

Before the start of the 2012 breeding season facilities were requested to set aside any infertile or early embryo death eggs for my project. Infertile or early embryo death eggs were used in this study for three main reasons: 1. it is ONE procedure to candle eggs to determine the age and health of the embryo and any egg with an embryo, even if microbial growth is present, is surface sterilised and incubated until death can be confirmed. Therefore by using infertile or early embryo death ONE eggs in this study, it meant that the eggs were not sterilised or incubated; 2. Eggs with advanced growth have small volumes of yolk and albumen, which are often difficult to extract without contamination {Cook, 2003 #382}; and 3. Dead eggs have been used when examining the incidence of trans-shell infection in other birds, it is a conservative test of microbial infection as it is unlikely that dead eggs have a greater defence against infection that living eggs {Cook, 2003 #382}. All eggs were collected and transported to the laboratory site within 48 hours of arriving at the ONE facility (see section 1.6.2; figure 1.4).

Table 1.4: A list of the North Island Brown Kiwi eggs used in this study. Operation Nest Egg (ONE) facility is the location where the egg was collected from. Note N/A = not applicable as egg 9 was not from a ONE facility but a wild population. Field site/egg origin is the location where the egg was laid. Date of collection is the date when the egg was taken from the nest in the wild. Egg age is the age when the egg was taken from the nest. Unknown = details not known.

Egg #	ONE facility	Field site/egg origin	Date of collection	Egg age (days)
1	Pukaha, Mt Bruce	Mt Bruce, wild	4-Aug	60
2	Kiwi Encounter, Rainbow Springs	Kuaotunu Peninsula, wild	23-Aug	Unknown
3	Kiwi Encounter, Rainbow Springs	Maungataniwha, wild	2-Sep	Unknown
4	Pukaha, Mt Bruce	Wairarapa, wild	17-Aug	41
5	Kiwi Encounter, Rainbow Springs	Maungataniwha, wild	24-Sep	Unknown
6	Kiwi Encounter, Rainbow Springs	Maungataniwha, wild	30-Sep	58
7	Kiwi Encounter, Rainbow Springs	Maungataniwha, wild	30-Sep	58
8	Kiwi Encounter, Rainbow Springs	unknown	10-Oct	55
9	Auckland Zoo	Auckland Zoo, captive	12-Oct	1
10	N/A	Ponui Island, wild	10-Oct	Unknown
11	Pukaha, Mt Bruce	Mt Bruce, wild	30-Nov	65

Figure 1.4: Example of packing used in this study to transport a North Island Brown Kiwi egg to the laboratory from an Operation Nest Egg facility.

1.6.4 Methods used for literature research

Because the direct impact on NIBK hatching success by each of the microbes in this study could not be determined (see section 1.5), their potential impact was instead determined through extensive literature research. The information below relates to the literature searches on bacteria in chapters three and five, the method for fungi in chapter three is unique to that chapter so is provided there (see chapter four, section 4.2.2.1).

Discover, Scopus, the Web of Science, the Web of knowledge, the National Centre for Biotechnology Information and Google Scholar, as well as the library catalogues from Massey University, the University of Newcastle, the University of Technology Sydney, the University of Wollongong, the University of Ballarat, the University of Melbourne, La Trobe University, Victoria University of Wellington, Deakin University, Edith Cowan University, Murdoch University, Queensland University of Technology and Griffith University were all used to find information regarding the microbes isolated in each of the studies.

The potential microbes have to cause both positive and negative impacts on NIBK hatching success was researched for each of the genera identified. Because there has been no work done on the impact of certain bacteria on the NIBK embryo no bacteria can be ruled out as pathogen. Therefore, I considered:

1. if the bacterial genus is pathogenic or beneficial to other avian embryos, as it could impact the developing NIBK embryo in a similar way.

2. if the bacterial genus was found to be pathogenic in birds in general, as well as in other animals and humans. As the genera could also be a potential pathogen of NIBK embryos.

3. if the bacterial genus was known to produce antibiotics or bacteriocins, substances that impacts the growth of other bacteria. The production of such substances has proven benefits for birds and avian embryos (McCabe, 1965, 1967) and could benefit the NIBK embryo.

4. if the bacterial genus was resistant to lysozyme. Lysozyme is present in extremely high concentrations in NIBK eggs (Osuga and Feeny, 1968; Prager and Wilson, 1974). The ability of a bacterium to resist the action of lysozyme increases its chances of surviving in the egg contents (Salton, 1957; Imoto, 2009) and increase its potential to impact the embryo as lysozyme breaks down the cell wall of bacteria (Shawkey et al., 2008).

To address the points above, four main areas were researched for each of the bacteria found: 1. known pathogenicity, in general, in avian adults and chicks and in eggs and embryos; 2. lysozyme resistance; 3. known benefits, in general, in avian adults and chicks and in eggs and embryos; and 4. antibiotic or bacteriocin production.

As a large number of studies were carried out on the domestic chicken as early as the 1920's, and work has been published in wild birds as late as this year, I did not restrict the search by dates. Instead I used a series of key words (see table 1.5) together with each of the bacteria genera for the four main areas looked at. After downloading the manuscripts I also retrieved any references from them that dealt with the subject.

Some of the bacteria in this study were isolated to species level; however, the literature research was only done to genera level because little information regarding the species-specific impacts could be found. There is a bias in the literature on bacterial impacts on avian egg hatching towards human pathogens such as *Staphylococcus aureus* (Bruce and Drysdale, 1994). As none of the species isolated in this study are serious human pathogens this may be a reason why species-specific information was lacking.

Table 1.5: List of the key words used in the literature research for the pathogenic and beneficial impacts of the bacteria isolated in this project. Searches were done by using 'general' plus 'avian' or 'egg' keywords on the table for each bacterial genera found in this study * = search for all words that start with the letters given. $ = search for word, and word plus plural. ^= also used the name of the antibiotic or bacteriocin as a key word.

Pathogenic key words			
Lysozyme resistance	General	Avian	Egg
Lysozyme, resist*, sensitive,	Pathog*, negative, harm*, impact, infect*, ill*, disease	Avian, bird*, poultry, chick*, aves	egg$, embryo*, shell$

Beneficial key words			
Antibiotic / bacteriocin production	General	Avian	Egg
Antibiotic*, bacteriocin	Benefi*, positive, good, prevent, protect*, mutuali*, ^	avian, bird*, poultry, chick*, aves, ^	egg$, embryo*, shell$, ^

1.6.5 Ethical and intellectual property issues

All work in this thesis was carried out under Department of Conservation permits 34821-RES and AK 28039 FAU. All stages of egg handling followed the Department of Conservation Kiwi Best Practice Manual (Robertson, 2003) and all egg handlers had taken the Kiwi Egg Candling course.

All photos and figures were created by me unless stated on the image itself.

Chapter Two: Literature review; the avian egg and the impact of microorganisms

2.1 Review of the avian egg

2.1.1 Avian incubation

In birds, incubation is the transfer of heat between parent and embryo (Turner, 2002). All birds, apart from the Megapodes (Megapodiidae), use some form of contact incubation; the heat is transferred by direct contact of the brood patch against the surface of the egg (Deeming, 2002a; Turner, 2002). The purpose of incubation is to provide a warm, steady egg temperature, which is necessary for successful development of the embryo. Embryonic development is initiated by incubation temperatures; therefore, through incubation birds have control over the development of their offspring (Deeming and Ferguson, 1991; Stoleson and Beissinger, 1995; Cook et al., 2003). Birds influence the development of the eggs by determining when to initiate incubation (Stoleson and Beissinger, 1995). As birds cannot lay more than one egg daily, this control becomes important in regards to the hatching intervals between young (Perrins, 1996). There are two main patterns birds follow when incubating; early onset or delayed onset of incubation.

Delayed onset of incubation is when birds withhold incubation from initial laid eggs until the clutch is complete (Deeming, 2002b). This pattern leads to the clutch hatching synchronously, with chicks of the same size and stage of development (Wang and Bessinger, 2009). This synchronous hatching reduces the monopolisation of resources by older siblings, minimises sibling rivalry and reduces nest predation as offspring leave the nest together (Clark and Wilson, 1981; Clark et al., 2010). Synchronous hatching is believed to be the primitive incubation strategy in birds and asynchronous hatching a derived, adaptive behaviour that benefits the parents and the first laid offspring (Clark and Wilson, 1981; Hébert, 2002).

Early onset of incubation occurs when incubation is initiated before the whole clutch is complete (Bollinger et al., 1990). Early incubation results in the clutch hatching at different times, as the first laid egg begins embryonic development earlier than the later laid eggs (Deeming, 2002b). This leads to hatchlings of different sizes and often directly contributes to the death of the younger, smaller chicks (Mock et al., 1990). The ‘paradox

of hatching asynchrony', is the seemingly maladaptive trait of having the ability to control birthing intervals, yet creating a clutch in a way that jeopardises the later-hatched young. (Stoleson and Beissinger, 1995).

The 'brood reduction hypothesis' was proposed to explain hatching asynchrony (Stoleson and Beissinger, 1995). It states that parents initiate incubation before clutch completion to produce chicks of different sizes, as this increases food competition between siblings (Lack, 1947, 1954). This means that if food resources become limited, the smallest chick is likely to receive the least amount of food and thus the parents will lose the nestlings they have invested the least amount of time and energy in. Studies have since shown that selection pressures during the laying period, other than food scarcity, can be important factors for hatching asynchrony, such as predation and thermal stress (Amundsen and Stokland, 1988; Bollinger et al., 1990).

Un-incubated eggs experience reduced viability and the 'egg viability hypothesis' is another proposed hypothesis for asynchronous hatching (Arnold, 1993; Cook et al., 2005a). This hypothesis states that initiating incubation before clutch completion maximises hatchability of all eggs in the clutch as un-incubated eggs have been shown to experience decreased viability. The decreased viability was originally believed to be a consequence of incubation temperatures (Arnold, 1993), but incubation also decreases microbial contamination (Cook et al., 2003; Cook et al., 2005a). It has been proposed that early incubation is a trade-off between the benefits of an increased clutch size, and the costs to the viability of early laid eggs from delaying incubation (Cook et al., 2005b).

Technically all birds exhibit asynchronous hatching to some extent, as all eggs cannot hatch exactly at the same time (Clark and Wilson, 1981; Stoleson and Beissinger, 1995). As such the definition of the two forms is: if chicks in a clutch hatch less than 24 hours apart, incubation is defined as synchronous, and more than 24 hours as asynchronous (Stoleson and Beissinger, 1995). But the initiation of incubation is highly variable in birds and depends on the species, the clutch size and even the individual bird itself (Wang and Bessinger, 2009). Although early onset and late onset of incubation are the two main categories, this simple dichotomy ignores another incubation strategy: partial incubation. Partial incubation is when the egg is incubated, but not to the extent of full

incubation. For example, the female Great Tit (*Parus major*) during the laying period will only incubate for short periods and then will remain in the nest cavity without maintaining brood patch contact with the eggs. Once the clutch is complete she initiates full incubation, with direct brood patch contact for extended periods of time, effectively warming the eggs to incubation temperature (Haftorn, 1981). Partial incubation has been documented in most bird species that have had incubation patterns extensively monitored, including North Island Brown Kiwi (NIBK, *Apteryx mantelli*) (Wiebe et al., 1998; Persson and Goransson, 1999; Poussart et al., 2000; Colbourne, 2002; Beissinger et al., 2005).

2.1.2 General avian egg structure

Most work on the structure of the avian egg has been done on the domestic chicken (*Gallus gallus domesticus*); however the basic structure is similar across the class Aves (Romanoff and Romanoff, 1949). An avian egg has three main layers: the shell plus associated membranes, the albumen and the yolk (Romanoff and Romanoff, 1949; Burley and Vadehra, 1989) (see figure 2.1).

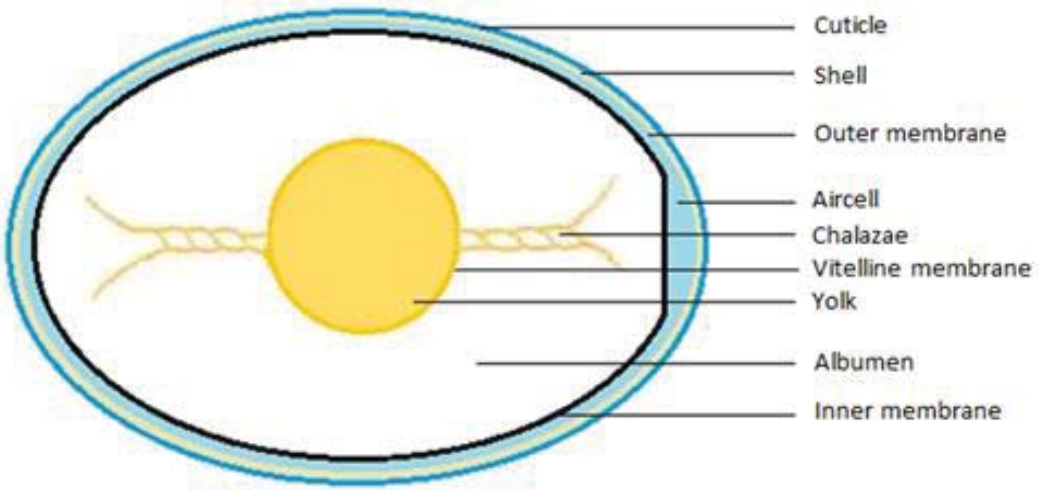

Figure 2.1: Generalised drawing of structures of an avian egg, modified from Burley and Vadehra (1989).

2.1.2.1 The eggshell

The eggshell is the outer most layer of the egg; it is the developing embryo's main protection against external threats (Solomon et al., 1994). The shell itself consists of

three distinct layers (see figure 2.2). A vertical crystal layer, which is composed of short thin crystals running in a vertical direction; the palisade layer or spongy layer which is a very dense, hard, calcified calcium carbonate layer which together with magnesium and collagen forms a spongy matrix; and finally mammillary knobs, which are in contact with the outer shell membrane and are densely packed so as to harden the shell (Okubo et al., 1997). This whole structure is covered and protected by the cuticle. This is a thin protein layer that regulates water infiltration and loss and limits microbial penetration (Solomon et al., 1994; Messens et al., 2005). However, the protection the cuticle provides is not always constant across the shell surface. The cuticle of chicken eggshells is on average 10µm thick, but is not always evenly distributed (Solomon et al., 1994; Okubo et al., 1997). The cuticle in chicken eggs has been shown to be easily removed by washing, or by rough, abrasive handling (Okubo et al., 1997). In some birds the structure of the shell leads to the eggshell being "self-cleaning"; microscopic cones over the surface of the shell make the shell hydrophobic and any water that falls on the surface of the shell forms droplets that take dirt and potentially bacteria off the shell (Gill, 2013). The shell is permeable via pores, which allows gas exchange to occur. In the chicken, these are funnel-shaped, small holes with a diameter of 10-30 µm (Okubo et al, 1997). The canals are situated between the palisade layers (see figure 2.2) and each egg contains around 10,000 pores (Okubo et al, 1997). The pores are the developing embryo's contact with the outside environment, thus they are permeable to air and moisture (Solomon et al., 1994) and under some conditions these pores allow bacterial penetration as far as the shell membranes (Stadelman, 1995).

There are two membranes associated with the shell; the outer membrane which is attached to the shell and the inner membrane which is next to the albumen (Burley and Vadehra, 1989). These membranes consist of a network of randomly orientated, branched keratin and mucin fibres, sometimes described as entangled threads (see figure 2.3) (Masshoff and Stolpmann, 1994; Okubo et al., 1997). In the chicken the thickness of the outer membrane is 50 µm, whilst the inner membrane is thinner at 15 µm (Okubo et al., 1997). These membranes act as a temporary filter to bacterial penetration (Haines and Moran, 1940; Board and Fuller, 1974; Burley and Vadehra, 1989).

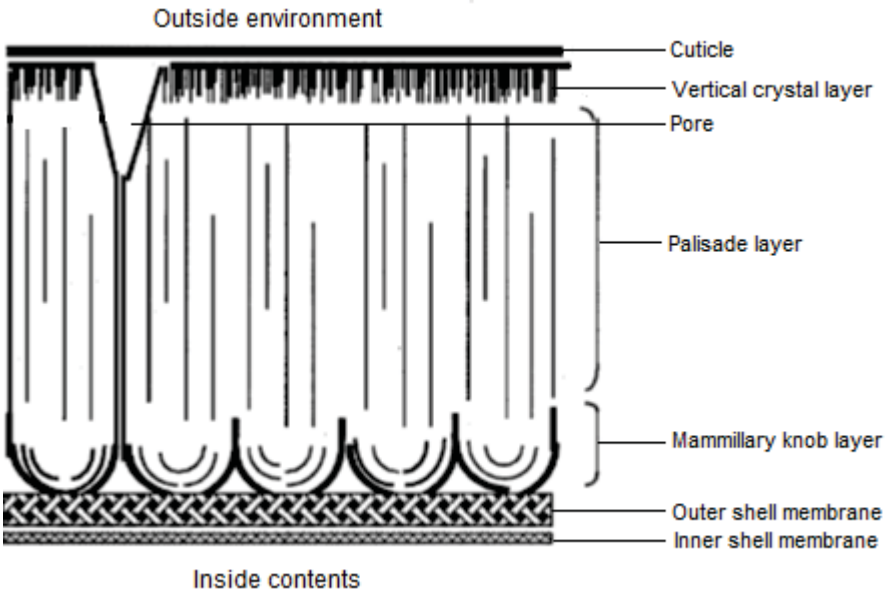

Figure 2.2: An illustrative representation of the structure of a chicken eggshell. Modified from Okuno, Akachi, & Hatta (1997).

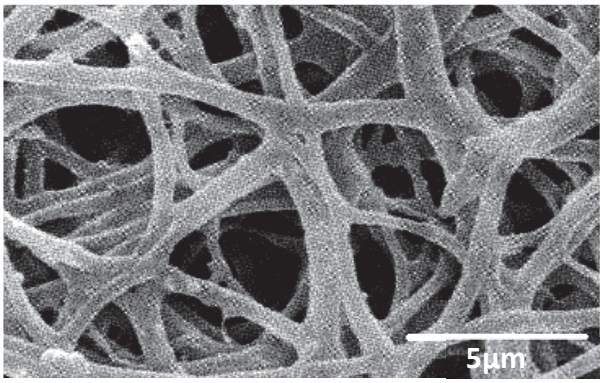

Figure 2.3: Scanning electron microscopic photograph of the chicken's shell membrane. Taken from Okuno, Akachi, & Hatta (1997).

2.1.2.2 The egg contents

The albumen (egg white) provides both physical and chemical protection to the yolk and developing embryo (Andrews et al., 1990). The albumen together with the chalaza, a thick rope like structure, keeps the yolk in the centre of the egg (see figure 2.1) (Okubo et al., 1997). The albumen is highly viscous, which provides physical protection to the embryo by absorbing shocks (Palmer and Guillette, 1991). Board and Fuller (1974) suggested that the high viscosity could also impede microbial growth.

Table 2.1: List of some of the antimicrobial proteins in avian egg albumen and their function. Modified from Kobayashi, Gutierrez, & Hatta, 1996.

Protein	Function
Ovotransferrin	Chelates Fe^{2+},Cu^{2+},Mg^{2+},Zn^{2+} (making them unavailable for bacterial growth)
Ovomucoid	Anti-protease: Inhibits trypsin (making it unavailable for bacterial digestion)
Lysozyme	Hydrolyses glycol moiety of peptidoglycans to kill Gram positive bacteria
Ovoinhibitor	Anti-protease: Inhibits trypsin, chymotrypsin, subtilisin, elastase (making them unavailable for bacterial growth and function)
Flavoprotein	Binds riboflavin (making it unavailable for bacterial growth)
Ovomacroglobulin	Inhibits trypsin and papain (making them unavailable for bacterial growth and function)
Avidin	Binds biotin (making it unavailable for bacterial growth)
Cystatin	Inhibits papain, ficin, bromelain (making them unavailable for bacterial growth)

The chemical defence of the albumen comes in the form of antimicrobial proteins (see table 2.1) (Burley and Vadehra, 1989). These proteins have various functions; some inhibit trypsin, a protease necessary for digestion by some microbes; others bind to necessary elements such as iron and zinc; others attack the bacteria themselves and break down the cell wall (Board and Fuller, 1974; Board and Tranter, 1995; Weinberg, 2009). Activity of these defensive proteins is optimal at incubation temperatures (34-37^{o}C) and their action increases with protein concentrations (Salton, 1957; Tranter and Board, 1984).

The yolk provides lipids, proteins and other substances to the growing embryo (Burley and Vadehra, 1989) and it is also the origin of several structures involved with embryo development such as the embryonic disc (Burley and Vadehra, 1989). The vitelline

membrane encircles the yolk and maintains its shape and position in the egg, keeping the yolk and the albumen separate (see figure 2.1) (Bellairs et al., 1963).

2.2 Avian embryo health

The embryonic development is predetermined and hereditary (Romanoff and Romanoff, 1972). However, the period of embryo growth is important, delicate and hazardous (Romanoff and Romanoff, 1972) and during the course of incubation the egg is vulnerable to external influences. Although the egg is equipped with a myriad of defences there are still multiple factors that can lead to reduced hatching success. For example, poor parental care, for any reason, can lead to negative consequences in the growth and survival of the offspring (Bearse and Miller, 1937; Romanoff and Romanoff, 1972; Deeming, 2002b). Environmental conditions such as changes in temperature and humidity can also affect growth and survival (Romanoff & Romanoff, 1972; Stoleson & Beissinger, 1999). As well as this, predation, disease, toxins and pathogens can all result in decreased growth or survival of avian embryos (Bruce and Drysdale, 1994; Fry, 1995).

2.2.1 The effect of parental care on embryo health

Parents can affect embryo health in several ways, both before and after laying. Poor parental preparation for egg formation can be a significant factor in lowered hatching success. The health and fertility of the parent birds directly affects the potential for the egg to hatch. Also as all the developing embryo's nutritional requirements are laid down by the mother during egg formation (Romanoff and Romanoff, 1972; Blackburn, 1999), any deficiency in the necessary compounds can seriously impact embryonic growth (Bearse and Miller, 1937; Romanoff and Romanoff, 1972). Therefore, parental health before laying the egg is important for both egg fertility and egg formation. As mentioned, parental incubation is necessary for embryonic growth (Deeming, 2002b), changes or disturbances in the normal patterns of incubation interrupts the natural growth patterns and can be detrimental for the embryo. For example, a delay in incubation, of as little as 3-5 days, has been shown to reduce the viability of eggs through both increased microbial infection and the impact of inadequate incubation temperatures (Cook et al., 2005b) (see section 1.4.1 incubation for egg defence).

Intermittent incubation can have similar effects, due to the egg experiencing a cyclic warming and cooling embryo development may be disrupted or halted (Deeming, 2002a). During incubation the parents also turn the egg; unturned eggs have higher mortality rates because of the risk of the embryo sticking to the shell membranes and also the potential for embryonic malposition (Deeming, 1995b). The developing embryo is highly susceptible to mechanical injury, especially during the early stages of formation (Romanoff and Romanoff, 1972). Harsh movements like shaking, jarring, or bumping can hinder further development and result in embryo malposition and mortality. Thus, parents during the process of incubation have to balance turning the egg to support proper embryo growth, with avoiding mechanical injury. Parent birds can also affect the embryos health by playing a protective role to the egg itself. This protection can range from deterring predators by distraction displays, to camouflaging the nest, to outright attack of the intruder (Skutch, 1954; Gottfried, 1979; Kreisinger and Albrecht, 2008). Parental care in some species is also required to maintain the incubation environment within suitable levels, as the developing embryo is easily disturbed by the physical components of the atmosphere. Behaviours such as shading the eggs and brood-patch wetting can alter the eggs environment and counteract the negative impact caused by unsuitable conditions (for unsuitable conditions see section 2.2.2 the effect of environmental factors on egg health). Overall, any reduction in the care normally provided by the parent can potentially contribute directly and indirectly to reduced hatching and other negative health effects in the egg and embryo.

2.2.2 The effect of environmental factors on egg health

Avian embryonic development is easily disturbed by environmental conditions outside the optimum range for a species (Romanoff & Romanoff, 1972). Incubation temperature is considered one of the most critical environmental conditions affecting egg viability (Romanoff and Romanoff, 1972; Webb, 1987; Stoleson and Beissinger, 1999). In most birds optimum incubation temperature is between 36-38^{o}C. However, embryonic growth can begin at temperatures above 25-27^{o}C, also known as physiological zero (Drent, 1975; Webb, 1987). If eggs are exposed to temperatures above physiological zero but below optimal incubation temperature, embryonic development continues but deformities often occur (Webb, 1987; Meijerhof, 1992). This is because only some

tissues begin to develop at these sub-optimal temperatures which results in uneven growth, abnormal development and embryonic mortality (Romanoff and Romanoff, 1972; Deeming and Ferguson, 1991). Viability of chicken eggs has been shown to decline after 7-15 days when exposed to temperatures below 25^{o}C (Miller and Wilson, 1976; Fasenko et al., 2001). Exposure for over a week to temperatures below physiological zero but above freezing has been shown to increase albumen pH and decrease albumen viscosity, both leading to a decline in the viability of the egg (Arnold, 1993; Fasenko et al., 2001). Temperatures higher than optimum incubation temperatures may cause acceleration in development which can lead to gross abnormalities and death of the embryo (Romanoff and Romanoff, 1972). High temperatures also pose a problem as avian embryos are particularly susceptible to death from overheating (Stoleson and Beissinger, 1999).

In some species the impact of temperature depends not only on the temperature itself, but also on the duration of exposure and the stage of development at which a given temperature occurs. For example chicken eggs fail to hatch if exposed continuously to temperatures above 40.5^{o}C and below 35^{o}C (Lundy, 1969). They also experience periods of increased susceptibility to high temperatures at about the fourth, 11th and 19th days of incubation (Romanoff and Romanoff, 1972). For wild birds, little is known about the effects of temperature on embryonic growth and mortality and much of the knowledge in this area is based on studies on domestic poultry (Stoleson and Beissinger, 1999). Temperature is known to be important during incubation but exactly how it affects development and mortality is poorly known (Stoleson and Beissinger, 1999) and further research is needed in this area.

Humidity is another important environmental factor influencing the development of the embryo (Romanoff and Romanoff, 1972). Birds can use incubation to maintain optimal temperatures for the eggs; however the ability to compensate for ambient humidity is limited (Walsberg, 1980, 1983). In poultry, the maximum egg hatchability is achieved when humidity levels are 80-90 per cent (Meijerhof, 1992). The effects of humidity are varied; low levels of humidity increases desiccation, whereas high ambient humidity can lead to impacts such as restricted pulmonary respiration (Simkiss, 1980). High humidity can also lead to increase microbial penetration, as microbes need water present to cross

the shell barrier (Board and Tranter, 1995) (see section 2.3.2 Factors affecting microbial contamination). Parental incubation can reduce the moisture levels on the shell surface, reducing the impacts of high ambient humidity (Board et al., 1979; Berrang et al., 1999).

2.2.3 The effect of predation on egg health

Predators are another reason for low hatching success. Avian eggs can form a large part of a predators diet as they are relatively easy prey (Drever et al., 2000). In many avian species predation can be the main cause of mortality, and the eggs are threatened by multiple predators (Fontaine and Martin, 2006). In New Zealand, in a review of 13 endemic and endangered forest birds, 46 per cent of nests failed due to predation (Innes et al., 2010). In some birds, such as the Brown Creeper (*Mohoua novaeseelandiae*) and Tomtit (*Petroica macrocephala*), predation accounted for over 70 per cent of egg losses (Moors, 1983; Brown, 1997). In New Zealand birds, predation of eggs is mainly by introduced mammals such as rats (*Rattus rattus* and *R. norvegicus*) and possums (*Thrichosurus vulpecula*) (Moors, 1983; Kiwis For Kiwis, 2012).

2.2.4 The effect of disease, toxins and pathogens on egg health

Disease, toxins and pathogens can also contribute to lowered hatching success (Cooper, 1989, 1993). Dichlorodiphenyltrichloroethane (DDT) is a well-known example of how a chemical, specifically an organochloride pesticide, can impact egg health. All over the world DDT was widely and aggressively used, and while the chemical itself is not highly toxic to birds, the bioaccumulation of it in prey species is. The effect of this bioaccumulation was devastating to exposed birds which presented with embryo mortality, reduced hatching, eggshell thinning, skeletal abnormalities, reproductive and nervous system deformations; and in adult birds; reduced fertility, suppression of egg formation and impaired incubation and rearing behaviours (Fry, 1995). In New Zealand, a Landcare Research report in 2013 found that Australasian Harriers (*Circus approximans*), a native bird of prey, had significant levels of a metabolite of DDT (Landcare Research, 2013), showing that it is still a problem for birds today. Another example of a negative effect of a chemical on eggs is lead contamination. Lead has been shown to cause decreases in clutch and egg size and direct mortality of embryos. Lead can also increase adult bird mortality and interfere with hiding and escape behaviour

which can have an indirect effect on the eggs as it reduces the parental care (Cooper, 1989).

Many infectious agents can have an impact on the developing embryo (Romanoff and Romanoff, 1972; Cooper, 1993). Viruses, fungi and bacteria have all been shown to cause dramatic reduction in hatching success of chicken embryos. For example chicken embryos have been shown to be highly susceptible to viruses like hepatitis (Hwang, 1965), have up to 79 per cent mortality with some fungi (Yamamoto and Ortmayer, 1966) and to have 100 per cent mortality with bacteria such as *Streptobacillus moniliformis*, *Staphylococcus aureus* and *Proteus mirabalis* (Buddingh, 1944; Bruce and Drysdale, 1991). Microbial infection of egg contents can occur quickly (Bruce and Drysdale, 1994), with most microbial penetration in experimentally infected chicken eggs occurring in four to five days (De Reu et al., 2006). Microbial infection of the egg contents in wild birds has been recorded after only three days in the nest (Cook et al., 2005a; Cook et al., 2005b).

2.3 Microbial impact on avian egg health

The interaction between the microbial flora of the environment and the avian egg has been extensively researched in the domestic chicken (Bruce and Drysdale, 1994; Baggot and Graeme-Cook, 2002). Microbes have been shown to significantly impact chicken hatching success (Bruce and Drysdale, 1991, 1994). Microbes have the ability to decrease hatching in poultry, though mechanisms such as embryo mortality and alteration of the egg contents (Board, 1968); and increase to hatching through competitive exclusion of egg pathogens (Ribble and Shinefield, 1967).

In wild birds the interaction between microbes, the environment and the egg has been considerably under researched (Baggot and Graeme-Cook, 2002; Cook et al., 2003). Thus, most of the information provided in regards to the causes and impacts of microbial infection on the egg and embryo is based on research on the domestic chicken. However, microbes have been highlighted in many studies as a potentially significant factor in hatching success and failure of wild birds (Cook et al., 2003; Cook et al., 2005a; Cook et al., 2005b; Boyer, 2010; Peralta-Sánchez, 2010; Potter et al., 2013).

2.3.1 Methods of contamination

There are two major ways bacteria can contaminate eggs: contamination via the reproductive tract of the laying bird, occurring prior to oviposition; or trans-shell contamination after the egg is laid (Bruce and Drysdale, 1994).

In chickens, certain poultry and human diseases such as salmonellosis have been shown to be transmitted vertically through the ovary prior to oviposition. In order for an egg to become contaminated from the mother, a microorganism not only has to survive and persist in the hen, but penetrate and survive in the tissues of the egg-producing organs and then penetrate and survive in the egg itself (Barrow, 1994). *Salmonella* spp., *Mycoplasma* spp., *Staphylococcus aureus* and *Pasteurella* spp. have been shown to contaminate eggs via the reproductive tract (Mayes and Takeballi, 1983; Humphrey, 1994). In general, there is little evidence for extensive vertical microbial transmission in the domestic chicken. The microbiology of the chicken's oviduct has been studied multiple times and in all cases the microbial flora recovered from the oviduct differed significantly from that found in eggs (Harry, 1963; Jacobs et al., 1978; Bruce and Drysdale, 1991). This indicates that in healthy hens the majority of contamination occurs after the egg is laid and it is largely accepted in the literature that contamination via the reproductive tract does not have a significant impact on the bacterial species found in the egg (Bruce and Drysdale, 1994). Of the few studies done on wild birds, conclusions are similar; eggs are laid with little infection and that primary source of contamination is the trans-shell route (Cook et al., 2005b).

2.3.2 Factors affecting microbial contamination

Contamination after the egg is laid can be affected by three main factors: the temperature differential between egg and environment; the presence of moisture on the eggs surface; and the presence and type of contamination on the egg and in the environment, including the parent bird (Bruce and Drysdale, 1994; Baggot and Graeme-Cook, 2002).

