Microbial Infection of Avian Eggs: A Threat to All Synchronously Incubating Species? Case Study of New Zealand's Little Blue Penguin (*Eudyptula minor*)

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Abstract

Microbial infection of eggs was originally investigated in terms of human health only. Recently, however, it was found that it can also cause early embryo mortality in birds, mainly through trans-shell infection prior to incubation. Trans-shell infection is highly dependent upon environmental conditions, egg temperature and egg properties such as shell quality and antimicrobial defences. Microbial infection of eggs is more likely to occur in synchronously incubating species as first laid eggs can be exposed for up to several days prior to full incubation. One example of a population that seems at particular risk of egg microbial infection is New Zealand's little blue penguin (*Eudyptula minor*) from Tiritiri Matangi Island. This bird lays two eggs on average three days apart, and is believed to begin full incubation only after the second egg has been laid. Both eggs are laid in particularly humid and soiled nests and contain only low levels of lysozyme, an important antimicrobial protein. The aims of this study were therefore to 1) obtain a first examination of the rates of shell and trans-shell microbial infection of chicken eggs in New Zealand and assess the effects of cleaning on those rates, 2) investigate the role of microbes in hatching failure of little blue penguin eggs and 3) investigate other factors affecting little blue penguin egg viability. This study revealed that shell infection in chicken eggs significantly increased with exposure and significantly decreased with cleaning; however, trans-shell infection was only marginally affected by exposure and cleaning. On Tiritiri Matangi Island, Hauraki Gulf, New Zealand, nest type, egg order and shell cleaning did not affect hatching success, suggesting that nest conditions and microbial infection prior to incubation were not a major cause of egg mortality in this population. Temporary abandonment during incubation, however, was very frequent in the second half of the breeding season and

fatal to most eggs. These temporary abandonments seemed to be caused by resource limitations, an aspect that should be investigated in future studies.

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1.1 INTRODUCTION

Birds, unlike many other animals, can influence the initiation of development of their offspring through the onset of incubation (White and Kinney 1974; Drent 1975; Carey 1980; Cook *et al.* 2003). As they can lay no more than one egg daily, parents can either initiate incubation before clutch completion or delay incubation until the last egg is laid (Clark and Wilson 1981; Stoleson and Beissinger 1995; Hebert 2002). Most studies have previously focused on the assumption that synchronous incubation and hatching is the normal condition and asynchrony is a deviation or an adaptive behaviour that provides a fitness benefit to first-hatched nestlings or their parents (Amundsen and Slagsvold 1991; Clark and Wilson 1981; Stoleson and Beissinger 1995). An alternative idea is that asynchronous incubation might be the result of a trade-off between the benefits of a large clutch size and the costs of delaying incubation (Cook *et al.* 2005b).

The first and most common hypothesis proposed to explain hatching asynchrony is known as the 'brood reduction hypothesis', first proposed by Lack and Lack (1951). This hypothesis states that parents begin incubation before clutch completion in order to produce siblings of different ages, which will increase food competition (Lack and Lack 1951; Lack 1954; Lack 1968; Howe 1976; Howe 1978; O'Connor 1978). If food is scarce it is likely that the smallest individuals will have access to the least amount of food and thus the nestlings lost will be those in which the parents have invested the least amount of time and energy (Howe 1978; Clark and Wilson 1981; Stoleson and Beissinger 1995). Initiating incubation before clutch completion can also provide protection to the eggs from predators (Hussell 1972; Bollinger *et al.* 1990), brood parasitism (Wiley and Wiley 1980; Romagnano *et al.* 1990), and interspecific or intraspecific competitors searching for nest sites (Beissinger and Waltman 1991;

Beissinger *et al.* 1998). It has also been shown that delayed incubation can reduce egg viability (Hussel 1985; Arnold *et al.* 1987; Veiga 1992); known as the 'egg viability hypothesis'. This hypothesis states that initiating incubation before clutch completion maximises hatchability since un-incubated eggs are more vulnerable than incubated egg and thus their viability declines with exposure time (Arnold *et al.* 1987; Ewert 1992).

Temperature is generally thought to be the major factor affecting the viability of un-incubated eggs (Stoleson 1999; Webb 1987); however, other factors such as gaseous environment, egg orientation, egg turning and humidity can also significantly affect these eggs (Wilson 1991; Deeming 1992; Meijerhof 1992). Temperature is critical to egg viability as eggs can begin developing before normal incubation temperatures have been reached. Indeed, while optimal incubation temperatures range between 36°C and 38°C, some embryonic tissues can begin to develop in the absence of incubation if the temperature exceeds physiological zero (23°C–27°C). This can cause unsynchronised growth, leading to lethal teratogenic abnormalities (Romanoff and Romanoff 1972; Webb 1987; Wilson 1991; Deeming 1992; Deeming and Fergusson 1992; Ewert 1992; Fasenko *et al.* 1992; Meijerhof 1992). There is some evidence that neurological and brain tissue development is particularly sensitive to prolonged exposures to these temperatures (Webb 1987).

Relative humidity, in contrast, is less a problem on its own, but is important mainly in terms of microbial infection. Water not only promotes the growth of bacteria and fungi but is also the medium of transport by which they can pass through shell pores (Board and Halls 1973; Board *et al.* 1979). Indeed, under appropriate ambient temperatures and humidity, bacteria and fungi present on the eggshell can multiply and cause damage to the shell, creating even more unplugged pores through which microbes

can enter the egg (Board and Halls 1973; Board *et al*. 1979; Baggott and Graeme-Cook 2002). This process, known as trans-shell infection, has recently been shown to be an important cause of hatching failure (Cook *et al.* 2003; Beissinger *et al.* 2005; Cook *et al.* 2005a; Cook *et al.* 2005b). By incubating the eggs shortly after laying, parents physically protect the embryo from microbial infection by reducing moisture on the eggshell and by increasing temperatures to levels at which most microbes stop growing and many antimicrobial proteins present in the albumen begin to function optimally (Board and Tranter 1986).

As described in the following chapter, antimicrobial proteins can destroy or limit the growth of certain bacteria. Although it is thought that most antimicrobial proteins function optimally at incubation temperatures (Board and Tranter 1986), they may still provide an extra protection to eggs prior to full incubation. Birds could therefore potentially increase the chances of egg survival by increasing the concentrations of these proteins, especially in first-laid eggs that are more vulnerable to infection. This was found to be the case in a population of barn swallows (Saino *et al.* 2002), where lysozyme levels were higher in first-laid eggs. In a more recent study involving the analysis of over 400 eggs from 8 species of birds, however, it was found that overall levels of antimicrobial proteins varied greatly between species but did not generally vary with egg order (Shawkey *et al.* 2008), suggesting that producing higher concentrations of antimicrobial proteins for first laid eggs might not be an optimum choice, possibly because it would cause a decrease in other aspects of albumen quality.

1.2 CONSERVATION SIGNIFICANCE OF THIS RESEARCH

Low hatching success has often been described as an important cause of population decline in New Zealand's native species, its primary cause being inbreeding resulting in infertility (Briskie and Mackintosh 2003; Mackintosh and Briskie 2005; Jamieson *et al.* 2006). However, inbreeding is not the only factor that can cause early embryo death. Microbes such as bacteria and fungi are capable of passing through the eggshell before incubation begins and infecting the developing embryo (Cook *et al.* 2003; Beissinger *et al.* 2005; Cook *et al.* 2005a; Cook *et al.* 2005b). These infections are more likely to occur in humid environments as moisture promotes microbial growth on the eggshell and transports microorganisms through the shell pores (Board and Halls 1973; Board *et al.* 1979; Bruce and Drysdale 1994; Sparks 1993).

As outlined earlier, species that delay incubation until several or all eggs in a clutch have been laid are at particular risk. In New Zealand this includes species such as the weka (*Gallirallus australis*), the pied oystercatcher (*Haematopus ostralegus finschi*), the variable oystercatcher (*H. unicolor*), the black stilt (*Himantopus novaezealandiae*), the shore plover (*Thinornis novaeseelandiae*), the fantail (*Rhipidura fuliginosa*), the silvereye (*Zosterops lateralis*) and the bellbird (*Anthornis melanura*). This also includes most penguin species such as the yellow eyed penguin (*Megadyptes antipodes*), the rockhopper penguin (*Eudyptes chrysocome*), the Fiordland crested penguin (*E. pachyrhynchus*), the Snares crested penguin (*E. robustus*) and the little blue penguin (*E. minor*).

The little blue penguin (LBP) population of Tiritiri Matangi Island, New Zealand, was chosen as a study species owing to the fact that they have a relatively low hatching success, a high rate of egg abandonment and breeding patterns that include

many factors thought to increase the risk of embryo infection (Jones 1978; Miyazaki and Waas 2003; Geurts 2006). Eggs are generally laid in very humid burrows which are often covered in faeces and are reused within and between years. In addition, the first laid egg is thought to be incubated only after the second egg has been laid (1-5days) and both can be left exposed to ambient conditions for up to several days during incubation (Gales 1984; Kemp and Dann 2001; Geurts 2006). In terms of chemical defence, it has been shown that LBP eggs possess low levels of lyzozyme, a major antimicrobial protein in avian eggs (Manwell and Baker 1973). All these factors thus lead to the assumption that LBP eggs are particularly vulnerable to infection and would provide an interesting model to study the effects of microbial infection and cleaning on hatching success.

If microbial infection is shown be a cause of hatching failure in this population, and cleaning is shown to increase hatching success, then it is very likely that this could be the case in many other populations experiencing similar nesting conditions. This could also be the case for newly translocated individuals or populations. Although species are generally translocated into areas within their historical range, the possibility of novel microbial environments evolving over time, and the variation in nest conditions and microbial environments between source and new sites could pose a threat to their eggs. Management plans of such populations or species would thus benefit from the inclusion of studies on microbial infection at various sites.

1.3 AIMS OF THIS RESEARCH

This study aims to determine whether microbes within the nesting environment can affect the viability of certain declining New Zealand species, and more specifically, the viability of LBP eggs on Tiritiri Matangi Island. An experimental study was first conducted on chicken eggs over the summer/autumn period of 2009, followed by a field study on LBP eggs over the 2009-2010 austral breeding season (August-January).

The aims of this research are the following:

1. Understand avian egg structure, infection and anti-microbial properties (Chapter 2).

2. Identify pathogenic groups known to infect egg contents in chicken eggs and examine their rate of shell and trans-shell infection (Chapter 3).

3. Determine whether cleaning eggs (using 70% ethanol) can reduce external microbial loads and rates of trans-shell infection (Chapter 3).

4. Determine the relationships between temperature, relative humidity and shell thickness and the risk of shell and trans-shell infection (Chapter 3).

5. Identify whether microbial infection affects LBP egg viability, and whether cleaning eggs (using 70% ethanol) can increase their hatching success (Chapter 4).

6. Identify other possible causes of egg mortality such as lay date, nest type, and egg size (Chapter 4).

7. Determine the causes and consequences of short-term egg abandonment during incubation (Chapter 4).

8. Discuss conservation and management implications of findings (Chapter 5).

CHAPTER 2.

Avian egg structure and microbial infection

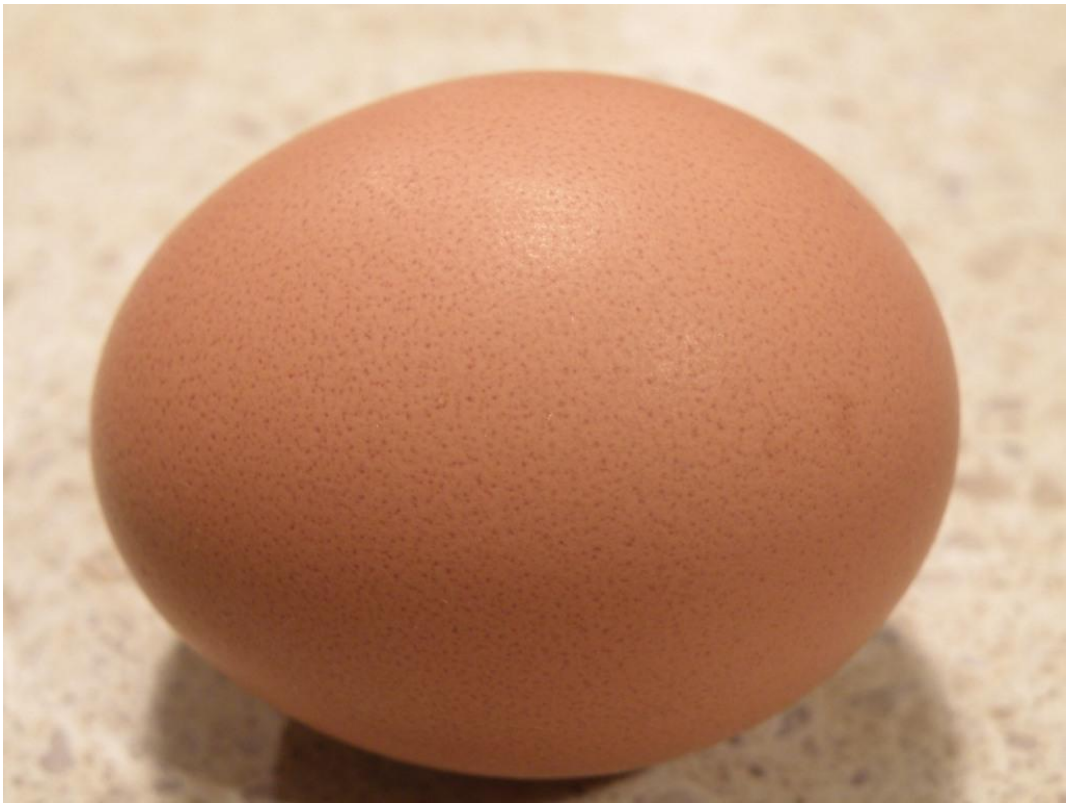

Plate 2.1 Chicken egg and its porous shell. Photograph by A.S. Boyer.

Abstract

Microbial infection of avian eggs was originally investigated in terms of human health only. Recently, however, it was found that it can also be a cause of reduced hatching success in wild birds, depending on the environmental conditions. Infection by pathogenic microbes can occur either by the trans-ovarian or trans-shell routes. In the case of trans-shell infections, microorganisms enter the shell through the pores and must penetrate the shell membrane before entering the albumen. In the albumen, proteins such as lysozyme and conalbumin are capable of destroying certain bacteria or inhibiting their growth. However, environmental factors such as temperature and humidity, and egg properties such as shell porosity and cuticle quality can increase or decrease the probability of trans-shell infection.

2.1 INTRODUCTION

Microbial infection of chicken eggs was first investigated in relation to the possible health issues affecting humans from their consumption (Gayon 1873; Haines 1939**;** Romanoff and Romanoff 1939). Human salmonellosis, for example, has had a long history of association with eggs, initially mainly duck eggs, but rapidly followed by chicken eggs (Anon 1944). In the 1970's, salmonellosis cases began to increase worldwide, beginning with the United Kingdom (St Louis 1988). By the late 20th century, the number of human salmonellosis cases was estimated at approximately 1.4 million annually in the USA only (Mead *et al.* 1999), 80% of which were attributed to eggs or egg products (Gast and Beard 1992). *Salmonella enteritidis* is generally thought to be the most pathogenic serovar for humans, however, *S. typhimurium* is also a pathogen of concern, being responsible for over 23% of all cases of human

salmonellosis in the USA and in Germany (Rabsch *et al.* 2001). Salmonella spp however, are not the only human pathogens found in eggs. Other bacteria including *Campylobacter jejuni*, *Listeria monocytogenes* and *Yersinia enterocolotica* have also been isolated from egg shells or contents and can potentially be a threat to human health (Humphrey 1994).

More recently chicken eggs have also been used as surrogates to examine the effects of exposure to microbes on the hatching success of various domestic and wild avian species. The main advantage of using chicken eggs has been that their susceptibility to infection in terms of number of pores and water conductance is similar to that of other birds (Bruce and Drysdale 1993). Other advantages are that their microbiology is familiar (Board and Fuller 1974) and they hatch synchronously which means that they may have evolved greater resistance to microbial infection than species that hatch asynchronously (Cook *et al.* 2003). This last aspect of egg microbial infection is of particular importance for endangered or declining species with a low hatching success. While many other factors can contribute to high rates of egg failure, microbial infection is one potentially important factor that is rarely investigated. To do so, it is important to first understand the structure of the avian egg and its physical and chemical defences.

This chapter will provide a brief summary of the general structure of avian eggs, the possible causes and routes of infection, and the physical and chemical defences available against microbial infection.

2.2 EGG STRUCTURE

2.2.1 Shell

The shell is the developing embryo's main protection against external threats. It must be solid enough to withstand the weight of the incubating parent, but soft enough to be broken by the hatching chick. It must be rigid enough to resist distortion yet flexible enough to dissipate energy from shocks. It must also be permeable enough to allow gas exchanges with the exterior while impermeable enough to avoid microbial contamination (Solomon *et al.* 1994).

The shell is composed of five morphologically distinct regions (Fig 2.1) known as the mammillary knob layer, the cone, the palisade, the vertical crystal layer and the cuticle (Solomon *et al.* 1994). It is composed of approximately 95% calcium carbonate, the remaining 5% being mainly an organic glycoprotein and proteoglycan matrix (Hincke *et al.* 1999; Mann and Seidler 1999). The cuticle is the most external layer. It is a very thin cover, consisting of approximately of 86% protein, 4% carbohydrates, 3% fat and 3.5% ash (Wedral *et al.* 1974) and is secreted over the eggshell immediately prior to oviposition (Mayes and Takeballi 1983). Its main function is to protect the external surface, thus preventing the obstruction of pores by nesting material, regulating water infiltration and loss and limiting microbial penetration (Solomon *et al.* 1994). However, it can sometimes be distributed irregularly around the egg or even completely absent (Board and Halls 1973). The degree of coverage is highly dependent upon the hen's age, strain and environment (Solomon *et al.* 1994). Underneath the cuticle are the vertical crystal layer and the palisade which fuse during early development. The earlier they fuse, the more resistant the shell will be to bacterial penetration (Bain 1991). The vertical crystal layer is a thin monolayer of crystals found on top of the palisade layer.

The palisade layer is a protein/polysaccharide complex with calcium binding properties (Mine *et al.* 2003). The pores are funnel-like openings formed as a result of the incomplete fusion of cones (Schmidt 1966). In the chicken egg, their total number ranges from 7000 to 17000 and they are most concentrated around the equator or blunt end of the egg (Tyler 1953; Solomon *et al.* 1994). The mammillary layer is situated beneath the pores, separating the true shell from the membranes. It is composed primarily of calcite and provides the foundation for additional shell formation. A strong bond between these two layers often results in less shell formation, thus thinner true shells (Solomon *et al.* 1994).

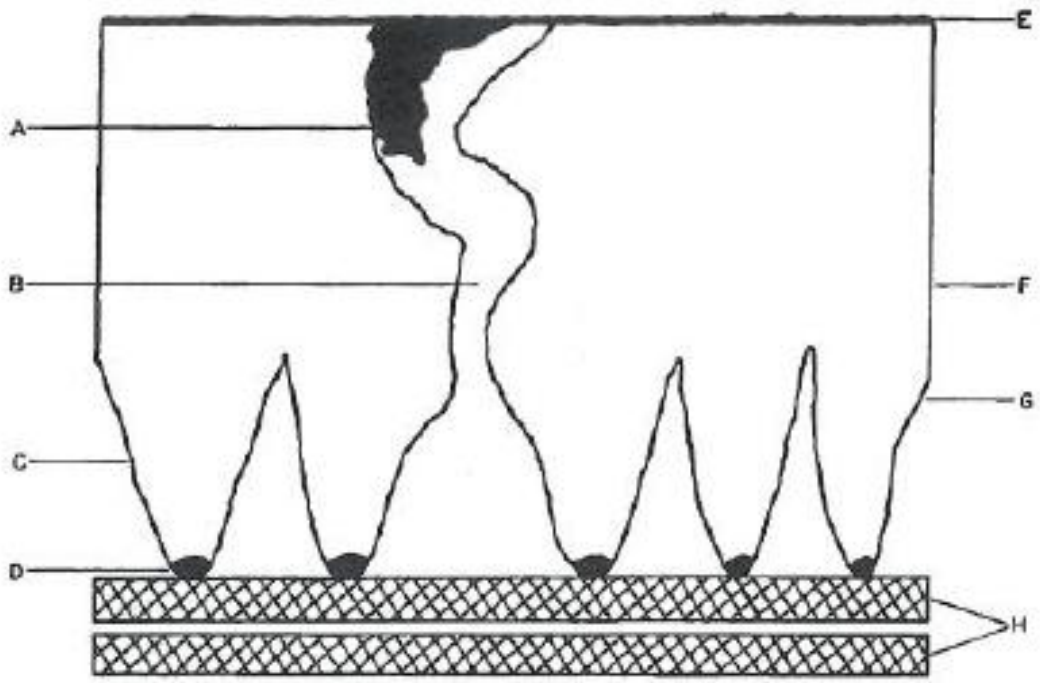

Figure 2.1. Drawing of a radial section of an egg shell showing the cuticular plug (A), pore (B), mammillary knob (C), mammillary core (D), cuticle (E), spongy matrix composed of the palisade and the vertical crystal layer (F), mammillary matrix (G), shell membranes (H). Figure taken from Board (1966).