The temperature differential between an egg and its environment is created when a warm egg cools down (Bruce and Drysdale, 1994; Berrang et al., 1999). Because of the

shell's porosity, when a warm egg cools a negative pressure is created. This can result in contaminants being drawn through the pores and into the egg contents (Bruce and Drysdale, 1994; Berrang et al., 1999). Therefore avian eggs are particularly vulnerable at the point of laying, as this is when the egg is very warm from the oviduct and has contact with the outside environment and the environment's microbial flora for the first time (Berrang et al., 1999). The warming and subsequent cooling of eggs also occurs during incubation, when a solo incubating parent leaves the nest to feed (Colbourne, 2002). Temperature also can impact what microbes can grow and survive on and in avian eggs (Board and Tranter, 1995). Whilst incubation temperatures of most birds are the temperatures at which antimicrobial proteins function optimally, they are also similar to optimum growth temperatures of a variety of microbes (Cowan and Steel, 1993).

Another factor affecting microbial contamination is the presence of water, as water is essential for microbial penetration through the pores of the eggshell (Board et al., 1979; Berrang et al., 1999). The level of microbial contamination in chicken eggs has been found to be positively correlated to the amount of moisture present in the air at the time of laying (Graves and MacLaury, 1962; Bruce and Drysdale, 1994). The combination of high humidity and temperature change significantly increases microbial contamination (Haines and Moran, 1940; Board and Halls, 1973; Bruce and Drysdale, 1994; Peralta-Sánchez et al., 2012).

The environment into which the egg is laid is vitally important, as this dictates what microbes are present to contaminate the egg (Bruce and Drysdale, 1994). The environment includes the nest as well as the parental bird's microbes, and some habitats have more diverse or more numerous microbes than others. High levels of contamination in an environment are usually correlated with high levels of contamination on the egg (Harry, 1963; Baxter-Jones, 1991; La´Baque et al., 2003). For example, soiled nests have high levels of microbial contamination (Graves and MacLaury, 1962; Drysdale, 1985). Dirty eggs, covered in mud and faeces, also have higher microbial contamination than clean eggs (Harry, 1963; Baxter-Jones, 1991; La´Baque et al., 2003). Faecal material is a significant source of microbes and faecal contamination of avian eggshells increases penetration through the shell and shell

membrane (Jahantigh, 2010). In chickens, faecal contamination of eggs is considered to be one of the most important sources of yolk sac infection (Rajesh et al., 2001). The nesting environment can also affect the bacterial load on wild birds. Peralta-Sánchez et al. (2012) found that the eggshell bacterial load of 24 species of birds differenced significantly, both within and between species, over a two year period. They suggest that as well as temperature and humidity, life history traits such as nest type and nest lining affected the bacterial density on avian eggshells.

The presence of external contaminants on the eggshell does not necessarily mean reduced hatchability of the egg. A multitude of factors determine if a microbial species will actually penetrate the shell, grow in the egg contents, and cause problems for the developing embryo (Bruce and Drysdale, 1994). The type of contaminating flora is a major factor in determining if the species will affect the embryo. Only some species have the ability to penetrate the defences of the egg and survive in the adverse conditions of the egg contents (Bruce and Drysdale, 1994). For example; it has been shown that pseudomonads and fungi can digest the cuticle layer, destroy the water resistant properties of the egg and increase the number of unplugged pores available for bacterial penetration (Board and Halls, 1973; Baggot and Graeme-Cook, 2002; Cook et al., 2003). Of the microbes that can overcome the egg's defences and penetrate into the egg contents, only certain groups appear important in regards to infection and embryo mortality (Bruce and Drysdale, 1991, 1994; Cook et al., 2005b). Some species such as S*taphylococcus aureus* and some *Streptococcus* are more effective in reducing hatching in chicken embryos than for example *Enterobacter aerogenes* or *Micrococcus* spp. (Bruce and Drysdale, 1991, 1994).

2.4 Egg defence against microbial attack

Although bird eggs develop outside their mother's body, and are exposed to numerous threats in the external environment, they are not defenceless. Parental care plays a large role in the defence of eggs, especially through the process of incubation (Lack, 1954), however, it is important to acknowledge that the egg itself has a series of complex defensive barriers. Each layer in the egg structure provides another obstacle for

microbes to overcome in order to penetrate the egg and infect the embryo (Board, 1966; Board and Fuller, 1974). The avian egg has both physical defences and chemical defences (Board & Fuller, 1974). Another type of egg defence that is only recently being considered is the defence provided by beneficial bacteria through the production of antimicrobial substances (Peralta-Sánchez, 2010; Potter et al., 2013). That avian eggs have evolved numerous, complex defences against microbial contamination suggests that microbes have been an important factor shaping avian evolution and embryo viability, and supports the idea that microbes are a significant threat to the avian egg (Peralta-Sánchez, 2010). Avian eggs of different species differ both in structure and in nest conditions, and these can affect their defence against a microbial threat. A recent study by (Soler et al., 2011) compared the bacterial load both on and in the eggs of the Great Spotted Cuckoo (*Clamator glandarius*), a nest parasite, and it's host, the Magpie (*Pica pica*). They found that the Cuckoo eggs has significantly less bacterial contamination, on the shell and in the contents of the eggs, when compared with Magpie eggs in the same nest. The authors suggest that the parasitic Cuckoo eggs are better adapted to dealing with the high microbial threats in a nest environment.

2.4.1 Parental behaviours as a defence

Incubation has been shown to be important in the defence of eggs for several reasons; it keeps the eggs warm, dry and can reduce microbial load. In both the domestic chicken and in wild birds, the viability of eggs quickly declines without incubation (Drent, 1975; Arnold, 1993; Stoleson and Beissinger, 1995; Cook et al., 2003; Beissinger et al., 2005). For example, in as little as 3-5 days without incubation eggs of the Green-Rumped Parrotlet (*Forpus passerinus*) experienced a significant reduction in hatching success (Stoleson and Beissinger, 1995) and in the Pearly-Eyed Thrasher (*Margarops fuscatus*) the length of time the eggs went without incubation was the most significant factor impacting hatching success (Beissinger et al., 2005). Incubation has been shown to lower the levels of microbes on the shells of eggs and significantly lower pathogenic microbes (D'Alba et al., 2010). The bacterial community on un-incubated eggs changes significantly over time to become dominant in potentially pathogenic species (Shawkey et al., 2009). For example, incubated Pearly-Eyed Thrasher eggs harbour less pathogenic microbes than eggs left exposed (Cook et al., 2005a). Incubation alters the egg's

environment in two major ways, increased temperature and reduced humidity, both of which could contribute to the decline in pathogenic microbes. Incubation warms the egg, this not only supports proper embryo growth but the temperatures reached allow for optimal functioning of the antimicrobial proteins (Board and Fuller, 1974; Kang et al., 2006). Incubation also reduces the water on the surface of the egg (D'Alba et al., 2010). Moisture is necessary for bacterial growth and penetration into the contents (Board et al., 1979) thus keeping the eggs dry can have a significant defensive role. Parental behaviours such as providing specific nesting material may also have a defensive benefit: material used can alter the thermal conditions which indirectly impacts nest and egg bacteria; or material can have anti-bacteria properties and reduce microbial contamination directly (Clark and Mason, 1985; Peralta-Sánchez, 2010) (see chapter two, section 2.4.1). Another defensive behaviour is shown by the Hoopoe (*Upupa epops*), a bird that spreads uropygial gland (preen gland) secretions that contain antimicrobial compounds, onto its eggshells which increases hatching success (Soler et al., 2008).

2.4.2 Physical properties of egg defence

The pores are the only way microbes can penetrate in an intact egg (Board, 1966; Bruce and Drysdale, 1994; Messens et al., 2005). The pores are essential as they allow gas exchange with the external environment, yet there is a trade-off as an increase in pore density potentially increases the risk of microbial infection (Bruce and Drysdale, 1994). The cuticle, which covers the external shell and therefore the pores, is the egg's primary defence against microbial penetration (Bruce and Drysdale, 1994; Messens et al., 2005). However, the cuticle is not even over the shell and cuticular cover maybe lacking in some areas (see below). So the crucial factor determining bacterial penetration is not the number of pores *per se*, but the numbers that are not plugged by the cuticle and therefore open to microorganisms (Bruce and Drysdale, 1994).

An intact cuticle is an effective barrier to microbial penetration, however the structure of the cuticle can be impacted in a number of ways (Solomon et al., 1994; Kobayashi et al., 1996). For example, initially after oviposition the cuticle is far less effective against penetration as it is wet and immature (Sparks and Board, 1985). As well as this, the

cuticle is not always evenly distributed over the shell and large areas of the shell can actually be left uncovered and unprotected (Nascimento et al., 1992; Bruce and Drysdale, 1994). In addition, any mechanical abrasion, such as parental egg turning during incubation, can reduce the cuticular cover of the shell (Board and Halls, 1973), and fungi and *Pseudomonas* have been shown to easily penetrate the cuticle if conditions are suitable, e.g. high humidity, which then facilitates bacterial penetration by opening the pores (Board and Halls, 1973). All these factors not only affect the structure of the cuticle, they can lead to the pores remaining open and the egg being vulnerable to bacterial invasion.

The shell itself is the next barrier to microbial penetration (Kobayashi et al., 1996). The thickness of the shell can increase the eggs resistance to microbial penetration (Messens et al., 2005) as the thickness often correlates with longer, often more spiralled pores which make it harder for bacteria to invade the egg contents (Mayes and Takeballi, 1983). Any fracture in the shell considerably enhances the likelihood of infection (Bruce and Drysdale, 1994; Messens et al., 2005). Many factors affect ability of the eggshell to resist penetration: the individual hen, the hen's age, environment, diet, stress and disease (Nascimento and Solomon, 1991; Roberts and Brackpool, 1994; Messens et al., 2005; Fasenko et al., 2009).

The last physical barriers in microbial defence are the shell membranes (Kobayashi et al., 1996). Studies have shown these two layers of randomly orientated fibres are of major importance to the egg, with the inner membrane the most important due to its smaller diameter (Kraft et al., 1958; Lifshitz et al., 1964; Board and Tranter, 1995; Messens et al., 2005). Their role is as a bacterial filter, however this is only temporary (Bruce and Drysdale, 1994; Berrang et al., 1999). Some studies indicate that the membranes could provide protection for the egg for up to 15-20 hours (Walden et al., 1956), while other studies recovered bacteria from the inner membrane after only a minute (Bean and MacLaury, 1959). Neither of these timeframes is long enough to provide protection for the entire avian incubation period.

Both the eggshell and the shell membranes also have antimicrobial proteins within them, however, the albumen is the main site of chemical defence (Gautron et al., 2007; Hincke et al., 2008).

2.4.3 Chemical properties of egg defence

Multiple proteins with antimicrobial activity have been identified from the avian egg. The location of the shell as the primary point of contact with the environment suggests it plays a strong role in microbial defence (Hincke et al., 2008). Proteins extracted from eggshells of various bird species have been found to have antimicrobial functions (see table 2.1) (Wellman-Labadie et al., 2008a, b); for example, ovocalyxin-36 is an antimicrobial protein found in chicken eggshells (Gautron et al., 2007; Hincke et al., 2008). The shell membranes also have iron binding proteins which can have a major impact on bacterial penetration (Tranter et al., 1983; Bruce and Drysdale, 1994). The presence of iron greatly enhances the growth of bacteria and fungi, therefore by limiting the availability of iron bacterial growth is restricted (Board, 1968; Clay and Board, 1991; Weinberg, 2009). Although both the eggshell and the shell membranes have antimicrobial proteins within them, the albumen is the main site of chemical defence (Gautron et al., 2007; Hincke et al., 2008).

Several classes of antimicrobial protein have been isolated from inside avian eggs (see table 2.1), but the two that have been most investigated are ovotransferrin and lysozyme (Shawkey et al., 2008). Both play key roles in the defence of the albumen (Messens et al., 2005); ovotransferrin chelates iron, copper, magnesium and zinc and thus deprives bacteria of these essential elements (Ibrahim et al., 2000) and lysozyme, which has been found in a range of avian eggs, breaks down the peptidoglycans within the cell wall of bacteria (Ibrahim et al., 2000; Wellman-Labadie et al., 2008a, b). The majority of bacteria found on the eggshell are Gram positive, yet inside the contents Gram negatives dominate (Board, 1966; Bruce and Drysdale, 1994; Cook et al., 2003; Cook et al., 2005a; Cook et al., 2005b). The cell wall of Gram negative bacteria has a thinner peptidoglycan layer, therefore Gram negative bacteria are more resistant to the action of lysozyme, which may explain their high numbers in spoiled eggs (Barrow, 1994).

Both the viscosity and the pH of the albumen can provide defence against bacteria. The basic pH (9-10) of the albumen has a negative effect on bacterial growth (Sharp and Whitaker, 1927; Tranter and Board, 1984; Kang et al., 2006). The high viscosity causes bacteria to remain localised, which helps prevent contamination of the yolk (Bruce and Drysdale, 1994; Fang et al., 2012).

2.4.4 Beneficial bacteria as a mechanism of egg defence

An often overlooked form of egg defence is that provided by beneficial bacteria (Lombardo et al., 1996; Soler et al., 2008; Soler et al., 2010; Peralta-Sánchez, 2010). Bacteria have been shown to inhibit the growth of other microorganisms and in some cases deter predators and parasites, research suggests that beneficial, symbiotic bacteria may be common in birds (Moreno et al., 2003; Soler et al., 2010).

Bacteria can inhibit other microorganisms using a number of mechanisms (McCabe, 1967) such as antibiotic and bacteriocin production (Tagg et al., 1976; Gordon et al., 2007; Heng et al., 2007), exhaustion of essential nutrients (Troller and Frazier, 1963) and competitive exclusion (Nurmi and Rantala, 1973).

One of the most well studied areas of bacterial benefits in birds is in regards to the bacteria of the gut. Studies on chickens have shown that introduction of bacteriocin producing bacteria into the feed can reduce the presence and negative impact of pathogenic bacteria (Jin et al., 1997; Patterson and Burkholder, 2003; Stern et al., 2005; Jozefiak and Sip, 2013). In wild birds beneficial bacteria have been shown to increase growth (Moreno et al., 2003) and survival (Mills et al., 1999) of chicks, provide defence against feather-degrading bacteria (Shawkey et al., 2003; Shawkey et al., 2008; Martín-Vivaldi et al., 2010) and even potentially protect the eggshells (Soler et al., 2008).

For example in Pied Flycatcher (*Ficedula hypoleuca*) nestlings, the presence of *Enterococcus faecium* in the cloaca was positively associated with nestling mass and size shortly before fledging. The authors suggested that *E. faecium* may act as a growth promoter due to competitive interactions with pathogenic bacteria in the gut (Moreno et al., 2003). In Tree Swallows (*Tachycineta bicolor*) nestlings the presence of Gram negative bacteria in the gut was positively correlated with a greater degree of wing

asymmetry; these microbes indirectly affected survival of the nestlings, because wing symmetry aids flying ability, a critical survival skill for these aerial insectivores (Mills et al., 1999). Some birds, like Hoopoes, may defend themselves against feather-degrading bacteria by using secretions from the uropygial gland which contain symbiotic, non-pathogenic, antibiotic producing bacteria (Shawkey et al., 2003; Martín-Vivaldi et al., 2010; Ruiz-Rodríguez et al., 2013). As the secretion is also spread all over the eggs, the authors have suggested that these bacteria may also protect the egg from pathogenic bacterial contamination (Soler et al., 2008).

However, the majority of evidence for bacterial inhibition on birds eggs is mainly known from research on poultry (Potti et al., 2002). Non-virulent strains of *Staphylococcus* have been shown to provide protection to chicken embryos by preventing subsequent infection with virulent staphylococci, *Diplococcus* spp., *Salmonella* spp., *Escherichia coli*, *Proteus mirabilis* and even one strain of the influenza virus (McCabe, 1965, 1967). Certain strains of *Salmonella* (Ribble and Shinefield, 1967) and *Pseudomonas* (Bird and Grieble, 1969) have also been shown to reduce subsequent infection of chicken embryos through competitive exclusion and bacteriocin production.

It has been suggested that in wild birds eggs similar mechanisms could operate (Baggot and Graeme-Cook, 2002; Cook et al., 2005a). It is then possible that non-pathogenic bacteria present on wild avian eggs could interfere with any pathogenic bacteria and prevent embryo infection, as is suggested for the Hoopoes (Soler et al., 2008). More work is needed before any conclusions can be drawn on how, why and when bacteria act as pathogens or mutualists in the wild (Frank and Jeffery, 2001; Moreno et al., 2003).

2.5 Review of previous studies of the microbes of avian eggs

The majority of work that has been done on the interactions between avian eggs and microbial flora has occurred in the domestic chicken (Board, 1965, 1966; Bruce and Johnson, 1978; Mayes and Takeballi, 1983; Bruce and Drysdale, 1991, 1994; Baggot and Graeme-Cook, 2002).

2.5.1 Microbial contamination of domestic bird's eggs

Studies from the shells of domestic chickens show a dominance of Gram positive bacteria, with *Micrococcus* spp., *Bacillus* spp. and *Staphylococcus* spp. being dominant (Bruce and Johnson, 1978; Board and Tranter 1986; Mayes and Takeballi, 1994; Baggot and Graeme-Cook, 2002). Gram negatives, such as *Pseudomonas* spp. and members of the Enterobacteriaceae family like *Escherichia* spp. and *Serratia* spp., are also commonly isolated from domestic chicken eggshells (Board 1965, 1966; Bruce and Johnson, 1978; Bruce and Drysdale, 1991; Baggot and Graeme-Cook, 2002).

The contents of rotten or failed chicken eggs show that Gram negative bacteria dominate, with *Pseudomonas* spp. and the Enterobacteriaceae genera *Escherichia* spp., *Proteus* spp. and *Serratia* spp. being frequently isolated. Gram positives from the genera' *Micrococcus* spp. and S*treptococcu*s spp. are also regularly found inside chicken eggs (Mayes and Takeballi, 1983; Board and Tranter 1986; Bruce and Drysdale, 1991; Bruce and Drysdale, 1994). Other domestic bird species such as turkey, duck and waterfowl also have a dominance of Gram negatives within the egg contents of un-hatched eggs (Mayes and Takeballi, 1983; Bruce and Drysdale, 1994).

2.5.2 Microbial contamination of ratite eggs

Four ratite relatives of the NIBK, the Ostrich (*Struthio camelus*), the Emu (*Dromaius novaehollandiae*), the Greater Rhea (*Rhea americana*) and the Lesser Rhea (*Pterocnemia pennata*) are also bred domestically (Folch, 1992). Studies on the hatching success of these birds have shown that domestic production is poor. In both the Greater Rhea (Navarro et al., 1998; Navarro and Martella, 2002; La´Baque et al., 2003) and the Ostrich (Deeming, 1995a; Jahantigh, 2010) hatchability of all eggs is around 43 per cent, in the Lesser Rhea hatchability of fertile eggs is 67 per cent (Chang-Reissig et al., 2004) and in the Emu hatching success be can anywhere from 36 - 76 per cent (Szczerbińska et al., 2003).

A few studies have been done on the amount of bacteria present within the eggs of domestic Ostrich and Rheas. Results show that the amount of bacteria varies between species, but on average: 13 per cent of Greater Rhea (Navarro et al., 1998), 20 per cent

of Ostrich eggs (Deeming, 1995a, 1996) and up to 30 per cent of Lesser Rhea eggs had microbial contamination present (Chang-Reissig et al., 2004). The microorganisms isolated from all three domestic ratite species examined were similar to those found in chicken eggs with *Pseudomonas* spp. and members of the Enterobacteriaceae, such as *E. coli,* being the most common bacteria found (Deeming, 1995a, 1996; Moore, 1996; La´Baque et al., 2003; Chang-Reissig et al., 2004). Fungi were also isolated from the domestic ratite eggs; *Mucor* spp., *Fusarium* spp. and *Aspergillus* spp. have all been isolated from multiple Ostrich (Deeming, 1995a, 1996) and Greater Rhea eggs (La´Baque et al., 2003).

Microbial contamination was highlighted as a significant factor in hatching failure of some domestic Ostrich populations (Deeming, 1995a, 1996; Jahantigh, 2010), but not in other Ostrich populations or in the Lesser Rhea (Moore, 1996; Chang-Reissig et al., 2004). More research is needed to determine what microorganisms can reduce hatchability in domestic ratites (La´Baque et al., 2003), as in chicken embryos not all microorganisms have the same impact (Bruce and Drysdale, 1991) and this is expected to be the same for other species eggs.

Microbial contamination is not the only factor contributing to low hatching success on ratite farms (Deeming, 1995a; Moore, 1996; Deeming, 1996; Cooper, 2001; Chang-Reissig et al., 2004). Low fertility, embryonic mortality and post-hatching leg deformities are all significant factors in low hatching success in domestic Ostriches (Hastings, 1991). These factors, including microbial contamination, can be caused by poor farming management (Deeming, 1995a; La´Baque et al., 2003). For example, Shane and Tully (1996) have shown that a hatchability rate of up to 80 per cent can be achieved in well managed Ostrich farms.

Domestic ratites experience considerably different conditions to wild ratites. For example, domestic ratites have veterinary care and the eggs are normally hatched in sterile, temperature controlled incubators (Hicks-Alldredge, 1996). Because of such differences conclusions about wild hatching cannot be inferred from the studies on domestic birds; studies need to be undertaken in the wild, in natural conditions in order to determine the factors affecting wild ratite hatching success. Although there are very

few studies, wild ratites can experience low hatching success. Wild Lesser Rhea were found to have between 60-90 per cent annual hatching success, while wild Greater Rhea had only around 30 per cent hatching (Navarro and Martella, 2002; Barri et al., 2009). Up to 63 per cent of Cassowary (*Casuarius casuarius*) eggs (Bentrupperbaumer, 1997) and around 45 per cent of Emu eggs successfully hatched in a year (Marchant and Higgins, 1990; Folch, 1992). Hatching success in wild Ostrich is extremely low, with only 10 per cent of eggs hatching successfully (Folch, 1992).

In the Lesser Rhea the majority of hatching failure in the wild has been attributed to both human disturbance and predation (Barri et al., 2009). In the Greater Rhea most hatching failures were caused by the males deserting the nest although both predation and environmental conditions, such as high rainfall, significantly contributed to nest desertion and therefore lowered hatching success (Fernandez and Beboreda, 1998). Microbial contamination was also noted in some eggs, which also lead to nest desertion (Fernandez and Beboreda, 1998). In both the Ostrich and the Cassowary, no studies have been undertaken to explain the low hatching success in the wild. In all five ratites, no studies have been done on the levels of microbial contamination in the wild, or the types of microbial contamination present.

2.5.3 Microbial contamination of wild bird's eggs

Overall research into microbial flora of wild avian birds has been scant (Baggot and Graeme-Cook, 2002) (for a full list of microbial isolates on wild birds eggs see appendix one), yet research that has been done shows the prevalence of bacteria and fungi on the shells of wild birds, indicating the role they could play in hatching success of birds around the world. There has been no experimental infection of wild eggs to determine the exact role of microbes on hatching. The research to date highlights the variation in bacterial composition between species and the need for more research into the impacts microbes have on avian hatching success. In order to determine the possible role microorganisms have on embryonic development, it is first important to understand the composition of the microbial community present on and in wild eggs (Potter et al., 2013).

Above I discussed the information known on microbes of wild ratite's eggs. Other wild birds also experience hatching failure, and like in domestic birds, presence of pathogenic microorganisms has been highlighted as a potentially important factor influencing egg mortality and hatching success in some wild bird populations (Kozlowski et al., 1991b; Stewart and Rambo, 2000; Cook et al., 2005a).

Eggshells and egg contents of wild birds have been shown to harbour a wide range of bacteria. As with chicken eggshells, Gram positives, Gram negative enterics and Gram negative fermenters are commonly isolated off the shells of wild birds, although community composition differs between avian species.

Both Gram positive *Staphylococcus* and Gram negative enterics from the *Enterobacteriaceae* family were common isolates off Barn Swallow (*Hirundo rustica*) eggshells (Peralta-Sánchez et al., 2010). Gram positive bacteria dominate the eggshells of the Pearly-Eyed Thrashers (Cook et al., 2005a; Cook et al., 2005b). Gram positives, such as the genera *Bacillus* and *Staphylococcus*, also dominate the shells of Western Bluebird (*Sialia mexicana*), Tree swallows and Violet-Green Swallows (*Tachycineta thalassina*). In these species there was also an abundance of genera that favour dry conditions and a distinct lack of *Pseudomonas* and *Streptococcus* and most Enterobacteriaceae (Wang et al., 2011). In both the Pied Flycatcher (Ruiz-de-Casaneda et al., 2011) and the House Wren (*Troglodytes aedon*) (Potter et al., 2013) *Pseudomonas* dominated eggshells but there was a lack of Gram positives and Enterobacteriaceae.

The egg contents of some wild bird species have also been analysed and as with the contents of chicken eggs, Gram negatives dominate. Gram negatives such as *Pseudomonas* and members of Enterobacteriaceae, like *Enterobacter* and *Escherichia,* dominate the flora of Pearly-Eyed Thrasher eggs (Cook et al., 2005b), House Sparrows (*Passer domesticus*), Tree Sparrows (*Passer montanus*) (Kozlowski et al., 1991b), Swainson's Hawk *(Buteo swainsoni*), Ferruginous Hawk *(Buteo regalis)* and the Great Horned Owl *(Bubo virginianus)* (Houston et al., 1997).

The composition of the bacteria on avian eggshells is not only different between species (Peralta-Sánchez et al., 2012), but it has been shown to be dynamic and change significantly over time upon individual eggshells (Shawkey et al., 2009; Potter et al.,

2013). Both incubation (Potter et al., 2013) and environmental conditions (Shawkey et al., 2009; Peralta-Sánchez et al., 2012) have been shown to impact the composition and density of microbial genera found on shells. During the incubation period of the House Wren the types of bacteria genera isolated differed. Six genera were isolated only during the pre-incubation stage, 14 genera only during the early incubation stage and 10 genera only during the late incubation stage (Potter et al., 2013). In the Pearly-Eyed Thrasher, bacteria composition was also dynamic. Bacterial genera that were identified as opportunistic pathogens and linked with addled eggs were shown to increase on eggs that were exposed to environmental conditions and to decrease on eggs which were incubated (Shawkey et al., 2009).

Chapter Three: Identification of bacteria on the shells of live, wild North Island Brown Kiwi eggs and potential impacts on hatching success

3.1 Introduction

Microbial infection in domestic chicken (*Gallus gallus domesticus*) eggs has been shown to both increase hatching success by preventing embryo infection (McCabe, 1967; Ribble and Shinefield, 1967) and cause significant hatching failure by pathogenic action (Bruce and Drysdale, 1991). Similar bacteria to those found associated with poultry have been found on the shells and contents of both domestic ratites (Deeming, 1995a, 1996; Moore, 1996; La´Baque et al., 2003; Chang-Reissig et al., 2004) and wild birds eggs (Kozlowski et al., 1991b; Houston et al., 1997; Cook et al., 2003; Cook et al., 2005a; Cook et al., 2005b; Wang and Bessinger, 2009). This suggests that microbes may be important factors in the hatching success of birds other than chickens (Cook et al., 2005a; Cook et al., 2005b; Peralta-Sánchez, 2010).

Avian eggs are highly suitable for bacterial growth, they have a high nutrient content and the optimum temperature necessary for avian incubation is also the optimal temperature for the growth of most bacteria (Board and Fuller, 1974; Peralta-Sánchez, 2010). The avian egg is not defenceless, it has multiple complex defences, both physical and chemical, to counter microbial attack (see chapter two, section 2.4). As mentioned in chapter two (see section 2.4) the evolution of such defences, throughout the avian clade, suggest that microbes are a significant threat to the avian egg (Peralta-Sánchez, 2010).

The North Island Brown Kiwi (NIBK, *Apteryx mantelli)* is an endangered species and even with intensive management it has been experiencing significant population decline (see chapter one, section 1.3) (Holzapfel et al., 2008). The majority of New Zealand's management focuses on the predator-vulnerable chick life stage. The main conservation strategies in place are predator control and Operation Nest Egg (ONE); where eggs are taken from the wild and hatched in captivity until juveniles reach the predator proof size of one kilogram (Butler and McLennan, 1991; Holzapfel et al., 2008).

Predation may not be the only factor in NIBK population decline as 60 per cent of eggs in the wild fail to hatch (McLennan et al., 1996) and while predation and infertility do not seem to be significant factors (McLennan, 1988; Potter, 1989), microbes have been

noted inside a large number of un-hatched eggs (McLennan, 1988; Potter, 1989; McLennan et al., 1996; Ziesemann et al., 2011). Several factors make NIBK eggs susceptible to microbial infection are: its large, thin, and porous shell; that they are laid into humid, re-used burrows; and the long incubation period, with the male leaving the nest for extended periods each night (Andrews et al., 1990; Folch, 1992) . Currently no knowledge exists on the bacterial flora of NIBK eggs in the wild, nor how it could impact hatching failure.

As discussed in chapter one (section 1.5) due to the endangered status of the NIBK, restrictions are placed on the type of research that can be carried out. Before invasive studies are undertaken, knowledge is needed to determine whether bacteria could indeed pose a threat or benefit to wild NIBK. Therefore, the aim of this study was to determine if there is bacteria on the shells of wild NIBK eggs and if so whether they have the potential to impact hatching success.

Based on published studies of other bird species I predicted that there would be high levels of microbes present on the NIBK eggshell, and that the taxa found would be similar to those highlighted as important on the shells of other wild bird species (e.g.: Cook et al., 2005a,b). The results of this study are discussed in regards to conservation work on NIBK and NIBK egg hatching success.

3.2 Methods

Study site, laboratory site and study population are as described in chapter one (see section 1.6).

3.2.1 Data collection

Sampling for this study took place during the 2010-2011 breeding season in conjunction with study where nests were monitored for research into parental investment (I. Castro and S. Jamieson pers. comm.). Twenty eggs from 11 different males were swabbed (hereafter known as first swab group). Eleven of these 20 eggs were swabbed a second time later in the incubation period (hereafter second swab group). On average, the second swabs occurred 20 days after the first swab (see table3.1), which is a quarter of the incubation period. The nests were approached at night, when the males were out

feeding. The eggs were extracted from the nest, using a fresh pair of latex gloves on each sampling occasion. Eggs were swabbed from round to blunt end and then, eggs were candled following best practice to ascertain age (Bassett, 2012). The eggs were placed back into the nest in the same orientation as they were found. No nest desertions occurred as a consequence of this sampling procedure. After collection the swabs were placed into a charcoal medium (Copan Italia, Italy) to increase bacterial survival (Human and Jones, 2004). Swabs were stored at 4°C until they could be sent from the island to the laboratory. Consequently, due to the distance between the field site and the lab, samples were cultured between two and five days after being taken (see section 3.4.6 for consequences and advice for future studies).

Upon arrival at the laboratory samples were given a unique egg identification code (eggID) consisting of the name of the egg's putative father and a number indicating the order of the swabbed egg (first or second egg in the clutch). Swabs were streaked onto Blood and MacConkey agars (Fort Richards, Auckland, New Zealand), the Blood agar was placed into a CO_2 enriched environment, and then both agar types were incubated at 36^{o}C for 48 hours (see figure 3.1). The combination of these two plate types adequately characterises the bacterial groups known to cause infections of avian eggs. Blood agar is a general media suitable for the growth of many organisms, including Gram positives. Blood agar also allows for the growth of fastidious organisms and can detect hemolytic activity, which is often linked with pathogenicity (Elsner et al., 2000).

MacConkey agar differentiates Gram negative enteric and fermenter bacteria, which are known to be fast-growing yolk pathogens. Other studies looking at pathogenic bacteria in avian eggs have used similar media (Cook et al., 2003; Cook et al., 2005a; Cook et al., 2005b; Ruiz-de-Casaneda et al., 2011). After 48 hours of incubation bacterial types were visually differentiated and colonies were given a lab ID and sub-cultured onto a new agar plate. Once purity of the isolates was assured the sample was placed in a glycerol broth and stored at -80^{o}C.

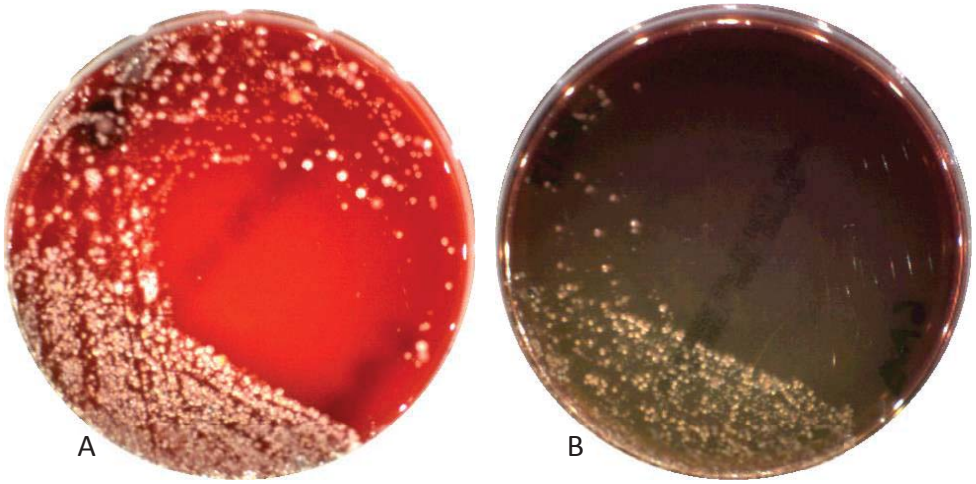

Figure 3.1: Example of (a) Blood and (b) MacConkey agar plates with a swab from a wild North Island Brown Kiwi eggshell after 48 hours incubation at 36°C.

Due to money constraints, only a subset of eggs could have their bacterial isolates identified. Thirteen out of twenty eggs had the bacterial isolates from the shell identified to the most exclusive taxa possible (see table 3.1).

3.2.2 Method design

3.2.2.1 Bacterial identification

Bacteria can be identified in two main ways; phenotypic characteristics and DNA sequencing (Janda and Abbott, 2007). In this study, bacterial identification was initially done phenotypically using the Omnilog® bacterial identification system (Biolog, CA, USA). The system identifies a broad range of Gram negative and Gram positive bacteria using 94 phenotypic tests. A "phenotypic fingerprint" of the microorganism is created using carbon sources and chemical sensitivities. This fingerprint can be used to identify the isolate to species level. The exact method used followed that outlined in Omnilog® user guide.

The Omnilog® bacterial identification system was initially used as it provided a convenient way of testing a large number of phenotypic reactions, the results however provided only identification to genera. Thus, sequencing of the 16S rRNA gene was used in an attempt to get species identification. This is the most common sequence used to study bacterial phylogeny and taxonomy as it is present and conserved in almost all

bacteria (Janda and Abbott, 2007). Details about extraction and PCR optimisation are in Appendix two.

3.2.2.2 Determining potential impact on North Island Brown Kiwi embryos

Ideally, to test the impact of each bacterial isolate on NIBK embryos, the bacteria would be tested by infecting a number of NIBK eggs with a bacterial isolate and recording its effect on hatching success compared with uninfected eggs. However, due mainly to ethical constraints this could not be done and the identified bacteria off the shell were instead researched thoroughly in the literature to identify if any genera could be a potential threat or benefit to NIBK hatching success. I paid particular attention to published information regarding lysozyme resistance, known pathogenicity, production of antimicrobials and known beneficial impact for each genera found to highlight potentially important bacteria in the hatching success of NIBK eggs (see chapter one, section 1.6.4).

Table 3.1: List of wild North Island Brown Kiwi eggs from Ponui Island swabbed for bacteria in 2011. ID'd = ✓ indica identified. EggID = a unique code to identify each egg consisting of the father's name and the order of the egg (either f egg was swabbed. All 20 eggs had a first swab taken, but only 11 of these had a second swab taken later in incubation.

General information				First swab				
ID'd	eggID	Lay date	Hatched	Swab date	Age (days)	Plate date	Bacteria present	Swab
✓	Bel1	25 July	Yes	1 August	7	6 August	Yes	22 Aug
	Bel2	22 August	Yes	22 August	0	26 August	Yes	
✓	Clel1	5 August	No	6 August	1	8 August	Yes	
✓	Dale1	1 July	No	5 August	35	8 August	Yes	
✓	Dale2	23 July	Yes	5 August	13	8 August	Yes	
✓	Dario1	16 July	Yes	1 August	16	6 August	Yes	22 Aug
	Dario2	7 August	No	22 August	15	26 August	Yes	
	George1	20 July	Yes	1 August	12	6 August	Yes	
✓	Ivan1	15 June	Yes	2 August	48	6 August	Yes	23 Aug
✓	Ivan2	3 July	Yes	2 August	30	6 August	Yes	23 Aug
✓	Martin1	before 23 June	No	1 August	15 +	6 August	Yes	22 Aug
	Martin2	23 June	No	22 August	60	26 August	Yes	
✓	Max1	3 June	No	2 August	60	6 August	Yes	4 Aug
	Max2	12 June	No	2 August	51	6 August	Yes	4 Aug
✓	Murphy1	22 June	No	1 August	40	6 August	Yes	22 Aug
✓	Murphy2	27 June	Yes	1 August	35	6 August	Yes	22 Aug
✓	Pouni1	14 June	Yes	1 August	48	6 August	Yes	22 Aug
✓	Pouni2	2 July	Yes	1 August	30	6 August	Yes	22 Aug
	Roy1	13 July	No	2 August	20	6 August	Yes	
	Roy2	18 July	No	2 August	15	6 August	Yes	

3.3 Results

3.3.1 Bacterial presence

All 20 wild NIBK eggshells sampled had bacteria present (see table 3.1) and 105 distinct colonies in total were sub-cultured from these 20 eggs. In total I attempted to identify 80 isolates (76%) using a combination of Omnilog® and DNA sequencing, and 59 of these (73%) had a positive ID, the remaining isolates could not be identified (see section 3.4.6).

3.3.2 Bacterial identification

Bacteria from three phyla, 12 families and 14 genera were isolated from the 13 eggs studied (see table 3.2). Most isolates were Gram positive cocci from the phylum Firmicutes, with *Staphylococcus* and *Macrococcus* being the most common genera. *Pseudomonas* spp. also occurred multiple times on eggshells.

Enterobacter was isolated from three separate eggshells, while *Acinetobacter* and the Actinomycetes *Corynebacterium* and *Dermacoccus* were all isolated from two separate shells. The genera *Serratia* was isolated twice off one eggshell. The remaining eight genera (*Buttiauxella, Pantoea, Sporichthya*, *Brevibacterium, Micrococcus*, *Bacillus, Raoultella* and *Streptococcus*) were isolated once. Four families and six genera found in this study have not previously been documented from wild bird eggshells (see table 3.2).

3.3.3 Hatching success of the North Island Brown Kiwi eggs in this study and the presence of bacteria on them

Of the 13 eggs examined in this study, seven hatched (54%) and six did not (46%). Three genera, *Streptococcus*, *Serratia* and *Corynebacterium*, were isolated solely from eggs that did not hatch. Five genera, *Acinetobacter*, *Enterobacter*, *Macrococcus*, *Pseudomonas* and *Staphylococcus,* were isolated off the eggshells of eggs that both hatched and did not hatch. The remaining eight genera were isolated only on eggs that hatched (see table 3.3).

Table 3.2: List of all bacteria identified off wild North Island Brown Kiwi eggshells from Ponui Island. EggID = a unique code to identify each egg consisting of the father's name and the order of the egg (either first or second laid). Age = age when egg was swabbed (+ = maybe more). All 20 eggs had a first swab taken, but only 11 of these had a second swab taken later in incubation. Some shells had multiple isolates from the same genera, these are included in the table as I cannot exclude them being from different species. * = genera or species found for the first time on wild eggshells.

	First swab		Second swab	
EggID	Age (days)	Bacterial Identification	Age (days)	Bacterial Identification
Bel1	7	*Staphylococcus kloosii**	*28*	*Pseudomonas stutzeri*
		*Macrococcus** *spp.*		
		*Sporichthya** *spp.*		
Clel1	1	*Acinetobacter spp.*		
		Enterobacter spp.		
Dale1	35	*Staphylococcus spp.*		
		Corynebacterium spp.		
Dale2	13	*Staphylococcus spp.*		
		*Macrococcus** *spp.*		
		Micrococcus spp.		
Dario1	16	*Staphylococcus hyicus**	*37*	*Pseudomonas stutzeri*
		*Macrococcus** *spp*		
		*Macrococcus** *spp.*		
Ivan1	48	*Enterobacter amnigenus*	*69*	*Staphylococcus kloosii*
		Enterobacter spp.		*Macrococcus** *equipercicus**
Ivan2	30	*Buttiauxella** *agrestis**	*51*	*Enterobacter amnigenus*
		Staphylococcus spp.		*Macrococcus** *spp.*
		*Raoultella** *planticola**		
		Dermacoccus spp.		
Martin1	15 +	*Macrococcus** *spp.*	39 +	*Pseudomonas stutzeri*
		Staphylococcus spp.		*Staphylococcus spp.*
		Serratia spp.		
		Serratia spp.		
Max1	60	*Staphylococcus spp.*	*62*	*Macrococcus** *spp.*
		*Macrococcus** *spp.*		*Streptococcus** *spp.*

EggID	First swab		Second swab	
	Age (days)	Bacterial Identification	Age (days)	Bacterial Identification
Murphy2	35	*Staphylococcus hyicus** *Macrococcus* spp.* *Macrococcus* spp.* *Dermacoccus spp.*	*56*	*Staphylococcus hyicus**
Ponui1	48	*Pantoea spp.* *Staphylococcus spp.*	*69*	*Pseudomonas stutzeri* *Bacillus spp.*
Ponui2	30	*Brevibacterium spp.* *Staphylococcus sciuri**	*51*	*Acinetobacter spp.*

Table 3.3: List of bacterial genera isolated off shells of wild North Island Brown Kiwi eggs that hatched and did not hatch. ✓ = genera was present on egg type; no tick means genera was not present. Frequency indicates the number of isolates from that genera found on the shells.

Genus	Present on			
	Hatched	frequency	Un-hatched	frequency
Acinetobacter	✓	1	✓	1
Bacillus	✓	1		
Brevibacterium	✓	1		
Buttiauxella	✓	2		
Corynebacterium			✓	2
Dermacoccus	✓	2		
Enterobacter	✓	3	✓	1
Macrococcus	✓	8	✓	5
Micrococcus	✓	1		
Pantoea	✓	1		
Pseudomonas	✓	3	✓	3
Raoultella	✓	1		
Serratia			✓	2
Sporichthya	✓	1		
Staphylococcus	✓	9	✓	5
Streptococcus			✓	1

3.3.4 Potential pathogenic impacts on North Island Brown Kiwi eggs from bacteria isolated

The genera isolated showed mixed characteristics in regards to lysozyme resistance and records of pathogenicity (see table 3.4). Twelve of the 16 genera (75%) have lysozyme resistant members; *Micrococcus* was the only genera with known lysozyme sensitivity.

Records of lysozyme resistance could not be found for all the genera on the shells of NIBK eggs, but because lysozyme works by attacking peptidoglycans found in the cell walls of bacteria, it is ineffective against most Gram negative bacteria due to their thin cell wall (Board, 1966; Ibrahim et al., 2000). Therefore, to be conservative all Gram negatives have been assumed to have resistance to lysozyme. The effect of lysozyme on three other genera (*Buttiauxella*, *Raoultella* and *Sporichthya*) could not be found.

The majority (75%) of the genera found in this study have been shown to be pathogenic in humans or animals, 11 genera (69%) have been identified as avian pathogens and eight (50%) have been shown to cause avian embryo death (table 3.4).

Table 3.4: Results of literature search of potential pathogenic factors of bacterial genera isolated off wild North Island Brown kiwi (*Apteryx mantelli)* eggshells. Lysozyme resistance indicates if members of genera are resistant to the action of lysozyme; resistant = all members are resistant to lysozyme; mixed = some members show resistance to lysozyme; likely resistant = no information but genera is Gram negative, which are typically resistant to the action of lysozyme due to the cell wall structure; sensitive = all members are sensitive to lysozyme. Known pathogenicity indicates if records exist of genera being pathogenic; general = records of pathogenicity in any animal or human; avian = records of pathogenicity in any bird species; egg = records of pathogenicity in avian embryo and avian eggs. ✓ = records present, x = no instance of pathogenicity, * = no information found. References indicates source used.

Genus	Lysozyme resistance	Known pathogenicity			References
		General	Avian	Egg	
Acinetobacter	Resistant	✓	✓	*	(Thorne et al., 1976; Cowan and Steel, 1993; Fudge, 2001; Juni, 2005; Muller et al., 2010)
Bacillus	Mixed	✓	✓	✓	(Dockstader, 1952; Peckham, 1959; Cole, 1990a; Cowan and Steel, 1993; Logan et al., 2007)

Genus	Lysozyme resistance	Known pathogenicity			References
		General	Avian	Egg	
Brevibacterium	Resistant	✓	✓	*	(Reinert et al., 1995; Pascual and Collins, 1999)
Buttiauxella	*	✓	*	*	(Carter and Chengappa, 1990; Cowan and Steel, 1993; Kampfer, 2005)
Corynebacterium	Resistant	✓	✓	*	(Hirasawa et al., 2000; Potti et al., 2002)
Dermacoccus	Resistance	*	*	*	(Becker et al., 2003)
Enterobacter	Likely resistant	✓	✓	✓	(Peckham, 1959; Carter and Chengappa, 1990; Bruce and Drysdale, 1991; Cowan and Steel, 1993; Fudge, 2001; Grimont and Grimont, 2005b; Abbott, 2007)
Macrococcus	Resistant	*	*	*	(Götz et al., 2006)
Micrococcus	Sensitive	✓	✓	✓	(Thorne et al., 1976; Bruce and Drysdale, 1991; Cowan and Steel, 1993; Bruce and Drysdale, 1994; Becker et al., 2003)
Pantoea	Likely resistant	✓	✓	✓	(Grimont and Grimont, 2005c; Gibbs et al., 2007)
Pseudomonas	Resistant	✓	✓	✓	(Bruce and Johnson, 1978; Brittingham et al., 1988; Carter, 1990; Bruce and Drysdale, 1991; Cowan and Steel, 1993; Bruce and Drysdale, 1994; Silvanose et al., 2001; Fudge, 2001; Blondel-Hill et al., 2007)
Raoultella	*	*	*	*	(Peckham, 1959; Abbott, 2007; Morais et al., 2009)
Serratia	Likely resistant	✓	✓	✓	(Peckham, 1959; Izawa et al., 1971; Carter and Chengappa, 1990; Fudge, 2001; Grimont and Grimont, 2005a; Grimont and Grimont, 2006; Abbott, 2007)
Sporichthya	*	*	*	*	
Staphylococcus	Mixed	✓	✓	✓	(Thorne et al., 1976; Brittingham et al., 1988; Cole, 1990b; Cowan and Steel, 1993; Olsen, 2000; Silvanose et al., 2001; Fudge, 2001; Götz et al., 2006; Bannerman and Peacock, 2007)
Streptococcus	Mixed	✓	✓	✓	(Brittingham et al., 1988; Cole, 1990c; Bruce and Drysdale, 1991; Cowan and Steel, 1993; Bruce and Drysdale, 1994; Olsen, 2000; Fudge, 2001; Spellerberg and Brandt, 2007)

3.3.5 Potential beneficial impacts on North Island Brown Kiwi eggs from bacteria isolated

The genera isolated in this study show mixed characteristics in regards to antibiotic or bacteriocin production and records of beneficial impact (see table 3.5). Fourteen of the genera (87.5%) isolated from NIBK eggs have members that produce antibiotics or bacteriocins. Three genera (19%) have proven to be beneficial to some avian species and all three have been shown to provide bacterial protection for chicken embryos. Information could not be found on the production of potentially beneficial substances for two (12.5%) of the genera (*Sporichthya* and *Buttiauxella*).

Table 3.5: Results of a literature search on the potential beneficial factors of the bacterial genera isolated off wild North Island Brown Kiwi eggshells. Antibiotic or bacteriocin production indicates if members of the genera are known to make these substances. Known benefit indicates if records exist of genera being beneficial; general = any instance of beneficial impact on animals or humans, avian = records of benefit in adult/young of any bird species, egg = records of benefit in avian embryo and avian eggs. ✓ = records present, * = no information found. References indicates source used.

Genus	Antibiotic /bacteriocin production	Known benefit			References
		General	Avian	Egg	
Acinetobacter	✓	*	*	*	(Andrews, 1986)
Bacillus	✓	✓	✓	*	(Lechevalier, 1975; Tagg et al., 1976; Lim and Kim, 2009; Soler et al., 2010)
Brevibacterium	✓	*	*	*	(Irie et al., 1960; Lechevalier, 1975; Collins, 2006)
Buttiauxella	*	*	*	*	
Corynebacterium	✓	✓	*	*	(Tagg et al., 1976; Lina et al., 2003)
Dermacoccus	✓	*	*	*	(Abdel-Mageed et al., 2010)
Enterobacter	✓	*	*	*	(Gordon et al., 2007)
Macrococcus	✓	✓	*	*	(Wongkattiya, 2008)
Micrococcus	✓	*	*	*	(Tagg et al., 1976)
Pantoea	✓	*	*	*	(Clardy et al., 2006)
Pseudomonas	✓	✓	*	✓	(Bird and Grieble, 1969; Lechevalier, 1975; Chavan and Riley, 2007; Cogen et al., 2008)
Raoultella	✓	*	*	*	(Fleming et al., 2010)
Serratia	✓	*	*	*	(Lechevalier, 1975; Grimont and Grimont, 2006; Gordon et al., 2007; Chavan and Riley, 2007)
Sporichthya	*	*	*	*	
Staphylococcus	✓	✓	✓	✓	(McCabe, 1965; Tagg et al., 1976; Cogen et al., 2008)
Streptococcus	✓	✓	✓	✓	(Ribble and Shinefield, 1967; Sprunt and Leidy, 1988; Cogen et al., 2008)

3.4 Discussion

3.4.1 Bacterial presence

All of the eggshells in this study had microbes present. This level of contamination was higher than that reported for other wild birds' eggs, as was predicted. While 80 per cent of Pearly-Eyed Thrasher (*Margarops fuscatus*) eggs had microbes on the shell surface, only 60 per cent of Pied Flycatcher (*Ficedula hypoleuca*) eggs had (Cook et al., 2005b; Ruiz-de-Casaneda et al., 2011). Although bacteria have been shown to be present on wild eggs in New Zealand (Little Blue Penguin (*Eudyptula minor*) (Boyer, 2010), this study is the first to identify bacteria present on the shells of wild birds in New Zealand to a taxonomic level.

That all NIBK eggs examined had bacterial contamination on the eggshell supports the initial hypothesis that factors specific to NIKB make them susceptible to microbial contamination. The large, thin shell means there is a large surface area, short pores and higher chance of hairline fractures. The eggs are laid in winter into humid, reused burrows. The eggs experience an extremely long, intermittent incubation period and first laid eggs can be left exposed for up to 30 days (see chapter one, section 1.5).

3.4.2 Bacterial identification

Not all bacteria are harmful to the developing embryo; as such, the presence of bacteria on the shell alone does not mean there will be an impact on hatching success. Identification of the bacteria allows for a better understanding of the potential impacts on NIBK hatching success.

Sixteen genera were present on wild NIBK eggs in this study. Most common were Gram positive cocci from the genera *Staphylococcus* and *Macrococcus* and the Gram negative *Pseudomonas*. Members from the Enterobacteriaceae family were also commonly isolated. The results of this study support the findings of other studies on eggshell bacteria (appendix two). Gram positives, *Pseudomonas* spp. and members of the Enterobacteriaceae have been recorded on the eggshells of chickens (Mayes and Takeballi, 1983; Bruce and Drysdale, 1994; Board and Tranter, 1995), domestic ratites (Deeming, 1995a, 1996; Moore, 1996; La´Baque et al., 2003; Chang-Reissig et al., 2004)

and wild passerines (Cook et al., 2005a; Cook et al., 2005b; Peralta-Sánchez et al., 2010; Wang et al., 2011).

This study used similar methods to the studies above to isolate the bacteria on avian eggshells. Because this method selects culturable bacteria and the growth media is targeted towards bacteria known to be egg pathogens, the presence of similar bacteria is to be expected. Likewise, all avian eggshells have similar composition and structure (see chapter two, section 2.1.3) limiting the groups of bacteria that can survive on an eggshell and this may also account for similarities between studies and species (Peralta-Sánchez, 2010).

Although the bacteria are similar between species studied, they are not identical. Five genera and six species isolated in this study had not previously been documented on the shells of wild birds' eggs (see table 3.2). One of these genera, *Streptococcus*, has been isolated from domestic chicken eggshells where it is both a known pathogen (Board and Tranter, 1995) and shown to increase hatching success (Ribble and Shinefield, 1967). The other four genera *Macrococcus, Buttiauxella* (Carter and Chengappa, 1990), *Raoultella* (Abbott, 2007) and *Sporichthya* (Tamura et al., 1999) are known as environmental inhabitants and little is known about their role in avian hatching. As little information exists on the impact of particular bacterial species on avian eggs (as discussed in chapter one, section 1.6.4), I focused on genera and the six new species are not discussed further.

Climate, nest type and parental care can all impact bacterial contamination of eggshells (see chapter two, section 2.3.2). The variation seen in bacterial types are likely due to the different sources of contamination of the eggs of different species face. Most other birds studied are either tropical, and/or cavity nesting passerines, with partial incubation of a three-four egg clutch, such as Pearly-Eyed Thrashers (appendix one) (Cook et al., 2005a; Cook et al., 2005b). NIBK in contrast are burrow-nesting birds, that breeding in temperate winter, have extremely long, intermittent incubation and have a first laid egg that can be left up to 15 days un-incubated.

Wang et al. (2011) studied passerine eggshells in a temperate climate and compared the results with the microbes identified from tropical passerine's eggshells (Cook et al.,

2005a; Cook et al., 2005b). Over 90 per cent of the genera identified were not previously identified on the tropical bird's eggshells, majority (76%) orders that favoured dry conditions, such as Actinomycetales and Bacillales. The Passerine eggs in temperate climates also lacked *Pseudomonas*, *Streptococcus* and most Enterobacteriaceae.

All studies also identified the bacteria found to different taxa, some identified species, some genera, and some only general groups such as Gram negative fermenters. This has implications when trying to compare studies (see chapter one, section 1.6.4) and as not all species in a genus are pathogenic (see section 3.4.6) it effects the conclusions that can be drawn as to microbial impacts on avian hatching success.

3.4.3 Potential threats to North Island Brown Kiwi embryos and hatching success from bacteria found

Because the effect of each bacterial genera on the hatching success of the NIBK embryo could not be experimentally examined due to ethical issues (but also see chapter 6) two factors that could contribute to pathogenicity were investigated in the literature: lysozyme resistance and any known records of pathogenicity. As well as these two factors, the presence of the bacterial genera on the shells of eggs that did not hatch was noted, as presence of the bacteria is needed before it can cause hatching failure (Bruce and Drysdale, 1994). Finally, I use information of bacterial impact on other avian species, mainly domestic chickens to suggest bacteria that have the potential to impact the hatching success of NIBK.

When the results of these four factors are combined, it can be seen that NIBK eggs harbour potentially pathogenic bacteria. Fifty per cent of genera isolated in this study have been shown to cause declines in avian hatching success. Of these genera, 90 per cent are lysozyme resistant and over 63 per cent were isolated from un-hatched eggs. The combined results highlight *Pseudomonas, Staphylococcus, Streptococcus, Bacillus, Micrococcus, Enterobacter, Pantoea* and *Serratia* as potential threats to NIBK hatching success.

As well as causing direct impacts to embryos, Gram negatives such as *Pseudomonas* spp. have been shown to indirectly impact the avian embryo by facilitating penetration and infection of the egg contents. *Pseudomonas* spp. can digest the eggshells protective cuticle and dramatically increase the number of open pores (Board and Halls, 1973; Board et al., 1979).

Other genera, although not linked with avian hatching success, cannot be excluded as potential pathogens towards NIBK eggs. Because no research onto the impact of specific bacteria on NIBK embryos has been undertaken, none of the genera isolated can be ruled out as potential pathogens and further work is needed before conclusions on pathogenicity can occur.

3.4.4 Potential benefits to North Island Brown Kiwi embryos and hatching success from bacteria found

The beneficial role of bacteria in avian hatching in general has received less attention than pathogenic impacts (Moreno et al., 2003; Soler et al., 2010). It is not surprising therefore that, as with pathogenic bacteria, benefits provided by certain bacterial genera have not been investigated for NIBK.

The majority of the genera (87.5%) in this study have been shown to produce substances that restrict the growth of other bacteria, such as antibiotics or bacteriocins. Members from three of the genera isolated, *Staphylococcus* (McCabe, 1965, 1967), *Streptococcus* (Ribble and Shinefield, 1967) and *Pseudomonas* (Bird and Grieble, 1969) have been shown to directly provide benefits to developing chicken embryos. The presence of the genera *Staphylococcus*, when injected into the allantoic membrane of chicken embryos caused significant protection and increased hatching (McCabe, 1965, 1967). Both *Streptococcus* spp. (Ribble and Shinefield, 1967) and *Pseudomonas* spp. (Bird and Grieble, 1969) also limited or prevented the colonisation of other bacteria in the chicken egg and resulted in increased hatching success.

The results of this study highlight *Staphylococcus*, *Streptococcus* and *Pseudomonas* as important genera that have the potential to provide benefits to NIBK eggs. More work

needs to be done and experimental studies conducted, to conclusively determine the beneficial impact of bacteria on NIBK eggs.

3.4.5 Impact for North Island Brown Kiwi conservation from the results of this study

The second aim of this study was to use the results to support and guide the direction of future work on NIBK egg hatching success. This study provides the first evidence of what bacteria are present on the shells of wild NIBK eggs and the results suggest that microbes could play a part in NIBK hatching success.

In regards to NIBK conservation, I believe that future work needs to focus on the egg stage. Predators are not the only factor in NIBK mortality and the results of this study show that wild NIBK eggs harbour potentially pathogenic bacteria. Currently ONE is used as a temporary tool to protect the predator vulnerable chick stage and it is to be phased out when predator control is sufficient to protect the majority of wild chicks. But ONE is also protecting the egg stage, which has a 64 per cent mortality rate in the wild (McLennan et al., 1996). Thus, based on the findings of this study, in my opinion ONE should not be phased out until more research into the causes of NIBK hatching failure and strategies to deal with this are developed.

I suggest that NIBK handlers consider microbes when dealing with birds and eggs; improper handling not only risks the spread of pathogens between birds and eggs, but also puts the handler at risk. The Kiwi Best Practice Manual (KBPM) states that gloves should be worn and hands and equipment disinfected between each individual NIBK egg (Robertson and Colbourne, 2003). However, the KBPM also advises that when extracting ONE eggs from the nest, as the eggs are cleaned upon arrival at the ONE facility (Bassett, 2012) dry bare hands are preferable to gloved ones, because bare hands increases the sensitivity to holding the egg (Robertson and Colbourne, 2003). The results of this project support the initial statement and instead emphasise that gloves should realistically be worn at all stages of egg and bird handling (also see chapter seven, section 7.2.2). All equipment, where possible, should be sterilised in the field between individual birds and/or eggs.

The impact of the shell microbes on the NIBK chicks is also an important factor to consider for future NIBK conservation and research. In the wild, NIBK chicks are exposed to these eggshells and the bacteria present on them, both during and after hatching. The chicks can remain in the nest for up to 27 days (Wilson, 2013) therefore these eggshells are a reservoir of potential microbial contamination for the chicks. We do not know if the pathogens present on the shell can infect the chick.

Some avian chicks, like the domestic chicken, also acquire their mutualistic gut bacteria directly from the eggshells (Schneitz, 2005; Minson, 2012; Stanley et al., 2013). The development of a healthy gastrointestinal microbiota early in life can be critical to future survival (Stanley et al., 2013). Stanley et al. (2013) investigated the gastrointestinal microflora of domestic chicken chicks, and found it to be highly variable between individuals. They suggest that normally a hatching chick would be exposed to the microbial flora of the eggshell and the nest environment; which are largely derived from the parent bird. It is through this expose that the gut is colonised. However, as commercial chicken hatching facilities have such high hygiene standards the shells and nests are often washed and fumigated. Because of this the chicks are not exposed to the natural microbial flora, and instead their gastrointestinal tracts are colonised by the random bacteria encountered (Stanley et al., 2013). NIBK chicks acquire gut microbial bacteria over the first few weeks of life, and the acquisition of positive gut microbes from the eggshell has been suggested to occur in this time (Minson, 2012).

Both these factors, the shell pathogens and the positive gut microflora, may have impacts for the captive breeding of NIBK as eggs are disinfected and chicks are not exposed to the natural bacterial flora. Although ONE chicks survive well in the wild (Bassett, 2012), we have little understanding of the impacts of shell bacteria on chicks in the wild and more research is needed in this area. Observations of the contact wild chicks have with the shells and the gut microflora in recently hatched wild chicks, would both be beneficial as future research.

3.4.6 Study limitations and future work

This study was successful in determining what bacteria are present on the shell and their potential to impact hatching success; possible new paths for future projects were also discovered. However, there were restrictions with this study and the results should be used to guide future work.

Some of the isolates could not be identified using the Omnilog® or DNA sequencing. Both methods use a database of known microbial isolates to obtain an identification of the unknown microbial isolate. That some isolates in this study could not be identified may be for a number of reasons, the isolate: 1. may be known species that are not present on the database; 2. may be genera in the database with species characteristics so different to those in the database that the system couldn't recognise them, this may be due to them being New Zealand isolates; 3. may not have been prepared accurately; or 4. may in fact be new species. The Omnilog® database is biased towards human pathogens; this could also impact the lack of identification of the environmental bacteria found on the shells of the NIBK eggs. Although extreme care was taken at every step of preparation, the lack of identification of some microbes could be due to preparing error.

Due to the distance between the field site and the laboratory there was a delay in getting samples prepped for testing. This could affect the results, as there may have been some bacterial death during transit. A charcoal medium was used in this study to lower the risk of bacterial death, however quicker plating would have been preferable. The distance from the laboratory also meant that the field sampling had to be carried out by other researchers during the main monitoring project on the island, as I had to be in the lab to process the samples; the consequence of this was that not all eggs could be swabbed twice. Future work should factor in the distance between field sites and the laboratory, and aim to plate up the samples as early as possible. Plating samples in the field is not recommended, as the lack of proper equipment such as biological safety cabinets increases the risk of infection to the handler.

Most studies have only provided genera identifications of eggshell bacteria and some only provide identification to group level. Although nothing is mentioned in these

studies about difficulties and restrictions with identification to more specific classification, the culture-dependent method used involves time and resources to isolate, prepare and identify each individual colony. Indeed, in this study, identification of the bacteria was restricted by budget; this meant that for majority of isolates only genera could be reliably confirmed. However, more information is needed on the specific species present on avian eggshells, as not all members in genus are pathogenic (Cowan and Steel, 1993). Future studies in avian egg microbiology need to understand the large budget involved with DNA sequencing, but also the necessity for accurate species identification. Some groups such as *Staphylococcus*, have both highly pathogenic species such as *S. aureus* and other more benign members such as *S. condimenti* (Bannerman and Peacock, 2007). The separation of these is vital for conclusions to be drawn about which bacteria are a threat to NIBK embryos, and for other avian embryos.

Pseudomonas, Staphylococcus, Streptococcus, Bacillus, Micrococcus, Enterobacter, Pantoea and *Serratia* are all highlighted in this study as bacterial genera that have the potential to impact NIBK hatching success. Future studies should now begin to expand and support our knowledge of NIBK hatching failure (see chapter seven, section 7.3) and begin to narrow down the list of bacteria above. Future work should identify the bacteria that are able to penetrate the shell, survive in the egg contents, and gain access to the embryo (see chapter six; and see chapter seven, section 7.3). Other studies could also narrow down the list of NIBK egg pathogens by directly testing the lysozyme resistance of the bacteria isolated, using NIBK lysozyme. NIBK lysozyme should be used in any tests conducted as it differs from chicken lysozyme (Osuga and Feeny, 1968; Prager and Wilson, 1974), (see chapter one, section 1.2.1). However, the difficulty and costs involved with isolating and purifying NIBK lysozyme may affect the feasibility of this experiment. Another experiment would be to test for virulence factors of the bacteria isolated off NIBK eggshells, the caterpillar of the Greater Wax Moth (Galleria mellonella) is commonly used to evaluate the virulence of a range of bacterial and fungal pathogens and would be a sensible method to use (Kavanagh and Reeves, 2004).