2.2.2 Membranes

The avian egg is comprised of two membranes, the inner shell membrane and the outer shell membrane which are held tightly together all around the egg except for the blunt

end where they are separated by an air sac. The outer shell membrane is attached to the true shell whereas the inner shell membrane surrounds the albumen (Mayes and Takeballi 1983). Both layers are composed of a network of randomly orientated branched fibres made mainly of keratin and mucin (Masshoff and Stolpmann 1961) and act as bacterial filters (Garibaldi and Stokes 1958). In the chicken egg, these two layers are accompanied by a third layer, the limiting membrane, which is a homogenous layer of electron-dense material (Tranter *et al.* 1983). The fibres composing the inner membrane are finer and more closely packed than those composing the outer membrane (Masshoff and Stolpmann 1961; Lifshitz *et al.* 1964).

2.2.3 Egg contents

The egg content is composed mainly of nutrients and water, the nutrients being stored essentially in the yolk and the water in the albumen (Board *et al.* 1994). The albumen is composed of three layers, the outer thin white, the albuminous sac and the inner thin white, and is separated from the yolk by a vitelline membrane (Fig. 2.2). The yolk is composed of 47% water, 33% lipids and 17.4% proteins (Shenstone 1968). It is non-uniform mass consisting of concentrically arranged bands of yellow and white yolk (Fig. 2.3), which are thought to correspond to a diurnal rhythm in its formation, the wide yellow bands being produced during the day and the narrower white bands during the night (Riddle 1911). Both yolk types are composed of yolk spheres which are composed of sub-droplets, larger and less numerous in the white yolk than in the yellow yolk (Bellairs 1961).

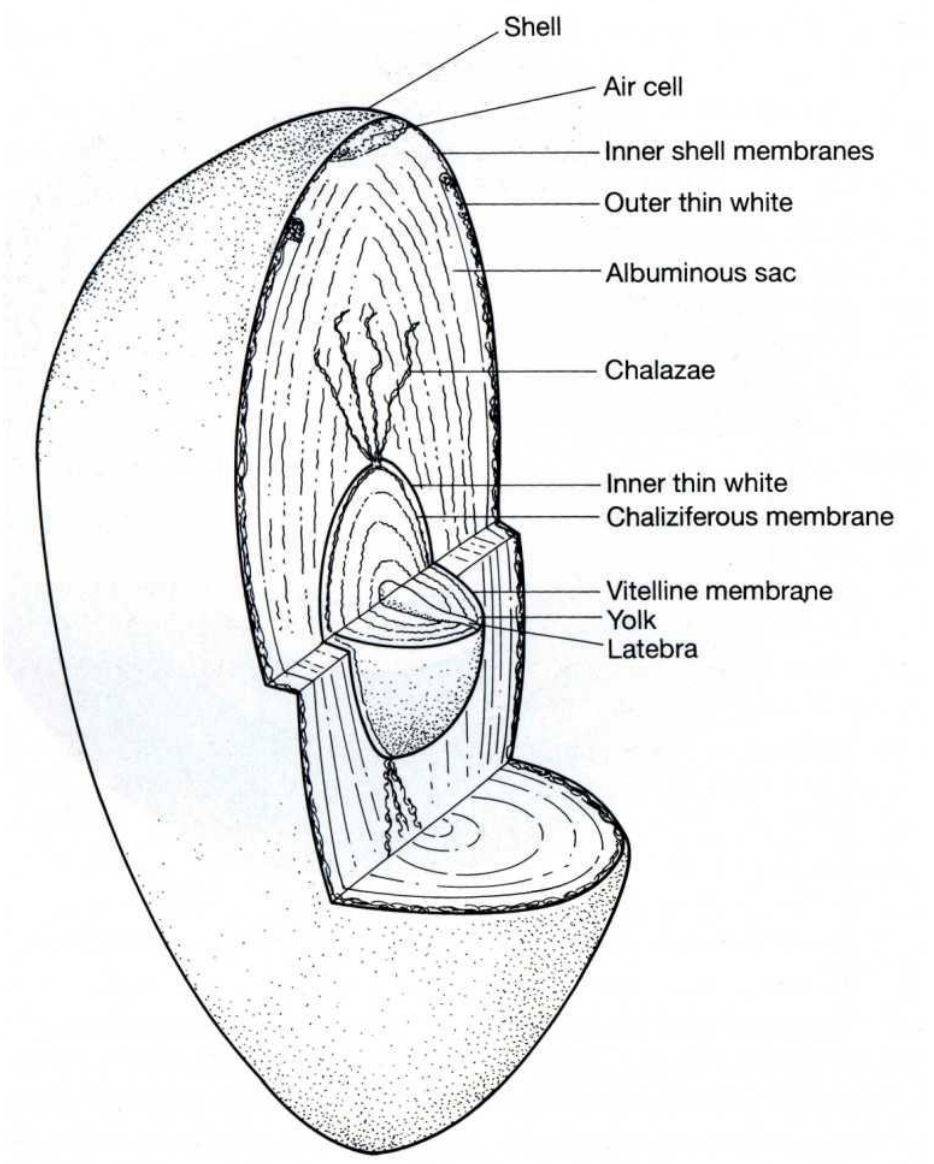

Figure 2.2. Drawing of cut-away sections showing the different layers inside a chicken egg. Figure taken from Board et al., (1994).

In addition to providing water to the developing embryo, the albumen acts as physical and chemical protection to the yolk. First of all, it acts as a shock absorber thus contributing to the protection of the vitelline membrane (Palmer and Guillette 1991). Second, the albumen contains various proteins that provide chemical defence to the egg (discussed in section 2.3.2.4).

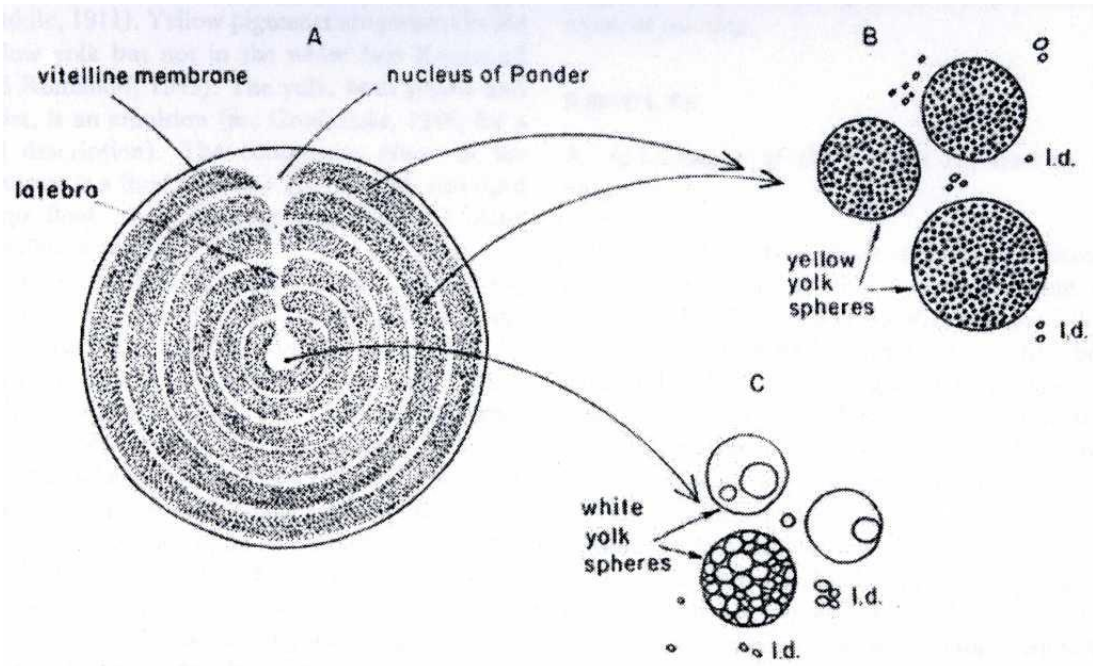

Figure 2.3. Diagram of the yellow and white yolk layers in an unincubated egg showing a longitudinal section (A), yellow yolk spheres taken from the yellow yolk layer (B) and white yolk spheres taken from the centre of the egg. l.d indicate free-floating lipid drops. Figure taken from Bellairs (1961).

2.3 MICROBIAL INFECTION

2.3.1 Routes of transmission

Egg contents can get infected via two routes known as vertical transmission and horizontal transmission. Vertical transmission is an infection prior to laying, and can be trans-ovarian or oviducal, as shown in Fig. 2.4. Horizontal transmission occurs post-laying where microorganisms infiltrate through the egg shell and infect the egg contents (Bruce and Drysdale 1994).

For chicken eggs, most studies have revealed that the bacterial flora in the hen's oviduct is noticeably different to that found in the eggs, suggesting that infection is most likely to occur post laying (reviewed by Bruce and Drysdale 1994). Some bacteria and viruses such as *Mycoplasma spp, Salmonella spp* and *Pasteurella spp,* however, can be transmitted vertically (Mayes and Takeballi 1983). In horizontal or trans-shell infection, microorganisms are thought to pass through the shell pores, and invade the outer thin white. Vadehra *et al.* (1970) showed that the air sac region or blunt end is the area most prone to such infections. Some of these organisms then pass through the albuminous sac and infiltrate the inner thin white (1) where they grow possibly due to nutrients provided by the yolk (2). When the inner thin white becomes heavily contaminated, microorganisms gradually invade the whole albuminous sac (3) and outer thin white (4) (Lock *et al.* 1992, Fig. 2.5).

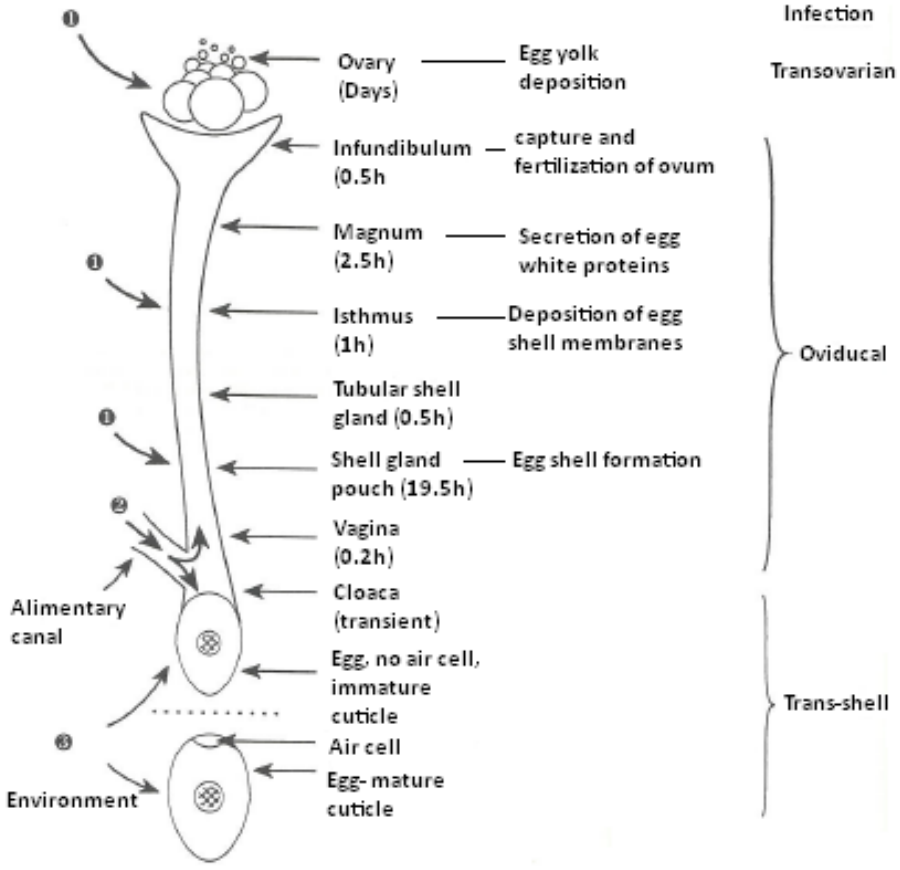

Figure 2.4. Diagram showing the stages of egg formation, time spent at each site and stages at which microbial infection can occur. Figure modified from Board *et al.* (1994) and Jonchère *et al.* (2010).

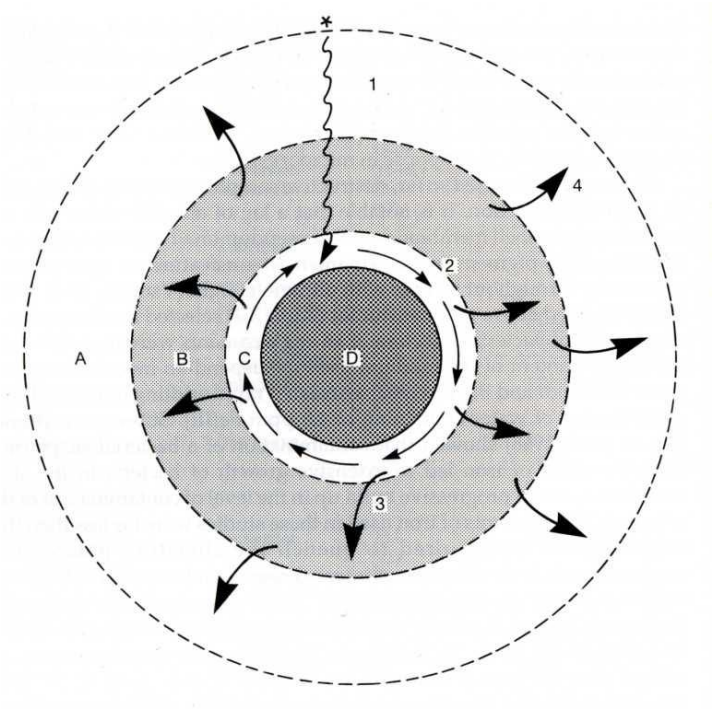

Figure 2.5. Sequence of events leading to the generalized infection of egg contents, using the example of *Pseudomonas putida*. Figure taken from Board *et al.* (1994).

2.3.2 Factors affecting infection

The quantity and type of microorganisms present are very important factors affecting microbial infection. Other environmental factors such as temperature and humidity, or egg properties such as shell thickness, porosity, membrane quality and chemical defences can also considerably impact upon bacterial infection rates.

2.3.2.1 Quantity and types

Eggs laid in clean environments exhibit less infection than eggs laid in heavily contaminated environments (Smeltzer *et al.* 1979). For example, studies have shown that eggs laid in roll away cages generally have a lower infection rate than eggs laid in nests, which also have a lower infection rate that eggs laid on the floor (Joyce and Chaplin 1978; Smeltzer *et al.* 1979; Bruce and Drysdale 1991; Jones 1991). Not all

micro-organisms, however, are capable of penetrating the egg shell and infecting the egg contents.

Studies comparing microbial infections of the shell and contents of chicken eggs revealed that different organisms have different functions and many of them cannot pass through the egg shell and survive in the albumen. Pseudomonads and fungi, for example, can digest the cuticle layer, thus increasing the number of unplugged pores available for trans-shell infection (Board and Halls 1973; Board *et al.* 1979; Baggott and Graeme-Cook 2002). Desiccation-tolerant Gram-positive rods generally dominate the eggshell microbiota, however, they are not the most predominant in the egg contents. The egg contents are generally infected by Gram-negative bacteria and opportunistic saprophytes (Board 1966; Mayes and Takeballi 1983; Board and Tranter 1986; Bruce and Drysdale 1991; Bruce and Drysdale 1994; Houston *et al.* 1997; Cook *et al.* 2003; Cook *et al.* 2005b).

According to different studies, the types of organisms dominating the eggs can differ within and between species. In a study by Seviour and Board (1972) for example, wildfowl and duck eggs were predominantly infected by different types of organisms (enterobacteria) than chicken eggs (micrococci). Also, duck eggs had a high incidence of *Pseudomonas* spp while the chicken and other waterfowl eggs did not contain any pseudomonads. In a study by Bruce and Jonhston (1978) however, chicken eggs were equally affected by micrococci and enterobacteria. Bruce and Drysdale (1991) have also shown that the presence of certain microorganisms alone (e.g. *Streptococcus* spp.) can reduce hatchability to zero while others (e.g. *Micrococcus* spp.) have a less radical effect.

2.3.2.2 Temperature and humidity

(Note: The effects of temperature and humidity are discussed more thoroughly in Chapter 3.)

Temperature was shown to affect trans-shell infection both at time of laying and during prolonged exposure post laying. At laying, warm eggs cool down creating a negative pressure which can cause microorganisms to be drawn through the pores (Haines and Moran 1940). Post laying, low temperatures can lead to the inactivation of certain antimicrobial proteins present in the albumen (Williams *et al.* 1968; Cook *et al.* 2005a). Likewise, humidity is a very important factor affecting trans-shell infection as it promotes the growth of bacteria as well as providing a medium of transport through the shell pores (Board and Halls 1973; Board *et al.* 1979). Moisture on the surface of the egg can be caused by high humidity in the air or nesting material surrounding the egg, as well as by the egg itself by swelling if temperatures are too high (Howard *et al.* 2003). A temperature differential along with high humidity is, therefore, the combination most likely to enhance trans-shell infection (Board and Halls 1973).

2.3.2.3 Shell and membrane quality

Several studies have revealed the existence of regional and temporal variations in shell thickness, shell conductance and pore structure in avian eggs, with increased shell conductance and pore density, and reduced shell thickness during incubation (Booth and Seymour 1987; Booth 1989; Balkan *et al.* 2006). Booth (1989), for example, showed that shell conductance was increased during incubation due to both an increase in pore density and thinning of the shell. Balkan *et al.* (2006), however, suggested that the increase in conductance was caused by the increase in pore density only and not the thinning of the shell. The same debate exists for the effect of shell thickness on bacterial

infiltration. While some studies have shown that shell thickness does not affect trans-shell infection (*e.g.* Williams *et al.* 1968; Smeltzer *et al.* 1979; Drysdale 1985), others have found that infection is higher when the shell is thinner (e.g. Sauter and Peterson 1974), which could be explained in part by that fact that thicker shells are accompanied by longer pores, which are likely to be more spiralled and thus, more difficult for bacteria with limited mobility to travel through (Mayes and Takeballie 1983).

During incubation, the increase in pore density is vital to the survival of the embryo as it allows gas exchanges with the external environment (Burton and Tullet 1983). Conversely, unplugged pores also provide the only route through which microorganisms can enter and infect the egg contents. An increase in pore density therefore potentially increases the risk of trans-shell infection. Despite this, a study by Drysdale (1985) showed no direct relationship between shell porosity and bacterial infection, and suggested that this was due to the presence of an effective cuticle layer. As mentioned previously however, the cuticle layer is not always evenly distributed and equally effective in all eggs and large areas of porous shell can be deprived of cuticular cover (Board and Halls 1973; Ball *et al.* 1975; Bruce and Johnson 1978). Furthermore, the efficiency of the cuticle is dependent upon the stage of the egg. At laying for example, the cuticle appears to be wet and soft for a few minutes, time during which bacterial penetration is found to be higher (Sparks and Board 1985). After a few days, the cuticle becomes subject to abrasion and can be partially or completely removed (Fromm 1963). Overall, the effects of shell thickness and pore density on trans-shell infection are highly dependent on the presence and quality of the cuticle.

Once microorganisms have penetrated the shell, they must pass through the shell membranes before entering the albumen. The shell membranes are important barriers to bacterial infiltration (Kraft *et al.* 1958), with the inner membrane more effective than

the outer membrane due to its tighter meshwork (Lifshitz *et al.* 1964, Fig. 2.6). Its efficiency, however, is only temporary and is said to last only up to 20h (Walden *et al.* 1956) depending on the age of the egg. The permeability of the inner membrane has also been shown to be influenced by its iron-binding properties. Strong iron-binding properties ensure a constant supply to the bacteria thus allowing it to grow inside the membrane (Tranter *et al.* 1983).