Chapter four: Identification of fungi on the shells of live, wild North Island Brown Kiwi eggs and potential impacts on hatching success

4.1 Introduction

Fungi have been shown to both facilitate bacterial penetration and cause hatching failure in domestic chicken (*Gallus gallus domesticus*) eggs (Bruce and Drysdale, 1994; Board and Tranter, 1995). Fungi present on the eggshells of both domestic chickens and domestic ratites (Deeming, 1995a; Shane and Tully, 1996) have been identified. However, for wild birds' eggs the majority of microbial studies have focused on bacterial contaminants and not on fungi (Brittingham et al., 1988; Houston et al., 1997; Baggot and Graeme-Cook, 2002). Although fungi have been noted on the eggs of wild Thrashers (Cook et al., 2003, 2005a, 2005b), Bluebirds and Swallows (Wang et al., 2011), to the best of my knowledge only wild House Sparrows (*Passer domesticus*) have had fungi identified from the eggs and embryo (Kozlowski et al., 1991a; Kozlowski et al., 1991b, c).

The avian egg has a myriad of defences to counter microbial attack; with the eggshell being the initial barrier (Solomon et al., 1994) (see chapter two, section 2.4). However, while these protective features are effective against bacteria, avian eggs have limited defences against fungi (Board and Halls, 1973). Avian eggs seem to lack any chitinases (proteins that break down fungal cell walls) (Board and Halls, 1973; Board et al., 1979). As well as this, some fungal species can also withstand the warm, dry conditions caused by incubation, thus overcoming this defence (Carlile et al., 2001). Finally, the shell cuticle is readily broken down by both hyphal growth and some fungal toxins which allows access to the egg contents by fungi and facilitates bacterial penetration, adding to the causes of egg hatching failures (Board and Halls, 1973).

The literature on fungal impacts on avian hatching in the wild primarily focuses on the fungal associations in the nest (Hubalek et al., 1973; Baggot and Graeme-Cook, 2002; Goodenough and Stallwood, 2010). Both pathogenic and non-pathogenic fungi have been found within wild birds' nests. Fungal isolates in the faeces of both wild and domestic birds have also been investigated and again pathogenic species have been found (Bangert et al., 1988; Fulleringer et al., 2006). Pathogenic species in both the nest and the faeces can have a significant impact of survival of both embryos and nestlings (Pinowski et al., 1994; Cook et al., 2005b; Goodenough and Stallwood, 2010).

The North Island Brown Kiwi (NIBK, *Apteryx mantelli*) is endangered and experiencing significant population decline even with intensive management (Holzapfel et al., 2008). The majority of this management focuses on the predator-vulnerable chick life stage. Predator control and Operation Nest Egg (ONE) are used extensively across the NIBK range, however, overall populations are still experiencing significant decline as NIBK chicks are not the only vulnerable life stage; over 60 per cent of eggs in the wild fail to hatch (McLennan et al., 1996). While predation and infertility do not seem to be significant causes of mortality, a large proportion of un-hatched eggs have microbial contamination (Potter, 1989; McLennan et al., 1996). In the previous chapter, I presented the bacteria genera present on the eggshells of wild NIBK eggs; these genera had the potential to affect hatching success. Currently no knowledge exists on the fungal flora of NIBK eggs in the wild, nor how it could impact hatching failure.

Information on which fungi are present on wild NIBK eggs and how these fungi impact hatching success is needed. The first aim of this study is to determine if fungi are present on wild NIBK eggs and if found, whether they have the potential to impact hatching success. I would expect higher levels of fungi to be present on NIBK eggs than on the shells of other wild birds studied (Cook et al., 2005a, 2005b; Wang et al., 2011), as NIBK have several factors that make them susceptible to microbial infection (see chapter one, section 1.2). The second aim of this study is to use the knowledge gained to make suggestions towards NIBK egg conservation.

4.2 Methods

The study site, study population and laboratory site are as listed in chapter one, section 1.6.

4.2.1 Data collection

The eggs used in this study were the same as per Chapter 3, section 3.2.1 (see table 4.1). Swabs from each sample were streaked onto Sabouraud Dextrose agar (Fort Richards, Auckland, New Zealand). Similar media has been used in other fungal studies examining the presence of fungi that can affect eggs on nests and in the soil (Hubalek et al., 1973; Pinowski et al., 1994; Cook et al., 2003, 2005b; Goodenough and

Stallwood, 2010; Korniłłowicz-Kowalska and Kitowski, 2013). Plates were then incubated in sealed containers with added humidity at 29^{o}C and checked regularly for fungal growth for up to three weeks. Each individual colony was given a laboratory identification code (LabID) and sub-cultured onto a new Sabouraud Dextrose plate. Once purity of each isolate was assured the sample was placed in glycerol broth and stored at -80^{o}C. Some fungi did not survive this step, and this is discussed later (see section 4.4.3).

Table 4.1: List of the fate and age of wild North Island Brown Kiwi eggs from Ponui Island swabbed for fungi. EggID = a unique code to identify each egg; the word at the beginning relates to the incubating male and the number indicates egg order. Egg age = age of egg when swabbed. All 20 eggs had a first swab taken, but only 11 of these had a second swab taken later in incubation.

EggID	Egg Fate	First Swab	Second Swab
		Egg age	Egg age
Bel1	Hatched	7	28
Bel2	Hatched	0	
Clel2	Failed	1	
Dale1	Failed	35	
Dale2	Hatched	13	
Dario1	Hatched	16	37
Dario2	Failed	15	
George1	Hatched	12	
Ivan1	Hatched	48	69
Ivan2	Hatched	30	51
Martin1	Failed	39	61
Martin2	Failed	60	
Max1	Failed	60	62
Max2	Failed	51	53
Murphy1	Failed	40	61
Murphy2	Hatched	35	56
Ponui1	Failed	48	69
Ponui2	Hatched	30	51
Roy1	Failed	20	
Roy2	Failed	15	

4.2.2 Method design

The first aim of this study was to determine if there are fungi present on the shells of NIBK eggs, and to identify them. The second aim was to use the results to direct future NIBK conservation and this was done by researching the potential for these fungi to impact hatching success and is in the discussion (see section 4.4).

4.2.2.1 Fungal identification

The Omnilog® fungal identification system was used initially as it performs 95 discrete tests simultaneously, providing information about the metabolic properties of each sample and comparing it to a database of over 297 yeast species and fungi from over 400 taxa and 120 genera. Methods followed that outlined in the Omnilog®. However, the Omnilog® identification system failed to identify all 10 isolates trialled (reasons for this are discussed in section 4.4.3). Thus, morphological characteristics were used to identify fungi to the most exclusive taxa possible. All filamentous fungi were stained with lactophenol cotton blue to allow the distinctive conidia and sporangia to be seen under the microscope (figure 4.1). Yeasts were unable to be classified further in this study due to budget constraints and lack of available expertise.

4.2.2.2 Determining potential impact on North Island Brown Kiwi embryos

Ideally, to test the impact of each bacterial isolate on NIBK embryos, the fungal genera would be tested by infecting a number of NIBK eggs with a fungal isolate and recording its effect on hatching success compared with uninfected eggs. However, due mainly to ethical constraints this could not be done and the identified fungi off the shell were instead researched thoroughly in the literature to identify if any genera could potentially impact NIBK hatching success. The search engines used are as described in chapter one (see section 1.6.4). As a large number of studies were carried out on the domestic chicken as early as the 1920's, and work has been published in wild birds as late as this year, I did not restrict the search by dates. Instead, searches were conducted for each fungal genera using key words (table 4.2). After downloading the manuscripts, I also retrieved any references from them that dealt with the subject.

As with the bacteria in chapter three (see section 3.2.2.2) the relationship between the fungi identified, and the resulting hatching success of the NIBK egg is also provided, as a microbe needs to be present to cause hatching failure (Bruce and Drysdale, 1994).

Table 4.2: List of the key words used in the literature research for the pathogenic and beneficial impacts of the fungi isolated in this project. This list was coupled with the fungal genera being researched. * = search for all words that start with the letters given. $ = search for word, and word plus plural.

key words		
General	Avian	Egg
Pathog*, negative, harm*, impact, infect*, ill*, disease, Benefi*, positive, good, prevent, protect*, mutuali*	General search terms plus avian, bird*, poultry, chick*, aves	General search terms plus egg$, embryo*, shell$

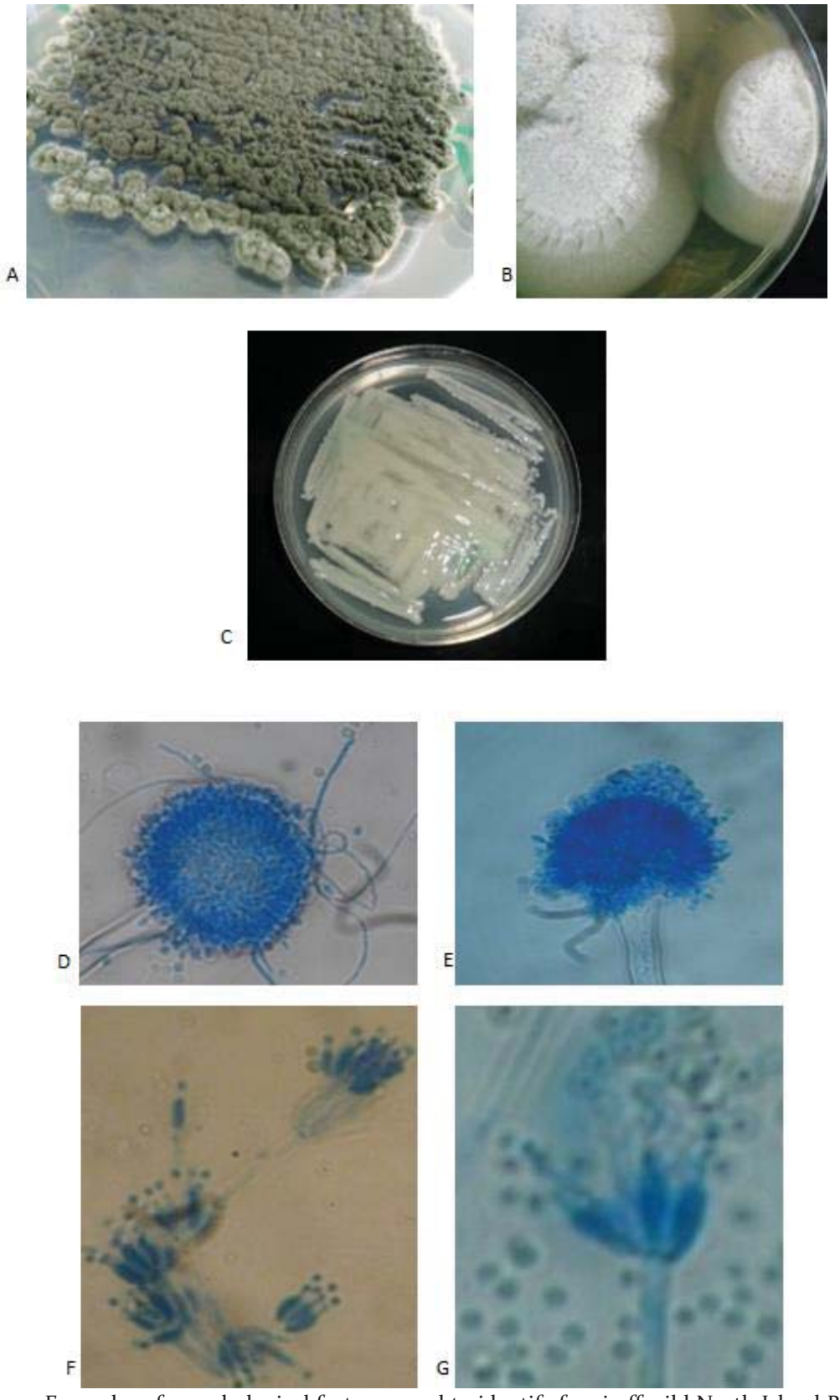

Figure 4.1: Examples of morphological features used to identify fungi off wild North Island Brown Kiwi eggshells. A) *Penicillium* growth B) *Aspergillus* growth C) yeast growth D) and E) *Aspergillus* conidia (50x) F) and G) *Penicillium* conidia (50x).

4.3 Results

Of the 20 NIBK eggshells sampled in this study, 13 had fungi isolated during some point in incubation (70%). Together, from these 13 eggs, 29 fungi were isolated and 23 of these were identified. Only fungi from the groups *Aspergillus, Penicillium* and yeasts were identified (see table 4.3). As mentioned, some isolates did not survive the -80^{o}C preparation and were not identified. Initial visual observation suggested that the isolates that did not survive were from different groups, as they had very different growth forms (i.e. both filamentous fungi and yeasts) and not just one species or genera died. Therefore, the results still represent a random, general fungal population isolated off these NIBK eggshells.

4.3.1 First swabs

The age of eggs when swabbed for the first time ranged from 0-60 days (see table 4.3). Of the 13 eggs that had fungal growth, only 10 had fungi isolated on the first swab. From these 10 eggs, 16 fungi were isolated and 13 were identified. *Penicillium* was the dominant genus on the eggshells, with 33 per cent of isolates being *Penicillium* growth. Twenty seven per cent of isolates in the first swab were from the genus *Aspergillus,* and 20 per cent were yeasts. Three of the isolates did not survive the -80^{o}C preparation and thus could not be identified.

4.3.2 Second swabs

The age of eggs when swabbed for the second time ranged from 28-60 days. Of the 11 eggs swabbed a second time, nine had fungal growth. Thirteen fungi were isolated from the second swabs, and 10 were identified. Yeasts (46%) and *Penicillium* (31%) were the dominant genera. Although *Aspergillus* spp. were isolated four times on the first swabs, they were not isolated on the second swabs. Three isolates did not survive the -80^{o}C preparation and thus could not be identified.

Table 4.3: List of the fungi isolated from wild North Island Brown Kiwi eggs of different ages from Ponui Island. EggID = a unique code to identify each egg; the word at the beginning relates to the incubating male and the number indicates egg order. Age = age when egg was swabbed. NG= no growth. X = fungal growth but isolate died before identification.

		First swab		2nd swab	
Egg ID	Fate of egg	Age (days)	Fungi ID	Age (days)	Fungi ID
Bel1	Hatched	7	*Penicillium*	28	*Penicillium*
Murphy1	Failed	40	*Aspergillus* yeast x	61	yeast
Ivan1	Hatched	48	*Aspergillus* *Penicillium* x	69	*Penicillium*
Ivan2	Hatched	30	*Aspergillus* yeast	51	*Penicillium*
Dario1	Hatched	16	*Aspergillus*	37	yeast
Max2	Failed	51	NG	53	yeast *Penicillium*
Murphy2	Hatched	35	NG	56	yeast yeast x x
Ponui2	Hatched	30	NG	51	x
Martin1	Failed	39	*Penicillium* yeast	61	NG
Ponui1	Failed	48	*Penicillium*	69	NG
Max1	Failed	60	x	62	yeast
Dale1	Failed	35	*Penicillium*		
Dale2	Hatched	13	yeast		

4.3.3 Hatching success of the North Island Brown Kiwi eggs in this study and the presence of fungi on them

As shown in table 4.3, seven (54%) of the NIBK eggs in this study hatched and six did not (46%). All three fungal genera were isolated from eggs that both hatched and did not hatch. Four of the failed eggs and two of the eggs that hatched had *Penicillium* isolated, while three hatched eggs and only one un-hatched egg had *Aspergillus* isolated. Four hatched and four un-hatched eggs had yeast isolated off the shells during some stage in the incubation period (see table 4.3). A larger sample size is needed before any conclusions can be drawn as to the correlation between fungal presence and hatching success of NIBK eggs (as discussed in chapter seven, section7.3.2).

4.3.4 Change in fungal composition (within and between eggs)

From the eggs swabbed twice, 11 (91%) showed a change in fungal composition during the incubation period, even over a short space of time. Of these 11 eggs, one had the same fungal genera as the initial swab, the remainder showed a change in the fungal genera between swabs (see table 4.3). Two of the eggs had only two days between first and second swab and one of these went from no growth, to two yeast species and a *Penicillium* species. The other egg could not be analysed fully due to the death of the first isolate.

4.4 Discussion

4.4.1 Fungal Presence

From the NIBK eggshells swabbed in this study, 70 per cent had fungi present. This level of contamination is higher than other wild birds' eggs, as was predicted. Cook et al., (2005a, b) four example found only between 40 -26 per cent of wild Pearly-Eyed Thrasher (*Margarops fuscatus*) eggshells had fungi present.

That a higher percentage of NIBK eggs had fungal contamination on the eggshell supports the initial hypothesis that factors specific to NIKB make them susceptible to microbial contamination (see chapter one, section 1.2).

4.4.2 Fungal identification

Aspergillus and *Penicillium* were the most common genera found on the shells of wild NIBK eggs, and yeasts were also isolated. All three groups of fungi have been found previously on the shells of the domestic chicken (Hadrich et al., 2013) and *Aspergillus* spp. have been isolated from the shells of domestic ratites (Deeming, 1995a; Shane and Tully, 1996). In wild birds, only fungi on sparrow eggs have been identified. On 139 House Sparrow and 24 Tree Sparrow eggs the only fungi identified were two *Candida* yeasts from two of the House Sparrow egg contents (see appendix one) (Kozlowski et al., 1991a; Kozlowski et al., 1991b). The presence of fungi has been noted, but no identification has been reported, on the shells of other wild birds (Cook et al., 2005b; Cook et al., 2005a; Wang et al., 2011). Therefore, this is only the third study to identify fungi present on wild birds' eggs, and the first to do so on living eggshells during active incubation.

More work has been done on the identification of fungi present in wild birds nest's and faeces. Fungi have been shown to be a substantial part of the microbial community within wild birds' nests (Goodenough & Stallwood, 2010). *Aspergillus*, *Penicillium* and yeasts, as well as a large number of other fungi, have all been isolated from the nests of wild birds (Hubalek et al., 1973; Goodenough and Stallwood, 2010). Cook et al (2003) placed freshly laid chicken eggs into the nests of wild Pearly-Eyed Thrashers (*Margarops fuscatus*). From these eggs, *Aspergillus* and yeasts were both identified, along with five other fungi genera and a large proportion of 'unknown' fungi. *Aspergillus*, *Penicillium* and yeasts have also all been isolated from the faeces of domestic and wild birds (Bangert et al., 1988; Fulleringer et al., 2006). Both the soil and the faeces of NIBK nests could be a source of the fungal contamination found on the NIBK eggs.

4.4.3 Potential impacts to North Island Brown Kiwi embryos and hatching success from Fungi found

Aspergillus and *Penicillium* can be a major source of mortality in adult birds, causing widespread death and disease (Pitt, 1979; Pier and Richard, 1992; Alley et al., 1999; Tell, 2005). However, the impact on the egg and the embryo has been less studied.

Although in chickens some species of *Aspergillus* and *Penicillium* have been shown to have severe impacts on the embryo (Pier and Richard, 1992; Jordan and Pattison, 1997; Olsen, 2000), in wild birds studies of the fungi present on the eggs has not been done. Fungi, including *Aspergillus*, *Penicillium* and yeasts have been identified from the nests of wild birds and are highlighted as potential pathogens for the embryo and nestlings (Hubalek et al., 1973; Kozlowski et al., 1991c; Pinowski et al., 1994; Tell, 2005; Goodenough and Stallwood, 2010; Kornikłowicz-Kowalska and Kitowski, 2013).

4.4.3.1 Potential impacts of *Aspergillus* and *Penicillium*

Some characteristics of *Aspergillus* and *Penicillium* make them particularly likely to cause infections and mortality of avian eggs. *Aspergillus* (Oglesbee, 1997) and *Penicillium* (Pitt, 1979) are extremely diverse and widespread genera found in soil, leaf litter and in the air, and although not every species within each genus is pathogenic, they are both characterised as having high pathological importance. Both groups contain many toxic species and many capable of causing cell destruction (Hubalek et al., 1973; Baker and Bennett, 2008; Geiser, 2009). Several characteristics of *Aspergillus* and *Penicillium* increase the likelihood of them impacting NIBK hatching success.

The morphology and growth of *Aspergillus* and *Penicillium* facilitates their penetration into the eggshell. The hyphal growth of both genera has been shown to have the potential to break through the protective egg cuticle and open the pores up to bacterial invasion (Board and Fuller, 1974). The conidia (asexually produced spores) of both genera are more than small enough to penetrate the pores of eggshells. For example, the conidia of *Aspergillus* spp. average 3-3.9 µm diameter (Hess and Stocks, 1969) and the conidia of *Penicillium spp.* 2-4 µm (Pitt, 1979), while the pores of NIBK eggs range from 10-100 µm (Silyn-Roberts, 1983).

Thermophily (the ability to persist at high temperatures) is another characteristic of some members within both fungal genera. This trait allows these fungi to persist at conditions outside the range of most fungi, with the optimal temperature for some species being the same temperature as incubated eggs, 35-37^{o}C. Some *Penicillium* species can survive at temperatures as low as 5^{o}C (Carlile et al., 2001), which means survival when the egg is un-incubated. *Aspergillus* and *Penicillium* species can tolerate,

grow and even thrive in dry and low nutrient conditions, and have the ability to enter dormancy in certain life stages (Carlile et al., 2001). Both these traits influence their virulence and facilitate their roles as a pathogen because they increase their ability to survive on the dry, potentially low nutrient eggshell.

Members of the *Aspergillus* genera have also been shown to have the ability to chelate, transport and store iron (Krappmann, 2008). This is a prerequisite for microbial pathogens as iron is essential for rapid growth (Haas, 2003). This trait could facilitate growth in the egg, as iron is limited within the albumen due to proteins such as ovotransferrin which chelate iron (Kobayashi et al., 1996; Krappmann, 2008).

Finally, there are the toxic secondary metabolites produced by both fungi groups, which increase their potential to negatively impact NIBK hatching success. Some of these toxins have been shown to cause death in young and adult birds but have also been shown to severely impact growth, development and survival of avian embryos (Scott, 1977; Todd and Bloom, 1980; Potchinsky and Bloom, 1993; Qureshi et al., 1998).

There are over 400 known secondary metabolites produced by *Aspergillus* species with over 70 of these being known toxins (mycotoxins) (Bartholomew, 2013). For example: aflatoxin, produced by some species of *Aspergillus*, is one of the most potent carcinogens known to man (Abundis-Santamaria, 2003). In the domestic chicken, exposure to aflatoxin has been shown to cause DNA damage in developing embryos, lead to reduced fertility, reduced hatchability of fertile eggs and increased embryo death (Todd and Bloom, 1980; Potchinsky and Bloom, 1993; Qureshi et al., 1998) . The *Penicillium* genus is also noted for the diverse array of mycotoxins produced by some species. Citreoviridin for example has been shown to lead to rapid death in a range of birds due to damage to the central nervous system (Scott, 1977); whilst penicillic acid has been shown to cause embryo death and also cell necrosis in several animal species (Ciegler et al., 1972; Scott, 1977). Some serious mycotoxins are produced by both *Aspergillus* and *Penicillium* species; for example, both genera produce citrinin and ochratoxin, which in turn cause serious health impacts to embryos and adult birds. Ochratoxin can cause liver and kidney damage in a range of animals and has also been

shown to be severely teratogenic (causes embryo malformations) in birds (Edrington et al., 1995). Ochratoxin causes rapid and excessive embryo cell death in chickens and has been linked with serious malformations in areas such as the spinal cord and hindgut (Wei and Sulik, 1996). Citrinin has been shown to cause kidney failure in chickens and can lead to embryo death (Flajs and Peraica, 2009).

Not all secondary metabolites have negative effects; the antimicrobial action of some of the secondary metabolites may in fact provide a benefit as they can restrict the growth of other, more pathogenic microbes that could increase egg health over the incubation period. Both groups also possess harmless species, which may have no impact on the egg or developing embryo. Benefits to the avian embryo, through mechanisms such as competitive exclusion, have been shown for bacteria (McCabe, 1967) (see chapter three), but no such work has been done on potential benefits of fungi. Fumagillin is a secondary metabolite produced by some *Aspergillus* spp. that is both an antibiotic and an antiprotozoal agent (Eble and Hanson, 1951). There are also secondary compounds produced by the *Penicillium* genera that have been shown to have anti-viral and anti-bacterial properties such as statolon (Kleinschmidt and Murphy, 1965; Pitt, 1979) and penicillin (Garrod, 1960). Although the fungi may exclude bacteria through the production of such antimicrobials, the hyphal growth could still compromise the shell structure and lead to a negative impact. The positive impact of fungi on avian eggshells is an area open to more research. However, the pathogenic characteristics of both *Aspergillus* and *Penicillium* species make them a threat to the hatching success of kiwi and further studies are needed to determine their impact.

4.4.3.2 Potential impacts of yeast

"Yeast" is the general term for two separate phyla of single-celled fungi, the Ascomycota and the Basidiomycota (Kurtzman, 1994). The yeasts are an extremely large and diverse group, the Basidiomycota alone has approximately 30,000 described species (Lachance, 2006; Kurtzman and Fell, 2006). Yeasts are present in almost every environment and are part of the natural flora of many organisms (Cole & Carter, 1990; Davenport, 1980); a large number of species are commensal or even mutualistic. Only

a few species of yeast have been implicated in animal disease (Cole and Carter, 1990). The main pathogenic yeasts encountered include: *Cryptococcus neoformans*, which has been shown to affect both the central nervous system and the respiratory system (Cole and Carter, 1990) and can cause cryptococcosis which is potentially fatal in both mammals and birds (Costa et al., 2010); and *Candida albicans*, which is prevalent on the skin and in the gut of many organisms yet can become pathogenic with symptoms ranging from mild irritation to death in immunocompromised individuals (Cafarchia et al., 2006). The yeasts in this study were not separated further due to budget and time constraints. Some yeasts have been shown to cause disease in birds, with the potential of being fatal (Cafarchia et al., 2006; Rippon et al., 2010). However, little research exists into the impact of yeasts on the avian embryo (Kozlowski et al., 1991c). Kozolwski et al. (1991c) examined the dead embryos of 49 House Sparrows and 19 Tree Sparrows; the only fungal infection was *Candida albicans* in one House Sparrow embryo. They suggests that the low, sporadic cases of infection by yeasts indicate that the avian eggshell provides a sufficient barrier to yeast penetration, they also state that the low penetration rate is a consequence of the lack of mobility and large size of most yeasts (Kozlowski et al., 1991c). However, before any conclusions can be drawn more research is needed to both identify the yeasts present on NIBK eggshells, and the impact of yeasts on avian embryos. Because of the lack of further identification, and the difficulties drawing conclusions about their potential impact, yeasts are not considered further in this study.

4.4.4 Change in fungal composition (within and between eggs)

The majority of the NIBK eggs in this study had a distinct fungal community composition, even though only three fungal types were identified. This community also seemed to be dynamic as the eggs swabbed twice over the incubation period showed community changes. No other studies to date have compared the change in fungal composition over time in wild birds' eggs; but this is the first evidence of how dynamic it may be.

Goodenough and Stallwood (2010) examined the interspecific variation in nest fungi in both the Blue Tit (*Cyanistes caeruleus*) and Great Tit (*Parus major*) and found that

there was considerable variability in the microbial assemblages in terms of presence and prevalence of microbes. If this is the case with NIBK nests then this may lead to the fungal composition changes on the egg; for this idea to be tested samples from the nest environment need to be examined. In addition, it should be acknowledged that the variation found in my study could be a consequence of the small sample size and more work is needed, with samples taken throughout the incubation period, to fully understand fungal composition over time (see chapter seven, section 7.3).

4.4.5 Impacts for North Island Brown Kiwi conservation from the results of this study

As NIBK are endangered there are restrictions on the type and amount of data that can be collected. Any study has risks that must be balanced against the positives that could be achieved. As no research into fungi and wild eggs in New Zealand has occurred, initial knowledge of the potential impact was important. This study has shown that fungi are present on the egg and that genera present have been shown to impact hatching success in other birds. The fungal genera found on NIBK eggs in this population could have significant impacts on hatching success, thus these results support further investigation into fungal growth on wild NIBK eggs.

Although these eggs are in a dirty environment and exposed to fungi regularly, this study also highlights the importance of sanitation when dealing with eggs of wild birds. Evidence suggests that the fungal communities are dynamic and could change in as little as two days. Thus, when handing eggs and birds in the wild, care needs to be taken to avoid cross contamination both from handlers and from other individual eggs and birds handled. We may be introducing new pathogenic fungi into that nesting environment. As well as a risk to other birds and eggs, these fungi also pose a risk to handlers. All three fungal groups have evidence of pathogenicity in humans (LoBuglio and Taylor, 1995; Loftus et al., 2005; Steinbach, 2008). These findings support the recommendations in the Kiwi Best Practice Manual (Robertson and Colbourne, 2003); care needs to be taken, and gloves worn when handling eggs and both hands and equipment needs to be sterilised between each egg and nest. However, the Kiwi Best Practice Manual also advises that when extracting ONE eggs from the nest, as the eggs

are cleaned upon arrival at the ONE facility (Bassett, 2012), dry bare hands are preferable to gloved ones, because bare hands increases the sensitivity to holding the egg (Robertson and Colbourne, 2003). The results of this project highlight that people working with NIBK need to recognise and acknowledge the potential impact microbes like fungi can have. Gloves should be worn at all stages of egg and bird handling (also see chapter seven, section 7.2.2).

4.4.6 Further advice for future work

Due to the distance between the field site and the laboratory there was a delay in getting samples plated. This could affect the results, as there may have been some fungal death during transit. Ideally, a laboratory closer to the study site should be chosen in future studies. However, most NIBK populations are far removed from cities and thus this may reduce the number of populations that may be available for this sort of research.

Fungal identification is notoriously difficult. Currently only 5-10 per cent of fungi species on earth have been named and scientifically described (Carlile et al., 2001). This study is one of the few to identify fungi from the shells of wild bird eggs, yet morphological characteristics only allowed for identification to the genus level. Initially, the Omnilog® fungal identification system was used which has a database of over 297 yeast species and fungi from over 120 genera, yet it failed to identify any of the isolates tested in it. *Aspergillus*, *Penicillium* and yeasts are all large groups, with many species within them. The reasons why the Omnilog® may have failed to identify the isolates are as discussed in chapter three, section 3.4.6. DNA sequencing methods for general fungi identification are still inaccurate and expensive (Ferrer et al., 2001) when compared to bacterial identification and this is why I did not attempt it in this study. Further identification is needed, as not all fungal species are pathogenic. However, this remains hindered by the lack of accurate and reliable methods.

A further issue faced in this study was fungi not surviving the glycerol preparation and -80^{o}C storage and therefore this method needs to be improved for any further studies. Higher fungi survival would improve sample size as each isolate is important and contributes significantly to the overall conclusions drawn from the data. Another

storage technique that could be trialled is the use of agar slants with a mineral oil overlay (McGinnis, 1980; Ramirez, 1982). One could also identify the isolates immediately once they have been ensured of purity and remove the reliance on storage. I suggest making multiple glycerols for each isolate to increase the chances of the isolate surviving. In this study most fungi survived the long-term storage method and this means they are available for identification in future work (see chapter seven, section 7.3).

As mentioned, some fungi have the ability to increase bacterial penetration into the egg contents (see chapter two, section 2.3.2) (Board and Halls, 1973; Baggot and Graeme-Cook, 2002; Cook et al., 2003). Future research could be undertaken to investigate if there is a correlation between fungal types on NIBK eggshells and the presence of microbes inside the eggs. This was not possible in this project as different NIBK eggs were used for shell and content analysis due to the restrictions on carrying out microbial work on the contents of NIBK eggs (see chapter five). Future work on the correlation of fungal presence and hatching success of NIBK eggs, with a larger sample size, would also be a beneficial line of research.

Chapter five: Identification of bacteria in the contents of un-hatched, wild North Island Brown Kiwi eggs and potential impacts on hatching success

5.1 Introduction

Bacterial infection inside the eggs of the domestic chicken (*Gallus gallus domesticus*) has long been known to cause both declines (Bruce and Drysdale, 1991) and increases in hatching success (Ribble and Shinefield, 1967). There is now growing evidence that bacteria could also be important factors reducing the hatching success of domestic ratites (Deeming, 1995a, 1996; Moore, 1996; La´Baque et al., 2003; Chang-Reissig et al., 2004) and wild birds (Kozlowski et al., 1991b; Peralta-Sánchez, 2010).

Avian eggs have a high nutrient content and the optimal temperature for growth of most bacteria is similar to the optimum temperature necessary for avian incubation, which make them highly suitable for microbial growth (Burley and Vadehra, 1989). Avian eggs are not defenceless, possessing several physical and chemical barriers against bacterial attack (see chapter two, section 2.4). In addition, parental behaviours such as incubation and providing specific nesting material may have an anti-microbial benefit (see chapter two, section 2.4.1) (Clark and Mason, 1985; Peralta-Sánchez, 2010). That avian eggs have several, complex defences against microbes suggests that microbes play an important role in avian evolution and supports the theory that microbes significant threat to the avian egg (see chapter two, section 2.4) (Peralta-Sánchez, 2010).

The North Island Brown Kiwi (NIBK, *Apteryx mantelli)* is endangered and experiencing significant population decline, even with intensive management (Holzapfel et al., 2008) (see chapter one, section 1.4). As discussed in previous chapters (see section 1.3 and 3.1) the main conservation strategies in place focus on predator control to protect NIBK chicks, yet only three out of every ten wild eggs successfully hatch. While predation and infertility are not high enough to explain the high hatching failure of NIBK eggs, previous studies have noted a high level of microbial contamination in contents of wild NIBK eggs (McLennan, 1988; Potter, 1989; McLennan et al., 1996; Ziesemann et al., 2011); and as shown in chapters three and four high levels of bacteria and fungi have been isolated off wild NIBK eggshells

However, having microbes on the shell does not indicate whether those microbes will be able to penetrate into the contents and affect the developing embryo. Bacteria

isolated from inside the egg pose more of a risk to the developing embryo than the bacteria isolated from the shell (Bruce and Drysdale, 1994). Thus, to fully understand the likely effect of microbes to NIBK eggs, it is necessary to investigate whether microorganisms can overcome the various defences of the egg and access the egg contents.

The aim of this study was to determine if bacteria are present inside NIBK eggs that could impact NIBK hatching success, the theory was that these bacteria would have a higher potential to impact the hatching as they have crossed the cuticle, shell and membrane barriers. I would expect higher levels of microbes present inside NIBK eggs than other wild bird's eggs studied due to the factors that make NIBK susceptible to microbial infection (see chapter one, section 1.2). In light of the results of this study, several suggestions are made in regards to NIBK conservation and egg hatching success.

5.2 Methods

Egg source and laboratory site are listed in chapter one (see section 1.2.2 and 1.2.3).

5.2.1 Justification for the use of un-hatched, infertile Operation Nest Egg eggs

I examined the bacterial contamination of NIBK eggshells (see chapters three and four) using living eggs from wild populations which were actively being incubated at the time of sampling. This was not possible for this study as it was the contents of the eggs, not the shell, which was being sampled. In order to test for contamination in the contents of NIBK eggs, the eggs must be opened in a way that would cause embryo death. As NIBK are an endangered species invasive techniques are unrealistic, therefore only un-hatched eggs were used in this study. By using un-hatched eggs insight was gained not only into what microbes can penetrate the defences of the NIBK egg, but as these eggs did not hatch, finding the bacteria in the contents could provide a link with hatching failure.

In wild populations, NIBK eggs are never removed from the nest if there is any possibility that they are fertile and alive. This increases the period that eggs are left in the wild after death, increasing the chance of secondary contamination. Therefore, to have access to eggs as early as possible, infertile or early embryo death eggs from ONE were used in this study.

Eggs are brought in to Operation Nest Egg (ONE) as early as 20 days of age and fertility is determined on site. By using infertile or early embryo death eggs I had access to the eggs as soon as they arrived at the facility. Early embryo death or infertile eggs are not sterilised or incubated upon arrival at the ONE facility, which meant that any microorganisms present were maintained as they were in the wild. However, there is always the possibility of contamination from handling and transport. While the Kiwi Best Practice Manual recommends latex gloves be worn during handling, and that transport occurs in a clean six-litre chillybin packed around with shredded paper, they also recommend that when ONE eggs are extracted from the nest bare hands be used as this increases the sensitivity to holding the egg (Robertson and Colbourne, 2003). Therefore, human contamination during handling and transport cannot be excluded (see section 5.4.5 for more consequences and 5.4.6 for solutions).

Using infertile or early embryo death eggs also is positive for my study because eggs with advanced growth have small volumes of yolk and albumen, which are often difficult to extract without contamination (Cook et al., 2005b). Cook et al. (2005b) used dead Pearly-Eyed Thrasher (*Margarops fuscatus*) eggs when examining the incidence of egg infection, they considered a conservative test of microbial infection because it is unlikely that dead eggs have a greater defence against infection that living eggs.

5.2.2 Method design

The aim of this study was to determine if the bacteria present in the contents of NIBK eggs could impact hatching success. This was achieved in two steps; identifying the bacteria present and researching the potential for those bacteria to impact hatching. The latter was achieved by looking at published studies of the microorganisms found and focussing on known pathogenicity and lysozyme resistance, which increases

survival in the egg contents (see chapter one, section 1.6.4). A list of the eggs and the ONE facilities they were provided by is listed in chapter one, section 1.6.3.

5.2.2.1 Bacterial isolation

All work was done in a biological safety cabinet in the laboratory. After the eggs arrived at the ONE facility, they were collected, taken to the laboratory and opened within 48 hours (see section 1.6.4).

All eggs had swabs taken from four locations; the shell, the outer membrane, the contents (albumen and yolk) and the inner membrane (see figure 5.1). As bacteria have different ability to penetrate the shell, and do so at different rates, these four swabs were taken from the various layers where the egg has defences to track the penetration of the bacteria. For example, presence of a bacterial isolate only on the shell and outer membrane would suggest limited penetration of that isolate. Due to budget restrictions, only the contents and the inner shell membrane isolates could be identified and the results of those are the only ones presented herein. However, the bacteria isolated from the other layers have been stored and are available for further work when funding becomes available (see chapter seven, section 7.3).

The swab of the shell was the first taken, to reduce contamination. The egg was then candled to determine if an embryo was present and to determine state and position of air cell. Weight, width and diameter of the egg were all measured. The torch and the tape measure used to do this was sterilised with 80 per cent ethanol between each egg to reduce cross contamination.

A hole was made over the air cell of the egg to open it. Sterile forceps were used to make a small crack and pull off the shell fragments (see figure 5.2). This was when the swab of the outer membrane was taken, from against the air cell. Then this membrane was pierced and two swabs were taken from the inside of each egg; the first from the contents, the second from the inner shell membrane.

Egg contents were then emptied and any evidence of an embryo was looked for to send for a *post mortem*, the egg contents were also visually described.

As mentioned, only swabs from the contents and the inner shell membrane were analysed further. Both content swabs were plated onto blood agar (Fort Richards, Auckland, New Zealand), placed in a CO_2 enriched environment and incubated at 36^{o}C; observations of growth were noted at 24 and 48 hours. Distinct bacterial types were visually determined and a colony re-plated onto a new blood agar plate. Once purity of these isolates was assured the sample was placed in a glycerol broth and stored at -80^{o}C.

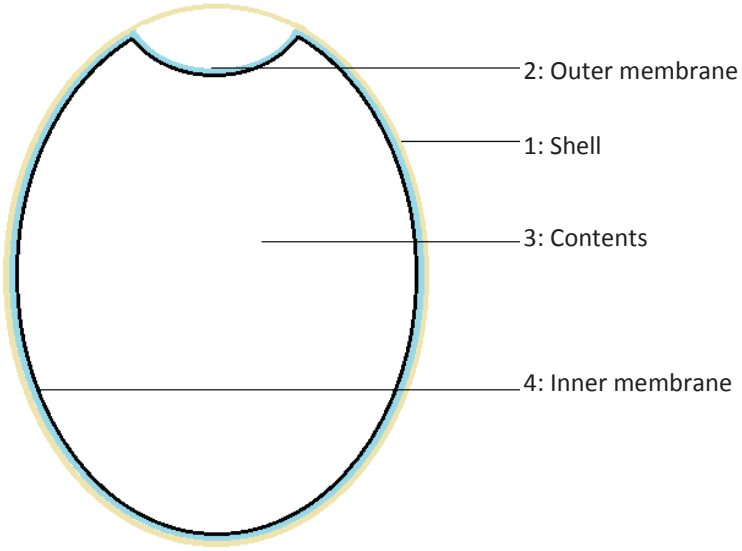

Figure 5.1: Locations of the four swabs taken from eggs for this study. Numbers indicate order swabs are taken in.

For eggs 9 and 10 the identification of the bacteria was not undertaken as the eggs were not suitable for this study. Egg 9 was from a captive bird thus outside the scope of this study. Egg 10 was cracked during transport and had been delayed in arriving to the laboratory by over a week; therefore, contamination from handling and transport could not be excluded. However, the information regarding these two eggs measurements, characteristics of their contents, and presence of bacterial contamination is given (see table 1.4).

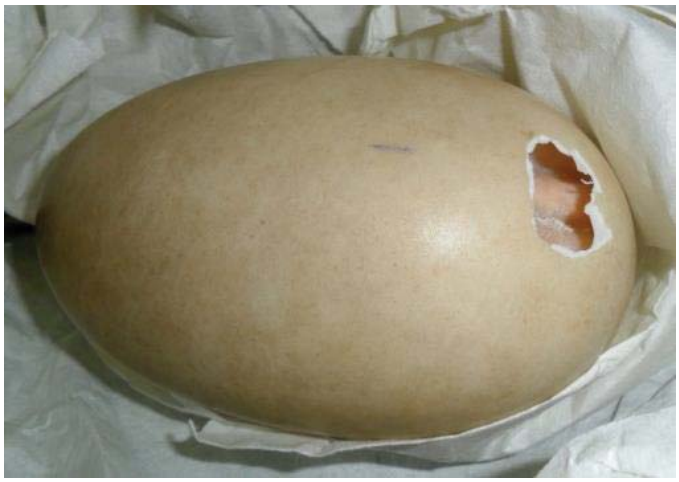

Figure 5.2: Example of the opening made to swab the contents of North Island Brown Kiwi eggs for bacteria. The opening is made over the air cell and the outer membrane is visible.

5.2.2.2 Bacterial identification

To identify the bacterial isolates sequencing of the 16S rRNA gene was used. This is the most common sequence used to study bacterial phylogeny and taxonomy as it is present and conserved in almost all bacteria (Janda and Abbott, 2007). Other studies on the bacteria isolated from NIBK eggshells used this method to effectively identify bacteria isolates to genera (see chapter three). Details about extraction and PCR optimisation are presented in Appendix two.

5.2.2.3 Determining potential impact on North Island Brown Kiwi embryos

Because each bacteria isolated from the contents could not directly be examined in regards to its effect on the hatching success of the NIBK embryo (see chapter one, section 1.5), two factors that could contribute to pathogenicity were investigated; lysozyme resistance and any known records of pathogenicity. Two factors that could lead to the bacteria being beneficial to the embryo were also investigated: any known records of a beneficial impact and the production of antibiotics or bacteriocins (see chapter one, section 1.5 and 1.6).

5.3 Results

5.3.1 Egg descriptions

Eleven eggs were obtained during the 2012 breeding season. The average weight was 411g (± Standard deviation (SD) = 47), length 32cm ((± SD = 2), width 24cm (± SD = 3) and volume 383ml (± SD = 54) (see table 5.1). None of the 11 eggs had any sign of an embryo, thus there were no *post mortems* carried out. Note that these eggs could be infertile or the embryo could have died before it was detectable, this was not tested in this study as it was beyond the budget but could be considered for future work. The contents of the eggs varied from looking 'normal' with clear albumen, bright yellow yolk and an intact air cell; to contents with degraded yolks, visible microbial infection and no air cells (see table 5.2).

Table 5.1: The size of eleven North Island Brown Kiwi eggs obtained for this study. Age and location of eggs is provided in table 1.4.

Egg #	Egg Size			
	Dimensions			Volume (ml)
	Weight (g)	Length (cm)	Width (cm)	
1	457	25	16	450
2	412	33	25	375
3	396	32	25	325
4	375	33	26	350
5	500	31	23	475
6	424	33	26	400
7	423	33	26	400
8	347	32	24	320
9	345	31	23	319
10	348	32	25	322
11	425	34	26	410

Table 5.2: Visual description of the egg contents of eleven North Island Brown Kiwi eggs obtained for this study. None of the eggs in this study had visible embryos present. Albumen: A normal/healthy looking albumen is clear/fluid; milky = albumen is a cloudy white colour with a watery consistency; watery = albumen; chalky = albumen is a grey colour, fluid but with gritty texture; chalky clumps = distinct clumps of solid, grey material; viscous = albumen has a thick sticky consistency. Yolk: A normal/healthy yolk is bright yellow and in a distinct intact sphere; viscous = yolk is intact but has a thick sticky consistency; pale yellow = yolk is intact but is a dull, pale yellow; thick black growth = intact yolk with distinct clumps of solid, black, furry material throughout; chalky clumps = intact yolk with distinct clumps of solid, grey material. Air cell: A normal/healthy air cell is intact, attached to the shell membranes and does not move; floating = air cell has detached from shell membranes and moves about egg contents; none = no air cell visible.

Egg #	Description		
	Albumen	Yolk	Air cell
1	Milky, chalky clumps	Viscous, pale yellow, thick black growth	None
2	Chalky	Pale yellow, viscous, chalky clumps	Floating
3	Clear/ fluid	Bright yellow, intact	Intact
4	Some clear, some chalky	Viscous, pale yellow	Intact
5	Viscous, chalky	No distinct yolk	Floating
6	Chalky liquid	Pale yellow, viscous, chalky clumps	Floating
7	Clear/ fluid	Bright yellow, intact	Intact
8	Chalky clumps	Bright yellow, intact	None
9	Clear/ fluid	Bright yellow, intact	Intact
10	Milky	Viscous, pale yellow	Floating
11	Milky, chalky clumps	Viscous, pale yellow, thick black growth	Intact

5.3.2 Bacterial presence

Of the 11 eggs swabbed in this study, nine eggs (82%) had bacteria isolated from both swabbed areas (see table 5.3). Only one of the 11 eggs had no bacteria isolated from either the contents or the inner membrane and one had bacteria on the inner membrane but not in the contents.

Ninety per cent of both the content and the inner membrane samples had one dominant bacterial type when plated (see table 5.3), the other bacteria on the plate were only present in one or two colonies (see figure 5.3). Of all the eggs showing growth, egg 10 was the only one with several colonies with heavy growth (see figure 5.3).

Table 5.3: The presence of bacteria from the contents (albumen and yolk) and the inner membrane of eleven un-hatched North Island Brown Kiwi eggs. Table shows if bacteria were isolated from the two areas, and if one bacteria type was dominant from that sample. ✓= yes, X = no.

Egg #	Bacteria isolated		Dominant growth	
	Contents	Inner membrane	Contents	Inner membrane
1	✓	✓	✓	✓
2	✓	✓	✓	✓
3	X	X		
4	✓	✓	✓	✓
5	X	✓		✓
6	✓	✓	✓	✓
7	✓	✓	✓	✓
8	✓	✓	✓	✓
9	✓	✓	✓	✓
10	✓	✓	X	X
11	✓	✓	✓	✓

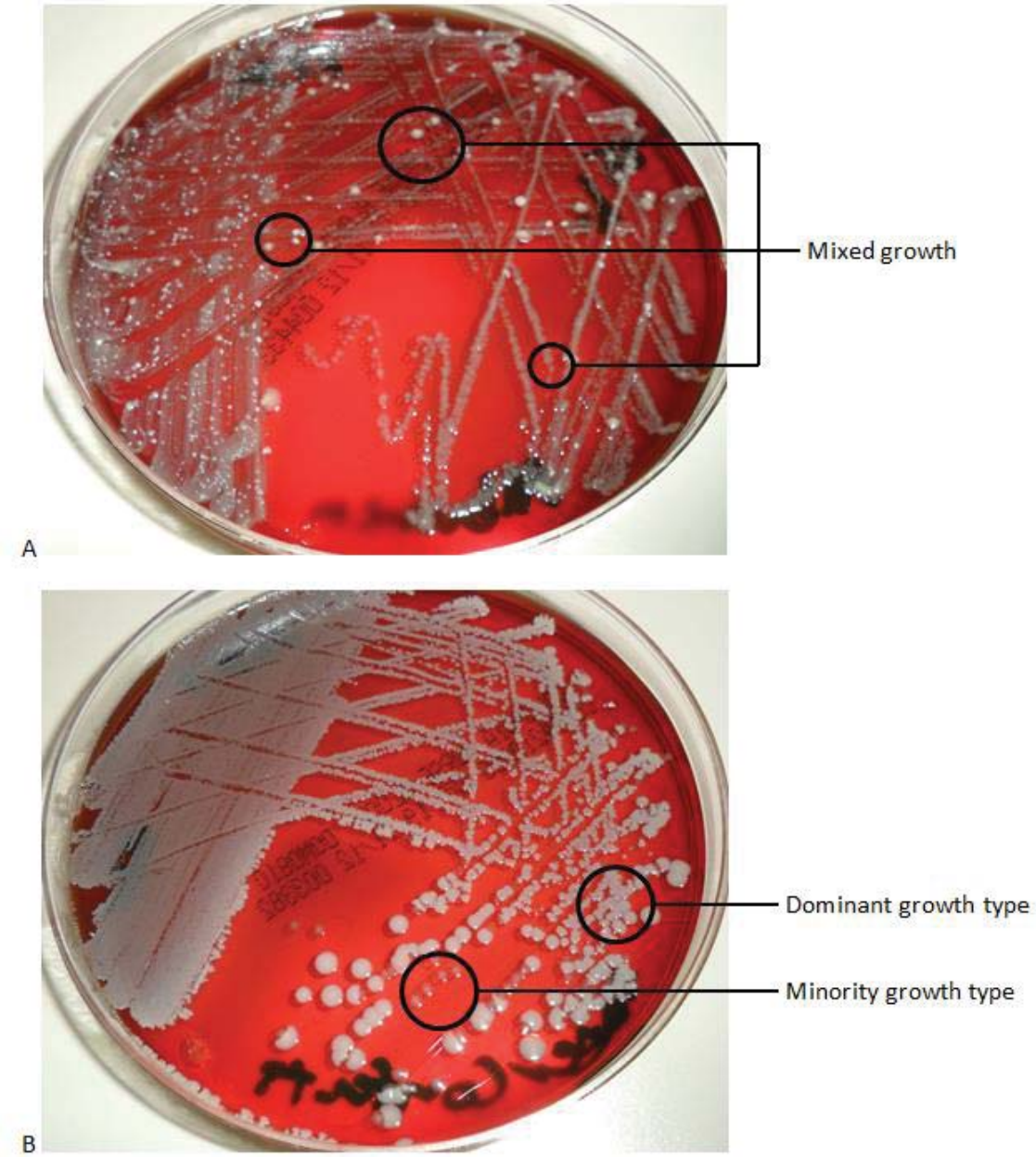

Figure 5.3: Blood agar plates showing comparison between A) dominant bacterial growth (egg 8) and B) mixed growth (egg 10) from the contents (albumen and yolk) of a North Island Brown Kiwi egg.

5.3.3 Bacterial identification

As discussed (section 5.2.2.1) eggs nine and 10 were not analysed, and egg three had no bacterial growth therefore only eight eggs had the bacteria isolated identified. From these eight eggs 20 bacterial colonies were isolated to the most exclusive taxa possible (see table 5.4). The bacteria isolated and identified were from two phyla, six different genera and four families. The most commonly isolated bacteria were Gram positive *Staphylococcus* and Gram negative enterics from the Enterobacteriaceae

family *(*genera *Escherichia, Serratia* and *Buttiauxella*). Gram positive rods of the genera *Bacillus* and *Paenibacillus* were both isolated from the contents of a single egg (see table 5.4). Two of the isolates could not be identified due to problems extracting the DNA and attempts to identify these had to be halted due to time and budget constraints.

Table 5.4: Bacteria isolated from the contents (albumen and yolk) and the inner membrane of eight un-hatched North Island Brown Kiwi eggs. Some shells had multiple isolates from the same genera, these are included in the table as I cannot exclude them being from different species Underlined bacteria = the bacteria type that showed dominant growth in the sample. NG = no growth from sample. X = sample died during culture and could not be identified. * = genera or species found for the first time in wild eggs.

Egg #	Contents	Inner membrane
1	*Staphylococcus caprae** *Buttiauxella* izardii**	*Staphylococcus condimenti** *Staphylococcus simulans**
2	*Escherichia coli*	*Escherichia coli*
4	*Staphylococcus* spp. *Serratia* spp.	*Serratia fonticola*
5	NG	*Bacillus* spp. *Paenibacillus* taichungensis** *Paenibacillus** spp.
6	*Staphylococcus hyicus**	*Staphylococcus hyicus**
7	*Staphylococcus hyicus**	*Staphylococcus hyicus**
8	*Staphylococcus* spp. *Staphylococcus* spp.	X X
11	*Escherichia* spp.	*Escherichia* spp.

5.3.4 Potential pathogenic impacts on North Island Brown Kiwi eggs from bacteria isolated

The genera isolated show mixed characteristics in regards to lysozyme resistance and records of pathogenicity (see table 5.5). Four of the genera isolated from inside NIBK eggs have lysozyme resistant members. Records of lysozyme resistance could not be found for two genera, *Buttiauxella* and *Serratia*, but because lysozyme works by attacking peptidoglycans found in the cell walls of bacteria, it is ineffective against most Gram negative bacteria due to their thin cell wall (Board, 1966; Ibrahim et al., 2000), therefore to be conservative these two Gram negative genera have been assumed to have lysozyme resistance.

All of the genera found in this study have been shown to be pathogenic in humans or other animals, and four of the six genera (67%) isolated have been shown to be avian pathogens that can also impact avian embryos (*Bacillus*, *Escherichia*, *Serratia* and *Staphylococcus*).

It should be noted that all bacteria were isolated from inside the egg contents. As all the eggs were intact, and had no large cracks, this means these bacteria had overcome the shell barrier and therefore pose a bigger risk to the developing embryo (Bruce and Drysdale, 1994).

5.3.4 Potential beneficial impacts on North Island Brown Kiwi eggs from bacteria isolated

The genera isolated show mixed characteristics in regards to antibiotic or bacteriocin production and records of beneficial impact (see table 5.6). Five of the genera (83%) isolated from inside un-hatched NIBK eggs have members that produce antibiotics or bacteriocins. Four genera (67%) have proven to be beneficial to some avian species and *Staphylococcus* spp. have been shown to provide direct protection for avian embryos from pathogenic bacteria. Information could not be found on the production of potentially beneficial substances for one of the genera (see table 5.6).

Table 5.5: Results of a literature search on the potential pathogenic factors of the bacterial genera isolated from inside un-hatched North Island Brown Kiwi eggs. Lysozyme resistance indicates if members of genera are resistant to the action of lysozyme: resistant = all members resistant; mixed = some members show resistance to lysozyme; likely resistant = no information but genera is Gram negative, which are typically resistance to the action of lysozyme due to the cell wall structure. Known pathogenicity indicates if any records exist of genera being pathogenic: general = records of pathogenicity in any animal or human; avian = records of pathogenicity in adult/ young of any bird species; egg = records of pathogenicity in avian embryo and avian eggs. ✓ = records present, x = no instance of pathogenicity, * = no information found. References indicates source used.

Genus	Lysozyme resistance	Known Pathogenicity			References
		General	Avian	Embryo	
Bacillus	Mixed	✓	✓	✓	(Dockstader, 1952; Peckham, 1959; Cole, 1990a; Cowan and Steel, 1993; Logan et al., 2007)
Buttiauxella	Likely resistant	✓	*	*	(Carter and Chengappa, 1990; Cowan and Steel, 1993; Kampfer, 2005)
Escherichia	Resistant	✓	✓	✓	(Brittingham et al., 1988; Carter and Chengappa, 1990; Cowan and Steel, 1993; Barrow, 1994; Olsen, 2000; Silvanose et al., 2001; Fudge, 2001; Scheutz and Strockbine, 2005)
Paenibacillus	Mixed	✓	*	*	(Logan et al., 2007)
Serratia	Likely resistant	✓	✓	✓	(Peckham, 1959; Izawa et al., 1971; Carter and Chengappa, 1990; Fudge, 2001; Grimont and Grimont, 2005a; Grimont and Grimont, 2006; Abbott, 2007)
Staphylococcus	Mixed	✓	✓	✓	(Thorne et al., 1976; Brittingham et al., 1988; Cole, 1990b; Cowan and Steel, 1993; Olsen, 2000; Silvanose et al., 2001; Fudge, 2001; Götz et al., 2006; Bannerman and Peacock, 2007)

Table 5.6: Results of a literature search on the potential beneficial factors of the bacterial genera isolated from inside un-hatched North Island Brown Kiwi eggs. Antibiotic or bacteriocin production indicates if members of the genera are known to make these substances. Known benefit indicates if records exist of genera being beneficial; general = any instance of beneficial impact on any animal or human; avian = records of benefit in adult/ young of any bird species; egg = records of benefit in avian embryo and avian eggs. ✓ = records present, * = no information found. References indicates source used.

Genus	Antibiotic /bacteriocin production	Known Benefit			References
		General	Avian	Embryo	
Bacillus	✓	✓	✓	*	(Lechevalier, 1975; Tagg et al., 1976; Lim and Kim, 2009; Soler et al., 2010)
Buttiauxella	*	*	*	*	
Escherichia	✓	✓	✓	*	(Robbins et al., 1957; Wooley et al., 1999; Gillor et al., 2004; Gordon and O'Brien, 2006)
Paenibacillus	✓	✓	✓	*	(Piuri et al., 1998; Stern et al., 2005)
Serratia	✓	*	*	*	(Lechevalier, 1975; Grimont and Grimont, 2006; Gordon et al., 2007; Chavan and Riley, 2007)
Staphylococcus	✓	✓	✓	✓	(McCabe, 1965; Tagg et al., 1976; Cogen et al., 2008)

5.4 Discussion

5.4.1 Bacterial presence

Previous studies have noted a high prevalence of microbial contamination inside wild NIBK eggs (McLennan, 1988; Potter, 1989; McLennan et al., 1996; Ziesemann et al., 2011). However, this study is the first to identify bacteria present in the contents of un-hatched wild birds in New Zealand.

Ninety per cent of the NIBK eggs examined had bacteria present in the contents. The amount of contamination in the infertile NIBK eggs in this study was higher than other infertile bird's eggs studied as was predicted. Sixty seven per cent of infertile House

Sparrow (*Passer domesticus*) eggs and 74 per cent of infertile Tree Sparrow (*Passer montanus*) eggs examined had bacteria present in the egg contents (Kozlowski et al., 1991b). In contrast, only 16 per cent of dead Pearly-Eyed Thrasher (*Margarops fuscatus*) eggs with little to no embryo development had bacterial contamination in the egg contents (Cook et al., 2005b).

5.4.2 Bacterial identification

I isolated bacteria from two phyla, four families and six different genera from the NIBK egg contents. Gram positive *Staphylococcus* were the most commonly isolated, Gram negative enterics from the Enterobacteriaceae family were also common within the contents.

Gram negative Enterobacteriaceae have been shown to be a dominant contaminant in other avian eggs and while *Staphylococcus* is present in the contents of other birds' eggs, the level of contamination recorded in NIBK eggs is higher than in other wild birds' eggs studied (Mayes and Takeballi, 1983; Bruce and Drysdale, 1991; Kozlowski et al., 1991b; Bruce and Drysdale, 1994; Board and Tranter, 1995; Deeming, 1995a, 1996; Cook et al., 2003, 2005b; Wang et al., 2011).

Many of the bird species investigated in previous studies are either domestic, or tropical cavity nesting passerines and all have short incubation periods (see appendix one) (Mayes and Takeballi, 1983; Bruce and Drysdale, 1991, 1994; Board and Tranter, 1995; Cook et al., 2003, 2005b). While in comparison NIBK are burrow nesting birds, breeding in temperate winter, have extremely long, intermittent incubation and the first laid egg can be left exposed for up to 15 days before it is incubated (Andrews et al., 1990; Folch, 1992). The differences in bacteria in the NIBK eggs, such as the high levels of S*taphylococcus*, could be a result of the different climates and nest conditions the eggs experience. In support of this concept, other studies that have been carried out in temperate climates have also found high levels of S*taphylococcus* in the egg contents of wild birds' eggs (Kozlowski et al., 1991b; Wang and Bessinger, 2009).

The dominance of Enterobacteriaceae such as *Escherichia* spp. has been seen in most wild birds' egg contents studied. Although similar, the bacterial types isolated from the

contents of wild birds' eggs are not identical between species. In this study there is a notable absence of the genus *Pseudomonas*, which has been highlighted as common bacteria in the eggs contents of domestic chicken (Mayes and Takeballi, 1983; Bruce and Drysdale, 1994), domestic ratites (Deeming, 1995a, 1996; La´Baque et al., 2003) and other wild birds' (Houston et al., 1997; Cook et al., 2005b). Gram positives were also frequently found from the genera *Micrococcus* and S*treptococcu*s inside wild bird's eggs (Mayes and Takeballi, 1983; Bruce and Drysdale, 1991; Kozlowski et al., 1991b; Bruce and Drysdale, 1994; Board and Tranter, 1995; Wang et al., 2011), but were absent in this study.

However, *Micrococcus*, S*treptococcu*s and *Pseudomonas* were isolated from wild NIBK eggshells (see chapter three, section 3.3.2). The absence of these genera from the contents may be because they lack the ability to penetrate the defences of the NIBK eggshells, or due to the small sample of this study. More research into the ability of certain bacteria to penetrate the NIBK eggshell is needed before conclusions about the absence of these genera can be drawn (see chapter six).

Two of the genera and six of the species isolated in this study have never been noted within the egg contents of wild birds (see table 5.4). The presence of different bacterial genera is not surprising due to differences in species; climate, nest type and parental care, as all three can all impact bacterial contamination of eggshells (see chapter two, section 2.3). As well as this, most previous studies have only identified bacteria to genera level, therefore little published information on the species present within wild birds eggs exist (see chapter one, section 1.6.4).

5.4.3 Potential threats to North Island Brown Kiwi embryos and hatching success from bacteria found

Almost 70 per cent of the genera isolated in this study have been shown to cause declines in avian hatching success and every genus isolated has lysozyme resistant members. Although no work has been done on the pathogenic impacts of the bacteria isolated on the hatching success of NIBK, the results of this study highlight *Escherichia, Bacillus, Staphylococcus* and *Serratia* as potential threats to NIBK hatching success.

Of the ten eggs that had bacteria growth, nine had dominant growth of one bacterial type. *Staphylococcus* was dominant in five of these cases. Dominance on the plate would suggest that there were more *Staphylococcus* colonies present on the swab from the egg contents than other bacterial genera, which suggests that this genus was dominant inside the egg also. This supports the conclusion that *Staphylococcus* is a genus that could have significant impacts on NIBK hatching success. However, my findings are not a direct measure of dominance and density studies should be undertaken in future work in order to fully understand the community dynamics within these wild NIBK eggs (see chapter seven, section 7.3).

It is hard to disentangle bacteria that are primary invaders and impacted the embryo and those that are secondary contaminates after the embryo had already died (Cook, et al., 2003, 2005a, 2005b). Until research onto the impact of specific bacteria on NIBK embryos has been undertaken, none of the genera isolated can be ruled out as potential pathogens. Further work is needed before conclusions on pathogenicity can occur (see section 5.4.6).

5.4.4 Potential benefits to North Island Brown Kiwi embryos and hatching success from bacteria found

The beneficial role of bacteria in avian hatching in general has received less attention than pathogenic impacts (Lombardo et al., 1996; Soler et al., 2008; Soler et al., 2010; Peralta-Sánchez, 2010). As with pathogenic bacteria, benefits provided by certain bacterial genera has not been investigated for NIBK.

The majority (83%) of the genera in this study have been shown to produce substances, such as antibiotics or bacteriocins, which restrict the growth of other bacteria. Antibiotics produced by four of the genera isolated have been shown to directly impact avian health. Members of the *Staphylococcus* genera have been shown to cause direct benefit to chicken embryos by competitive exclusion. Both *Escherichia* (Robbins et al., 1957; Wooley et al., 1999; Gillor et al., 2004; Gordon and O'Brien, 2006) and *Paenibacillus* (Soler et al., 2010) members have been shown to impede the establishment of pathogenic bacteria in avian guts. Finally, *Bacillus* members have

been suggested to prevent the establishment of pathogenic bacteria in the nest environment due to antibiotic production (Soler et al., 2010).

Therefore, the results of this study highlight *Staphylococcus, Paenibacillus*, *Escherichia* and *Bacillus* as having potential species that could provide benefits to NIBK eggs. This should be investigated in future work (see chapter seven, section 7.2.3).

5.4.5 Impacts for North Island Brown Kiwi conservation from the results of this study

As with chapter three, the result of this study suggest that microbes could play a part in NIBK hatching success and support the need for further research into microbial impact on NIBK hatching success.