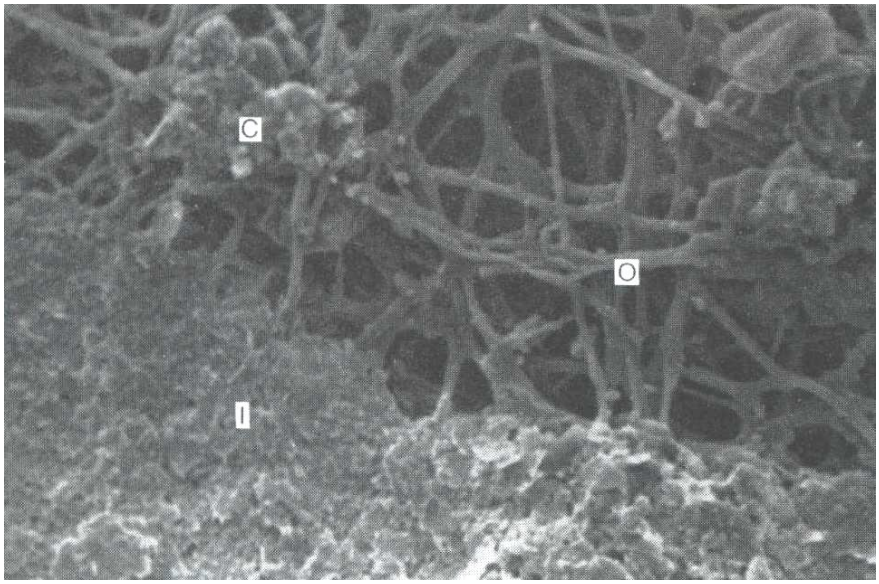

Figure 2.6. Image (x965) of the inner (I) and outer (O) shell membranes and calcium deposits (C) associated with the latter. Image taken from Solomon *et al.* 1994.

2.3.2.4 Chemical defences

The egg's albumen is composed of a wide variety of proteins and enzymes with antimicrobial functions, the three major enzymes being lysozyme, avidin and conalbumin (Board 1966; Board and Fuller 1974). Lysozyme can destroy certain bacteria by splitting the bond between the two major components of the peptidoglycan layer in the cell wall. This enzyme is particularly effective on Gram-positive bacteria due to their high percentage of peptidoglycan (Ibrahim 1998), which explains why

Gram-negative bacteria are more often found in the egg contents than Gram-positive bacteria. Avidin can slow the growth of many bacteria by binding biotin, a vitamin essential for their growth (Board and Fuller 1974). Conalbumin (ovotransferrin) is a protein capable of chelating iron thus reducing free iron available for bacterial growth, and is therefore thought to be one of the major factors preventing the growth of many lysozyme insensitive organisms (Board 1974). Conalbumin represents approximately 10% of the total solids content in the albumen (Alderton *et al.* 1946). The yolk however, contains large quantities of free iron (Kilic *et al.* 2002). Therefore, when bacteria manage to get near the yolk, the iron and other nutrients present in the yolk enable them to grow rapidly. In a study by Lock *et al.* (1992) it was shown that a piece of shell infected with salmonella placed in a sterile dish containing only albumen failed to grow, whereas a piece of this same shell placed in albumen surrounding the yolk was able to grow (Lock *et al.* 1992).

Although mainly seen as a physical barrier, the eggshell also possesses many proteins with antimicrobial functions, some of which have been discovered very recently (Jonchère *et al.* 2010). A study by Mine *et al.* (2003) revealed that egg shell matrix proteins may inhibit certain bacteria (*Pseudomonas aureginosa, Bacillus cereus,* and *Staphylococcus aureus*) by interacting with and disrupting their membranes. These proteins included lysozyme, ovalbumin and ovotransferrin, proteins also found in the albumen, as well as ovocleidin, the first shell specific matrix protein (Hincke 1995).

2.3.3 Consequences of egg infection

The consequences of egg infection have been studied mainly in regards to human health, the major issue being the consumption of eggs contaminated with human

pathogens, mainly *Salmonella spp*. However, microbial infection of eggs can also have considerable consequences on wild bird populations by reducing hatching success and thus population growth.

Infections by salmonellas can occur through both vertical and horizontal transmission, depending on the species (Humphrey 1994). With *Salmonella enteritidis*, the species generally implicated in human illness, infection of the egg is most likely to occur in the oviduct due to a colonization of the hen's intestinal and reproductive tract after consuming contaminated feed (Williams 1981a). Although contamination was sometimes recorded on the shells of infected eggs (Humphrey *et al.* 1989a; Humphrey *et al.* 1989b), this mode of transmission generally leads to a direct contamination of the egg contents, with no traces on the shells (Humphrey *et al.* 1989b; Humphrey *et al.* 1991). With species other than *S.enteritidis* however, infection is more likely to occur through the shell contaminated by the environment (Humphrey 1994). Once inside the egg, it seems that *S. enteritidis* can resist the albumen's chemical defences by producing siderophores which do not only chelate iron but also remove it from the conalbumin complex (Chart 1993). This ability to infect egg contents combined with its high pathogenicity for humans have been the cause of pandemics for over 25 years (Gantois *et al.* 2009).

In wild birds, microbial infection of eggs was first investigated by Cook *et al.* (2005b). They demonstrated that exposure to microbes prior to incubation can significantly reduce hatching success depending on environmental conditions, and that cleaning the shell of these eggs with ethanol can significantly reduce trans-shell infection and increase their hatching success. Exposure prior to incubation is common in many species, and is known as synchronous incubation. For these species, incubation

only begins when all or most of the clutch has been laid, and thus first laid eggs may be exposed to ambient conditions for up to several days. In a study on the barn swallow (*Hirundo rustica*), it was shown that lysozyme concentration was highest for first laid eggs. This was suggested to be due to the increased vulnerability of first laid eggs since they remain in the nest for longer and undergo more cycles of warming and cooling as the mother lays the other eggs (Saino *et al.* 2002). However, in a later study examining eggs from eight different species, it was shown that overall microbial protein levels were not affected by egg order suggesting that increasing antimicrobial protein concentrations in first laid eggs might not be an optimum choice for many bird species. In the following chapters I will thus investigate the consequences of exposure and microbial infection of domestic chicken eggs and wild LBP eggs, eggs that have been shown to possess low levels of lysozyme (Manwell and Baker 1973) and are laid in relatively humid environments in New Zealand. These studies will provide a first indication of microbial infection of avian eggs in New Zealand and assess whether this infection is a cause of decline in little blue penguin egg viability on Tiritiri Matangi Island.

CHAPTER 3.

Shell and trans-shell microbial infection of chicken eggs

Plate 3.1. Chicken eggs and datalogger in artificial burrows at Massey University, New Zealand. Photograph by A.S. Boyer

Abstract

Initiating incubation before clutch completion is common in avian species, and often results in a competitive hierarchy within the brood which can be lethal to the last hatched chicks. Incubating the eggs as soon as they are laid also provides protection to the early laid eggs, otherwise left exposed to ambient conditions for several days. In New Zealand, many native avian species exhibit low hatching success of unknown causes which result in population declines. In this study, I therefore explored the effects of exposure of chicken eggs to ambient conditions and cleaning on the rate of shell and trans-shell microbial infection. I exposed 54 newly laid chicken eggs to ambient conditions in artificial underground burrows for up to 5 days. Half of the eggs were cleaned twice daily while the other half was left uncleaned and microbial communities (colony counts) were compared pre- and post- treatment from shell swabs. Post treatment colony counts from internal egg samples with different exposure periods and cleaning treatments were also compared. Exposure significantly increased shell infection and cleaning significantly reduced shell infection. In contrast, trans-shell infection was only marginally affected by exposure and cleaning. This study provides the first examination of the rates of shell and trans-shell infection in cavity nests in New Zealand.

3.1 INTRODUCTION

The effects of trans-shell infection on the viability of early staged embryos was first investigated in chickens (*Gallus gallus domesticus*) and other commercial species (Board and Tranter 1986; Bruce and Drysdale 1993; Deeming 1995), and only more recently in wild species (Beissinger *et al.* 2005; Cook *et al.* 2005a; Cook *et al.* 2005b). For example, a study with commercial ostrich (*Struthio camelus*) eggs showed that early microbial contamination was a significant problem affecting hatching success (Deeming 1995). In this study, embryonic mortality was high at the beginning and end of incubation and contamination accounted for 22.8% of early deaths. This was found to vary between eggs from different farms, and nest management was recommended. In 2003, Cook *et al.* investigated the effects of ambient conditions and microbes on domestic chicken eggs in tropical environments, which was then followed by similar studies on wild pearly-eyed thrasher (*Margarops fuscatus*) eggs in these same environments in 2005 (Beissinger *et al.* 2005; Cook *et al.* 2005a; Cook *et al.* 2005b). These studies targeted microbial groups known to live on egg shell and infect egg contents (see Chapter 2).

In their studies, Cook (2003), Beissinger *et al.* (2005) and Cook *et al.* (2005a, 2005b) showed that temperature and microbial infection of egg content can equally and independently reduce hatching success. They also showed that incubation reduces growth of bacteria and fungi on egg shells, and that this difference is more marked at cooler and more humid sites (Cook *et al.* 2003; Cook *et al.* 2005a; Cook *et al.* 2005b). Pathogenic microbes were increasingly dominant on exposed eggshells, but these groups were less obvious on incubated eggs, which were dominated by more benign microbial groups (Cook *et al.* 2005a). The studies by Cook *et al.* (2003, 2005b) also

showed that cleaning eggs twice daily with ethanol reduced microbial growth, and increased hatching success at the coolest and most humid site, a site at which hatching success was higher overall regardless of the treatments (Cook *et al.* 2003; Cook *et al.* 2005b). Conversely, in the study by Beissinger *et al.* (2005), hatching success was shown to be higher at the warmer and less humid site. In that study, minimum temperature only significantly affected hatching success, with both mean temperature and proportion of time above physiological zero being only marginally important. In a subsequent study by Godard *et al.* (2007) on chicken eggs, hatching success was shown to increase with the number of intervals above 27°C, a temperature at which antimicrobial proteins are thought to become more active (Williams *et al.* 1968; Cook *et al.* 2005a). Cleaning eggs did not increase hatching success, although it did reduce microbial loads on the egg shell. These studies clearly show that temperature and relative humidity can potentially have strong effects on trans-shell infection of microbes and egg viability of avian species. However, these factors do not seem to have clearly defined and predictable effects and may vary greatly according to the type of environment and possibly the antimicrobial properties of each egg, an aspect that was not investigated. These studies also show that depending on environmental conditions and nest types, cleaning has the potential to significantly increase hatching success. It is thus clear that more studies are necessary to increase our knowledge of the factors affecting egg viability. This is of particular importance in countries like New Zealand where low hatching success is an identified cause of population decline.

3.2 AIMS

The aim of this study is to further investigate the effects of microbes, temperature, relative humidity and cleaning on shell and trans-shell infection, in relatively cold and very humid environments in the Auckland region, New Zealand.

3.3 METHODS

3.3.1 Study species, site and time

In this study, I used four domestic chickens (*Gallus gallus domesticus*) from a farm in Dairy Flat, 30km north of Auckland, New Zealand. These free range chickens were moved to Massey University Albany campus, 10km south of Dairy Flat on the 16th of March 2009 until the 17th of May 2009, for the purpose of this study (Fig. 3.1). All four chickens were at point of lay and did not moult during the study.

Figure 3.1. Map of New Zealand showing the location of the study site. Modified from ©2010 Google Imagery.

3.3.2 Egg collection and artificial nests

Eggs were collected every morning between 9am and 11am, within 2h of laying. After each egg was labelled and swabbed it was placed in one of the three artificial burrows dug in the humid soil in the forest fragment on the Massey University Albany campus. All three burrows were approximately 20cm wide, 20cm high and 30-40cm deep. As in Godard *et al.* (2007), wire mesh was placed at the entrance of the burrows to prevent

predation and interference from surrounding cavity nesting species. Burrows were positioned relatively close to each other (2-5m) to ensure similar environmental conditions and aligned along the river, 1-2m above water level. A data logger (Hobo Pro, Onset Computer Corp.) recording temperature and relative humidity every 30 min was placed in each burrow (Plate 3.2).

Plate 3.2. Artificial burrows used for this study. Nest 1 (A), nest 2 (B) and nest 3 (C). Photographs by A.S. Boyer.

3.3.3 Experimental Design

A total of 54 eggs were used in this study. On the day of laying, eggs were removed from their original nest in pairs, and randomly assigned a treatment (cleaned or uncleaned), an exposure period (1, 3 or 5 days), and one of three replicate artificial nests (following Cook *et al.* 2003 and 2005) as shown in Table 3.1. They were labelled with a xylone-free marker and swabbed to obtain a pre-treatment sample of the microbiota on the eggshell. Eggs were labelled with: Egg number, Treatment (C for cleaned, N for not cleaned), Exposure period (1, 3 or 5), and Date. Ex: 1. C1 16/03. Eggs were then placed in their assigned nest and left exposed to ambient conditions until the end of their predetermined exposure period, where they were then swabbed again to obtain a post-treatment sample of the microbiota on the eggshell. Once swabbed, the eggs were placed in sealable bags and taken to the laboratory where they were opened to sample

the egg contents. Each week one egg was swabbed and opened at laying as a control for vertical transmission of micro-organisms. The proportion of eggs infected and the number of colony forming units (CFU) on the egg shell and inside the eggs were then compared between treatment groups to assess the effects of prolonged exposure, cleaning, temperature and relative humidity on microbial infection. The number of CFU associated with each egg corresponds to the number of CFU counted pre-treatment subtracted from the number of CFU counted post-treatment in order to calculate the number of microbes that were added to the egg during the treatment.

Table 3.1. Distribution of chicken eggs between cleaning treatments, exposure periods and nests.

Treatment Period	NEST 1		NEST 2		NEST 3	
	Cleaned	**Uncleaned**	**Cleaned**	**Uncleaned**	**Cleaned**	**Uncleaned**
1 DAY	C1	N1	C1	N1	C1	N1
	C1	N1	C1	N1	C1	N1
	C1	N1	C1	N1	C1	N1
3 DAYS	C3	N3	C3	N3	C3	N3
	C3	N3	C3	N3	C3	N3
	C3	N3	C3	N3	C3	N3
5 DAYS	C5	N5	C5	N5	C5	N5
	C5	N5	C5	N5	C5	N5
	C5	N5	C5	N5	C5	N5

3.3.3.1 Egg swabbing

To ensure consistency, the eggs were swabbed in the field on the morning of laying and at the same time on the morning of the last day of treatment. They were swabbed over a 3cm diameter area at the blunt end, as this is the area most prone to bacterial penetration

(Vadehra *et al.* 1970). The area swabbed was marked so that future swabs were from the same area. The swabs were then placed in a tube filled with 5ml of physiological saline (Ringer's solution) as described in Cook *et al.* (2003); Cook *et al.* (2005a); Cook *et al.* (2005b) and Godard *et al.* (2007) and cultured within 1h of collection. All swabs were kept in a cooled chilly bin until they were plated.

3.3.3.2 Egg cleaning

Eggs assigned to the cleaning treatment were cleaned twice daily with 70% alcohol to kill microbes on the shell surface and prevent trans-shell infection. Eggs were not cleaned on the last day of their treatment period and thus the post-treatment swab was taken approximately 15-17 h after the last cleaning. Previous experiments have demonstrated that this cleaning treatment is unlikely to negatively affect the shell cuticle (Cook *et al.* 2003; Cook *et al.* 2005a).

3.3.4 Egg content analysis

The methods used to open and sample the eggs followed Cook *et al.* (2003; 2005a; 2005b) and Godard *et al.* (2007), however for practical reasons quantities sampled were ten times higher, as explained below.

3.3.4.1 Egg opening

After the final swab, eggs were transported in sealed bags to the laboratory to be opened. The shell surface of each egg was sterilised with 95% ethanol, and broken open at the blunt end. The egg shell was then peeled away with sterile forceps and a 3cm diameter piece of inner shell membrane was removed from the air-sac region. The

opening was then enlarged and the content of the egg poured into a Petri dish. A 0.1ml aliquot of albumen was removed with a 200µl pipette. The yolk surface (perivitelline membrane) was then sterilised with 70% ethanol and opened aseptically, and a 0.1ml aliquot of yolk was removed in the same way as the albumen. As with the shell swabs, these samples were placed in a tube containing 5ml of sterile physiological saline (Ringer's solution).

3.3.4.2 Targeted microbes

Characterising the whole microbial community living in and on the chicken eggs was beyond the scope of this study, rather the approach here was to detect the most common groups of bacteria known to inhabit bird eggs and also those known to reduce the viability of early-staged embryos, based on extensive studies of bacteria on domestic fowl eggs and some more recent studies on wild bird eggs (Board and Tranter 1986; Kozłowski *et al.* 1989; Bruce and Drysdale 1991; Bruce and Drysdale 1994; Houston *et al.* 1997; Cook *et al.* 2003).

The two media chosen for this study were Tryptose soya agar (TSA), considered to be one of the best general media for both aerobic and anaerobic soil organisms, and MacConkey agar (MAC), which grows only Gram-negative bacteria and differentiates lactose fermenters and non-fermenters. As MAC Conkey plates were incubated at 34°C, the microbes found on these plates were those that can resist such high temperatures, , and thus can potentially remain alive in the egg until full incubation has begun.

3.3.4.3 Plating and Colony counts

Tubes containing the shell swabs and egg content samples were vortexed for 60 seconds to allow the microbes to detach from the swabs and mix homogenously within the solution. When necessary, serial dilutions were made to obtain a countable number of microbes on the plates. A subsample of 0.1ml of saline supernatant from each sample (original sample or diluted sample) was then plated onto each of the two growth media. MAC and TSA plates were then incubated aerobically for 48 h at 34-35 °C and 23 °C, respectively. The number of bacterial colonies (i.e. colony forming units, CFU, per 0.1ml) was then counted on each plate (Plate 3.3). The presence or absence of fungi on each plate was also noted.

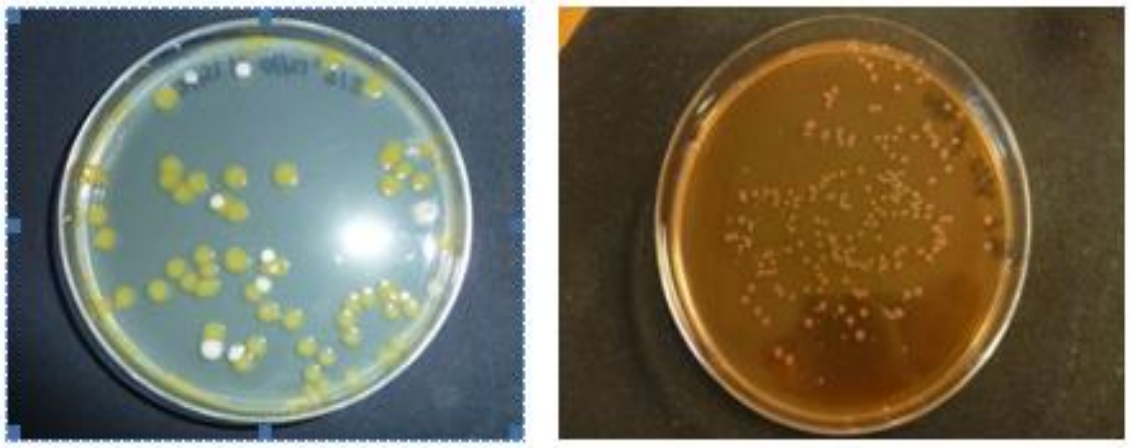

Plate 3.3. Example of a TSA plate (left) and a MAC plate (right). Photographs by A.S. Boyer.

3.3.5 Shell size and thickness

The length and width of each egg was measured using 150mm callipers. In addition, three pieces of shell were taken from the blunt end of each egg, when the egg was

opened. The thickness of each piece of shell was measured using a digital micrometer with a precision of 0.001mm and an average shell thickness was calculated for each egg.

3.3.6 Statistical analysis

Colony count data were log transformed when values within a treatment were too far apart to be observable on the same graph. CFU numbers were then compared within and between treatments using a Mann-Whitney test to assess the effects of cleaning and exposure on shell and trans-shell microbial infection. The shell thickness of eggs that were infected in their contents was also compared to that of eggs with no apparent trans-shell infection using an unpaired T-Test. Variations in temperature and relative humidity were compared within and between nests using a Kruskal-Wallis (KW) one way ANOVA test, and compared to variations in colony counts to determine whether variations in shell or trans-shell infection can be linked to small variations in temperature and relative humidity. The correlation between membrane and shell colony counts was assessed using a Spearmen rank correlation test. Values are expressed as means ± Standard Errors.

3.4 RESULTS

3.4.1 Nest conditions

The daily temperatures over the whole study period (16th March- 17th May 2009) ranged from 11°C to 17.2°C with a mean daily temperature of 14.8°C (±1.5°C). Physiological zero was therefore never reached. The daily relative humidity ranged from 75% to 100% with a very high mean daily relative humidity of 98.1% (±3.3%). Temperature and relative humidity were not correlated during the time of this study (Spearman Rank correlation r_{su}=0.205, p=0.15) and did not significantly vary between nests (T°C: KW=0.9451,n_1=n_2=52, n_3=45, p=0.62, RH: KW=2.655, n_1=n_2=52, n_3=45, p=0.26, Fig.3.2).