In regards to NIBK conservation in New Zealand, the conclusions of this chapter are the same as in chapter three and four (see section 3.4.5 and 4.4.5); future work needs to focus on the egg stage. Predator control alone is not the answer to NIBK conservation and the results of this study again stress that ONE should not be phased out based on solely predator control. This study also emphasise the need for gloves to be worn at all stages of egg and bird handling (also see chapter seven, section 7.2.2).

The results of this study also have implications for NIBK chicks, as with the bacteria found on the shell (see chapter three, section 3.4.5), the bacteria found within the NIBK egg are a potential source of contamination for the chick. As well as growing and hatching from the egg contents, NIBK chicks retain a large yolk sac that they use for nourishment (Prinzinger and Dietz, 2002). This would suggest that the chick would be in direct contact with any infection of the egg contents, and indeed infections of the yolk sac have been shown to kill NIBK chicks in the wild (Wilson, 2013). More work is needed to determine the impact pathogenic shell microbes have on the chick, and I would suggest future studies identify the bacteria found in NIBK chick yolk sac infections and compare them with the bacteria found in this study to see if similar genera and species are present.

5.4.6 Study limitations and future work

Because of the endangered status of NIBK, two major restrictions were placed on the eggs that could be collected. One such restriction was the age of the eggs when they were collected. Most eggs in this study were collected at 40 plus days of age and it is unknown at what age these eggs died. ONE eggs are typically left in the nest until 41-57 days to allow for embryo development to occur uninterrupted, as studies have shown that collecting eggs at this age increase hatching in captivity as it reduces embryo malposition's and developmental issues (Colbourne et al., 2005; Robertson et al., 2006). However, even though the hatchability of NIBK eggs in captivity increase with age at collection, these eggs are at constant risk, and the longer they are left in the wild the higher the chance of bacterial contamination. The presence of potentially pathogenic bacteria within these ONE eggs indicates NIBK eggs are at risk while in the wild, and supports the need for more research into NIBK incubation so that the recommended age of 41-57 days could be reduced and the risk of egg losses in the wild lowered (Robertson, 2003).

As with any study on bacteria in wild birds, drawing conclusions as to if the bacteria caused embryo death is difficult, as it is hard to distinguish between primary and secondary contamination (Romanoff and Romanoff, 1972; Cook et al., 2003, 2005b; Cook et al., 2005a; Peralta-Sánchez, 2010). As with the NIBK eggs used in chapters three and four, there is the risk of human contamination from the handler. However, potential primary invaders can be identified by looking at factors such as: dominance, abundance and evidence from other studies. This is study used dominance and evidence from other studies, but more work is needed to draw definitive conclusions about primary and secondary invaders. *Post mortems* can be undertaken to test for bacterial contamination within the embryo and this would be what I would suggest for future work. In this study no *post mortems* could be carried out on embryos as all eggs were deemed infertile or early embryo death however, future studies need to consider the age of the eggs and the possibility of secondary contamination before they interpret their results (see chapter seven, section 7.3 for more future work).

The classification of the bacteria within this study was also restricted by budget. However, most other avian egg studies have only provided identifications of avian egg bacteria to genera, and some only provide identification to group level (see chapter one, section 1.6.4). As discussed in chapter three (see section 3.4.6) future work on avian egg microbiology needs to focus on the specific species present, as not all members in genus are pathogenic (Cowan and Steel, 1993) (see chapter seven, section 7.3). To expand the results of this study, future research is needed to fully understand the microbial impacts on NIBK hatching (see chapter seven, section 7.3). The ability of the bacteria to overcome the NIBK eggs defences is a logical place to start (see chapter six).

Chapter six: Initial steps in the development of a non-destructive method to test the penetration of bacteria through the North Island Brown Kiwi eggshell

6.1 Introduction

Some bacteria and fungi have long been known to impact the avian embryo; yet not all microbes have the same effect on hatching success. Some microbes cause high embryo mortality (Bruce and Drysdale, 1991, 1994) and some cause increased hatching success (McCabe, 1965, 1967), but the impact of most is unknown. Domestic chicken (*Gallus gallus domesticus*) eggs have been used to experimentally investigate microbial impact on hatching success. The majority of studies measure the impact of serious human pathogens like *Salmonella* and *Staphylococcus* directly on the embryo. In these studies, the effect on the embryo was measured by injecting the bacteria into the egg contents, or into the embryo itself (Vadhera et al., 1970; Bruce and Drysdale, 1991). The resulting reduction in hatchability provides an indication of pathogenicity, for example, *Staphylococcus aureus* reduced hatchability to zero when placed into the air cell of chicken's eggs (Bruce and Drysdale, 1991).

The presence of a microbe on the shell of a bird may not have any influence on hatching success (Bruce and Drysdale, 1991). Several characteristics influence the ability of an organism to impact on the health of the embryo: does it produce toxins or antibiotics? Can these interrupt embryo development, affect the growth of other bacteria or alter the content conditions? However, these factors are less significant if the bacteria in question cannot penetrate the egg's primary defensive barrier: the shell.

The ability of bacteria to penetrate through the shell has been investigated in domestic chicken's eggs. Typically whole eggs are submerged into a bacterial suspension, incubated for some time and the growth of the target organism in the contents noted (Haines and Moran, 1940; Bruce and Drysdale, 1991). Other studies have used whole eggs filled with agar and a tetrazolium solution (Triphenyltetrazolium chloride, TTC) (Board and Halls, 1973; Sparks and Board, 1985; Neill et al., 1985; Baxter-Jones, 1991; De Reu et al., 2006). These eggs are exposed to microbes on their shells and incubated. Microbial growth reduces TTC to formazan, which is a distinctive red colour and is used to indicate microbial penetration. A shell fragment method for testing the penetration of *Salmonella* through the domestic chicken's eggshell has also been developed. In this

method, fragments of shells that were placed on top of agar were exposed to bacteria. Penetration is then measures using scanning electron microscopy (SEM) to determine the precise location of the bacterial cell within the shell (Nascimento, 1992).

Microbes are a concern for wild birds too, yet there is little research in this area. Microbes are present on wild live eggs (see chapters three, four and five) (Cooper, 1993; Houston et al., 1997; Cook et al., 2005b; Beissinger et al., 2005) and hatching success has been shown to increase when eggshells are cleaned (Cook et al., 2005b). The direct impact of any specific bacteria or fungi on wild bird embryos is poorly understood. This is not surprising, as the methods used for chickens rely on large sample sizes and freshly laid eggs with low levels of contamination (Williams and Whittemore, 1967). Most involve opening the egg and other invasive techniques which compromise the embryo e.g.: Neill et al. (1985); De Reu et al. (2006). In order to assess the impact microbes have on embryos of wild birds, a different approach than that used for chicken eggs needs to be taken. The use of invasive techniques is not only unethical but there are stricter controls around what can be done on wild birds, especially in endangered species where each egg is vital to population growth and therefore unavailable for experimentation. Furthermore eggs in the wild are exposed to far more contaminants than domestic eggs in controlled conditions; as such uncontaminated eggs are an unlikely expectation (Soler et al., 2010).

North Island Brown Kiwi (NIBK; *Apteryx mantelli*) are endangered (Holzapfel et al., 2008) and as such each egg is vital to the population. As discussed in chapter one, eggs in the wild experience high hatching failure and microbial infection has been proposed as an explanation (McLennan, 1988; McLennan et al., 1996). Chapters three, four and five in this study showed that microbial genera shown to impact hatching success in other birds have been found on the shells and in the contents of wild NIBK eggs. However, the impact of these microbes on the hatching success of NIBK embryos is as yet unknown.

Placing potential pathogens on the shells of fertile NIBK eggs to research hatching success, as has been done in chickens (Neill et al., 1985; De Reu et al., 2006), is impossible because each NIBK egg is important to the population. For this reason, the

only eggs that are available for research are un-hatched eggs coming from Operation Nest Egg (ONE) (see chapter one, section 1.3). Working with ONE eggs has its own difficulties such as: limited sample size, variable egg condition, variable age of eggs, and unknown time and cause of death (see chapter five, section 5.2.2.1). Any method must acknowledge and adapt to these difficulties. Because of this, the aim of this study was to develop a non-destructive method to determine if a given microbe could penetrate through the NIBK eggshell and thus have the potential to affect the embryo and hatching success.

I wanted to develop a method that not only would acknowledge the difficulties of working with ONE Kiwi eggs but would also be easily repeatable by Kiwi researchers and be cost effective. The idea was that this method would allow researchers to determine which microbes have the highest chance of impacting hatching success and therefore would be an indication of where future work efforts should lie in the field. This would allow for a focus on the most potentially significant microbes and could lead to a reduction in the need for invasive techniques, as well as a reduction in the number of bacteria identifications as these can be difficult, time consuming and costly (see chapters three and five).

In this chapter, I describe the various techniques I used in trying to develop this method and the finding that impeded my ability to accomplish the ultimate task of having a finalised, useable method. I have included this information as a chapter in this thesis, although a method was not fully developed, for two reasons; one it occupied a large amount of my research time and two it showed the degree of contamination that is present in wild NIBK eggs, which is central to this thesis.

6.2 Method development

6.2.1 Shell fragment method

The idea of this method is simple: a fragment of NIBK shell will be placed onto a dish of agar, the target microbe is placed on top of the shell, the plate incubated and the growth monitored (see figure 6.1). If the microbe was capable of passing through the shell, I would find it in the agar after a period of incubation. The value of this technique is that each egg can be used to test for multiple microbes as different fragments can be assigned different treatments; also using fragments allows for the inclusion of cracked or damaged eggs, which couldn't be used if a whole egg method was in place. Using egg fragments is a way of adapting to the limited sample size and variable egg conditions of ONE eggs. This method was modelled off the shell fragment method designed by Nascimento (1992). Nascimento (1992) used SEM to investigate penetration of salmonella into chicken eggshells. However, this method had to be adjusted to the use of NIBK eggshells, the use of multiple bacteria and also the budget of this study. Where possible I have explained the differences between the methods, for example I used visual identification of penetration instead of SEM due to the restricted budget.

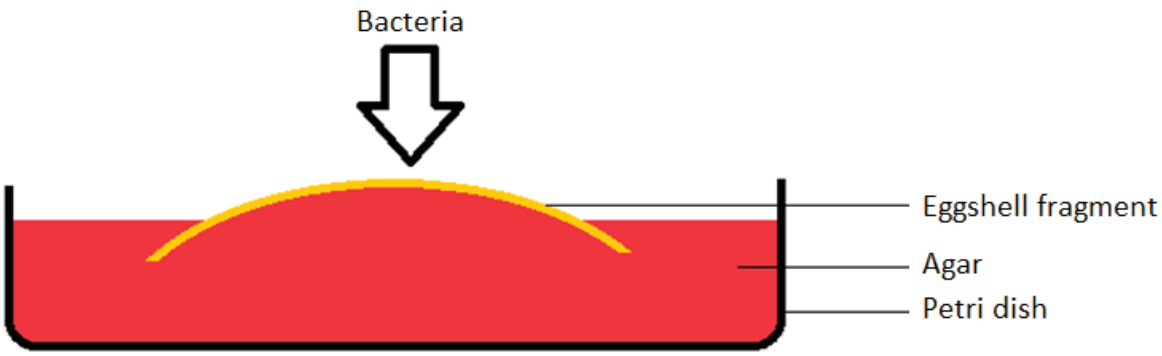

Figure 6.1: Layout of shell fragment method. Indicating ideal shell fragment placement in agar and bacterial placement onto shell.

All work was carried out in the laboratory as outlined in chapter one (see section 1.3.2). Where possible, all work was done within a biosafety cabinet (BSC) except for example: transport between autoclave and other machines used such as the water

bath. The eggs were those used in chapter five and were sourced from the ONE facilities outlined in chapter one (see section 1.3.4).

6.3 Pilot trials

To develop this method I undertook eight pilot trials, one for each of the various steps needed in the process. I will next provide a justification and description of each of these trials and their outcomes.

6.3.1 Trial 1: Agar concentration

6.3.1.1 Justification

Because the shell fragment was set into the agar, pre-poured plates could not be used. Agar must be made up and then poured into the petri dish before the shell fragment could be placed on top. The consistency of the agar needed to be liquid enough to pour easily and flow under the shell fragment without bubbles forming. The agar also needed to be solid enough that the shell did not sink and the shell surface remained above the agar. Nascimento (1992) used a concentration of 0.8 per cent agar, however, as NIBK eggs have a different structure to chicken eggs (Silyn-Roberts, 1983) the agar concentration had to be tested for NIBK eggs. Note that in some trials, blood unavailable at the time that the NIBK eggshells came into the lab, therefore it was omitted and sterile water used to maintain the correct agar concentration. This did not affect the results as bacteria still grew on the non-blood agar plates.

6.3.1.2 Method

Nutrient agar, brain heart infusion (Fort Richards, Auckland, New Zealand) and horse blood (Venous Supplies, Waikato, New Zealand) were used to create the blood agar. The concentration of the agar and sterile water was varied to determine consistency; however, the brain heart broth (3.7%) and the blood (5%) were kept at the same concentrations to ensure nutrient levels were equal across tests.

The agar (of various concentrations, see table 6.1) and the broth were mixed together with water and sterilised in an Getinge steam steriliser autoclave (Getinge Group, Auckland, New Zealand). The agar was cooled to 20^{o}C before the horse blood was

added and then the blood agar mix was poured into sterile petri dishes. The amount of time taken for the agar to set, as well as the consistency of the agar when poured, were the factors used to decide the best agar concentration. Each concentration was trialled three times.

This method resulted in sterile agar. I tested for contamination, of the petri dishes, the agar alone with no blood, the horse blood and the blood agar mixture as a whole by incubating at 36^{O}C. No growth after 48 hours was used as an indication of sterility.

6.3.1.3 Results

Both 0.8 and 0.7 per cent agar were too runny to use, the agar just flowed over the top of the shell fragments. The best consistency was the 1.4 per cent agar; it was solid and held the shell fragment well but set too quickly, which made it hard to work with. The 1.2 per cent agar had a similar consistency to 1.4, being solid and holding the fragment in place well, however as it set a little slower, it allowed more time to ensure correct placement of the shell fragment and thus was deemed the best concentration to use (see table 6.1).

Table 6.1: Comparison of different the agar concentrations in regards to setting time and consistency. Each are ranked in order of suitability (1=most suitable, 5= least suitable).

Agar concentration (%)	Setting time rank	Consistency rank
1.4	3	1
1.2	1	2
0.9	2	3
0.8	4	4
0.7	5	5

6.3.2 Trial 2: Emptying egg contents

6.3.2.1 Justification

Before the shell could be used for this experiment, the contents had to be removed. This had to occur in a way so that the contents would not contaminate the shell, and with a method that would not overly damage the shell structure. Nascimento (1992) used a dental drill (NM 3000, Nouvag, Switzerland) to open his chicken eggshells and the contents were simply poured out. This could not be done for the NIBK eggshells as the drill could not be obtained.

6.3.2.2 Method

Initially I developed a suction method to remove the contents from chicken eggshells. A vacuum flask and sterile 1ml pipette were used to suck out the egg contents, the pipette had 5 mm of the tip cut off using a sterile scalpel blade to create a bigger hole (see figure 6.2a). A small hole was created at the sharp end of the egg using a blunt chisel and forceps (see figure 6.2b) where the pipette could be inserted. This method allowed for a smaller opening to be made than would be required for tipping out the egg contents and reduced the amount of egg content present on the membranes, combined these reduced the chance of contamination from opening the egg. To test if this method caused contamination, four chicken eggs, which had had their contents removed via the suction method, were filled with agar and incubated at 36^{o}C for 48 hours.

Although the suction method above resulted in little contamination, it could not be trialled for NIBK eggs as the NIBK eggs used in this study were also used to determine bacterial presence in the egg contents (see chapter five) because the sample size for both experiments was already small. Therefore, these eggs had to have their contents emptied in a way that allowed for embryo growth to be observed and swabbing of the contents to take place as described in chapter five. Thus, the suction method was not suitable and instead, the NIBK eggs were opened in a BSC using the blunt chisel and forceps like above. At this stage a swab was taken from the egg contents (see chapter five, section 5.2.2), then instead of being 'sucked out', the contents were poured into a

beaker to look for embryo development. These shell fragments where then immediately used for this experiment.

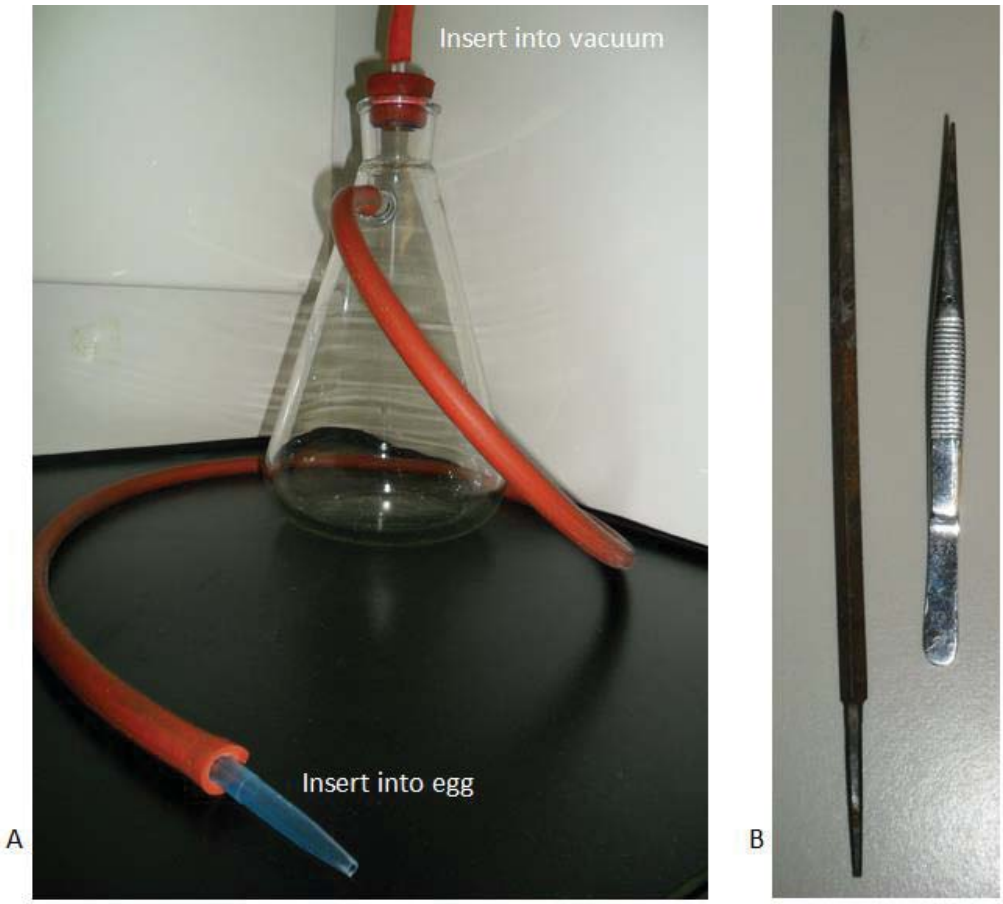

Figure 6.2: Tools used to suck egg contents out of whole eggs. A) Suction flask to connect to vacuum and pipette with 5mm of the tip cut off to insert into eggs. B) Tools used to make opening in egg.

6.3.2.3 Results

Three (75%) out of the four of chicken eggs that had their contents removed via the suction method showed no microbial growth, indicating the suction method did not introduce excess contamination into the eggshell or membranes. The fourth egg had mixed growth of two colonies, which could have been in the egg already or due to handling error.

It should be said that some contamination of the eggshells might have occurred when the eggs were opened. However, the use of sterile tools and the BSC reduced contamination, and the fragments were sterilised later (see Trial 6: Egg cleaning).

6.3.3 Trial 3: Eggshell fragment size

6.3.3.1 Justification

The size of the fragment to be used in penetration studies of eggshells was important: it had to be large enough to allow for the bacteria to be placed on the upper surface and small enough to fit in the petri dish with a closed lid. An egg has three distinct regions in the shell, with different curvatures; the blunt end, the equator and the sharp end (see figure 6.3). The curvature of the shell needed to be considered as the more curvature in the shell, the smaller the fragment that can be cut to fit the fragment into the petri dish.

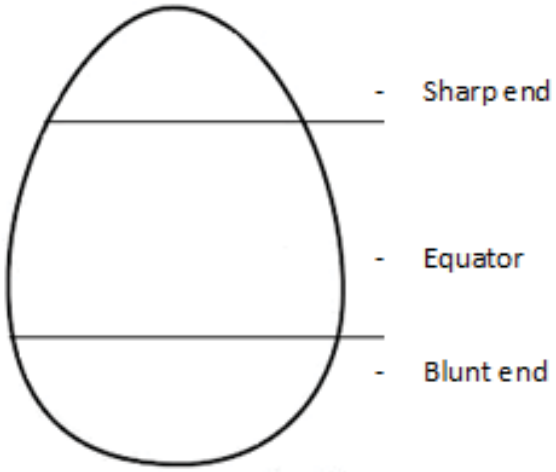

Figure 6.3: Diagram of the three distinct areas of avian eggshells.

6.3.3.2 Method

Five chicken eggs were used to trial fragment size. Nascimento (1992) used 1cm^2 fragments of chicken eggshells from the equatoral region, however I wanted to determine the size that would best fit the petri dishes I was using, and also provide a larger surface area than Nascimento (1992) used.

Therefore, fragments were taken from the three regions and had differing sizes ranging from 1-5cm^2. Using the results from the chicken egg trial, one NIBK egg was also used to trial fragment size. In both cases, the fragments were placed on petri dishes to determine the size that would fill the two criteria.

6.3.3.3 Results

The best size fragment was taken from the equator of the chicken's egg and was 3-4cm^2. When NIBK eggshells were trialled, due to the larger size of the egg around the equator there was less curvature, so fragments could be made smaller to obtain a larger sample size per egg (see figure 6.4). However, this resulted in the shells being more difficult to cut and therefore I considered that 3cm^2 was still the best size for an eggshell fragment. This size was easy to cut, large enough to place bacteria on the surface, and small enough to allow the petri dish lid to close.

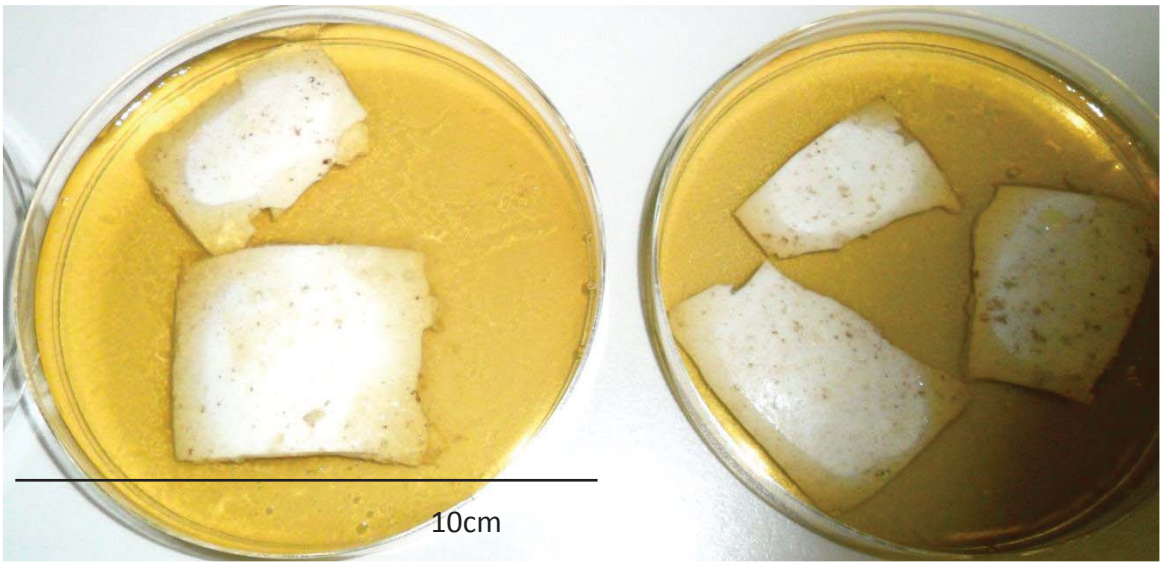

Figure 6.4: Comparison of different sized North Island Brown Kiwi eggshell fragments used to determine best size for shell fragment method.

6.3.4 Trial 4: Cutting the eggshell fragment

6.3.4.1 Justification

When determining the best method for cutting the shell fragment I had to ensure that the right size fragment could be cut, while also avoiding shattering the shell or causing any cracks. Damage to the shell alters the structure and could alter results by increasing penetrability. Cutting the eggshell cleanly with reduced cracking minimises the impact on shell structure, but any cutting is potentially compromising the shell structure and this must be factored into any conclusions drawn from the results. Nascimento (1992) again used the dental drill for this stage, but I had to trial tools that were avaliable to me.

6.3.4.2 Method

Chicken eggshells were used initially and several tools were trialled (see table 6.2). Chicken eggshells are thinner and more fragile than NIBK eggshell and this was considered when trialling tools.

All cutting occurred in a BSC, latex gloves were worn at all stages. The blades of all tools were placed in ethanol and flamed before cutting the shells and all tools were wiped down with ethanol after use.

To test if egg contamination occurred by cutting, open agar plates were placed in the BSC while eggs were being cut. Plates were then incubated at 36^{o}C for 48 hours. This was a way of measuring contamination within the BSC and also if any contamination was occurring by the technique itself, i.e. the eggshell cutting.

6.3.4.3 Results

The Ozito™ 170W rotary tool kit was the best tool to cut NIBK eggshells. It cut cleanly, was easy to use and allowed for small fragments to be cut (see figure 6.5). No growth occurred on any of the plates from in the BSC, indicating that during this process the eggs were not being exposed to environmental bacteria.

Table 6.2: Comparison of tools used to cut both chicken and North Island Brown Kiwi (NIBK) eggshells. Rank indicates suitability (1=most suitable, 4 = least suitable). Only top three chicken egg tools were trialled on NIBK eggshells (- =not trialled).

Tool trailed	Chicken eggshells	Rank	NIBK eggshells	Rank
Scissors	Crushed shell completely	3	Difficult to use, caused large cracks	3
The renovator deluxe™ multi-tool	Power kept dropping making clean cutting difficult. Clean line when cut.	2	Did not cut though well, caused large cracks	2
Ozito™ 170W rotary tool kit	Easy to use, minimal cracking	1	Cut clean lines, easy to use, no visible cracking	1
Bosch™ PMF 180E Multi	Blade too wide made cutting of small fragments difficult	4	-	-

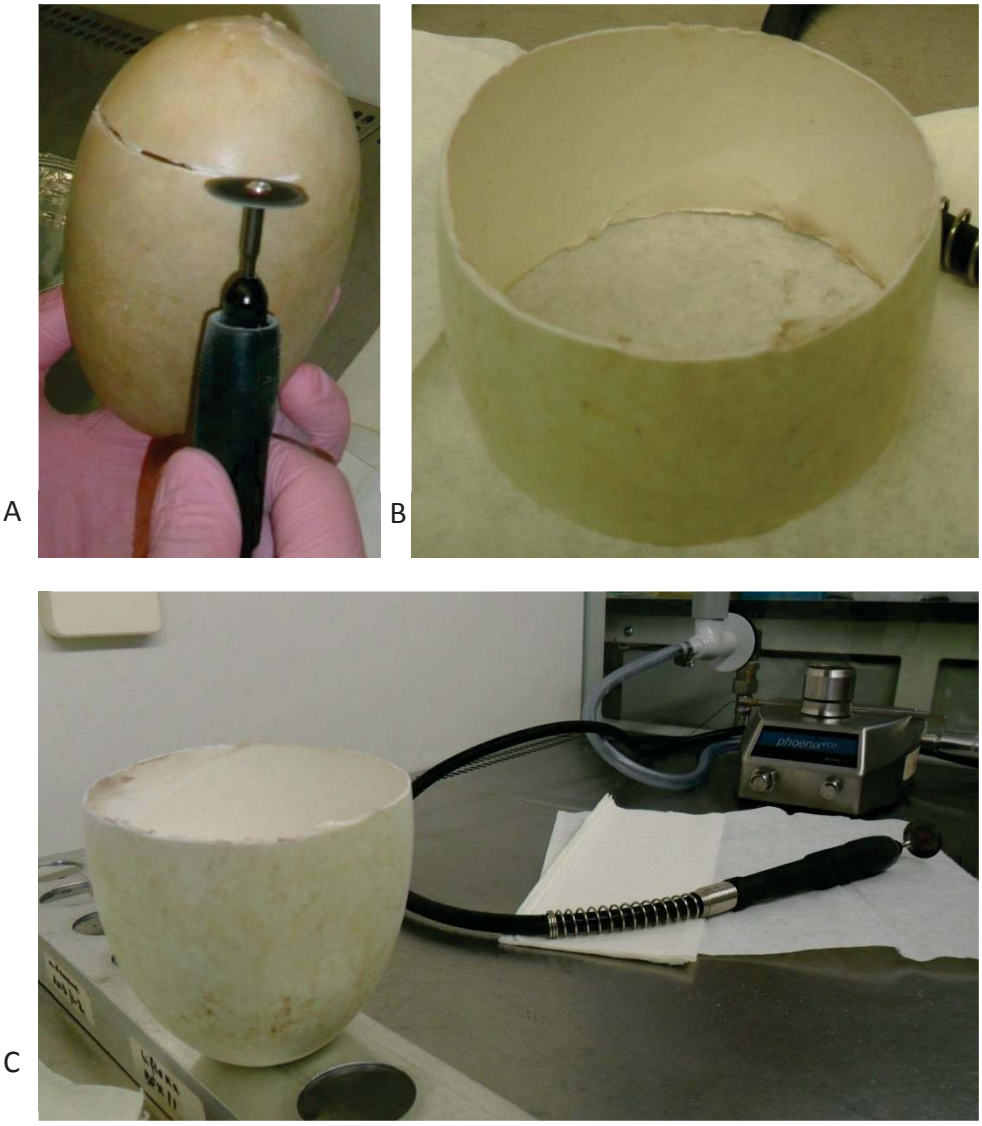

Figure 6.5: Example of the cutting of a North Island Brown Kiwi eggshell using the Ozito™ 170W rotary tool kit in a biologica biosafety cabinet (BSC). A) initial cut of the shell, note the use of latex gloves and the thin, clean line obtained with the tool B) example of the equatorial region of the eggshell that has been cut out and will be divided into fragments and C) example of the blunt end of the eggshell removed, note the BSC and the rotary tool.

6.3.5 Trial 5: Fragment placement

6.3.5.1 Justification

The fluid blood agar was poured into petri dishes and the eggshell fragment placed on top (cuticle side up). Nascimento (1992) mentioned that air bubbles needed to be elminated but did not elaborate on how the shell fragment should be placed. In this study the placement of the fragment had to meet two criteria: 1. to minimise air

bubbles, and 2. ensure that no agar touched the upper surface of the shell, because if agar is coating the shell surface then microbes could grow down this surface agar layer and not through the shell.

6.3.5.2 Method

Several attempts were made to place chicken eggshell fragments onto the fluid agar without air bubbles forming and without the surface of the shell being covered in agar.

6.3.5.3 Results

The best method to reduce the amount of air bubbles the under shell was to tilt the shell when placed into the agar. In the end, one side of the fragment was placed down first and then the shell was lowered into the agar so that the agar coated the bottom surface of the shell and stopped air bubbles forming. Care was needed to ensure that agar did not get onto the upper surface of the shell. To ensure no bubbles were present, as a final stage I checked underneath the plate. If small bubbles were present, tapping the petri dish on the bench or lifting the edges of the shell removed them.

6.3.6 Trial 6: Egg cleaning

6.3.6.1 Justification

Nascimento (1992) used *Salmonella* as the target microbe in his experiment. In his study the eggs were just rinsed in distilled water, as *Salmonella* can be easily isolated and distinguished as it differs from the normal microflora of chicken eggs they did not need to be sterile. However, in my study the bacteria being tested were ones that had been isolated from wild eggs. Therefore, in order to determine that the bacteria placed on the shell was the same bacteria that grew through the shell, and not for example a microbe already within the shell, the egg fragments needed to be sterilised prior to use. Effectively, any method used to clean eggshells could potentially compromise the shell structure and increase the penetrability by the bacteria being tested. For example harsh, abrasive cleaning has been shown to remove the cuticle layer (Sparks, 1994; Rideout, 2012). The cleaning method chosen in this study ideally should not overly compromise the shell structure.

6.3.6.2 Method

Several eggshell cleaning methods were initially trialled using chicken eggshell fragments and then NIBK eggshell fragments (see table 6.3 and table 6.4). I used two controls, unclean shells and shells washed using distilled water, I expected both of them to allow the growth of bacteria. Ethanol, ultraviolet light and a combination of both were used, as these methods are often used to disinfect equipment and surfaces (Macpherson, 2004). Autoclaving and pressure cooking were also trialled because these methods are known to be able to sterilise medical equipment (Rutala and Weber, 1999). ONE uses Incusan™ to clean the NIBK eggs they receive (Bassett, 2012), This was not available to me in this study however, chemical cleaning, such as ethanol and Incusan™ may only disinfect, and not sterilise, the shell fragment (A. Midwinter pers. comm.).

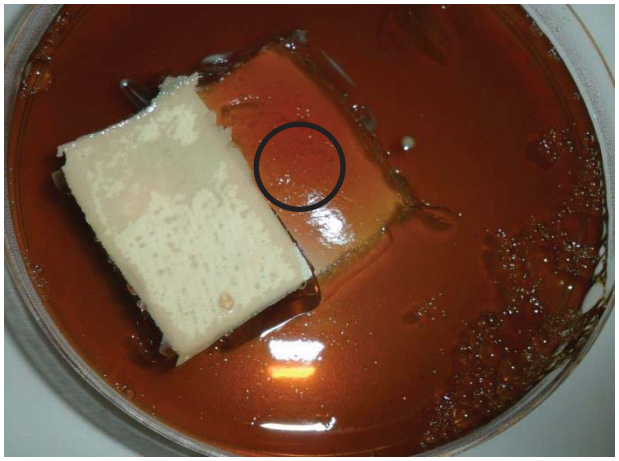

Figure 6.6: Photo of a North Island Brown Kiwi eggshell fragment being checked for contamination after a sterilising trial. The shell fragment has been taken off the agar, the black circle indicates where to swab under shell to check for contamination.

Once the shells had been cleaned by the designated method, they were set into agar using the methods described in trials one and five and incubated at 36^{o}C for 48 hours. The shells were visually checked after 24 and 48 hours incubation. However, visually observing growth on the surface of the agar was not enough to determine penetration, as microbial growth is not always visible on the surface (see trial eight). The agar under the shell fragments needed to be swabbed and re-plated to be certain no growth occurred (see figure 6.6). Therefore, after 48 hours incubation each shell fragment was

removed from the agar and the surface of the agar under the shell was swabbed (see figure 6.6) and plated onto a new blood agar plate. This re-sub was incubated for further 48 hours at 36°C to confirm bacterial growth.

Table 6.3: List and description of the eight methods trialled in this study to attempt to clean eggshell fragments.

Cleaning method trialled	Description of method
No cleaning	No cleaning attempt was made
Sterile water	500ml of water was squirted onto both sides of the shell fragment using a sterile wash bottle
Ethanol	500ml of 80% ethanol was squirted onto both sides of the shell fragment using a sterile wash bottle
Ultra-violet light (UV)	Shell fragment was placed in a petri dish and held under UV light for 15 minutes
Ethanol and Ultra-violet light (UV)	500ml of 80% ethanol was squirted onto both sides of the shell fragment using a sterile wash bottle. The shell fragment was then placed in a petri dish and held under UV light for 15 minutes
Autoclave (dry run)	Shell fragment was sealed in an autoclave sterilisation bag and autoclaved in a Getinge steam steriliser (Getinge Group, Auckland, New Zealand) for 20 minutes at 121°C
Autoclave (fragments set in agar)	The shell fragment was set in agar in a petri dish, this was then autoclaved in a Getinge steam steriliser for 20 minutes at 121°C
Pressure cooker	Shell fragment was placed into a beaker then put in an Model 925 All American Pressure Cooker (All American Cooker, Hillsville, Virginia, USA), for 20 minutes at 15 psi and 120°C

To determine if contamination was occurring due to handling error, swabs were also taken from two of the NIBK shell fragments directly after coming out of the pressure cooker trial.

6.3.6.3 Results

Initial trials using un-cleaned eggs resulted in growth on the agar for both chicken and NIBK eggshells as expected. Sterile water failed to clean chicken eggshells and bacterial growth was seen after incubation; for this reason, I did not use cleaning with distilled

water on NIBK eggs. Ethanol, ultraviolet light and a combination of both resulted in a reduction in bacterial growth on the chicken eggs, but there was still heavy growth on the NIBK shell fragment. When the chicken eggshells were place straight into the autoclave without agar, the shell membranes visibly shrived and pulled away from the shell. When the NIBK eggshell was placed into the autoclave without agar, the membranes did not visibly dry out but heavy growth still occurred when the agar under the shell was swabbed after 48 hours. The chicken eggs that were set in agar before being placed in the autoclave did not dry out, but the agar bubbled up vigorously over and around the shell fragment. This bubbling could result in the agar being pushed through the pores, which would compromise the results of this study as it could increase the penetration of bacteria, thus this method was abandoned. Instead, a pressure cooker was used, as in my experience this creates a wetter environment than the autoclave. The pressure cooker successfully sterilised the chicken eggs however NIBK eggs still showed growth after 48 hours (see figure 6.7). Both NIBK eggshell fragments that were swabbed directly after the pressure cooker treatment showed heavy growth, indicating that the contamination was present before cleaning took place.

Figure 6.7: Comparison of the bacterial growth present after attempts to sterilise North Island Brown Kiwi eggshells. Fragments were pressure cooked at 15 psi for 20 minutes, then set into agar and incubated at 36°C for 24 hours and 48 hours.

Due to restraints around sample size, time and budget, attempts to sterilise NIBK eggs had to be halted at this stage. Identification of the bacteria that grew after the cleaning trials was out of the budget of this study, but samples have been kept in storage for future work (see chapter seven, section 7.3).

Table 6.4: Comparison of the sterilising treatments used on North Island Brown Kiwi (NIBK) and chicken eggshell fragments. Fragments were treated, set in agar and incubated. Observations of bacterial growth were noted visually after 24 hours, then checked by a re-sub of the agar under the shell after 48 hours. - = not tested.

Treatment	Chicken eggshells		NIBK eggshells	
	24 hour (visual check)	48 hour (under shell agar swab)	24 hour (visual check)	48 hour (under shell agar swab)
No cleaning	Growth	Growth	Heavy growth	Heavy growth
Sterile water	Growth	Growth	-	-
Ethanol	Growth Less than control	Growth	Heavy growth	Heavy growth
Ultra-violet light	Growth Less than control	Growth	Heavy growth	Heavy growth
Ethanol and UV light	Growth Less than control	Growth	Heavy growth	Heavy growth
Autoclave (dry run)	No growth Membranes dried out completely	No growth	No growth	Heavy growth
Autoclave (fragments set in agar)	No growth Agar bubbled over shell surface	No growth	-	-
Pressure cooker	No growth	No growth	No growth	Growth

6.3.7 Trial 7: Bacterial infection method

6.3.7.1 Trial 7.1 containing the bacteria on the shell

6.3.7.1.1 Justification

The bacterial suspension had to be placed on the eggshells in such a way that it was contained and did not run off onto the agar. The containment method needed to be sterile and maintain its integrity during incubation. Nascimento (1992) used a dental elastomer syringe to place a ring of silicone high vacuum grease (Espe Gmbh, Germany) onto the chicken eggshells. This was not avaliable for this project therefore attempts were made to re-create the grease ring using media avaliable.

6.3.7.1.2 Method

Three containment methods were trialled (see table 6.5). Each method was ranked according to four criteria: 1. how easy it was to apply; 2. if it could be autoclaved to sterilise; 3. did it hold a bacterial suspension; and 4. did it hold it continue to hold the bacteria suspension at 36^{o}C, which was the incubation temperature in all other trials.

Candle wax and Vaseline were initially trialled in an attempt to replicate Nascimento's (1992) method. A ring of each was placed onto the surface of chicken eggshell fragments. The candle wax was placed on hot and allowed to cool on the shell. The Vaseline was placed into a syringe (5cc/ml) then autoclaved before piping onto the shell.

Filter paper discs (15mm diameter) (Whatman, Sigma-Aldrich, Auckland, New Zealand) were also trialled on both chicken and NIBK eggshells. The discs were autoclaved then placed onto the shells with sterilised forceps.

6.3.7.1.3 Results

Both candle wax and Vaseline were ineffective for the majority of the criteria (see table 6.5). Candle wax was messy to apply and it did not form a solid ring easily. It also cooled very quickly making it hard to apply evenly. The wax melted during incubation,

which created a layer between the bacterial suspension and the shell. This could have affected the results by creating an artificial barrier to bacteria penetration.

Vaseline in a syringe was easier to apply than the wax, yet still did not form a clean, solid ring as any air bubbles caused the Vaseline to come out unevenly from the syringe. Autoclaving the Vaseline also changed its texture, making it softer and harder to work with even after it had cooled. Like the wax, Vaseline melted during incubation. Filter paper was the most effective method for containing the bacteria on the shell; it was easily autoclaved and could be put on the shell simply with forceps.

To determine if contamination occurred using this method, autoclaved filter paper was placed onto agar and incubated at 36^{o}C for 48 hours. No growth occurred indicating that the filter paper is sterile. To ensure that the bacteria could survive on, and grow through, the filter paper during incubation 15µl of an *E. coli* suspension was placed onto an autoclaved filter paper disc that was placed onto agar; this was incubated at 36^{o}C for 48 hours. The filter paper with bacteria showed growth of the target bacteria, indicating that bacteria can survive and grow through the filter paper.

Table 6.5: Comparison of methods used to contain a bacterial suspension on an eggshell fragment. A ring was made with both the wax and the Vaseline to hold the suspension on the shell. The four criteria used to determine most suitable method were how easy it was to apply, did it hold the bacterial suspension, could it be autoclaved, and did it hold its shape through incubation at 36^{o}C.

Containment method	Ease of application	Does it hold bacteria?	Can it be Autoclaved?	Does it hold its shape at 36^{o}C
Wax ring	Hard- messy to form ring and cools too quickly	No - gaps form	Yes	No – melted over shell
Vaseline ring	Moderate- syringe made it easier to form a ring but still messy	Yes - ring is solid	Yes - but changes texture, becomes softer	No – melted over shell
Filter paper disc	Easy - can place with forceps	Yes	Yes	Yes

6.3.7.2 Trail 7.2: Quantity of bacteria

6.3.7.2.1 Justification

Two factors needed to be considered: the amount of bacteria suspension to place on the filter paper; and the concentration of bacteria in the suspension.

The amount of bacterial suspension that can be placed on the filter paper needed to fulfil two criteria: 1. the volume had to be a large enough to saturate the filter paper to ensure the bacteria was in contact with the shell; this was because I wanted to test if the bacteria could grow through the shell, not through the filter paper; and 2. the bacteria suspension had to be contained by the filter paper, and did not run off the filter paper onto the shell. Because the containment method differed to that of Nascimento (1992; see trial 7) the amount of suspension could not be modelled from his method and had to be determined for by this trial.

In regards to the concentration of the bacteria suspension, most studies have used concentrations of bacteria that poorly represent wild conditions to test bacterial penetration (Brown et al., 1965; Williams and Whittemore, 1967), including Nascimento (1992). In his method Nascimento (1992) diluted the *Salmonella* by a factor of 10^{-6} with saline, which gave approximately 30 colony forming units (C.F.U.) per 10µl of suspension. Ideally known densities from wild NIBK shells should be used for this type of experiment, but these are unknown (see chapter seven, section 7.3). In this study, I used moderate concentrations of bacteria, which I hoped would be representative of wild conditions. This allows method to be developed and then the density adjusted when work on bacterial densities in the wild has been done.

6.3.7.2.2 Method

Bacterial suspensions were made by serial dilution with Phosphate-Buffered Saline (PBS) (0.01M). To test what volume of bacterial suspension should be placed on the filter paper, water was added in 5µl increments from a pipette until the two criteria above were met.

The tests for density were undertaken using the volume chosen in the above test. Two factors were considered: 1. the density of bacteria when the amount of bacterial solution was spiral plated, as this gives an indication of the amount of CFU; 2. the amount of bacterial growth when the volume of bacterial solution was placed onto filter paper on agar and incubated at 36^{o}C for 48 hours, after which growth was observed. Dilutions from 10^{8}-10^{2} were trialled (see table 6.6).

6.3.7.2.3 Results

The result revealed that 25µl of bacterial suspension was the best volume that could be applied to each filter paper disc, fully saturating it but without any of the liquid running off onto the shell. Two filter paper discs were used on each shell fragment, as this both increased the CFU and covered more surface area (see figure 6.8). Therefore, 25µl was the amount of bacterial suspension that was used in the density trials.

The density of 10^{4} yielded the best results on both spiral plates and when filter paper was placed onto agar. Higher concentrations resulted in a large number of CFU, whilst lower concentrations resulted in very little growth.

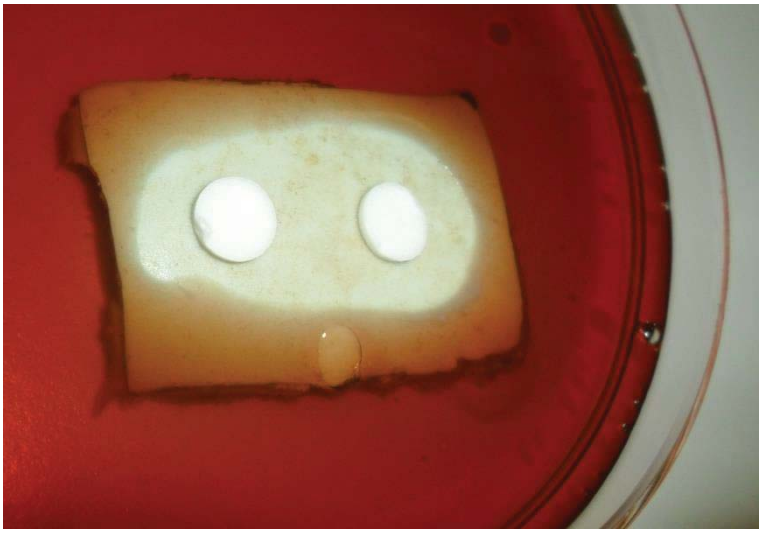

Figure 6.8: Example of filter paper placement on North Island Brown Kiwi eggshell fragment. Each disc was 15mm in diameter, and the shell fragment was set into blood agar.

Table 6.6: A comparison of bacterial suspension concentrations used for the shell fragment method. Concentrations were both spiral plated and placed onto agar to test suitability (see text for details). Rank indicates suitability (1 = most suitable, 5= least suitable).

Concentration	Spiral Plate Result	Agar and filter paper trial
10^8	3	3
10^6	2	1
10^4	1	2
10^3	4	4
10^2	5	5

6.3.8 Trial 8: Identifying if the bacteria penetrated the eggshell

6.3.8.1 Justification

The testing for this did not occur as an individual trial; instead, observations were made during the other trials to determine the best way to identify if the target bacteria could grow through the eggshell. Therefore, the information is not provided in a methods and results layout as with other trials, and is instead discussed below.

As mentioned Nascimento (1992) used SEM to determine if the target bacterium penetrated the eggshell, which was not possible in this study. Instead, visual observation of the growth of the target bacterium on the agar under the shell was used to confirm eggshell penetration in this study. However, throughout this method development it became clear that even if the agar under the shell looks free of contamination, when a swab is taken from the surface of the agar and plated, growth was present.

Therefore, to identify if growth occurred I made two observations: 1. a visual observation of growth at stages throughout the trial incubation period; and 2. at the end of the trial period I took a swab from the agar under the shell, and incubated it to observe any growth (see trial six).

In order to identify if the bacterial growth is the target bacterium, the eggshells need to be sterile, which was not possible in this study (see trial 6).

6.3.9 Example of a design for additional trials testing temperature alteration using the shell fragment method

In this section, I describe an experiment that I intended to carry out but was unable to because of the issues presented in the previous sections (trial 6 and trial 7).

6.3.9.1 Justification

The eggshell method for penetrability testing has the potential to be altered to model more accurately what NIBK eggs experience in the wild. One such alteration is the change in incubation temperatures. NIBK exhibit partial incubation until clutch completion (see chapter one, section 1.2.2), meaning that incubation is not fully initiated until all eggs in the clutch are laid. The first laid egg can be left un-incubated for up to 15 days. Once incubation is initiated, it is intermittent as the male leaves the nest every night to feed. Thus, during the 80 day incubation period, eggs experience a range of temperatures and a range of warming and cooling cycles. Warming and subsequent cooling of egg contents has been shown to create a pressure differential that sucks bacteria into the egg contents (Bruce and Drysdale, 1994). The shell fragment method can be varied to investigate potential increase in penetration due to such temperature changes. Below is an example of the set-up of a method that could be used once the sterilisation of NIBK eggs is achieved. The temperatures in this trial were selected based on the incubators I had available, and were designed to model the egg being incubated (36^{o}C), the egg un-incubated (20^{o}C), and the egg cold (3^{o}C).

6.3.9.2 Method example

Four trials should be undertaken over a period of five days (see table 6.7). Three temperature adjustment trials should be undertaken to model NIBK incubation: trial one (modelling an un-incubated egg being incubated): incubation at 20^{o}C for 24 hours then at 36^{o}C for the final four days; trial two (modelling a cold egg being incubated): incubation at 3^{o}C for 24 hours then at 36^{o}C for final the four days; trial three (modelling extreme periods of warming and cooling): incubation at 3^{o}C for 24 hours, then at 36^{o}C for 24 hours, this change, from 3^{o}C to 36^{o}C, is repeated every 24 hours for the remaining time.

A control should be placed at a constant temperature of 36°C for the whole five days, as this is optimal temperature for bacterial growth and will indicate if bacteria have survived.

The filter paper method described in trials 7.1 and 7.2 (section 6.3.7.1 and 6.3.7.2) should be used. The trial bacteria should be one isolated from the contents of an un-hatched NIBK egg, such as one of the *Staphylococcus* spp. isolated in chapter five. A bacterium known to penetrate chicken eggshells, such as *Escherichia coli*, should be used as a positive control. A negative control, using no bacteria should also be done to ensure no preliminary contamination. Growth should be checked for at 48 hours and then at the end of the five days (120 hours) by swabbing under the shell as described in trial eight (section 6.3.8).

Table 6.7: The layout of a trial to test effect of temperature change on penetrability of bacteria through avian eggshell fragments. A control plus three temperature alteration trials are shown. 48 and 120 hours are the times each treatment is checked.

Treatment	Control		Trial one		Trial two		Trial three	
	Constant 36°C		20°C for 24 hours, then moved to 36°C		3°C for 24 hours, then moved to 36°C		3°C to 36°C to 3°C (24 hour rotations)	
	48hr	120hr	48hr	120hr	48hr	120hr	48hr	120hr
Trial bacteria (*Staphylococcus)*								
Positive control (*Escherichia coli)*								
Negative control No bacteria								

6.4 Summary of method so far

Blood agar should be made up with sterile Milli-Q (MQ) water to a concentration of 3.7 per cent brain heart broth, 1.2 per cent agar and five per cent horse blood. Follow the packet instructions to make agar; mix water, brain heart broth and agar powder

together and then heat to dissolve. Autoclave this agar mix and place in a 50^{o}C water bath to keep fluid. The blood is added later to avoid cell lysing due to the heat of the agar.

All work should be carried out in a BSC with latex gloves worn at all times. The eggs should be opened by creating a small hole in the sharp end of the egg (see figure 6.1b). The contents can be sucked out (see figure 6.1a) or poured out depending on the use of the eggs in other studies. A 3cm^{2} fragment should be cut from the equator of the egg using a rotary tool (Ozito 170W rotary tool kit). The rotary tool should be wiped down the ethanol and the blade should be dipped in ethanol and flamed before and after each use.

Take the agar mix from the water bath and pour in the blood. Once mixed, the blood agar should be poured to half fill a petri dish. Immediately place the shell fragment into the agar ensuring agar does not touch the shells upper surface. The fragment should be placed one side first, then lowered slowly to reduce air bubbles. If any bubbles are present, moving the fragment and lifting the sides should remove them. There should be a space on the shell surface that the agar does not cover.

Autoclave two 15mm filter paper discs, then place on the open shell space using forceps. A 10^{4} dilution should be made up of the target bacteria and PBS. 25µl of this bacterial dilution should be placed on each disk; this should be done slowly to ensure no liquid runs off the disc. The petri dish should then be incubated at the desired temperature and subsequent growth observed.

This method can be modified to adjust for incubation temperatures, times and other conditions (see additional chicken egg trials). Remember to swab under the shell fragment after final day of incubation, as not all growth is visible.

6.5 Discussion

The aim of this study was to develop a method to determine if any given bacteria could penetrate the NIBK eggshell. Through a range of pilot trials the initial stages of this method were developed. However, unexpected difficulties with getting the NIBK eggshells sterile meant that the method was not finalised.

Despite this, the shell fragment method initiated in this study offers a way of investigating penetration, allows for sample size to be maximised from minimum eggs, is cost effective and it is easily modified with regards to modelling incubation conditions and egg composition. Time, budget and the lack of eggs available for experimentation meant that this method could not be further investigated within this study; but I believe that this method could be successful with a small amount of additional research. Other studies have used antibiotics in the agar and antibiotic resistant strains of the microbe in question. Yet as two of the goals of this study were to design a method that was low cost and easily replicated for conservation purposes and to design a method that could reduce the microbes needed to be identified, using antibiotic resistant strains was impractical.

The inability to sterilise NIBK eggs is in itself an important and unexpected result. That NIBK eggs contain bacteria within their shells, even after several attempts at cleaning, supports the idea that microbes have the ability to impact hatching success. It indicates that some bacteria may have the ability to penetrate the shell partially and then complete penetration when egg defences are lower or when conditions are more suitable for bacterial growth, such as when the egg is not being incubated and the antimicrobial proteins are not at optimal functioning temperature (see chapter two, 2.3).

I noticed something in support of this hypothesis during the development of this method. As mentioned in 6.2.1 each NIBK egg in this study was used for two experiments; this method and for another study identifying the bacteria within the egg contents (see chapter five). Together these studies resulted in swabs being taken from the shell and the contents of these eggs. All of the NIBK eggs had heavy growth isolated off their eggshells and most NIBK eggs had heavy growth isolated from within the contents. One NIBK egg however, had no growth isolated from the contents. Growth on the shells but not within the egg contents was also observed for two of the chicken eggs used in this study.

During this study, as part of the method development, un-cleaned fragments of these three eggshells (the one NIBK and the two chicken eggs) were placed onto agar. After

48 hours, very heavy growth was observed on the plates. Thus, although no growth was found within the contents of the intact eggs, heavy growth was seen after the shell was cut up and placed on agar. As prior tests revealed the cutting of the eggshells and the agar were not contaminated, and the agar did not touch the surface of the shell fragments, the growth on the agar suggests that the bacteria were already within the pores of the eggs and grew through the shell once conditions were suitable.

I think that the most conservative explanation for why the bacteria grew through the shell onto the agar, but not through the shell into the egg contents, is that the egg contents were acting as an antimicrobial barrier. This would suggest that the bacteria are surviving in the shell, for example within the pores, but cannot grow into the egg contents, as they are stopped by the egg content's defences, such as lysozyme. However, the protection afforded by the contents is broken down when the shell is placed onto agar and incubated. To test if this hypothesis is true, it would be necessary to identify which bacteria grew through the shell into the agar. Identification of these bacteria was out of the budget of this study, but they have been kept in storage for future work (see chapter seven, section 7.3). It should also be noted that this shell method was designed to allow for modelling of the egg contents defences; therefore, to test the above hypothesis one could also add lysozyme to the agar and observe whether bacteria could then grow in it.

As discussed in previous chapters (see chapters three, four and five, sections 3.4.5, 4.4.5, and 5.4.5) the Kiwi Best Practice Manual (KBPM) recommends that as ONE eggshells are cleaned upon arrival at the ONE facility (Bassett, 2012), bare hands should be used to handle NIBK eggs in the wild. However, the results of this project show that NIBK eggshells are harbouring bacteria, even after medical grade sterilisation. Therefore, I suggest that gloves be worn at all stages of egg and bird handling, for the wellbeing of the egg and the handler (also see chapter seven, section 7.2.2).

Future work needs to look at reasons for these eggs having bacteria present, even after medical grade cleaning. One possible reason for this survival may be the shell structure of NIBK eggs. Silyn-Roberts (1983) examined the eggshell structure of one

complete and two incomplete NIBK eggshells, and found that the shell structure of NIBK eggs was different to the domestic chicken eggshell in a range of factors. NIBK shells were thicker, more complex and had larger pore shape and size characteristics that could increase the ability for bacteria to survive sterilising as the complex structure could be protecting the bacteria from the effects of the sterilisation process. Crushing the eggshell and removing this structure before autoclaving would help identify if it is indeed the shell structure that is preventing sterilisation, as opposed to the bacteria being resistant. Although care was taken throughout this study the possibility of contamination of the eggshells in the lab is a possibility and this cannot be ruled out completely. To account for this, swabs were taken from two eggshells directly after the cleaning to test for contamination. These swabs had heavy growth present, supporting the idea that the microbes were present before the cleaning took place.

The shell structure of NIBK eggs needs to be more thoroughly investigated so we can understand how bacteria can penetrate the shells and thus begin to understand how they can survive through medical grade cleaning. Scanning electron microscopy (SEM), with a larger sample size than three, would be an effective tool to use for this.

Once we learn how to successfully sterilise NIBK eggs, it opens the door for a range of possibilities with this method. For example, this method can be modified to include the factors of NIBK that could affect microbial penetration, such as adding lysozyme to the agar, or mimicking the intermittent incubation the egg experiences like suggested in trial 6.3.9.

Another factor in penetration is the defensive antimicrobial proteins within the egg contents. The fact that for some eggs swabs of the contents did not show any growth, yet vigorous growth occurred when the shell was placed on agar, suggests these proteins play a key role. Studies into the level of lysozyme have shown that NIBK have ten times the amount expected from a bird their size (Osuga and Feeny, 1968; Prager and Wilson, 1974). This also suggests they play a large role in defence. The agar can be adjusted to include these proteins and thus investigate their impact. In order for this to

occur however, more information about the levels of these proteins found in NIBK eggs is also needed.

Chapter seven: Discussion and conclusions

Over 60 per cent of NIBK eggs in the wild do not hatch (McLennan et al., 1996) and the reasons for this mortality are unknown. This low hatching success is contributing to NIBK overall population decline. Infertility and predators are unlikely to be major factors in NIBK egg mortality. Eighty five per cent of NIBK eggs are fertile (Potter, 1989), this is reflected in the high hatching success of Operation Nest Egg (ONE; see chapter one) eggs with up to 90 per cent of eggs successfully hatching (Department of Conservation, 2012). The main conservation strategies in place in New Zealand, ONE and intensive predator control, are focused on the predator vulnerable chick stage. However, predation on wild NIBK eggs is relatively low as NIBK eggs are extremely resistant to predators (McLennan et al., 1996). Consequently, the predator control efforts in place do little to protect eggs in the wild. Mclennan et al., (1996) suggested that microbes might be involved in egg mortality. NIBK eggs have several factors that make them vulnerable to microbial infection (see chapter one, section 1.5), and previous studies have noted a high prevalence of microbial contamination in wild NIBK eggs (McLennan, 1988; Potter, 1989; McLennan et al., 1996; Ziesemann et al., 2011). Because of these two factors, the overall hypothesis of this project was that microbes are contributing to NIBK hatching failure, and I expected the results of my chapters to reflect this.

7.1 Conclusions about the impact microbes have on hatching success in North Island Brown Kiwi

The first aim of this project was to determine if NIBK eggs harbour microbes that could impact hatching success. Microbial infection of avian eggs is a dynamic process, many factors influence whether a microbe can penetrate though the shell and gain access to the embryo (Board, 1966, 1968). The four chapters in this thesis combine to give an overview of the relationships between microbes and NIBK eggs.

In chapters three and four, I identified the bacteria and fungi from the shells of wild NIBK eggs. These chapters provided an understanding of the types of microbes that were present on living eggs during active incubation. In contrast, in chapter five I used infertile eggs to investigate the bacteria present inside un-hatched NIBK eggs. Together the results in these chapters supported each other in terms of conclusions and gave an

understanding of the microbes present at different stages in NIBK egg development. Chapter six then investigated a method to determine if a target bacteria could penetrate through the NIBK eggs defensive shell. This method was not finalised, however this study showed that NIBK eggshells harbour bacteria that survived even through medical grade cleaning. The fact that bacteria could survive in the shell during adverse conditions may result in increased penetration when conditions do become suitable.

The combined results of this project strengthen the conclusion that NIBK eggs harbour bacterial genera that have the potential to impact the embryo, and that these genera may also be able to survive until conditions are suitable for penetration into the contents. Together these results support the overall hypothesis that microbes are contributing to NIBK hatching failure.

Four of the six bacterial genera (67%) isolated from NIBK egg contents (see chapter five, section 5.3.4) have been shown to cause embryo mortality in other birds. Three of these four potential embryo pathogens (75%) were isolated off the shells of the Ponui Island population (see chapter three, section 3.3.4). *Bacillus* and *Serratia* were only isolated off one NIBK eggshell and from the contents of one NIBK egg and *Escherichia* was the sole invader in the contents of two NIBK eggs, but was not isolated from the shells in this project. However, the fourth genera *Staphylococcus*, was the most common genera isolated in this project. *Staphylococcus* was isolated from the contents of 63 per cent of eggs and from 92 per cent of eggshells examined. Members of the genera *Staphylococcus* have been shown to significantly impact the hatching of other avian embryos. For example, some species have been shown to reduce hatchability to zero in domestic chicken (*Gallus gallus domesticus*) embryos (Bruce and Drysdale, 1991), while others have increased hatching success in chicken embryos due to competitive exclusion (McCabe, 1965, 1967; Ribble and Shinefield, 1967). The prevalence of *Staphylococcus* and its link with hatching in other birds, indicates that this genus it may be a significant factor in NIBK hatching success and/or failure and warrants further focused investigation (see section 7.3.1).

Micrococcus, *Pseudomonas* and *Streptococcus* are embryo pathogens found on the wild shells (see chapter three, section 3.3.2) but not the contents of NIBK un-hatched egg (see chapter five, section 3.3.3). These genera should not be ignored as both *Pseudomonas* and *Streptococcus* are significant embryo pathogens in other birds (Bruce and Drysdale, 1991; Board and Tranter, 1995). That they were not present in the un-hatched eggs may be due to the small sample size of the egg content study, not due to their inability to penetrate the NIBK eggshell.

Pseudomonas can be a significant embryo pathogen, but it has also been shown to increase microbial penetration into egg contents (Board and Halls, 1973; Bruce and Drysdale, 1994; Board and Tranter, 1995). Thirty per cent of the NIBK eggshells examined in this study had *Pseudomonas* isolated, and the presence of *Pseudomonas* on the shells of avian eggs has been shown to facilitate bacteria penetration into the egg contents as it can break up the shells defensive cuticle and open up the pores (Board and Halls, 1973). Some fungi have also been shown to open up the pores of avian eggs via hyphal growth, 70 per cent of eggs in this study had fungi present and 64 per cent of eggs that had fungi growth had *Aspergillus* and *Penicillium* present (see chapter four, section 4.3); both are fungal genera that have hyphal growth and could lead to an increased number of open pores (Board and Fuller, 1974). As mentioned in chapter four (see section 4.3.3 and 4.4.6) future studies should investigate the correlation between fungi present on NIBK eggshells and the both the resulting hatching success and the penetration of bacteria into the egg contents.

The positive impacts of microbes on the NIBK eggshell should not be ignored, as they could also be contributing to NIBK hatching success in the wild. The benefit of microbes for the avian embryo is understudied (Lombardo et al., 1996; Soler et al., 2008; Soler et al., 2010; Peralta-Sánchez, 2010). As mentioned, some species in the genera *Staphylococcus* (McCabe, 1965, 1967), *Pseudomonas* (Bird and Grieble, 1969) and *Streptococcus* (Ribble and Shinefield, 1967) have been shown to provide a benefit to chicken embryo and all were isolated off NIBK eggshells (see chapter three, section 3.3.5). While *Pseudomonas* and *Streptococcus* were only isolated off the shell, *Staphylococcus* was isolated from both contents and shell and was one of the most dominant bacteria isolated in this project.

While there is potential for bacteria both on and in the NIBK egg to have a positive impact on hatching success in the wild, it should be said that because of the high levels of hatching failure (over 60%), and the high presence of microbial contamination inside NIBK eggs (over 90%, see chapter five), I would suggest that microbes are most likely having a negative impact.