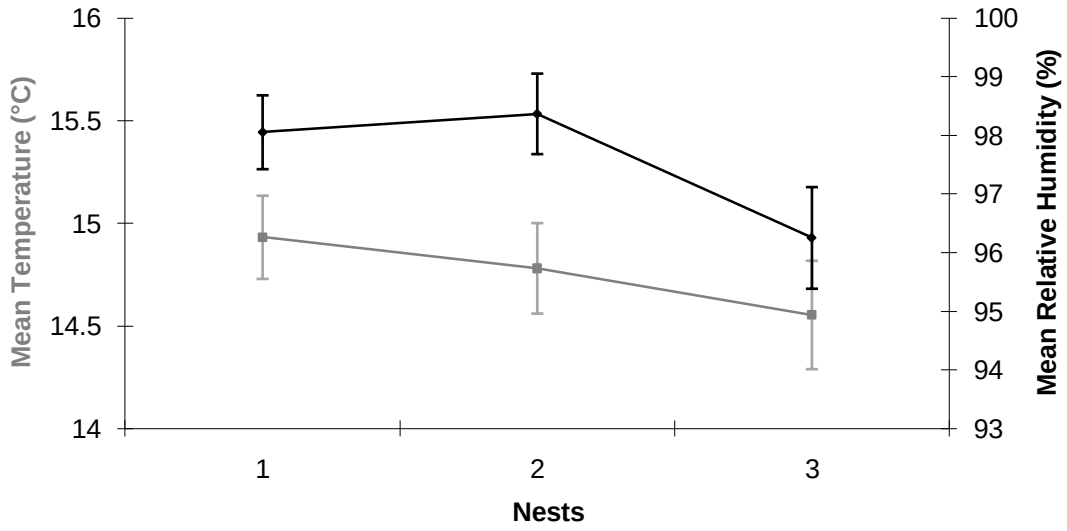

Figure 3.2. Mean temperature (grey) and relative humidity (black) in each nest, over the whole study period (± SE).

3.4.2 Infection at laying

Overall, 87% of the egg shells (n=47) were infected by bacteria at laying. The mean number of CFU at laying was 182.1 (± 83.36), however, this varied greatly between eggs (0 to over 7000). Bacterial growth was much more frequent on TSA plates (87% of the eggs, n = 47) than on MAC plates (20.4% of the eggs, n= 11) with CFU counts also much higher on TSA plates (358.2 ± 166) than on MAC plates (5.98 ± 3.52). The average number of CFU on the shell of control eggs opened at laying was relatively low (74.6 ± 26.4) with an average of 147.3 (± 51.4) CFU on TSA plates and 2 (± 1.68) on MAC plates. No control eggs were found to be infected in the membrane, albumen or yolk.

3.4.3 Treatments

3.4.3.1 Impact of exposure

3.4.3.1.1 Shell infections

A total of 40 eggs (74%, n=54) were infected during this study. The overall percentage of eggs infected on the surface of their shells increased with exposure, regardless of whether they were cleaned or not. All uncleaned eggs exposed for 3 and 5 days and nearly 78% of the uncleaned eggs exposed for 1 day had shell surface infections. The percentage of cleaned eggs infected increased from 44% to 55% to 66% in 1, 3 and 5 days respectively. The infection was recorded on TSA plates in 38 eggs and on MAC plates in 32 eggs (Fig. 3.3). The eggs that showed infection on the MAC plates were generally the same eggs that showed infection on TSA plates (all except one). The difference between post- and pre-treatment CFU counts on uncleaned egg shells significantly increased between 1 day (CFU TSA: 3574.2 ± 2740.8; MAC: 118 ± 88.8)

and 3 days (CFU TSA: 11318.4 ± 5033.9; MAC: 1027.6± 383.8) of exposure (Mann-Whitney Test: TSA U=14, $n_1=n_2=9$, p=0.0188; MAC: U=13.5, $n_1=n_2=9$, p=0.0192 two tailed) and 1 and 5 days (CFU TSA: 90107.6 ± 38385; MAC: 1251.1± 411) of exposure (TSA U=7, n1=n2=9, p=0.0019; MAC U=10.5, $n_1=n_2=9$, p=0.0091 two tailed), yet this increase was not significantly different between 3 and 5 days of exposure (TSA U=21, $n_1=n_2=9$, p=0.0939; MAC U= 39.5, $n_1=n_2=9$, p=0.965 two tailed, Fig 3.4).

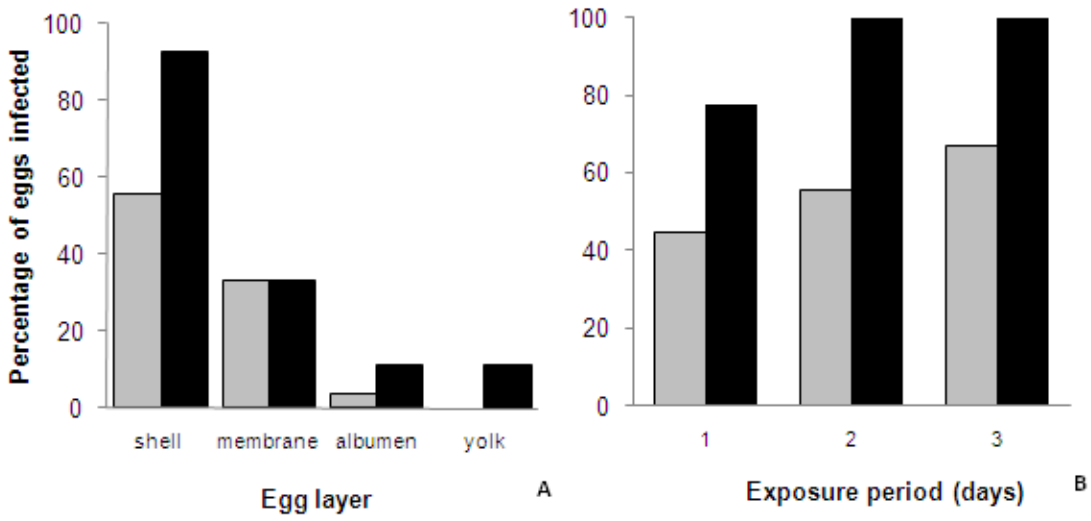

Figure 3.3. Percentage of cleaned (grey) and uncleaned (black) eggs showing infection on either TSA plates or MAC plates (A) in each layer, (B) after each exposure period.

On cleaned eggs, the number of CFU counts did not significantly increase as exposure increased (Mann-Whitney Test p> 0.05 for all exposure periods and plate types, see Appendix 3.6.1). Within MAC data, there was no correlation between Gram negative lactose fermenters and non fermenters (Spearman Rank Correlation r=0.7143, p=0.1361) and neither of these two groups became dominant as exposure increased.

3.4.3.1.2 Membrane, albumen and yolk infections

A total of 18 eggs (33%) were infected in the membrane, all of which were also infected on the eggshells. Of these 18 eggs, 4 showed growth on both TSA and MAC plates, one on MAC plates only and the remaining 13 on TSA plates only. The overall percentage of eggs infected on the membrane did not increase from day 1 (22.2%) to day 3 (22.2%) but did show an increase on day 5 (55.6%, see Fig 3.3). CFU were found on very few MAC plates and only after 3 days (n=1) and 5 days (n=4) of exposure, whereas TSA plates were infected more often and after all three exposure periods (n=4, n=4, n=9 for 1, 3 and 5 days, respectively). Within MAC plates, no significant difference was found between CFU counts of fermenting and non fermenting bacteria (Mann Whitney: U= 157.5, n1=n2= 18, p=0.8949). Comparisons between post and pre counts on the eggs' membranes revealed no significant difference between exposure periods within cleaned and uncleaned eggs (for all exposure periods and plate types, see Appendix 3.6.1, Fig 3.4).

A total of 5 eggs were infected in the egg yolk and albumen (on TSA plates only). All five eggs with infected egg contents had been through all three exposure periods.

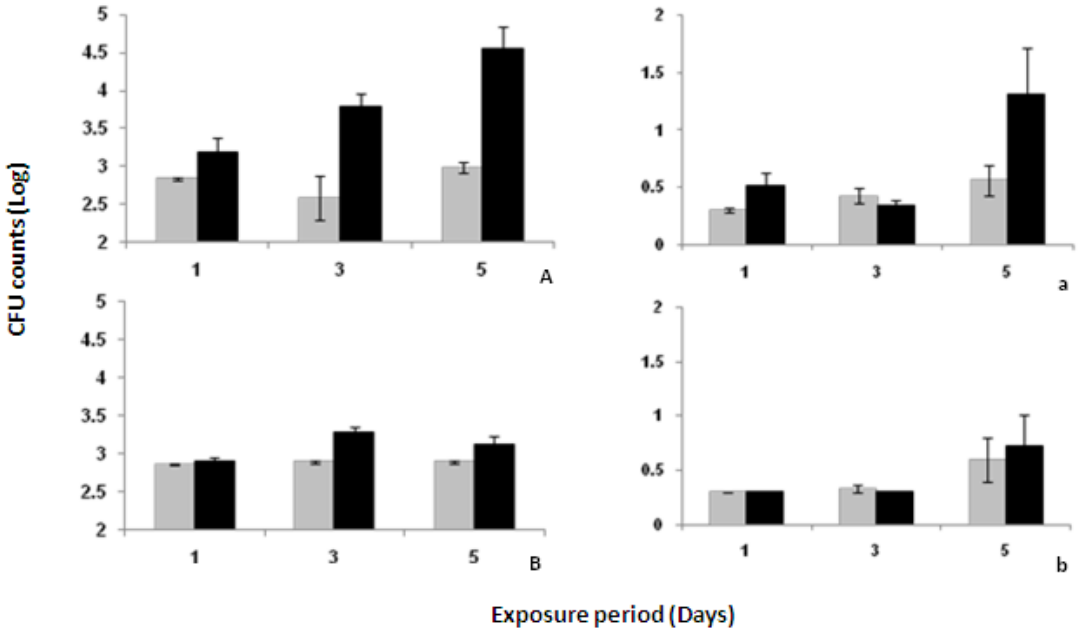

Figure 3.4. Effects of duration of exposure on microbial growth on the egg shell (A,B) and membrane (a,b) of cleaned (grey) and uncleaned (black) eggs. These graphs show the mean (± SEM) difference between post- and pre-treatment CFU counts on TSA plates (A,a) and MAC plates (B,b) after a log transformation.

3.4.3.2 Impacts of egg cleaning

3.4.3.2.1 Shell infections

Overall, the percentage of cleaned egg shells infected (55.6%, n= 27) was nearly half of that of uncleaned egg shells (92.6%, n=27). After one day of exposure 44% (n=9) of cleaned egg shells and 78% (n=9) of uncleaned eggs were infected on either TSA or MAC plates. After 3 days of exposure 56% (n=9) of cleaned and 100% (n=9) of uncleaned egg shells were infected. After 5 days of exposure 67% (n=9) of cleaned and 100% (n=9) of uncleaned egg shells were infected.

The number of CFU was significantly higher for uncleaned eggs than cleaned eggs after 1, 3 and 5 days of exposure (Mann Whitney U= 12.0 ,n1=n2=9, p= 0.0134; U=1.0, n1=n2=9, p<0.0001; U= 1.0, n1=n2=9, p<0.0001 for 1, 3 and 5 days of exposure, respectively) on TSA plates and after 3 and 5 days of exposure (Mann

Whitney U = 10.0, $n_1=n_2=9$, p=0.008; U=8.0, $n_1=n_2=9$, p= 0.0047 for 3 and 5 days of exposure respectively) on MAC plates (see Fig 3.3 and Appendix 3.6.1). No significant correlation was found between the number of CFU counted for cleaned and uncleaned egg shells of a same pair (Spearman Rank correlation TSA plates: r_{su}= 0.3254, p=0.3853; r_{su}= -0.2455, p=0.5206; r_{su}= 0.2098, p= 0.5809 for 1, 3 and 5 days, respectively; MAC plates: r_{su}= 0.1399, p=0.7081; r_{su}= 0.5295, p= 0.1475; r_{su}= 0.07429, p= 0.8432, for 1, 3 and 5 days, respectively, Fig 3.5).

3.4.3.2.2 Membrane, albumen and yolk infections

Overall, a third of both cleaned and uncleaned eggs were infected in the membrane. Cleaning, therefore, had no effect on the proportion of eggs infected in the membrane. By day 5, however, uncleaned eggs had on average nearly twice as many bacteria present in their membranes than cleaned eggs (Fig. 3.3), although this was not found to be significant due to great variations within this sample (Mann Whitney U= 28.0, n1=n2=9, p=0.2843; U= 38.5, $n_1=n_2=9$, p= 0.8904 for TSA plates and MAC plates, respectively). In addition, three out of the four eggs infected in the albumen and all three eggs infected in the yolk were from the uncleaned treatment. For these eggs, microbial growth was observed only on the TSA plates.

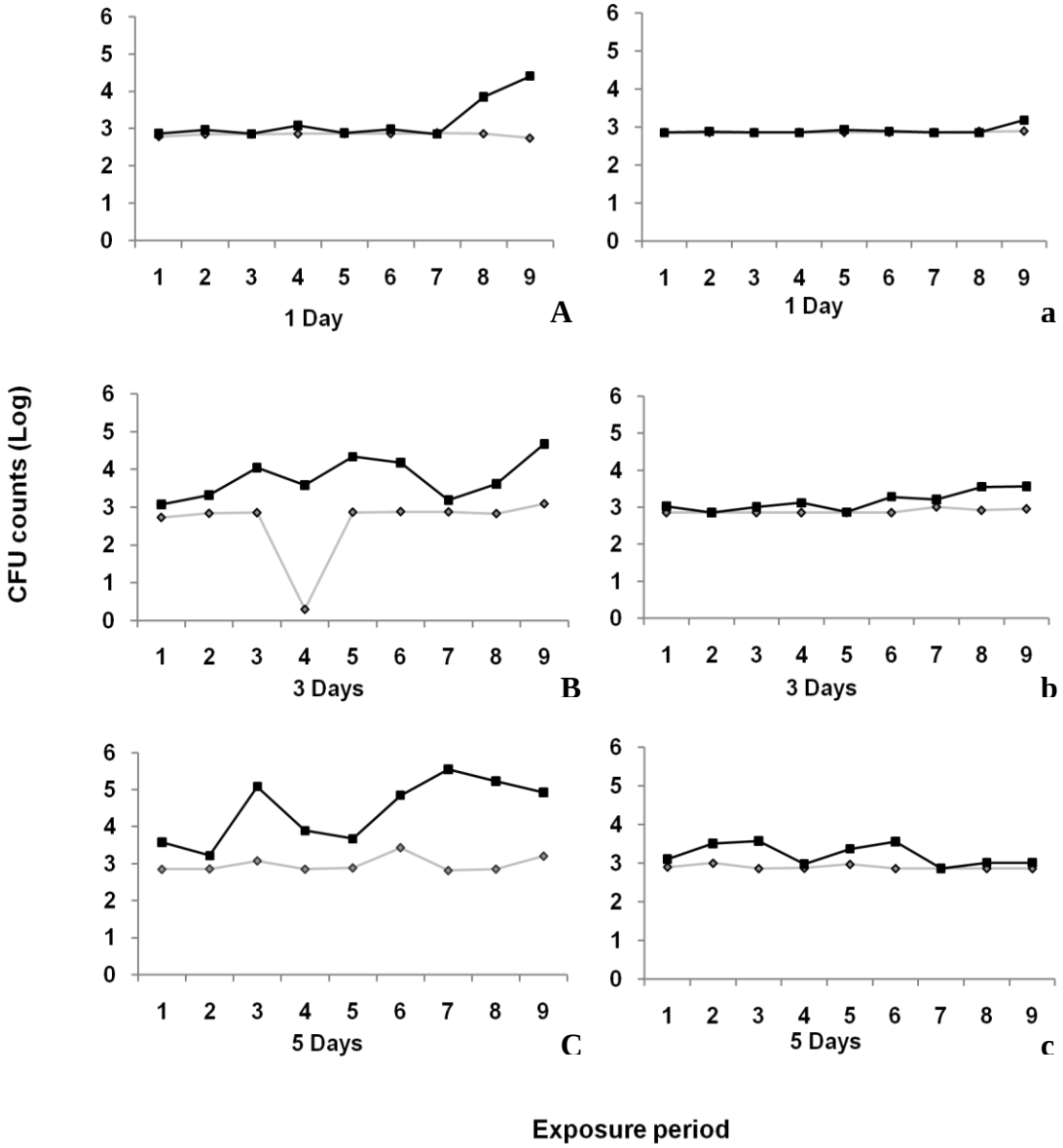

Figure 3.5. Microbial growth on egg shells on cleaned (grey) and uncleaned (black) eggs after 1 (A, a), 3 (B, b) and 5 (C, c) days of exposure. Capital letters correspond to CFU counts on TSA plates while lower case letters correspond to counts on MAC plates.

3.4.4 Shell and trans-shell infection

Membrane infection was observed on TSA plates only after one and three days of exposure, and on both plates after five days of exposure. There was no correlation found between CFU counts from egg shells and egg membranes, suggesting that infection in the membrane is not proportional to shell infection (Spearman rank correlation TSA

plates: r=0.3482, p=0.1568; r=-0.02411, p=0.9243; r=0.4179, p=0.0844 for 1, 3 and 5 days of exposure, respectively, Fig 3.6). All eggs infected in the membrane, albumen and yolk, however, were also infected on the shell, and 3 out of 5 eggs infected in the albumen and yolk also showed evidence of infection in the membrane.

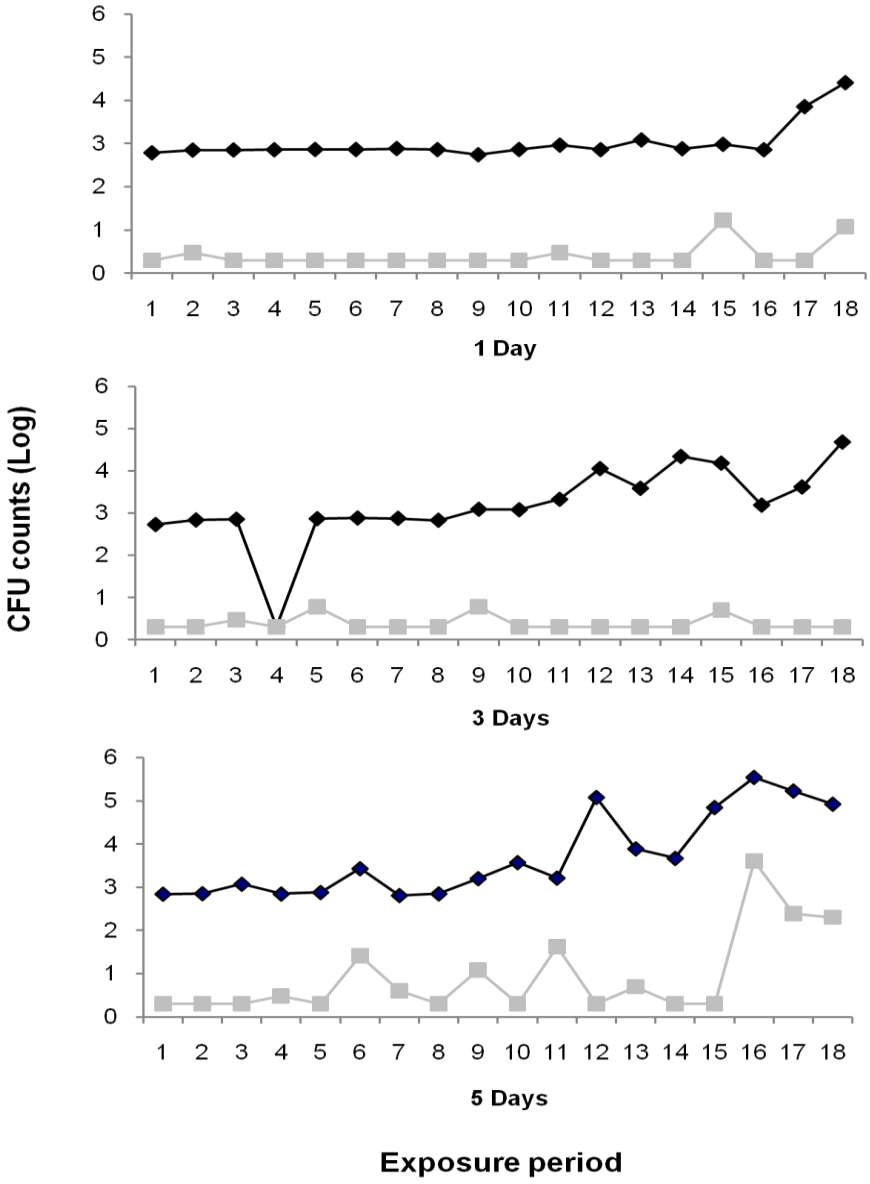

Figure 3.6. Shell (black) and membrane (grey) CFU counts after 1, 3, and 5 days of exposure.

3.4.5 Impact of temperature and humidity on shell and trans-shell infection

Temperature and relative humidity remained relatively constant throughout the whole study. No correlation was found between variations in either of these two parameters and variations in shell or membrane CFU counts (Spearman Rank correlation, Fig. 3.7, Appendix 3.6.2). All eggs infected in the albumen and yolk, however, were those exposed to the highest relative humidity (100% RH).

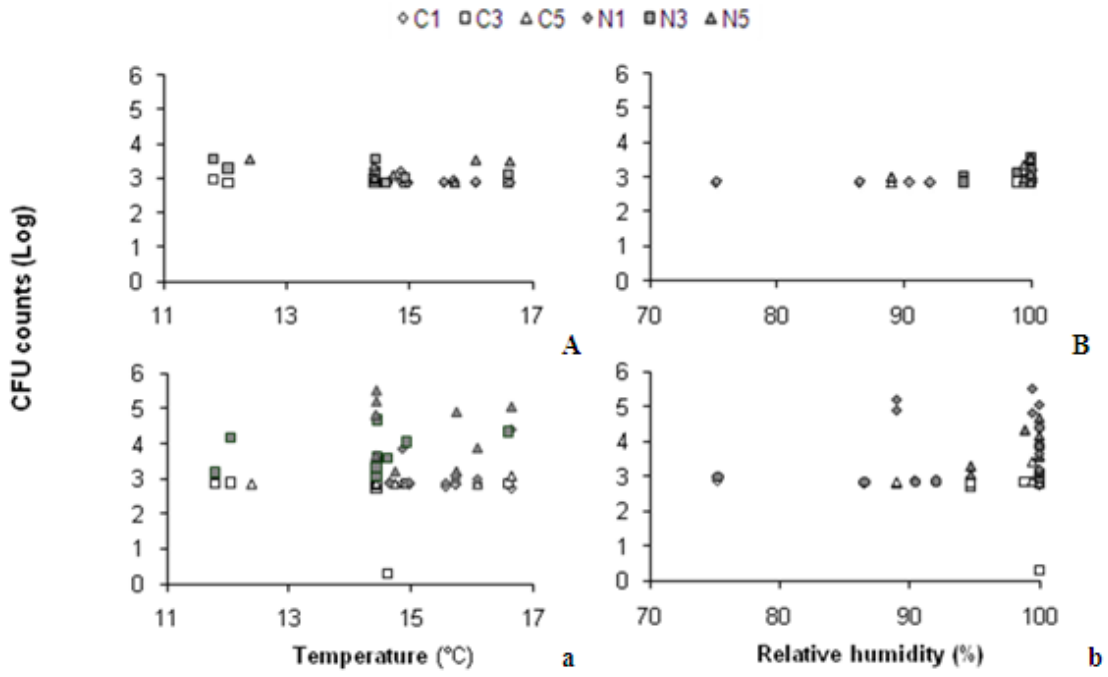

Figure 3.7. Relationship between mean CFU counts (log transformation) on egg shells of cleaned (C-white points) and uncleaned (N-grey points) and temperature (A,a) and relative humidity (B,b). Capital/Upper case letters correspond to CFU counts on TSA plates while lower case letters correspond to counts on MAC plates.

3.4.6 Shell size and thickness

The mean (± SE) egg size was 57.4mm (±3.6, range 52-66.4mm) in length and 43.3mm (±1.6, range 40.8-46.9mm) in width, with a shell thickness of 0.348mm (±0.039, range 0.221-0.409mm). Eggs that showed evidence of trans-shell infection were on average thinner (0.341 ± 0.009mm) than eggs with no sign of trans-shell infection (0.356 ± 0.006mm), although this result was not statistically significant for this sample size (Unpaired t-test t=1.422, p=0.1610). Cleaning did not significantly impact egg shell thickness (Unpaired t-test t=0.4709, p=0.6397).

3.4.7 Fungal infection

A total of 37% (n=54) of the eggs showed fungal infection on the eggs shell at some stage during the treatment. Of these eggs, 75% were infected by fungi on the day of laying and 90% by the last day of treatment. All but two eggs infected at laying were still infected post-treatment. Cleaning thus, did not reduce fungal infection. Of all eggs with trans-shell infection (n=18), only 44% showed fungal growth on the egg shell while 56% did not. The presence of fungi, therefore, did not increase the risk of trans-shell infection.

3.5 DISCUSSION

The aim of this study was to compare the effects of cleaning treatments, exposure periods, abiotic factors such as temperature and relative humidity and variations in shell thickness on the microbial infection of the egg shell and its content, within a specific environment. As with previous ecologically focused studies (Cook *et al*. 2003; Cook *et al*. 2005a; Godard *et al*. 2007), I did not identify specific pathogenic microbes but rather looked at the general effect of exposure and cleaning on groups of microbes known to pathogenically infect the egg and its content.

As found in Cook *et al.* (2003) the origin of microbes found within the eggs cannot be proven to be 100% trans-shell infection as opposed to vertical transmission from the mother. Trans-shell infection, however, is very likely to be the cause of albumen and yolk infections given that none of the control eggs opened at laying showed evidence of any content infection.

3.5.1 Exposure and cleaning

As expected, microbial growth on the egg shell of newly laid chicken eggs exposed to ambient conditions increased rapidly with increasing exposure. Consistent with previous studies (Cook *et al.* 2003; Cook *et al.* 2005b; Godard *et al.* 2007), I found that cleaning significantly decreased microbial infection on the egg shells even after only one day of exposure. Unlike Cook *et al.* (2003) however, who found that infections reached the inner membrane by day one, the albumen by day three and the yolk by day five, infections within the egg in this study were rarely past the shell membrane, and albumen and yolk infections were found at all three exposure periods. Cook *et al.* (2003) also found that cleaning reduced trans-shell infection, which was not the case in this study.

While it seemed to reduce the amount of bacteria penetrating the shell after five days of exposure, it did not reduce the overall proportion of eggs infected, as found in Godard *et al.* (2007).

Potential errors in the sampling technique resulting in a failure to detect some existing microbes cannot be excluded, however, they were limited by the use of the same methodology as previous studies with even higher quantities of content sampled. This suggests that in cases where no growth was observed, either microbes were absent or present in such low concentrations that they could not be detected, or they were unable to grow on the media provided and thus did not belong to the groups targeted.

3.5.2 Nest types and environmental conditions

Cavity nests have been found to be only marginally affected by exposure and cleaning of eggs in comparison to open cup nests (Godard *et al.* 2007). Indeed, with much lower microbial loads found on the shells of cavity nest eggs, the effects of cleaning and exposure on trans-shell infection were less noticeable. Hence, Godard *et al.* (2007) showed that cavity nests and open cup nests do not provide the same protection in terms of microbial infection. The nest type, however, is not the only factor affecting microbial infection rates. Temperature and relative humidity, for example, have been shown to be crucial factors affecting shell, trans-shell infection and egg viability in synchronously incubating species, with low temperatures and high relative humidity being the combination that is most likely to promote shell and trans-shell infection (Beissinger *et al.* 2005; Cook *et al.* 2005a; Cook *et al.* 2005b). Depending on the environmental conditions the effects of exposure and cleaning could thus potentially vary significantly within similar nest types.

In the present study, a much higher percentage of eggs was found infected inside the egg than in the study by Godard *et al.* (2007) (33%, n=54 *versus* 5.6%, n=18, respectively). This could be partly explained by relatively different environmental conditions in the study areas. Firstly, humidity, which is believed to promote microbial growth on the egg shell and facilitate trans-shell infection (Board and Halls 1973; Board *et al*. 1979), was on average much higher in this study than in the one by Godard *et al.* (2007) (98.1 ±3.3%, range of 75-100% compared to 85 ± 3.7% range of 58-96%, respectively). Second, temperatures recorded were much lower in the present study than in Godard *et al.* (2007) (14.8 ±1.5°C, range of 11- 17.2°C versus 20.9± 1.1°C, range of 15.2-28.7°C, respectively) with, unlike in Godard *et al.* (2007), no intervals above 27°C, a temperature at which antimicrobial enzymes are thought to be activated (Williams *et al.* 1968; Cook *et al.* 2005a).

The structure and constituents of cavity nests can also vary greatly between species, and this composition could potentially affect the likelihood of microbial infection. Godard *et al.*'s (2007) study used human made cavity nests (bluebird nest boxes) which had been cleaned with a bleach solution and covered with a sterile coconut fibre lining and grass material that had been previously frozen. In the present study, cavity nests were excavated in the ground 10 to 15 days prior to the experiment and were not cleaned or supplemented with sterile lining, in order to be more comparable to natural nests.

3.5.3 Shell thickness

Shell thickness was occasionally shown to be an important factor in trans-shell infection (Chapter 2), thinner eggs being possibly more susceptible to bacterial penetration. In

their earlier studies, Cook *et al.* (2003; 2005a) were the first to provide direct evidence that egg viability differs between avian species with altricial and precocial modes of development, and suggested that this could be due to their smaller egg size, thinner shell thickness and possibly differences in shell porosity (Cook *et al.* 2005a). Furthermore, Godard *et al.* (2007) showed that even within a given species, small variations in shell thickness can play an important role in hatchability. In the present study, eggs that showed evidence of trans-shell infection were also on average slightly thinner than eggs with no bacterial penetration, reinforcing the idea that thinner shells might be more likely to be penetrated by microbes.

3.5.4 Fungi

It has been shown in previous studies that fungi present on the eggshell can cause damage to the eggshell, creating more unplugged pores through which microbes can penetrate (Board and Halls 1973; Board *et al*. 1979; Baggott and Graeme-Cook 2002), thereby increasing the risk of hatching failure (Cook *et al.* 2003). In the present study, exposure increased the presence of fungi on the egg shells, although they were already common on day 0 eggs. Fungi, however, did not increase the risk or number of microbes penetrating the egg shell, suggesting that they may not play the most important role in trans-shell infection. This was also found in the study by Godard *et al.* (2007) in which fungi alone was found not to play a predictive role in trans-shell infection and hatchability. Cleaning did not reduce fungal infection either, suggesting that fungi can rapidly reinvade the egg shell after cleaning.

3.5.5 Conclusions

Overall, this study suggests that under certain environmental conditions, cleaning has the potential to reduce shell and thus possibly trans-shell infection of eggs left exposed for p to 5 days. However, the effects of exposure, cleaning and environmental conditions on microbial infection do not seem to have a clearly defined and repeatable effect on all eggs. Indeed, similar factors may affect eggs in different ways in different nest types as well as within nests of the same type, depending on environmental factors and egg properties such as shell thickness. Antimicrobial properties, an aspect that has not been investigated in this study, might also affect egg infection and should be considered in future studies. This study therefore suggests that the effects of microbial infection should be investigated for additional species experiencing low hatching success, and in various nesting sites, especially prior to translocations and when creating artificial nests.

3.6 APPENDIX

3.6.1 Shell and membrane microbial infection.

Comparisons of CFU counts between exposure and cleaning treatments and their significance (two tailed Mann-Whitney Test,* represent significant p values).

	MAC			TSA		
	Comparisons	**U value**	**P value**	**Comparisons**	**U value**	**P value**
Shell	C1 VS N1	25.0	0.1964	**C1 VS N1**	**12.0**	**0.0134 ***
	C3 VS N3	**10.0**	**0.0080 ***	**C3 VS N3**	**1.0**	**<0.0001 ***
	C5 VS N5	**8.0**	**0.0047 ***	**C5 VS N5**	**1.0**	**<0.0001 ***
	C1 VS C3	25.0	0.1813	C1 VS C3	39.0	0.9296
	C1 VS C5	22.0	0.1098	C1 VS C5	32.5	0.5077
	C3 VS C5	37.0	0.7890	C3 VS C5	27.0	0.2581
	N1 VS N3	**13.5**	**0.0192 ***	**N1 VS N3**	**14.0**	**0.0188 ***
	N1 VS N5	**10.5**	**0.0091 ***	**N1 VS N5**	**7.0**	**0.0019 ***
	N3 VS N5	39.5	0.9648	N3 VS N5	21.0	0.0939
Membrane	C1 VS N1	-	-	C1 VS N1	30.5	0.3853
	C3 VS N3	-	-	C3 VS N3	31.0	0.4067
	C5 VS N5	38.5	0.8904	C5 VS N5	28.0	0.2843
	C1 VS C3	-	-	C1 VS C3	30.5	0.3853
	C1 VS C5	-	-	C1 VS C5	25.5	0.1893
	C3 VS C5	35.0	0.6441	C3 VS C5	34.5	0.6208
	N1 VS N3	-	-	N1 VS N3	31.0	0.4067
	N1 VS N5	-	-	N1 VS N5	26.0	0.2107
	N3 VS N5	-	-	N3 VS N5	20.5	0.0799

3.6.2 Correlation between temperature, relative humidity and microbial infection

Correlations between mean values of CFU counted on the shell (A) and in the membrane (B) of eggs from each treatment and temperature and relative humidity the eggs were exposed at (Spearman Rank correlation).

A	Temperature				Relative humidity			
SHELL	MAC		TSA		MAC		TSA	
	R value	P value	R value	P value	R value	P value	R value	P value
C1	-0.4360	0.2499	-0.5672	0.1206	-0.0804	0.8432	-0.3907	0.2912
N1	-0.0183	0.9816	0.5690	0.1206	0.0479	0.9116	0.6033	0.0968
C3	-0.1657	0.6777	-0.2353	0.5517	0.5892	0.0968	0.3287	0.3853
N3	-0.4118	0.2696	0.5042	0.1618	0.4980	0.1777	0.3884	0.2912
C5	0.1185	0.7756	0.1681	0.6777	-0.0048	0.9816	0.1844	0.6436
N5	-0.0084	0.9816	0.2773	0.4630	0.4352	0.2499	0.5809	0.1080

B	Temperature				Relative humidity			
MEMBRANE	MAC		TSA		MAC		TSA	
	R value	P value	R value	P value	R value	P value	R value	P value
C1	-	-	0.2750	0.4630	-	-	0.3592	0.3363
N1	-	-	0.8253	0.0083 *	-	-	-0.0416	0.9116
C3	-	-	0.6629	0.0589	-	-	0.0655	0.8801
N3	-	-	-0.4143	0.2696	-	-	0.2455	0.5206
C5	-0.5178	0.1618	0.0368	0.9484	-0.3409	0.3586	-0.1414	0.7081
N5	-0.1036	0.8100	0.2458	0.5206	-0.7955	0.0138 *	-0.0405	0.2696

CHAPTER 4.

Effect of Nest Type, Microbial Infection and Exposure on the Viability of Little Blue Penguin (*Eudyptula Minor*) Eggs on Tiritiri Matangi Island, New Zealand

Plate 4.1. Little blue penguin incubating eggs in a soil nest on Tiritiri Matangi Island, New Zealand. Photograph by A.S. Boyer.

Abstract

In New Zealand, low hatching success has often been described as an important cause of population decline in native avian species, its primary cause being inbreeding. However, other factors such as nest type, ambient conditions and exposure to microbes have recently been found to significantly impact on the hatching success of wild birds in other countries, and have not yet been investigated in New Zealand. The goal of this study was therefore to investigate the role of nest type and exposure to microbes, as well as other factors such as lay date and parental behaviour, on the hatching success of little blue penguins (*Eudyptula minor*) on Tiritiri Matangi Island, Hauraki Gulf, New Zealand. This species, that seems to delay full incubation until both eggs are laid, breeds in particularly humid and soiled nests and possesses low levels of lysozyme, an important antimicrobial protein. This study revealed that nest type, egg order and shell cleaning did not affect hatching success, suggesting that nest conditions and microbial infection prior to incubation are not a major cause of egg mortality in this population. Temporary abandonment during incubation was very frequent in the second half of the breeding season and fatal to most eggs. These temporary abandonments seemed to be caused by resource limitations, an aspect that should be investigated in future studies.

4.1 INTRODUCTION

4.1.1 Background information on little blue penguins

The little blue penguin (*Eudyptyla minor*: Spheniscidae) is the smallest of all penguin species, averaging a height of 33cm and a weight of 1.1kg (Marchant and Higgins 1990). It is distributed along the New Zealand and southern Australian coasts, and can be found on both the mainland and offshore islands (Marchant and Higgins 1990). Little blue penguins feed exclusively at sea but must return to land to breed and moult.

4.1.1.1 Subspecies and status

Originally, *Eudyptula* penguins were divided into two species, the little blue penguin (LBP) *E. minor* (Forster 1781) and the white-flippered penguin *E. albosignata* (Finsch 1874). Later, the two *Eudyptula* penguins were reunited into one species but divided into six subspecies based on morphometric analysis (Kinsky and Falla 1976). These included *E.m novaehollonsiae* (South Australia), *E.m chathamensis* (Chatham Islands, New Zealand), *E.m minor* (Otago, Southland, and South Island's West Coast, New Zealand), *E.m albosignata* (Banks Peninsula and Motunau Island, New Zealand), *E.m variabilis* (southern North Island and Cook Strait) and *E.m iredalei* (northern North Island, New Zealand). More recent molecular analysis, also supported by morphometric and vocalisation data, has suggested that little blue penguins are in fact divided into two clades mixing both Australian and New Zealand populations (Banks *et al.* 2002), the first clade consisting of Otago and Australian populations, and the second consisting of North Island, Cook Strait, Chatham Island, and Banks Peninsula populations. In 2009, genetic analysis confirmed the existence of only one subspecies in Australia, however, was not consistent with the suggested subspecies distribution within New Zealand

(Peucker *et al.* 2009). Despite these new findings, it is the sub species classification by Kinsky and Falla (1976) that generally persists in the popular and scientific literature. Whether considering sub species or clades, no research has yet proven that any LBP are reproductively isolated from each other.

According to the IUCN classification (IUCN 2009), the LBP species is listed only "of least concern" due to its very large range. The entire population has been estimated at 350,000-600,000 breeding pairs (Dann 2006), although from their study on Philip Island, Australia, Dann and Cullen (1990) have established that pre-breeding individuals constitute around 57% of the population. This results in a total population estimate of around one million individuals. In New Zealand however, LBP populations are classified by the Department Of Conservation as "range restricted" to "nationally vulnerable" (Hitchmough 2007). The northern North Island subspecies *E.m iredalei* is classified as "in decline".

4.1.1.2 Breeding ecology

Breeding generally begins at the age of 2-3 years (Dann and Cullen 1990; Dann *et al.* 1995), although birds breeding under the age of 16 months have been reported in New Zealand (Perriman and Steen 2000). This species is considered monogamous (Marchant and Higgins 1990) however, mate fidelity can be low in populations with high mortality rates (Jones 1978). The breeding season begins during the austral winter and extends until summer (Marchant and Higgins 1990) and the initial lay date is highly variable within and between populations (Perriman and McKinlay 1995).

The main factor influencing the onset of LBP breeding is generally thought to be prey availability (Perriman and McKinlay 1995; Numata *et al.* 2000), which can be

influenced by variations in Sea Surface Temperatures (SST) and in the Southern Oscillation Index (Reilly and Cullen 1981; Michelson *et al.* 1992; Perriman *et al.* 2000). The Southern Oscillation Index (SOI) is the difference in atmospheric pressure between Tahiti and Darwin and is used as a descriptor of large scale climatic perturbations such as El Niño and La Niña events (Allan *et al.* 1996). These climatic variations can also affect overall breeding success and mortality (Perriman *et al.* 2000).

The LBP is the only penguin capable of laying and rearing two clutches in a year (Williams 1995). Clutches are generally comprised of two eggs, laid on average 3 days apart (Kinsky 1960; Gales 1984; Kemp and Dann 2001, Heber 2008). Eggs are incubated for 33-36 days (Reilly and Bamford 1975; Reilly and Cullen 1981; Chiaradia and Kerry 1999), and first laid eggs take significantly longer to hatch than second laid (Kemp and Dann 2001). This is thought to be due to a delay in brood patch development delaying full incubation until the second egg is laid (Gales 1984, Kemp and Dann 2001). Once hatched, chicks are guarded by parents for up to three weeks before entering a post-guard stage, where both parents forage at sea during the day, generally returning to feed the chicks at night (Williams 1995). After five weeks, chicks begin to wander out of their burrows (Reilly and Cullen 1981), before finally fledging around the age of 7-10 weeks, and at least 80% adult weight (Fortescue 1995; Fortescue 1999; Perriman *et al.* 2000; Renner and Davis 2001; Heber *et al.* 2008). At the end of the breeding season, adult penguins undergo moulting, a period at which they must stay ashore.