It should also be said that the results of chapter six show that bacteria can remain in the shell structure through adverse conditions. This may mean that genera, such as *Pseudomonas* and *Streptococcus,* could remain in the layers of the shell and penetrate into the contents when conditions are suitable, such as a drop in temperature causing a pressure differential, or another microbe altering the content conditions in a way that is more compatible to bacterial growth.

As mentioned in chapters three and five (see sections 4.4.5 and 5.4.5) contamination of the egg could impact the chick as not only does it grow from the egg, but NIBK chicks retain a large yolk sac that they use for nourishment (Prinzinger and Dietz, 2002). Microbial infections of this yolk sac have been shown to kill NIBK chicks in the wild (Wilson, 2013). The contaminated eggshells are also present in the nest when the chicks hatch and therefore may provide a reservoir of potential microbial contamination for NIBK chicks. NIBK chicks remain in the nest for up to 27 days (Wilson, 2013) giving any pathogenic microbe that survives egg incubation and hatching time to infect the chick, as has been suggested in other species (Baxter-Jones, 1991; Deeming, 1997). For example, *Aspergillus* has been shown to infect hatching Ostrich chicks at the time of piping (when the chick initially cracks or chips a hole through the shell) and eggshells infected with *Aspergillus* lead to exposure and infection of other chicks in the nest (Shane and Tully, 1996). Avian chicks may acquire their gut microflora directly from the eggshells (Schneitz, 2005; Minson, 2012; Stanley et al., 2013). The composition of the gut microflora can be vital for the health and survival of the chicks (Stanley et al., 2013). It has been suggested that in modern commercial hatcheries, as the eggshells and nesting environments are thoroughly cleaned, domestic chicks are not exposed to the correct microflora and their gastrointestinal tracts are instead colonised by random bacteria. NIBK have been shown to acquire gut microbial bacteria quickly over the first few weeks of life

(Minson, 2012), as NIBK remain in the nest for this time the eggshell and the nesting environment are likely natural sources for healthy, gastrointestinal microbiota. However, like domestic chicken eggs ONE NIBK eggs are disinfected and housed in sterile environments, thus chicks are not exposed to their natural bacterial flora. More work is needed to determine if microbes can be passed onto NIBK chicks from their eggs and the impact if they do.

7.2 Impacts of research

The second aim of this project was to use the results to direct future work and conservation efforts for NIBK. The finding that microbes are present and abundant on NIBK eggs has impacts for both fieldwork and NIBK conservation. This project opens the door into the potential causes of hatching failure in NIBK and highlights the urgent need for more research into egg mortality. By researching the microbiology of the NIBK egg in the wild, this project adds valuable knowledge to the field of wild avian egg microbiology.

7.2.1 Recommendations for North Island Brown Kiwi conservation and Operation Nest Egg

ONE is a successful conservation strategy; however, predator control alone is not the answer to NIBK conservation. The low hatching success of NIBK is contributing to their decline, as over 60 per cent of NIBK eggs in the wild do not hatch (McLennan et al., 1996). ONE was initially designed to protect Kiwi chicks but with 90 per cent hatching success, ONE is also indirectly protecting the NIBK eggs.

According to the NIBK recovery plan ONE is not a permanent conservation strategy; the end goal is to phase out ONE when predator control is deemed sufficient to protect the majority of chicks (Department of Conservation, 2012). While I do not disagree with this end goal, I would like to suggest that it be adjusted so that ONE is not phased out merely when predator control is deemed sufficient to protect chicks, but when there are long-term, cost-effective ways to keep NIBK populations self-sustaining. As said previously in this thesis, predators are not the only factor in NIBK

mortality and this project highlights the risk to eggs in the wild by pathogenic microbes such as *Staphylococcus* and *Aspergillus*.

Currently ONE is protecting NIBK numbers, and this is giving Kiwi researchers an opportunity to study the causes of population decline with less worry about population extinction (Bassett, 2012). This project highlights the need for more of the conservation related research to be on the hatching failure of NIBK in the wild. Although the chicks are extremely vulnerable, the eggs are also at risk and they need to be acknowledged in NIBK recovery plans. There is the risk that if ONE is phased out solely based on predator control and before an understanding of the causes of hatching failure is gained, NIBK populations will not increase as expected and populations will continue to decline.

7.2.2 Impacts for field work

A key result of my research is that microbes are present on NIBK eggs. This may seem an obvious statement, but in New Zealand, conservation efforts are focused on threats like predation and habitat destruction, as they are the obvious, visible issues. Nevertheless, microbial disease does play a major role. There have been several well documented cases of disease outbreaks in New Zealand birds which have links with human disturbance and interference (Austin, 1978; Alley et al., 1999; Alley, 2001; Derraik et al., 2008; Tompkins et al., 2013).

Handling of NIBK eggs is a human disturbance and interference; it could result in the spread of pathogenic microbes around and even between populations. Handling these eggs could also be spreading of microbes from the birds to the people handling them. NIBK are known to have dangerous and contagious pathogens in their blood and digestive tracts, such as *Cryptococcus* (Robertson, 2003) and through this research the potentially dangerous genera *Aspergillus, Staphylococcus*, *Streptococcus* and *Pseudomonas* (see chapters three, four and five) are added to this list.

As said throughout this thesis (see chapters three, four, and five, sections 3.4.5, 4.4.5, and 5.4.5), while the Kiwi Best Practice Manual recommends gloves be used only in some stages of NIBK egg handling (Robertson, 2003), because of the results of this

project I recommend that gloves should be worn at all stages of egg and bird handling, regardless of if they are ONE eggs. In addition, all equipment used should be cleaned between individual eggs; this includes callipers, candling torches and weighing bags.

As well as the evidence of the presence of bacteria, this project highlights how little we know about microbes in the wild. We do not know what exactly is on these shells or the impact they can have and because of this we need to be extra careful. One of the eggshells in this project had a *Bacillus* isolated off it. Initial DNA sequencing identified it as *B. anthracis*, the bacteria responsible for anthrax. Anthrax is a lethal disease; infection can occur via inhalation, ingestion, or body fluid transmission (Turnbull, 2002). Anthrax has been considered successfully eradicated in New Zealand after an intense eradication programme (Davidson, 2002). Needless to say, it was a shock finding it on a wild NIBK egg from an offshore island population. Further morphological tests revealed that it was not this species, but another closely related species of *Bacillus.* However, the point is still valid; we do not know what is on these eggs and until we do, care should be taken at all times when handling them.

7.2.3 Impacts for the field of wild avian egg microbiology

Research on the types of microbes inhabiting wild birds' eggs is scarce, therefore this project is important as it adds to, and improves, our understanding and knowledge in the field of wild avian egg microbiology. To date, only 12 previous studies have been published, from 12 different bird species, that identify the bacteria present on wild birds eggs (appendix one). Of these 12, all were on neognathous birds, 10 (83%) were from the family Passeriformes, four (33%) were in tropical climates and eight (67%) were in temperate climates in the Northern hemisphere. Not only does this project add a new bird species and family to the list of birds that have had egg bacteria identified, but it is also the first study to be undertaken on a paleognathous bird, and therefore it adds a completely new clade to the list of research. As well as this, it is the first study to be undertaken in a temperate climate in the Southern Hemisphere, thereby expanded our understanding of temperate conditions. This project also expands the list of bacteria that are present on wild birds' eggs with five new genera and six new species being identified off the eggshell (see chapter three, section 3.3.2),

and two new genera and six new species being identified from within the egg contents (see chapter five, section 5.33). The egg size and incubation period of the NIBK is also different to the 12 species of birds previously studied (appendix one), and so provides information on a large egg with a long incubation period, in contrast with the small eggs and short incubation periods of the 12 bird species previously studied. The average egg size of the 12 species ranges from 1.9-6.4 cm x 1.2-5.1 cm, whilst on average the NIBK egg is 12.8 x 7.8 cm. The average incubation period for the 12 species ranges from 12-35 days, whilst the NIBK incubation period is 80 days.

The information regarding the fungi present on wild birds' eggs is even less than for bacteria (appendix one). Kozlowski et al. (1991a, b) looked at the contents of dead eggs from the House Sparrow (*Passer domesticus*) and the Tree Sparrow (*Passer montanus*) and only two fungi, both *Candida* yeast were identified. Therefore, this project is only the third study to identify the fungi present on wild birds' eggs and the first to present the fungi on living eggs, during active incubation. This project also adds a new bird species and family to the list of birds that have had the fungi identified and it is the first study to be undertaken on a paleognathous bird, so again adds a completely new clade to the list of research. Both *Aspergillus* and *Penicillium* are new genera added to the list of fungi isolated off wild birds' eggs.

More work is needed in the field of wild avian egg microbiology, and this project, together with previous studies, shows that although there are methodological challenges associated with the study of microbes in natural conditions (Soler et al., 2010) there are strategies and methods to overcome these.

Future research should be undertaken on other Southern hemisphere birds, and other birds with large eggs and long incubation times to have a wider understanding the field. Sea birds such as Albatross (Diomedeidae) would be a good candidate for this research.

This project also emphasises the need for more research into the beneficial impacts bacteria can have on avian eggs and hatching success. Little work has been done on the positive impact that bacteria can have on avian embryos (Lombardo et al., 1996; Soler et al., 2008; Soler et al., 2010; Peralta-Sánchez, 2010), and the work that has

been done is focused on the domestic chicken (McCabe, 1965, 1967; Ribble and Shinefield, 1967; Bird and Grieble, 1969) (see chapter two, section 2.4.4). Although it has been suggested that benefical bacteria could increase hatching success in wild birds (Baggot and Graeme-Cook, 2002; Cook et al., 2005a; Soler et al., 2008), to date no definitive study has been undertaken. Future work is needed to investigate how, why and when bacteria act as pathogens or mutualists on avian eggs in the wild (Frank and Jeffery, 2001; Moreno et al., 2003). Future studies could investigate the antimicrobial spectrum of potential positive egg bacteria. The method Ruiz-Rodríguez et al. (2013) used to examine the antimicrobial spectrum of bacteriocins from symbiotic bacteria of the Hoopoe (*Upupa epops*) uropygial gland would be appropriate for such studies.

7.3 Future work in towards understanding North Island Brown Kiwi egg mortality

This project was designed to be a starting point into the research of NIBK egg mortality. It focused on microbes as they are a likely factor. The initial aim was to determine if microbes were present on and in NIBK eggs that could impact hatching success. This project was successful in this aim, yet more work is now needed to expand and support our knowledge on NIBK hatching success and failure. Future work is suggested below that will both further our understanding of NIBK hatching failure and support and expand the conclusions of this project. As mentioned in chapters three, four and five samples of the microbes isolated, including those not yet identified, were stored and therefore are available for future work.

7.3.1 Work that is complimentary to microbial studies

Disentangling the impact of environmental conditions and microbial contamination should be the primary focus of future work. ONE has shown that NIBK can experience high hatching success. Yet the conditions of eggs at ONE facilities are not like in wild settings; in ONE the eggs are disinfected and placed in a constantly controlled environment (Bassett, 2012). As temperature and humidity are adjusted to maximise hatching success and the eggs are disinfected, the eggs are protected not only from

outside contamination but also from the environmental fluctuations. Both microbes and unsuitable environments have been shown to impact avian hatching success (Board and Tranter, 1995; Cook et al., 2003; Cook et al., 2005a; Cook et al., 2005b). The impact of environmental factors and microbes are hard to disentangle (Cook et al., 2003; Beissinger et al., 2005; Cook et al., 2005a) as a change in environment may be affecting directly or indirectly on the embryo. For example, high temperature can directly affect the embryo by causing malformations or indirectly by causing increased microbial growth. The penetration method study in this project (see chapter six) was designed to allow for changing environmental conditions to be tested, which could help aid in determining differing effects. Although this method was not finalised in this study, I believe it still holds promise. Two future studies could be undertaken, in conjunction with the penetration method, to disentangle the impacts of environment and microbial infection: one study would be to compare the relationship between hatching success of wild birds and the environmental conditions of nests. Variables such as humidity, temperature, microbial load, nesting material type, faecal contamination and nest re-use could be compared with NIBK hatching success in that area. The second study would be an egg cleaning experiment to be carried out on wild NIBK eggs. Several studies have trialled this in other species, using ethanol to wipe the eggshells (Cook et al., 2005b; Godard et al., 2007; Boyer, 2010), with mixed results. Cleaned Pearly-Eyed Thrasher (*Margarops fuscatus*) eggs experienced higher hatching success than un-cleaned eggs, supporting microbial factors in hatching failure (Cook et al., 2005b). However, the eggs of the Little Blue Penguin (*Eudyptula minor*), another native New Zealand bird, showed no difference between cleaned and un-cleaned eggs (Boyer, 2010). It should be noted that with any invasive research on NIBK, such as egg cleaning, care and pilot trials need to be undertaken to minimise the disruption and disturbance to the incubating male.

The impact on the hatching chick by the microbes of the egg should also be investigated. NIBK chicks are extremely vulnerable, with some populations having up to 90 per cent chick mortality each year and not all of it is due to predation (Wilson, 2013). As mentioned, the chicks are exposed to the microbes of the egg when they hatch and also while they continue to live in the nest for up to 27 days. The microbes

identified in this project as being pathogenic or mutualistic could also have the same impact on the chicks as the embryo. This would be an interesting and beneficial line of future research and if positive associations with bacteria were found it would have consequences for how we manage captive chicks.

7.3.2 Additional microbiological work

Additional microbiological studies are needed that will expand the conclusions of this project, adding to the hypothesis that microbes are contributing to NIBK hatching failure. Microbes that have the potential to impact NIBK hatching success were highlighted in this project (see chapters three, four and five). Future studies should focus on these bacteria and begin to investigate their potential to impact the embryo. One way of investigating the impact would be to directly infect NIBK eggs with these bacteria and monitor the resulting hatching success. However, as discussed, this method has both ethical and conservation issues as it could result in embryo deformities and death. NIBK are endangered, and as such causing death of individuals should be avoided. Researchers should instead begin to narrow down the list that of bacteria provided by this study, and the method developed in chapter six was provided as an example of how this could be done. Because this method was not finalised (see chapter six, section 6.5), I suggest future studies continue from where this project left off. Another method for investigating microbial penetration is to identify the microbes present in each layer of the egg, as was proposed in chapter five (see section 5.2.2). By identifying the microbes present in separate layers of the egg it would be possible to track the penetration of that microbe. For example, the isolation of a certain bacteria only in the shell and inner membrane of NIBK eggs, but not the contents would suggest limited penetration of that species.

Additional future studies should also identify and quantify the bacterial load on wild eggshells throughout incubation and utilise both culture dependent and independent methods. The time taken to get the swabs from the wild into the laboratory needs to be considered for all methods as delays can be up to five days, as was seen in this project, which can affect the results.

The studies in this project used culture-dependent methods; the microbes were grown from swabs onto suitable medium. This is a selective method, as only around one per cent of microbes are cultivable (Peralta-Sánchez, 2010). This was suitable for the aims of the studies in this project, as the method was designed to target the microbes most likely to impact NIBK hatching success. However, culture independent techniques, such as DNA microarrays, would support the results of this project (Shawkey et al., 2009) and should be considered for future work.

In this project, the type of bacteria present on avian eggshells was used as an indication of the probability of embryo infection (see chapters three and five), as has been done in other avian egg studies (Cook et al., 2003; Cook et al., 2005a; Cook et al., 2005b; Shawkey et al., 2009; Peralta-Sánchez, 2010). However, the density of bacteria on avian eggshells has also been used to indicate the probability of microbial impact. Higher bacterial load on eggshells increases the probability of embryo infection (Bruce and Drysdale, 1991; Board and Tranter, 1995; Peralta-Sánchez, 2010). This study used dominance of a bacterial genus as an indication of dominance within the egg itself, however, future work should aim to both identify and exactly quantify the microbes found on NIBK eggshells as this would lend further support to the hypothesis that microbes are impacting hatching failure. Finding the densities of certain bacteria on the shells of wild NIBK eggs will also assist in determining the correct concentrations to place on the eggshell during the eggshell fragment method described in chapter six (see section 6.3.7.2.1).

The wild NIBK eggs in this project were all swabbed at different stages in the incubation period and only 55 per cent of eggs were swabbed for a second time. Even so, for both fungi and bacteria these second swabs were often different in composition from the first. Due to the small sample size it is unknown if this is a statistically significant difference but it does suggest that the microbial community is dynamic over time. The presence of certain microbes at certain stages of incubation may be important when investigating impacts these microbes have on the embryo. For example, there is a link between potentially pathogenic bacteria present on the eggshell at the start of incubation and the probability of embryo death (Peralta-Sánchez, 2010). Therefore, it would be advantageous to have an in depth

understanding of the microbial composition of NIBK eggshells at different stages of incubation and future studies should aim to swab eggs at multiple times throughout incubation period.

It should be said that some of the microbes identified in this project were only identified to genera level (see chapter one, section 1.6.4). Identification of all microbes to species level should be incorporated into the goal of any further work, as this would provide further support for the hypothesis that microbes are contributing to hatching failure in NIBK. This would allow clarification as to what impacts these microbes are having, as some species within a genus are known pathogens, while others are benign (Bannerman and Peacock, 2007) and some even provide a benefit (Soler et al., 2010). This work can be done using the stored samples from chapters three, four and five. Identification of the bacteria that survived the sterilising and grew after the cleaning trials should also be undertaken to help clarify if they are species that can survive medical grade cleaning or if they are contaminants from poor handling (see chapter six, section 6.3.6).

Some bacteria such as *Salmonella* spp., *Mycoplasma* spp., *Staphylococcus aureus* and *Pasteurella* spp. have been shown to contaminate chicken eggs before they are laid, via the reproductive tract (Mayes and Takeballi, 1983; Humphrey, 1994). And although it is accepted that contamination of most avian eggs occurs after the egg has been laid, (Board, 1968; Bruce and Drysdale, 1994; Cook et al., 2005b), contamination of NIBK eggs via the reproductive tract before they are laid would be an interesting avenue to explore. *Post mortems* on NIBK are common and future work could compare bacteria in NIBK reproductive tract with what is found on and in the shell in this project. The freshness of the carcass would need to be factored in, as *post mortem* contamination would impact on the results. However, if done correctly this type of investigation support the results of this project showing microbes have the ability to impact hatching success and would also provide insight into the mechanisms through which egg contamination occurs.

7.4 Summary

The results of this project have shown that microbes are a very likely contributor to NIBK egg mortality. NIBK eggs from several populations and locations harbour both potentially pathogenic and potentially beneficial microbes both on the shells and in the contents. These microbes may have the ability to persist in the shell until conditions are favourable. We still do not know definitively what is causing the 60 per cent hatching failure in NIBK but these results highlight the need for egg mortality and microbial factors to be considered in to NIBK conservation and recovery plans.

The intensive management of NIBK, through predator control and captive rearing, is actually contributing to NIBK vulnerable status. Because of this, ONE is a successful but not permanent conservation strategy. I support the eventual phasing out of extensive use of ONE, but as predators are not the only factors in NIBK mortality, I suggest that we need to re-evaluate when this phasing out occurs. Intensive management of NIBK should be phased out not only when predator control is deemed sufficient to protect majority of chicks, but when wildlife managers have a better understanding of what other factors contribute to NIBK mortality, at all stages of life. We need long-term, cost-effective ways to keep NIBK populations self-sustaining, and this will include protection of the eggs as well as the chicks.

This research has not only started to compile the list of potential pathogens present on NIBK eggs; but it has increased the list of potential human pathogens carried by NIBK. The results of this project also show that NIBK eggshells can harbour bacteria that survive even through medical grade cleaning. Because of this I recommend that gloves should be worn at all stages of egg and bird handling, regardless of if they are ONE eggs. In addition, all equipment used should be cleaned between individual eggs; this includes callipers, candling torches and weighing bags.

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Appendix one: List of studies of the microbial flora of wil

Appendix table 1: Table to show all the studies that could be found that identified the microbes present on the shells (s) and in the contents (C) of wild birds eggs. Average egg size and length of incubation was found in Hoyo, J.,
Other studies have tested specifically for the presence of mesophilic bacteria, Enterococcus, Staphylococus, and Gram negative Enterobacteriaceae both on and in avian eggs of several bird species (Soler et al., 2012; Peralta-Sán
found on which species' eggs. Because of this they are not included in this table.

	Bird						Avergage egg size (cm)	Average length of incubation (days)	Climate	Sample size (# of eggs)	Reference	
	Infraclass	Order	Family	Genus	Species	Common name						Gr
C	Neognathae	Falconiformes	Accipitridae	*Buteo*	*regalis*	Ferruginous hawk	6.4 x 5.1	32	Temperate, Northern hemisphere	21	Houston et al., (1997)	Gram-neg Gram-po Actino Gram-nega Gram-nega Actino Gram-neg Gram-nega Gram-negati Gram-nega Gram-pos Gram-pos
C	Neognathae	Falconiformes	Accipitridae	*Buteo*	*swainsoni*	Swainsons hawk	5.7 x 4.6	35	Temperate, Northern hemisphere	42	Houston et al., (1997)	Gram-nega Gram-neg Gram-po Actino Gram-nega Gram-nega Gram-nega Gram-po Actino Gram-nega Gram-nega Gram-negati Gram-nega Gram-pos Gram-pos
S	Neognathae	Passeriformes	Hirundinidae	*Hirundo*	*rustica*	Barn Swallow	2 x 1.4	17	Temperate, Northern hemisphere	33	Peralta-Sanches (2010)	Gram-negat Gram-pos Gram-negat
S	Neognathae	Passeriformes	Mimidae	*Margarops*	*fuscatus*	Pearly-eyed thrasher	3.2 x 2.3	15	Tropical	240	Cook et al., (2005a,b)	Gram-nega Gram-negati Gram-pos Gram-po
C	Neognathae	Passeriformes	Mimidae	*Margarops*	*fuscatus*	Pearly-eyed thrasher	3.2 x 2.3	15	Tropical	116	Cook et al., (2005b)	Actino Gram-nega Gram-negati Gram-pos Gram-po
S	Neognathae	Passeriformes	Muscicapidae	*Ficedula*	*hypoleuca*	Pied Flycatcher	1.8 x 1.3	12	Temperate, Northern hemisphere	50	Ruiz-de-Casaneda et al., (2011)	Gram-nega Gram-nega Gram-pos Gram-neg Gram-neg Gram-neg Gram-neg Gram-po Actino Gram-neg Actino Actino Gram-nega Gram-nega Gram-nega Actino Gram-pos Gram-pos

S	Neognathae	Passeriformes	Muscicapidae	*Ficedula*	*hypoleuca*	Pied Flycatcher	1.8 x 1.3	12	Temperate, Northern hemisphere	50	Ruiz-de-Casaneda et al., (2011)	
C	Neognathae	Passeriformes	Passeridae	*Passer*	*domesticus* and *montanus*	House sparrow and Tree sparrow	2.1 x 1.5 and 2 x 1.4	12	Temperate, Northern hemisphere	90 and 23	Pinowski et al., (1994); Kozlowski et al., (1991a)	
S	Neognathae	Passeriformes	Troglodytidae	*Troglodytes*	*aedon*	House wren	1.6 x 1.2	12	Temperate, Northern hemisphere	313	Potter et al., (2013)	

S	Neognathae	Passeriformes	Troglodytidae	*Troglodytes*	*aedon*	House wren	1.6 x 1.2	12	Temperate, Northern hemisphere	313	Potter et al., (2013)	Gram-neg Gram-neg Gram-ne Actin Gram-p Gram-p Gram-p Gran Gran
C	Neognathae	Strigiformes	Strigidae	*Bubo*	*virginianus*	Great horned owl	5.4 x 4.6	34	Temperate, Northern hemisphere	12	Houston et al., (1997)	Gram-ne Acti Gram-ne Gram-ne Gram-ne Acti Gram-neg Gram-ne Gram-p Gram-p
S	Neognathae	Passeriformes	Hirundinidae and Turdidae	*Tachycineta* and *Sialia*	*bicolor, thalassina* , and *mexicana*	Tree swallow violet-green swallow and western bluebird	1.9 x 1.3, 1.8 x 1.2, and 4 x 2	15	Temperate, Northern hemisphere	43	Wang et al., (2011)	Gram-neg Gram-n Actin Gram-p Actin Gram-p Gram-n Actin Actin Actin Actin Actin Gram-neg Actin Gram-p Actin Actin Actin Actin Gram-p Gram-neg Gram-p Actin Gram-neg Actin Gram-n Gram-p Gram-p Gran
C	Neognathae	Passeriformes	Hirundinidae and Turdidae	*Tachycineta* and *Sialia*	*bicolor, thalassina* , and *mexicana*	Tree swallow violet-green swallow and western bluebird	1.9 x 1.3, 1.8 x 1.2, and 4 x 2	15	Temperate, Northern hemisphere	25	Wang et al., (2011)	Acti Gram-p Acti Acti Actin Acti Acti Acti Gram-p Acti
S	Neognathae	Passeriformes	Mimidae	*Margarops*	*fuscatus*	Pearly-eyed thrasher	3.2 x 2.5	15	Tropical	12	Shawkey et al., (2009)	over 1492
C	Neognathae	Passeriformes	Passeridae	*Passer*	*domesticus*	House sparrow	2.1 x 1.5	12	Temperate, Northern hemisphere	38	Stewart and Rambo (2000)	Gram-n Gram-n

S	Palaeognathae	Apterygiformes	Apterygidae	*Apteryx*	*mantelli*	North Island Brown Kiwi	12.8 x 7.8	80	Temperate, Southern hemisphere	13	This project	Gram-r Gran Ac Gran Ac Ac Gram- Gram- Gram- Gram Gram Ac Gram-r Gram-n Gram-n Gran Gram- Gram Gram Gram Gram Gram
C	Palaeognathae	Apterygiformes	Apterygidae	*Apteryx*	*mantelli*	North Island Brown Kiwi	12.8 x 7.8	80	Temperate, Southern hemisphere	8	This project	Gran Gran Gram- Gram- Gran Gran Gram- Gram- Gram Gram Gram Gram Gram
C	Neognathae	Passeriformes	Passeridae	*Passer*	*domesticus*	House sparrow	2.1 x 1.5	12	Temperate, Northern hemisphere	90	Kozlowski et al., (1991a)	
C	Neognathae	Passeriformes	Passeridae	*Passer*	*domesticus*	House sparrow	2.1 x 1.5	12	Temperate, Northern hemisphere	49	Kozlowski et al., (1991b)	
S	Palaeognathae	Apterygiformes	Apterygidae	*Apteryx*	*mantelli*	North Island Brown Kiwi	12.8 x 7.8	80	Temperate, Southern hemisphere	13	This project	

Appendix two: DNA extraction and PCR optimisation method

Pure colonies of the target bacteria were grown up on Blood agar and then incubated at 36^{o}C for 48 hours. DNA was extracted using the following method:

1. 1ml of 2% chelex was placed in a 1.5ml eppendorf tube
2. Two-three pure colonies were taken off the agar and added to the tube
3. The eppendorf tube was vortexed to mix the bacteria and the chelex
4. The tube was frozen at -80^{o}C for at least 20 minutes
5. The tube was thawed, before it was heated in a AccuBlock™ Digital Dry Bath heat block (Total Lab Systems Ltd, Auckland, New Zealand) at 100^{o}C for 10 minutes
6. The tube was placed in a Sigma 1-14 centrifuge (John Morris Scientific Ltd, Auckland, New Zealand) for 3 minutes at 12000rpm
1. The tube was then frozen at -20^{o}C for 1 hour (or overnight)
2. The tube was heated at 65^{o}C in the heat block again for 1 hour
3. The tube was cooled to room temperature before it was placed in the centrifuge for 2 minutes at 12000 xg
    a. 300µl of the contents were then pipetted into a new 1.5ml eppendorf tube
    b. 200µl of phenol-chloroform was added
    c. The new eppendorf tube was centrifuged for 3 minutes at 12000 xg
4. The supernatant was then pipetted into a new 1.5ml eppendorf tube
    a. 20 µl of sodium acetate was added
    b. 1ml of 80% ethanol was added
    c. The tube was then inverted 8-10 times
5. The tube was frozen at -20^{o}C for 10 min before being centrifuged for 10 minutes at 12000 xg to pellet the DNA
6. The pellet was washed with 1ml of 80% ethanol, and the tube inverted 5 times
7. The supernatant was discarded by decanting, and the tube was air dried to remove the ethanol
8. 50µl of sterile water was added to the dry pellet, and left at room temperature for 30 minutes to rehydrate.

The extracted DNA was then nanodropped in a ND-100 Spectrophotometer (Thermo Fisher Scientific Ltd, Auckland, New Zealand) to indicate the amount of DNA present and also purity of the sample. The DNA was be amplified using polymerase chain reaction (PCR); therefore, low amounts of DNA were acceptable. The purity of the

sample is very important as the sequence being targeted, the 16S region, is present and conserved in almost all bacteria (Janda & Abbott, 2007). The 260/280 ratio was used to determine purity; it indicates the wave lengths associated with DNA and RNA. Values of 1.8-2 were used, any higher or lower and the sample may have large amounts of proteins, phenols or other contaminants. If the nanodrop revealed too little DNA or an impure sample, the bacteria was regrown and the DNA extracted again

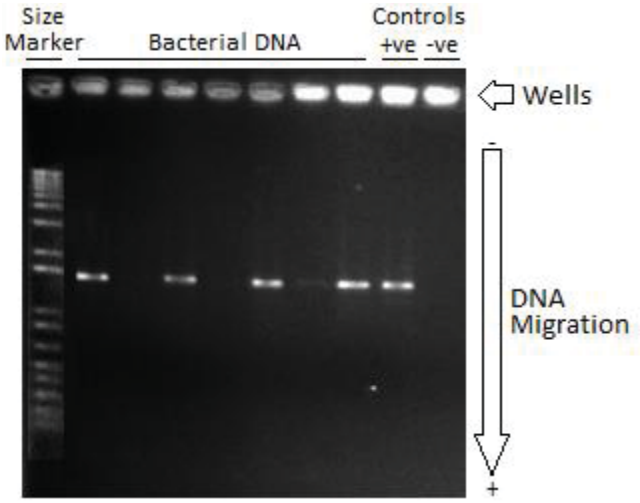
The DNA was amplified using PCR. The forward primer (27F) was (5' to 3') AGA GTT TGA TCC TGG CTC AG and the reverse primer (1494R) was (5' to 3') TAC GGC TAC CTT GTT ACG AC (Lane, 1991) (Invitrogen, Life Technologies, Auckland, New Zealand). The PCR reaction mixture (20µl) contained: 2µl 10X buffer, 1µl dNTP (2mM), 0.6µl $MgCl_2$ (50mM), 0.5µl Taq DNA polymerase (Invitrogen, Life Technologies, Auckland, New Zealand) 1µl of each primer, 11.9µl sterile MQ water, and 2µl of the bacterial DNA. Once combined, the PCR mixture was then placed into a thermo-cycler (SensoQuest Labcycler, dnature Diagnostics & Research, Gisborne, New Zealand) for amplification over 40 cycles. DNA mix was pre-heated at 94°C for 2 min. The cycles consisted of denaturation at 94°C for 20 seconds, annealing at 50°C for 20 seconds, and extension at 72°C for 2 minutes. The DNA was then held at 10°C until it was removed from the machine.

The size of the amplified DNA was determined using (1%) agarose gel electrophoresis. Positive and negative controls were also used to ensure accuracy of the method; the positive control consisted of a known DNA product, whilst the negative control contained no DNA. A band around the 1.5-2Kb area indicated a positive result (see Appendix figure 1).

The PCR products with the correct band were then washed:

1. 16µl of 20% PEG was added, and the tube was lightly flicked to get liquid to the bottom
2. The tube was heated at 37 ^{O}C for 15 minutes
3. The tube was centrifuged for 30 minutes at 13000rpm
4. The supernatant was pipetted off
5. 100µl of 80% ethanol was added, and the tube was again lightly flicked to get liquid to the bottom
6. The tube was centrifuged for 10 minutes at 13000rpm

7. The supernatant was pipetted off and the tube was left to air dry
8. 12µl of sterile MQ water was added to rehydrate the DNA



Appendix figure 1: 8 bacterial 16S rRNA segments run on a 1% w/v agarose gel, stained with ethidium bromide. Both positive (+ve) and negative (-ve) controls are used. The DNA size marker is a commercial 1 Kbp ladder. The position of the wells and direction of DNA migration is noted.

The washed DNA was then sent for sequencing at Massey Genome Services (Massey University, Palmerston North, New Zealand). The DNA sequences were trimmed, visually examined, and aligned using Geneious software (version 5.6.2, Biomatters Ltd, Auckland, New Zealand). BLAST software (Basic Local Alignment Search Tool from the National Center for Biotechnology Information (NCBI), accessed from http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC =blasthome) was used to identify the bacteria.

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