Although highly variable between species and years, hatching and breeding success are generally low in most penguin species (Williams 1995), and can be influenced by parents' incubation behaviour (e.g St Clair 1992), egg size (e.g Warham

1975), nest type (e.g Seddon and Davis 1989) and environmental conditions (e.g Perriman *et al.* 2000). In LBP populations environmental conditions, food availability (Cullen *et al.* 1992; Perriman and Mc Kinlay 1995), and nest site and type (Perriman and Mc Kinlay 1995; Bull 2000a; Perriman and Steen 2000; Miyazaki and Waas 2003a; Knight and Rogers 2004) seem to have the most impact on egg and chick viability.

4.1.1.3 Feeding ecology

LBP are considered top predators, although they are also preyed upon by sea mammals and sharks (Spellburg 1975). They are inshore feeders and feed primarily on small pelagic fish, squid, and crustraceans (Croxall and Davis 1999; Perriman *et al.* 2000). They can, however, adjust their diet depending on seasonality and prey availability (Klomp and Wooller 1988; Schreiber and Burger 2002). On Tiritiri Matangi Island, a study based on stomach regurgitation showed that major prey species included anchovy (*Engralis australis*), yellow-eyed mullet (*Aldrichetta forsteri*), red cod (*Pseudophycis bachus*), and arrow squid (*Nototododarus sloanii*) along with other unidentifiable fish (Geurts 2006). Also, stable isotope ratios from feathers supported by prey size ranges revealed that LBP feed mainly on juvenile prey (Geurts 2006).

Radiotracking studies showed that most LBP foraging trips during the year last one day (Weavers 1992; Collins *et al.* 1999). During incubation, however, these trips can last for up to several days. On Motuara Island, New Zealand, for example, the mean duration of trips recorded was up to 7.2 days, the longer the trip the higher the probability of nest desertion. (Renner 1998).

4.1.1.4 Threats

On the mainland and unprotected islands, predators and habitat loss or modification are thought to be the main causes of decline in LBP populations (Stahel and Gales 1987; Dann 1992; Dann 1994; Norman *et al.*1992). As LBP are highly philopatric, changes in habitat structure and vegetation can strongly influence the breeding success of these birds (Fortescue 1995; Bull 2000b). On predator free islands and protected areas, changes in climate and sea conditions leading to declines in food supply are thought to be the most important factors affecting LBP breeding and viability, a threat which also affects mainland populations (Perriman *et al.* 2000). On the Otago Peninsula and Oamaru, New Zealand, for example, it was reported that under La Niña conditions, LBP initiate breeding later, have less double breeders and a lower fledging success than under El Niño conditions and normal years (Perriman *et al.* 2000).

Another major concern in Australia and New Zealand is the mass beach wrecks that are thought to occur regularly (Geurts 2006). In Australia, they were found to be associated mainly with mass mortalities of one of their food species (Dann *et al.* 2000). This, however, has not yet been investigated in New Zealand.

4.1.2 Reasons for choosing this study species

As mentioned above, LBP from Titiriri Matangi Island belong to the subspecies *E.m iredalei*, which is currently classified as “declining”. Although the current state of the Tiritiri Matangi population remains unknown due to a lack of ongoing monitoring, the past few studies have revealed relatively low levels of hatching success. Identifying the possible causes of egg mortality therefore seems essential for the future management of LBP. In addition, this species exhibits particular nesting behaviours such as

synchronous incubation and laying in soiled and humid nests, that are believed to increase the risk of egg microbial infection, a cause of early embryo mortality. LBP eggs have also been shown to contain only low levels of lysozyme, an important egg-white antimicrobial protein, which could potentially lead to a lowered antimicrobial defence system (Manwell and Baker 1973).

4.2 AIMS

The aim of this study is to identify possible causes of little blue penguin egg failure on Tiritiri Matangi Island, and in particular the effects of nest type and prolonged exposure to ambient conditions and microbes.

4.3 METHODS

4.3.1 Study site

4.3.1.1 Study site and time

This study was conducted during the 2009 breeding season (July-December) on Tiritiri Matangi Island (36°36'S, 174°53'E), a 220ha island situated in the Hauraki Gulf, 3.5 km east of Whangaparoa Peninsula and 30km north of Auckland, New Zealand (Fig. 4.1). This island, originally occupied by Maori and European settlers for cultivation and farming, became a recreation reserve in 1970 (Rimmer 2004). In 1980 it was reclassified as a scientific reserve open to the public, and by 1993 goats (*Capra hircus*), rabbits (*Oryctolagus cuniculus*), cats (*Felis catus*), rats (*Rattus exulans*) and mustelids had been eradicated (Drey *et al.* 1982; Mitchell 1985). By 1994, a community restoration group had established around 400,000 trees over 60% of the island (Rimmer 2004; Brunton and Stamp 2007), creating a suitable environment for the translocation and reintroduction of threatened native fauna and flora. The island is now covered by remnant mature forests, replanted forests and patches of grassland. The coastline is composed of shingle beaches, large grey-wake boulders and rocky headlands (Jones 1978).

4.3.1.2 Sampling area

The main area surveyed for penguin burrows was the coastline, but areas of flax, forest and grassland were also checked. These areas were named according to their location on the island, as follows (Fig. 4.1 C):

1. Wharf North (WN): From the Wharf (through Hobbs beach) up to North West Point
2. Wharf South (WS): From the Wharf down to below the Wattle Valley area
3. Emergency North (EN): From Emergency Landing to Fisherman's bay
4. Emergency South (ES): From Emergency Landing to the Lighthouse area
5. North (N): Papakura Point
6. Cable Road (CR): Road joining Hobbs Beach and Ridge track
7. Wattle Valley (WV): Pipes and walkways in the Wattle Valley area.

Figure 4.1. Map of New Zealand (A) showing a close up of the Hauraki Gulf (B) and Tiritiri Matangi Island (C) with its surveyed areas. Grey lines represent the borders of each section. Modified from ©2010 Google Imagery.

4.3.2 Survey methods

4.3.2.1 Nest searches

Searches for LBP nests began in June 2009. These searches consisted of one to three day trips approximately every second week until mid August, followed by weekly trips until September 4th. From September 4th onwards, searches were conducted daily. In previous studies, LBP nest searches were often initially conducted at night by listening to penguin vocalisations to locate breeding sites (Miyazaki and Waas 2003b). This was not deemed necessary as the majority of accessible breeding sites had already been identified by previous studies (Jones 1978; Miyazaki and Waas 2003a; Chen 2004; Geurts 2006; Jansen van Rensburg 2010). Searches were thus conducted during the daytime, generally between 7am and 7pm depending on the tides. Day time searches are much more efficient on Tiritiri Matangi as many nests are located in areas relatively inaccessible at night. Nests that were active in previous years (2005-2006 or 2006-2007) and still potentially usable this year were automatically labelled as potential nests as nest faithfulness has been shown to be strong in this species (Kinsky 1960; Reilly and Balmford 1975; Jones 1978; Reilly and Cullen 1981; Waas 1990).

4.3.2.2 Potential and breeding nests

As in previous studies, two main types of nests were identified, Potential Nests (PN) and Breeding Nests (BN) (defined by Mattern 2001). A third nest category was added for nests where vocalisations could be heard but no penguins, eggs or chicks could be sighted (due to inacessability).

Potential Nests

Potential Nests were burrows that were not yet used for breeding but contained evidence of penguin activity suggesting that they could potentially be used for the upcoming breeding season. Evidence of penguin activity included the presence of one or two penguins, fresh faeces, feathers or nesting material. Burrows in which one or several adults could be regularly heard vocalizing but not seen nor their eggs or chicks were also classified as potential nests. In some cases, however, due to movements of the individuals inside the nest or of the nest itself (i.e dirt or rock movements after strong winds and storms), adults and their eggs or chicks became visible, and were thus then classified as breeding nests. All potential nests were flagged with red flagging tape and labelled according to their location.

Breeding Nests

When eggs or chicks were found in a nest, the nest was defined as a Breeding Nest (BN) and was flagged according to its location. Breeding nests were further classified as 'accessible' or 'inaccessible'. Accessible nests were nests in which the eggs were reachable and could be taken out to be swabbed and cleaned if needed. Inaccessible nests were nests in which we could see penguins and eggs but the eggs could not be reached and thus were neither swabbed nor cleaned. Within the accessible nests, two categories were defined: 'nests found at laying' and 'nests found after laying'. Nests found at laying were nests in which each egg was found on the day it was laid and thus could be swabbed and cleaned on that first day. Nests found after laying were nests for which the lay date of the eggs was unknown, or chicks were already present when the nest was discovered.

4.3.2.3 Nest types

Nests were assigned to one of four categories according to their substrate. The categories used were artificial, rock, soil and vegetation (Fig. 4.2), as described below in Table 4.1 (adapted from Renner 1998 and Geurts 2006).

Figure 4.2. LBP artificial nest box (A), soil nest (B), rock nest (C) and vegetation nest (D). Photographs by A.S. Boyer

The first artificial nest boxes were built in 1998 and were constructed of stones with clear glass tops allowing the public to see the LBP. In 1999, an additional 36 wooden nest boxes were placed around the island as part of a previous study and in late September 2009, an additional seven burrows covered with ceramic tiles were inserted on the west coast of the island, close to the stone nest boxes, on Hobbs beach and on Cable road. By the beginning of the 2009 breeding season, however, most of the wooden nest boxes and one stone nest box had totally or partially collapsed.

Table 4.1. Nest types used by LBP and their description and location on Tiritiri Matangi Island, New Zealand.

Nest Type	Description	Location
Artificial	Stone, wooden and ceramic nest boxes, drain pipes, under board walks	Wharf area, Cable road, Ridge road, Wattle Valley
Rock	Rock caves and crevises	Entire coastline
Soil	Burrows built in soil or dirt banks or dug in the ground	Higher in land, Papakura Pa, Emergency Landing area
Vegetation	Holes or tunnels within or under trees or other vegetation such as Flax or Bracken	Across the entire island

4.3.3 Data collection

4.3.3.1 Nest conditions

Waterproof data loggers (Hobo Pro and Hobo Pro V2, Onset Computer Corp.) were placed in different types of accessible potential and breeding nests to record average temperature and relative humidity over the laying and incubation period. Temperature and humidity were recorded every 30 minutes until both eggs had hatched, or until the eggs had been removed after being abandoned or cracked. The general aspect and condition of all potential and breeding nests were also noted during each visit. This included relative dampness of the nest, especially after heavy rain.

4.3.3.2 Nest monitoring

Potential and breeding nests were monitored by checking contents of the burrows using a torch or a headlamp. When the back of a burrow was not visible directly with a torch, it was checked with a flexible burrow scope (Provision 636, Technical Solutions Corp.). If no penguin could be seen or heard, the nest was recorded as empty.

Breeding nests

If present, the number of penguins, eggs or chicks was recorded. When a penguin was found in a nest, the presence of eggs or chicks was checked by gently lifting the penguin up using the flat end of kitchen tongs if they could not be seen directly. If an egg was newly laid, it was gently taken out of the nest, labelled and assigned to one of the treatment groups described in the next section. The egg was then placed back in its original position and the nest was checked again a few minutes later to ensure that the parent was also back in its original position. It took on average 5 to 10 minutes for the penguin to settle back in the nest. The nest was then monitored daily until the second egg was laid. Potential nests with regular indications of penguin activity were also monitored daily to increase the chances of finding the first egg on the day it was laid. During incubation, the parent in the nest was lifted once a week to ensure the eggs were in good condition.

Potential nests

The condition or surrounding of potential nests was a good indicator of penguin activity and thus potential breeding in the near future. This included the state of the nest (good condition, partially or totally collapsed), the presence, quantity and freshness of faeces or feathers in and around the nest and the dampness of the nest.

4.3.3.3 Egg or chick removal

When eggs from a breeding nest were found unattended, they were candled in attempt to determine whether the embryo was still alive. While it was often difficult to see through the egg due to the thickness of the shell and the lack of complete darkness, it was possible to see and measure changes in the size of the air sac. When eggs were

found unattended for over two weeks, cold and with no signs of development they were removed, and taken to the lab to check for signs of fertility, embryo development and measurements of shell thickness. Although it was very unlikely that the eggs would survive more than a few days without being incubated, they were left in the nest for this long to observe the parent's behaviour towards the egg upon its return. Similarly, when cracks were noticed on the eggs they were left in the nest for several days and the parents' actions were observed. When chicks were found dead they were removed if accessible and fresh and then frozen for future necropsies. Causes of chick mortality were then used to better understand causes of egg abandonment.

4.3.4 Definitions

4.3.4.1 Lay, hatching and fledging dates, and incubation period

Lay date

Nest checks were conducted during the daytime only, generally between 7am and 7pm and were dependent upon tide levels. Two checks of the same nest on consecutive days could thus be separated by up to 36 hours. However, if an egg was not present in a nest on one day and present on the next day, the lay date was recorded as the day the egg was found in the nest (Heber *et al.* 2008). If a new nest was discovered with one egg, it was monitored daily until the second egg was laid, and the first egg was later recorded as being laid 3 days prior to the second one, 3 days being the median laying interval found between the laying of two eggs that season. If a new nest was discovered with two eggs, the lay date was estimated by subtracting the average incubation period of 36 days to the hatching date (Marchant and Higgins 1990). If a new nest was discovered

with chicks, the lay date was estimated by comparing chick sizes with those of chicks of known lay or hatching dates.

Hatching date

As with lay dates, if a nest was found with eggs on one day and chicks on the next day, the hatching date was recorded as the day the chick was found out of its egg. If the hatching date was unknown, two methods were used to estimate the age of the chick. A first estimation was made by comparing the size of the chick with that of chicks with known hatching dates. If the chick fledged, a second estimation was made by subtracting the average nesting period of 54 days from the fledging date. An average of both estimations was then used to determine the age of the chick.

Fledging date

Chicks were considered to have fledged after reaching at least seven weeks, 80% adult weight and showing a complete adult plumage (Fortescue 1999; Renner and Davis 2001). Chicks that disappeared before seven weeks from hatching or before reaching an appropriate body weight and adult plumage were considered to have failed.

Incubation period

Incubation period was defined as the total number of days the egg was attended by at least one parent. As it was not possible to reach all eggs to determine their temperature, incubation was assumed to begin on the day the egg was laid if the parent was present in the nest. When an egg was found alone once or several times, the total number of days in between temporary abandonments was added to obtain the total incubation time.

4.3.4.2 Failed nests

A nest in which all chicks were found dead, or all eggs found broken or unattended and cold for over a week was considered to have failed. Eggs were, however, not removed from the nest for another week (as explained in section 4.3.3.3). The cause of death of both chicks and eggs were classified as follows:

Egg mortality

1. **Egg never incubated:** the egg was abandoned just after being laid, and thus never incubated.
2. **Egg abandoned:** the egg had initially been incubated but was then abandoned in the nest
3. **Egg cracked:** the egg was partially or completely cracked
4. **Egg out of the nest:** the egg had rolled or had been pushed out of the nest
5. **Weather effects:** the egg was abandoned, damaged or found out of the nest due to adverse weather conditions
6. **Unknown:** the egg disappeared or its cause of mortality was unknown

Embryo development

When possible, eggs that failed to hatch were taken to the laboratory to be opened and analysed. If present, the age of the embryo was determined by comparing it to photographs of Adélie penguin (*Pygoscelis adeliae*) embryos (Hebert 1967). Although Adelie Penguin eggs are larger than LBP eggs, their incubation lengths are very similar (Adelie penguins: 35days, Hebert 1967; LBP: 34 to 36 days Marchant and Higgins 1990). The goal of this analysis was to determine at what stage the embryo had stopped developing and whether its death was due to parental abandonment or occurred prior to

abandonment and thus was due to other causes. Embryos were thus classified as follows (modified from Geurts 2006, Fig. 4.3):

1. **Primary:** Little or no visible development (1-10days, Fig 4.3 A,B)
2. **Intermediate:** Eye pigmentation, allantoic bud and hindgut visible, soft limbs (11-21days, Fig 4.3 C,D,E)
3. **Tertiary:** Visible feather coverage, flexed limbs resting on body or over top of the head (22+ days, Fig 4.3 F,G,E).
4. **Unknown:** Rotten eggs or eggs at very early stages where fertility could not be confirmed.

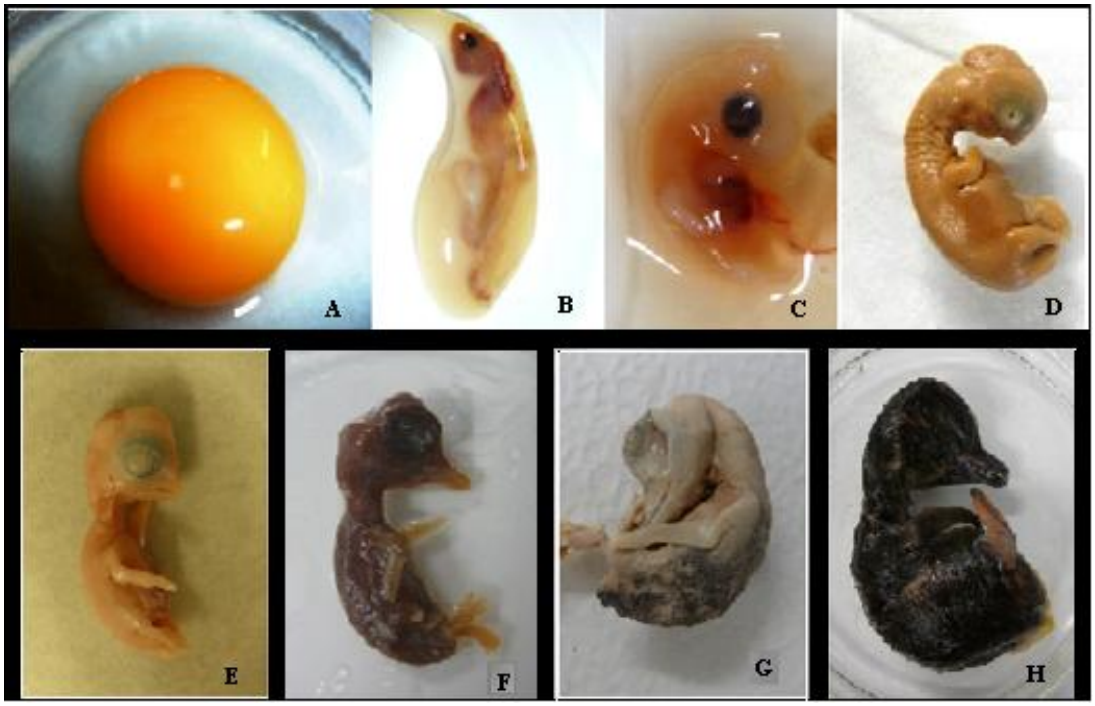

Figure 4.3. LBP embryonic developmental stages with a fertile egg (A), the end of primary development with a 9 day old foetus (B), intermediate stage with a 13 (C) and 15 (D) day old foetus and tertiary stage with a 22 (F) and 23day old foetus (G) and tertiary stage with a 30 day old foetus (H). Photographs by A.S. Boyer.

Chick mortality

Although not directly relevant to this study, the cause of death of chicks was used as an indicator of possible explanations for egg abandonment.

1. **Starvation:** chicks were rapidly losing weight and died underweight. When chicks were found dead out of their nest and were severely underweight it was assumed that they had left the nest in an attempt to find food and were thus considered as starving. Chicks dying of starvation were a good indication that parents might not be returning to their nests as often as needed, or with too little food.
2. **Abandonment:** young chicks were left alone or deliberately pushed away during guard stage and most likely died of hypothermia. Parents abandoning both chicks at a very young age or pushing one chick away from the nest while guarding the other was also a possible indication of food shortages.
3. **Disappearance/unknown:** chicks had disappeared from the nest prior to fledging date and their fate was unknown.

4.3.5 Experimental design

As in the previous chapter, this experimental design was modified from Cook *et al.* (2003; 2005a; 2005b). The goal was to determine whether nest conditions such as temperature and relative humidity, exposure and microbial infection could affect egg viability, and whether cleaning these eggs could increase hatching success by reducing microbial infection. In addition, this experiment investigated whether variations in egg size and weight influence hatching probabilities. To do so, each accessible egg found on the day of laying was taken out of the nest, labelled, swabbed, measured and weighed (Fig. 4.4) and attributed to one of two treatment, as described below.

4.3.5.1 Egg extraction, labelling, swabbing, measuring and weighing

A total of 48 eggs were used in this study. They were gently removed from the nest using long kitchen tongs covered in bubble wrap to avoid incurring any damage. Latex gloves were used to handle the eggs to avoid any microbial contamination from unclean hands. The eggs were first labelled with a xylene-free marker indicating their number, the letter 'a' for the first laid and 'b' for the second laid and the lay date. They were then placed in a small container which had been altered to cushion and prevent any movement of the egg and consequently weighed on a 200g electronic scale. Their maximum length and width were then measured using 150mm callipers. The eggs were then swabbed over a 3cm diameter area at the blunt end using sterile wooden cotton-top swabs, which were then placed in a tube filled with 5ml of physiological saline (Ringer's solution) as described in Cook *et al.* (2003; 2005a; 2005b) and Godard *et al.* (2007). All tubes were kept in a cooled container until they could be placed in a refrigerator. Labelling, measuring, weighing and swabbing were completed as quickly as possible, and throughout the entire process eggs were kept in the same position as they were found. Once sampled, eggs were placed back into the nest, in their original position.

Figure 4.4. LBP egg labelling, swabbing and weighing. Photographs by Chris Rodley.

4.3.5.2 Treatments

After being swabbed, eggs were either attributed to the cleaned or uncleaned treatment. First laid eggs in the cleaned treatment were wiped daily with 70% ethanol until the second egg was laid. Second laid eggs were not cleaned as full incubation was likely to begin on that day. All inaccessible eggs were added to the uncleaned category.

4.3.5.3 Targeted microbes and plating

Targeted microbes and plating techniques used here were the same as described in the previous chapter (see Chapter 3 for more details, sections 3.3.4.2 and 3.3.4.3). However, due to limited access to the study site, some samples had to be kept refrigerated on the island for up to three days before being plated. As experiments run at the start of this study showed that keeping such samples refrigerated for three days decreased the number of viable Colony Forming Units by over 99%, it was decided that all samples were to be kept in the refrigerator for three days before being plated. This ensured that all samples were treated as equally as possible.

4.3.5.4 Egg analysis

Volume

In addition to the length and width, the volume (ml) of each egg labelled was calculated using the following equation:

$$V = klb^2$$

where l is the egg's length (cm), b its breadth (cm) and k is a constant (Coulson 1963). The constant value was estimated at 0.514 (±0.002) by Kemp and Dann (2001) by measuring the volume of 34 LBP eggs by water displacement.

Shell thickness

The shell and membrane thickness of each egg collected was measured using a 1x0.001 mm digital micrometer. When possible, the shell of hatched eggs was also collected and its thickness was measured.

Embryo development

When possible, eggs were collected and opened to determine their embryo development and time of death (see section 1.4.3.3.2 for more details).

4.3.6 Data analysis

4.3.6.1 Defining reproductive success

Reproductive success was calculated using to the estimation method described in Heber *et al.* (2008). It is based on four reproductive parameters, as described in Table 4.2.

Table 4.2. Definitions of reproductive parameters of LBP from Tiritiri Matangi Island, New Zealand.

Reproductive parameter	Definition
Hatching success	Proportion of eggs that hatched relative to the number of eggs laid
Fledging success	Proportion of chicks that fledged relative to the number of chicks that hatched
Breeding success	Proportion of chicks that fledged relative to the number of eggs laid
Chicks per pair	Average number of chicks fledged per pair that produced eggs

4.3.6.2 Microbial infection

Microbial infection was quantified as Colony Forming Units (CFU) per 0.1ml of original sample after a 48h incubation period. This number was obtained by counting the number of CFU on a plate and multiplying it by the sample's dilution factor. CFU were then compared across all eggs to assess the effect of nest type, temperature and relative humidity on initial microbial loads and the effect of initial microbial loads on hatching success.

4.3.6.3 Statistical analysis

Colony counts were sometimes log transformed to achieve normality. Kruskal-Wallis tests were used to compare incubation lengths and the effects of nest type on microbial infection and breeding success. Paired and unpaired t-tests were used to compare egg properties. Fisher's exact tests were used to compare various parameters of breeding success between late and early nests and the effects of cleaning on hatching success. Mann-Whitney U- tests were used to compare microbial infection across cleaned and uncleaned eggs.

4.4 RESULTS

4.4.1 Lay date and conditions

Egg laying was recorded from the 6th of September to the 19th of November 2009. A total of 56 breeding attempts had a known lay date. Lay dates were bimodal and the season was divided into two six week periods (early and late). The majority of the eggs (75.4%) were laid early. During this period, the average two weekly Sea Surface Temperature (SST) varied between 12.2-16.6°C, with the lowest recorded in late October (Fig. 4.5). This year's mean (±SE) SST for the month of laying was 14.9 ±0.1°C, similarly to that in 1999, 2003 and 2005 (Fig. 4.6), and slightly higher than that of the previous month (14.3 ± 0.1°C) and following month (13.5 ± 0.8 °C). The mean SST for the whole breeding season was calculated at 15.5 ±0.8°C.

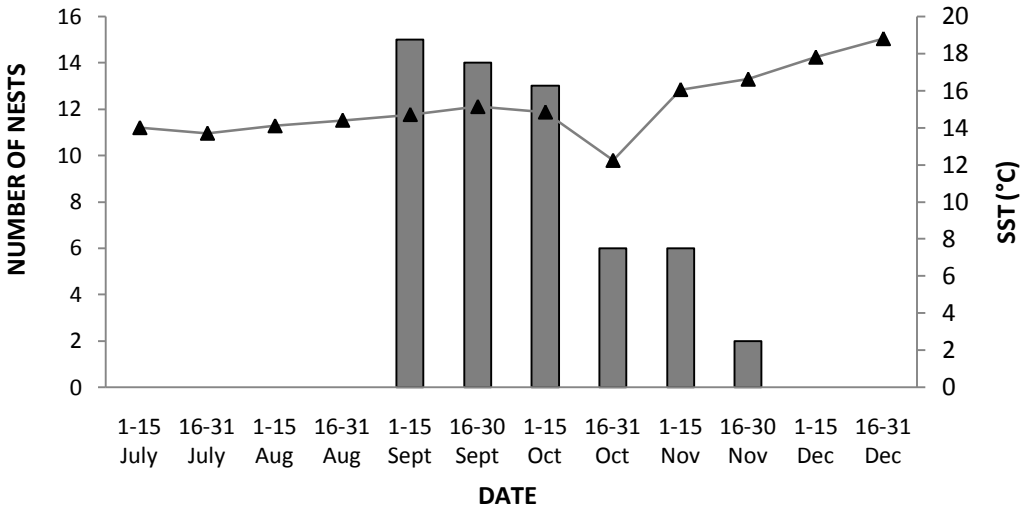

Figure 4.5. LBP nest count according to their lay date and average SST associated with each two-week period pre-laying, during laying and post laying.

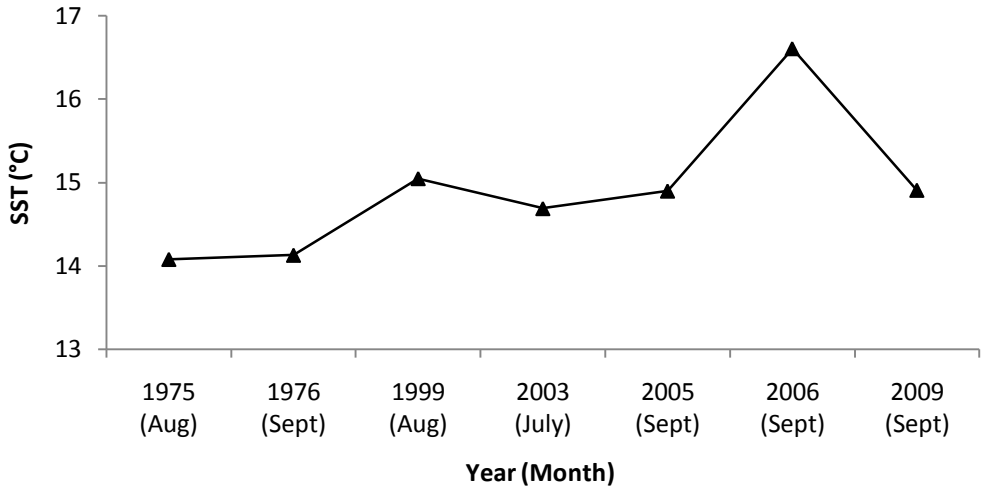

Figure 4.6. Monthly average SST corresponding to the initial lay date of LBP on Tiritiri Matangi Island from 1975 to 2009. Data from 1975 -1976 are from Jones (1978), 1999 from Miyazaki and Waas (2003a), 2003 from Chen (2004), 2005 from Geurts (2006), 2006 from Jansen van Rensburg (2010) and 2009 from this study.

4.4.2 Nesting attempts

4.4.2.1 Potential nests, breeding nests and RDB

A total of 55 breeding nests and 62 potential nests were found during the 2009 breeding season. Four (7.3%) of the 55 breeding nests were used for two breeding attempts (replacement double breeders, RDB), and one was used for three breeding attempts, resulting in a total of 61 nesting attempts for the season. These double and triple breeding attempts were assumed to be from the same pairs, however, due to a lack of bands on these individuals, this could not be confirmed. Second attempts began mid October, as first attempts ended (Fig. 4.7). Forty-six (75.4%) breeding attempts were found at the egg stage while the remaining 15 (24.6%) were found at the chick stage.

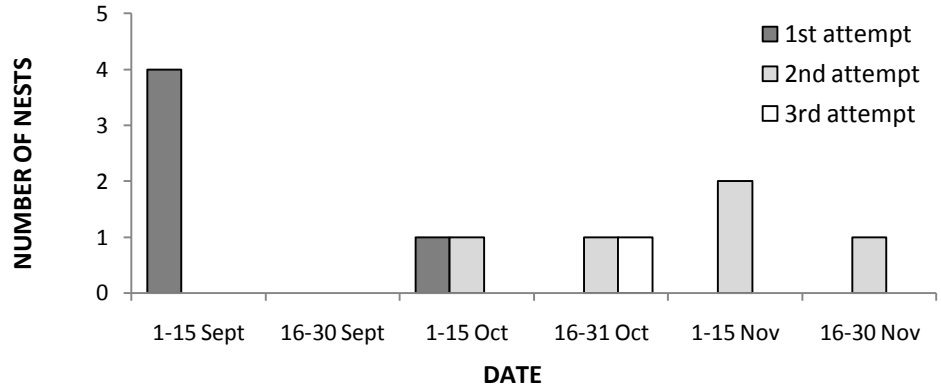

Figure 4.7. Dates of first, second and third breeding attempts of replacement breeding little blue penguins on Tiritiri Matangi Island, New Zealand (n=5).

4.4.2.3 Nest location and type

Out of the 61 nesting attempts, 31 were found on the Wharf North (WN) section, 12 on the Emergency South (ES) section, 7 on the Emergency North (EN) section, 6 on the Wharf South (WS) section, 3 on the North (N) section, 1 on Cable Road (CR) and 1 in Wattle Valley (WV) (Fig. 4.8).

Figure 4.8. Map of Tiritiri Matangi Island showing the location of LBP nests. Yellow triangles represent single nests, blue circles represent 2-3 nests and red circles represent 4-5 nests. Modified from ©2010 Google Imagery.

The most common nest type was rock (72 %), followed by vegetation (13%), soil (10%) and finally artificial (5%). Late nests were mainly found in the rocks (Fig. 4.9). Four out of the five RDB nested in rocks, while the fifth one used an artificial nest box.

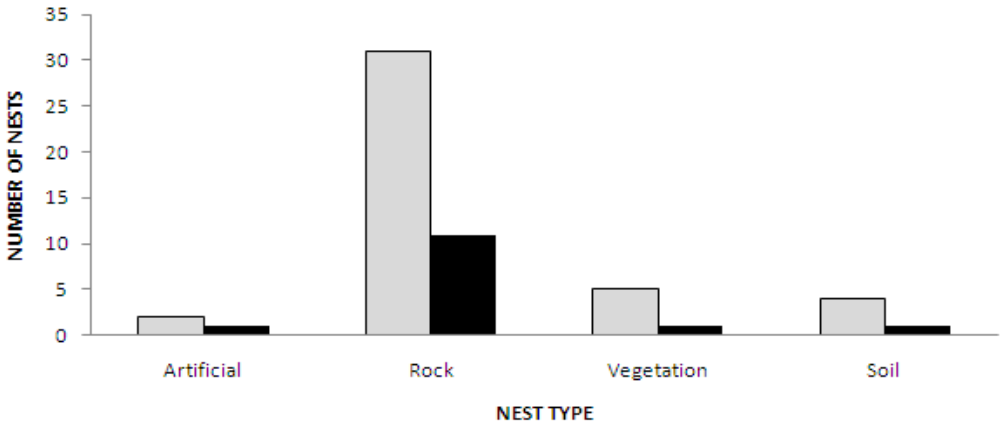

Figure 4.9. Comparison of nest types between early (grey) and late (black) LBP nests.

4.4.3 Egg laying and hatching

4.4.3.1 Incubation length

The mean incubation period for all hatched eggs with a known lay date (n=16) was 35.5 (±0.4) days, ranging from 33 to 39 days with a median of 35 days. In two-egg clutches, 1st laid eggs hatched on average after 36.5 (±0.6, n=8) days of incubation and 2nd laid eggs on average 34.5 days (±0.4, n=8). This difference in incubation time was significant (paired t-test: t=2.65, d.f.=14, p=0.0192). Although nest type did not significantly influence overall incubation length (Kruskal Wallis KW= 0.8795, n_1=4, n_2=10, n_3=2, p=0.6442), soil nests had the longest incubation length but also the greatest variability (Fig. 4.10).

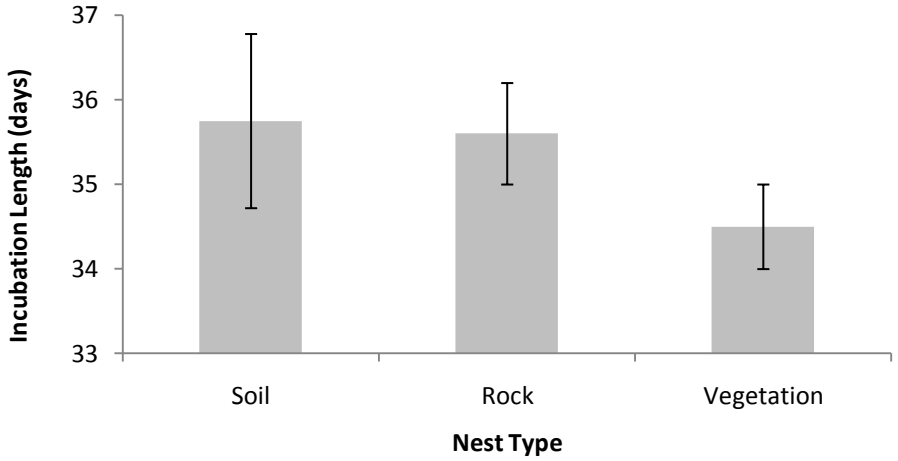

Figure 4.10. Average (±SE) LBP incubation length in soil nests (n=4), rock nests (n=10) and vegetation nests (n=2).

4.4.3.2 Laying and hatching intervals

In two-egg clutches, the second eggs were laid on average 2.7 (± 0.2, n=25) days after the first one, ranging from 1-5 days. The median laying interval was three days. The average hatching interval was 0.7 (±0.2, n=19) days, ranging from 1-3 days with a median of one day.

4.4.3.2 Egg properties

LBP eggs were on average 54.1 (±0.3, n=39) mm long and 41.5 (±0.2, n=39) mm wide, corresponding to an average volume of 47.8 (±0.7) ml. Average weight was calculated at 54 (±1.1, n=35) g. In two-egg clutches, first-laid eggs (eggs A) and second-laid eggs (eggs B) did not differ significantly in volume or weight, however eggs A were found to be significantly longer than eggs B (Table 4.3). Overall, this year's eggs were found to be slightly longer than previously recorded on Tiritiri Matangi Island (length: 52.4 (±0.7) mm, 52.4 (±0.3) mm and 52.9 (±0.6) mm; width: 40.8 (±0.5) mm, 40.9(±0.1) mm and 41.6 (±0.3) mm in 1975 (Jones 1978), 2005 (Geurts 2006) and 2006 (Jansen van Rensburg 2010) respectively).

Table 4.3. Comparisons of length, width, weight and volume of first-laid and second-laid eggs in two-egg clutches of little blue penguins on Tiritiri Matangi. Paired *t*-test: *, $P < 0.05$.

Measurement	Egg	n	Mean	*s.e.*	*d.f.*	*t*
Length (mm)	A	11	54.89	0.67	10	4.34*
	B	11	52.80	0.67		
Width (mm)	A	11	41.28	0.56	10	0.25
	B	11	41.35	0.60		
Weight (mm)	A	9	57.02	2.58	8	2.02
	B	9	55.03	2.92		
Volume (ml)	A	11	48.20	1.50	10	1.86
	B	11	46.56	1.67		

4.4.4 Nest conditions

The average daily temperature and relative humidity was recorded for a total of seven breeding nests and six potential nests. Two of the data loggers placed in breeding nests and two in potential nests, however, failed to recover the data. In breeding nests, temperatures varied between 11.6 and 30.6°C and relative humidity varied between 53.1 and 99.9% over the whole breeding season. In potential nests (empty nests) temperatures varied between 9.0 and 15.6°C and relative humidity varied between 77.9 and 100%.

4.4.5 Breeding success

A total of 99 eggs and 15 chicks were found between early September and late December, resulting in a minimum of 114 eggs laid on Tiritiri Matangi during the 2009 breeding season. These eggs originated from 53 two-egg clutches and eight one-egg clutches, corresponding to an average of 1.87 (±0.04) eggs per clutch. From those 99

eggs found, 43 hatched resulting in a total of 58 chicks recorded this breeding season. Of these 58 chicks, only 4 fledged (Table 4.4).

Table 4.4. Comparisons of LBP apparent breeding success on Tiritiri Matangi Island over the last five years. (2005 study by Geurts (2006); 2006 study by Jansen van Rensburg (2010))

	Breeding season		
	2005	**2006**	**2009**
Beginning of laying	9 September	10 September	6 September
End of laying	16-31 December	1-15 December	16-30 November
Nests found	87	65	61
Hatching success (%)	27	53.3	50.9
Fledging success (%)	27	37.8	6.9
Breeding success (%)	10	33.3	3.5
Chicks per pair	1.7	0.67	0.95

Lay date

Overall eggs laid early in the season had a significantly greater hatching success than eggs laid late in the season (Fisher's exact test: n=114, $p<0.0001$), but there was no significant difference in fledging success (n=58, p=1), breeding success (n=114, p=0.5708) or nest success (n=61, p=0.5599), with all parameters very low this season (Table 4.5, Table 4.4). Significantly more late than early nests failed at egg stage (n=61, p=0.001) and significantly more early than late nests failed at chick stage (n=61, p=0.0033, Table 4.5).

Nest type

Nest type did not significantly affect hatching (Kruskal-Wallis KW=6.766, n_1=44, n_2=6, n_3=8, n_4=3, p=0.0798), fledging (KW=0.169, n1=22, n2=5, n3=3, p=0.5574) or overall breeding success (KW=0.9886, n_1=44, n_2=6, n_3=8, p=0.61), however only rock nests fledged chicks.

Table 4.5. Little blue penguin reproductive success (% ± 95% Confidence Intervals) on Tiritiri Matangi Island, New Zealand, over the 2009 breeding season.

Nests	Hatching success	Fledging success	Chicks per pair	Breeding success	Nest success	Nests failed at egg stage	Nests failed at chick stage
All	50.9 (41.8-59.9)	6.9 (2.7-16.4)	0.07	3.5 (1.4-8.7)	4.9 (1.7-13.5)	50.8 (38.6-62.9)	44.3 (32.5-56.7)
Early	63.5 (52.9-73.0)	7.4. (2.9-17.6)	0.08	4.7 (1.9-11.5)	6.7 (2.3-17.9)	37.8 (25.1-52.4)	55.6 (41.2-69.1)
Late	13.8 (5.5-30.6)	0	0	0	0	87.5 (64.0-96.5)	12.5 (3.5-36.0)

4.4.6 Egg failure

Out of the 114 eggs laid, 56 (49.1%) failed to hatch. The majority were abandoned during incubation (57%), 16% were found cracked in the nest, 11% had rolled or been pushed out of their nest, 7% were laid either in a nest or on the rocks but never incubated, 4% were found out of the nest after a storm and 5% had disappeared for unknown reasons. The main cause of failure for eggs from late nests was abandonment (80%) while earlier nests failed due to multiple causes (Fig. 4.11).

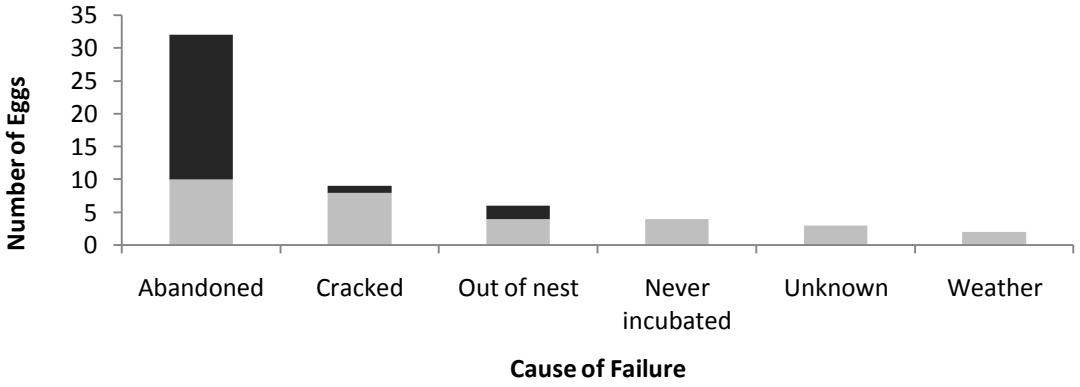

Figure 4.11. Causes of egg failure of early (grey) and late (black) LBP eggs (n=56) over the 2009 breeding season on Tiritiri Matangi Island, New Zealand.

4.4.6.1 Stage of failed eggs

A total of 28 failed eggs were collected and opened. Twenty-four of these eggs had been incubated while the remaining four were abandoned shortly after being laid. Two of these four eggs showed a clear large blastoderm, a good indication of fertility. The remaining two were cracked during their transport and their fertility could not be determined. They were thus classified as unknown. The stage of failure could not be determined for 12 eggs due to decomposition or cracks in eggs at a very early stage. For eggs with a known outcome (n=17), embryo death occurred most frequently at the intermediate stage (n=7) followed by primary stage (n=6) and finally tertiary stage (n=4). Eggs from early nests failed at all three stages, however, no eggs collected from late nests reached tertiary development (Fig 4.12). All eggs collected from artificial nests (n=4) failed at primary or unknown stages, most eggs (60%, n=5) from vegetation nests failed at intermediate stage and eggs from rock nests (n=16) failed at all three stages in similar frequencies (16%, 21% and 21% for primary, intermediate and tertiary stages, respectively).

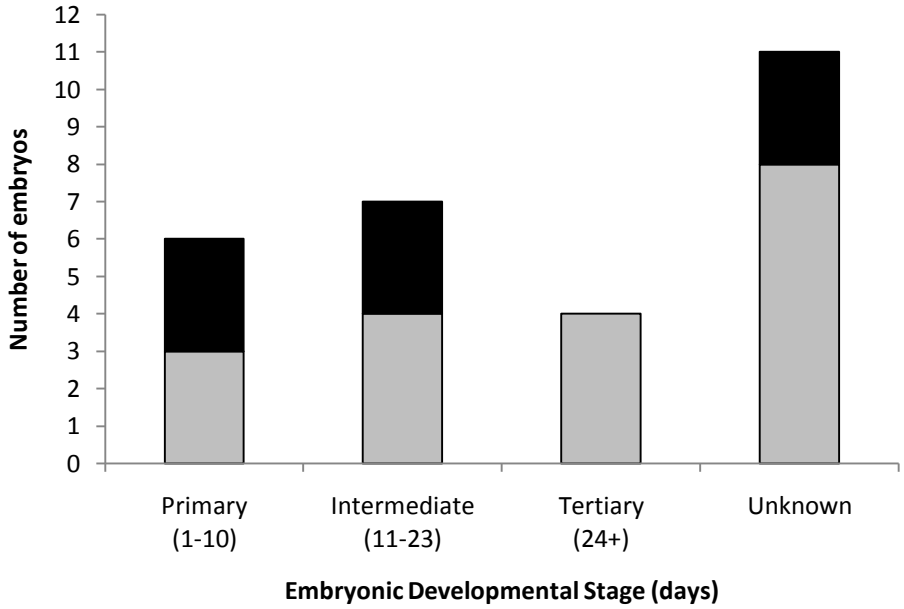

Figure 4.12. Embryonic developmental stage of early (grey) and late (black) failed LBP eggs.

4.4.6.2 Factors affecting egg failure

Egg order

Out of the 13 first laid eggs and 13 second laid eggs monitored, only five of each hatched, resulting in a 38% success for each group. Egg order thus did not influence hatching success.

Microbial infection and nest type

Microbial infection at laying varied greatly between eggs, and CFU counts from TSA plates and MAC plates were significantly correlated (Spearman Rank Correlation r=0.5992, p=0.0002). Nest type, however, did not influence microbial infection (Kruskal-Wallis KW=1.044, n1=6, n2=21, n3=2, n4=5, p=0.7905 for TSA plates; KW=0.2323, n1=6, n2=21, n3=2, n4=5, p=0.9722 for MAC plates; Fig. 4.13), nor hatching success (see section 4.4.5).

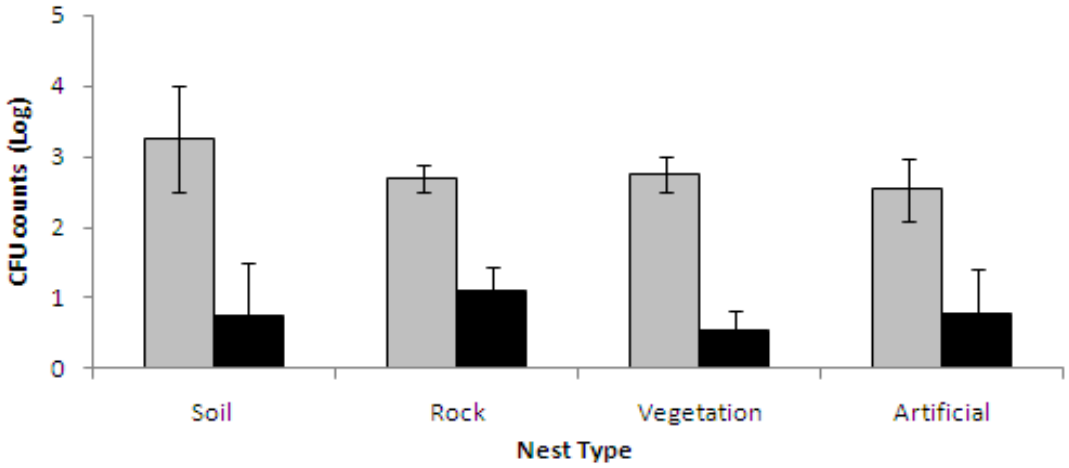

Figure 4.13. Average (±SE) initial shell CFU counts from TSA (grey) and MAC (black) plates for each nest type. N= 6, 21, 2, 5 for soil, rock, vegetation and artificial nests, respectively.

Cleaning and handling

A total of 48 eggs were labelled, half of which were cleaned and half were left uncleaned. Cleaning did not significantly increase the hatching success of these eggs, whether considering only labelled eggs (Fisher's exact test n=48, p=0.7661) or all eggs (n=114, p=0.0671). However, as the majority of eggs failed to hatch due to abandonment during incubation, it made more sense to assess the effects of cleaning in terms of developmental stage at which embryos ceased to grow. This revealed that cleaning did not reduce early embryo mortality, or mortality at any other stage. Within cleaned eggs, there was no significant difference between CFU counts of eggs that hatched and eggs that didn't hatch. However, within uncleaned eggs, those that hatched had a significantly higher infection at laying than those that failed (Table 4.6). Eggs labelled did not experience a significantly lower hatching success than eggs not labelled (n=114, p=0.0574), suggesting that handling and labelling did not considerably impact on egg viability.

Table 4.6. Comparisons of average CFU counts (Log) between cleaned and uncleaned LBP eggs that hatched and failed. * represents a statistical significance.

	Treatment	**Outcome**	**N**	**Mean CFU count (Log)**	**Mann-Whitney Test**
TSA	Cleaned	Hatched	8	2.8	U=57, p=0.8718
		Failed	15	2.7	
	Uncleaned	Hatched	3	3.2	U=3, p=0.0424*
		Failed	7	2.3	
MAC	Cleaned	Hatched	8	1.4	U=51, p=0.5792
		Failed	15	1.1	
	Uncleaned	Hatched	3	NA	NA
		Failed	7	NA	

Egg size and shell thickness

Egg length varied between 50-58mm. Egg width varied between 38-44mm. Egg volume varied between 38-56ml. Egg weight varied between 39-78g. Egg shell thickness varied between 0.391-0.676mm. None of these parameters varied significantly between eggs that hatched and eggs that failed (Table 4.7).

Table 4.7. Comparisons of length, width, weight, volume and shell thickness of hatched and failed little blue penguin eggs on Tiritiri Matangi Island, New Zealand. Unpaired *t*-test with Welsh Correction: *, $P < 0.05$

Measurement	**Outcome**	**n**	**Mean**	***s.e.***	***d.f.***	***t***
Length (mm)	Hatched	18	53.92	0.47		
	Failed	26	54.21	0.45	39	0.4427
Width (mm)	Hatched	16	40.92	0.25		
	Failed	26	41.78	0.35	39	2.005
Volume (ml)	Hatched	16	46.29	0.83		
	Failed	26	48.78	0.99	39	1.923
Weight (g)	Hatched	16	54.16	1.78		
	Failed	24	53.90	1.70	35	0.1047
Shell thickness (mm)	Hatched	4	0.4665	0.029		
	Failed	10	0.5045	0.026	8	0.9865

Parental abandonment

Fifty-seven percent (n=28) of all eggs collected had been monitored daily from laying. Two of these eggs had been abandoned at laying. Two others had been fully incubated without interruption and died while hatching. All other eggs had been unattended temporarily (1-7 days) during incubation, then incubated for a further 5-26 days before being finally abandoned. Of these eggs, all but three ceased to develop within 1-2 days of being left temporarily unattended. The remaining three eggs continued to develop until the nest was abandoned (Table 4.8). Of all eggs that hatched and were monitored daily since they were laid (n=22), none were found unattended during incubation.

Table 4.8. Number of days that eggs were incubated, temporarily abandoned, and incubated again and the estimated age at which the embryo ceased to develop (according to photographs of Adélie penguin embryos by Herbert, 1967). * represents embryos that survived the temporary abandonment.

Days from laying to temporary abandonment	Days left unattended	Days incubated upon return	Embryo development (days)
1	1	16	none
1	7	8	none
2	3	26	none
2	2	15	17-19 *
5	3	26	none
6	1+5	3+10	6-8
8	1+5	3+10	7-10
14	7	24	13-15
17	7	24	14-16
20	3	5	27-28 *
22	3	5	28-30 *
10	Abandoned after 10 days		10-12
35	Died while hatching		35
36	Died while hatching		3

4.4.7 Causes of chick mortality

Overall, 93% (n=58) of all chicks died before reaching fledging stage. Over 57.4% died of likely starvation during the post guard stage, 7.4% were abandoned during the guard stage and most likely died of hypothermia and starvation and 35.2% disappeared during post guard stage. The mean (±SE) age of dead chicks was 35.9 ±3.6 days (n=13, range 14-56 days) and the mean weight of dead chicks was 349.9 ±37.6g (n=12, range 139-540g). In two-chick clutches, the youngest chick always died simultaneously or prior to the oldest, suggesting an unequal caring for both chicks (Plates 4.2 and 4.3).

Plate 4.2. Evidence of weight differences between the youngest (right) and oldest (left) chicks from a same clutch that hatched one day apart. Photograph by A.S Boyer.

Plate 4.3. Little blue penguin that has pushed the youngest chick away from the nest and is brooding the other chick that is one day older. Photograph by A.S Boyer.

4.5 Discussion

4.5.1 Lay date and conditions

SST and the variations in atmospheric pressure known as Southern Oscillation Index (SOI) are generally thought to be important factors affecting the reproduction and survival of seabirds, by affecting prey abundance and food web structure (Vader *et al.* 1990; Hunt *et al.* 1991; Montevecchi and Myers 1997). As shallow divers, LBP are thought to be even more affected by changes in SST than larger pursuit diving species (Jarvis 1993; Montevecchi 1993). Michelson *et al.* (1992) for example, showed that the onset of breeding of LBP from Philip Island, Australia, is dependent upon food availability, and is thus likely to be affected by climatic variations. The hatching success of these birds, however, was not shown to be correlated with SST variations (Kemp and Dann 2001). Similarly, in New Zealand, studies have shown that a low SOI one year, representative of an El Niño event, can delay the onset of LBP breeding in the following year, without however, affecting their hatching success and overall breeding success (Perriman *et al.* 2000).

Little blue penguins from Tiritiri Matangi Island usually initiate breeding between the months of July and September (Jones 1978; Miyasaki and Waas 2003; Chen 2004, Geurts 2006; Jansen van Rensburg 2010), often at a relatively low SST for the year (Geurts 2006). Indeed, at low temperatures abundance of phytoplankton tend to be higher, therefore increasing food sources for higher trophic levels. This year, the mean SST for the month of laying was similar to that of previous years, however, unlike observed by Geurts (2006), it was slightly higher than that of the previous and following months. The overall laying phase was divided into two six-week periods. In the first

period, defined as early laying, over three quarters of the eggs were laid. Their hatching success was slightly higher than previously recorded on Tiritiri Matangi Island (Miyazaki and Waas 2003a; Geurts 2006; Jansen van Rensburg 2010), and overall comparable to many other LBP populations in New Zealand and Australia (Reilly and Cullen 1981; Bull 2000a; Perriman and Steen 2000; Kemp and Dann 2001; Chambers 2004; Heber 2008). Eggs laid during the second half of the laying period were significantly less numerous and had a significantly lower hatching success than early eggs. Surprisingly, this sudden drop in laying was accompanied with a drop in SST. Along with this decline in egg laying, a considerable series of temporary and permanent nest abandonments began. While the exact causes of this decline in laying and increase in abandonment remain uncertain, the most probable explanation would be a decline in food availability, forcing the parents to spend more time at sea and less time at shore.

4.5.2 Breeding attempts

A total of 61 nesting attempts were recorded during this study. However, given the size of the island and the importance of checking potential nests every day to find the eggs on the day they were laid, some of the most inaccessible and time consuming areas around the island were not monitored. Although this count cannot be considered as a full estimation for this population, the areas surveyed were similar to those surveyed in 2005 and 2006, with the exception of two areas, Northeast bay and Pohutukawa cove, in which only three and two nests were found in 2005 and 2006, respectively. Thus, the season's nest count is likely to be comparable with previous years.

4.5.3 Egg laying, hatching and incubation

Differences in incubation lengths between first and second laid eggs are found in many penguin species. In crested penguins (*Eudyptes*) for example, first laid eggs generally hatch after second laid eggs, despite a laying interval of 3-6 days (Williams 1981b; Lamey 1990). One explanation for this variation is the egg position within the nest. In rockhopper penguins (*E. chrysocome*) for example, higher temperatures were recorded from second laid eggs than first laid eggs with the latter being generally found in the anterior nest position where heating is less consistent causing delays in embryo development (Burger and Williams 1979). This is thought to be a brood reduction strategy from the parents, as the first egg serves mainly as an insurance against failure of the second egg, and is often ejected during incubation (Williams 1981b; Williams 1989; St. Clair and St. Clair 1996). It has also been suggested that these eggs serve as a signal to conspecifics that the nest is occupied (Johnson *et al.* 1987), as a visual indication of a young female's fertility or as a tactile stimulation for the development of the brood patch to ensure an optimal thermal environment for the second egg (St. Clair 1992).

In Yellow-eyed penguins (*Megadyptes antipodes*), eggs are also laid 3-5 days apart but both usually hatch synchronously (Darby and Seddon 1990), making the first egg's incubation time 3-5 days longer than the second. Unlike in any other bird species however, incubation was shown to decrease with increasing age of incubating parents (Massaro *et al.* 2004). While the exact reasons for this phenomenon are unclear, possible explanations could be that incubation becomes more efficient with age due to behavioural changes, a more rapid development of the brood patch, a better coordination of incubation spells and possibly a modification in egg properties (Massaro *et al.* 2004). Indeed with increasing age, it appears that females might be able to

produce eggs with more albumen and a more porous shell, which allows a faster embryonic growth.

The laying interval for LBP observed in 2009 (1-5 days) on Tiritiri Matangi Island was very similar to that observed in other LBP populations (Kinsky 1960; Gales 1984; Kemp and Dann 2001; Heber 2008). The incubation length (33-39 days) was also similar to previous findings (Reilly and Bamford 1975; Reilly and Cullen 1981; Gales 1984; Chiaradia and Kerry 1999; Kemp and Dann 2001; Heber 2008). As recorded in other studies, first laid eggs took significantly longer to hatch than second laid eggs, which is likely to be due to the brood patch only developing between the laying of the two eggs, and thus delaying incubation of the first egg (Gales 1984; Kemp and Dann 2001). This idea is reinforced here by the fact that accessible first-laid eggs were generally cold when taken out of the nest to be cleaned before the second egg was laid. Hatching success, however, did not differ between first and second laid eggs, although this was from a relatively low sample size.

4.5.4 Egg size

In some penguin species such as crested penguins, producing different sized eggs is a reproductive strategy. First laid eggs, which are thought to be mainly an insurance against the failure of the second egg as explained above, are much smaller (15-45%) than second laid eggs (Warham 1975). In LBP however, both eggs are generally similar in size. A difference in length between first and second laid eggs has been recorded in the present study as well as in previous studies from New Zealand and Australia (Kinsky 1960; Gales 1984; Kemp and Dann 2001) nevertheless, their overall volume and weight generally remained similar.

4.5.5 Nest type

Nesting habitat is an important factor to consider as it provides not only shelter but also a specific microhabitat where the eggs and chicks grow and develop. In yellow-eyed penguins and Humboldt penguins (*Spheniscus humboldti*) for example, nest type was shown to influence breeding success through variations in temperature and humidity inside the nests (Seddon and Davis 1989; Mauricio *et al.* 1999). In LBP populations, the effects of nesting habitat seem to vary according to their location. In New South Wales, nests built in the low woodland produced more fledglings than those in sandstone boulders (Knight and Rogers 2004). Similarly, on Bowen Island, Australia, nests found in the *Banksia* woodland had a higher success than those in the tussock (Fortescue 1995). On Matiu-Somes Island, New Zealand, Bull (2000a) found that nest type significantly affected the fate of the eggs, with higher success rates in the most robust vegetation nests. On Otago Peninsula, breeding success was higher in artificial nest boxes than in natural burrows (Perriman and Steen 2000).

On Tiritiri Matangi Island, the majority of LBP nests are usually found in the rocks, at mid to low elevation. In 2003, Miyazaki and Waas (2003a) reported that larger male penguins were generally associated with these mid to low elevation nests, suggesting that these sites were potentially better breeding sites, possibly due to the proximity of the sea. Conversely, later studies found that nest distance to the sea did not affect LBP breeding success (Geurts 2006, Jansen van Rensburg 2010). This year, only chicks from rock nests fledged, however very few chicks had hatched from other nest types. Hatching, fledging and breeding success was therefore overall not affected by nest type.

Temperature and humidity measures inside breeding burrows revealed variations of up to 3°C and 14% humidity across nests. Nest type did not seem to influence these measures, although only a very small number of nests could be sampled. The comparison of microbial infection across nests also revealed no significant influence of nest type, suggesting that nest infection may vary greatly within nest types.

4.5.6 Microbes and cleaning

As described in Chapter 2, under certain environmental conditions microbes can penetrate the avian egg shell and infect its contents. The shell cuticle, membranes and albumen antimicrobial proteins are the most effective barriers. Recently it has been shown that such infections can be an important cause of hatching failure in domestic and wild birds, and cleaning a potential solution to reduce this infection (See Chapter 3) (Cook *et al.* 2003; Beissinger *et al.* 2005; Cook *et al.* 2005a; Cook *et al.* 2005b Godard *et al.* 2007). Despite this, however, cleaning did not significantly increase hatching success nor decrease early embryo mortality in this study. In addition, first laid eggs did not experience a higher mortality than second laid, suggesting that microbial infection prior to incubation was unlikely to be a main cause of egg mortality. Also, eggs recorded with higher initial microbial loads did not have a lower chance of survival, suggesting that initial microbial infection might not influence the egg's outcome. This however remains inconclusive due to issues in the methodology discussed in section 4.5.8..

4.5.7 Parental abandonment and food limitations

In species where incubation and guarding duties are shared, coordination of nest relief between parents is essential to ensure successful breeding (Croxall and Ricketts 1983).

This coordination is thought to be determined mainly by body condition (McNamara and Houston 1996; Numata *et al.* 2000), the lower the body condition, the more time a bird may need to spend at sea, and the less time it may be able to fast (Yorio and Boersma 1994; Weimerskirch 1995; Tveraa *et al.* 1997). Therefore, if the foraging trip of one parent extends for too long, the incubating parent might be forced to leave its nest to feed before starving to death (Groscolas 1990). When left unincubated, embryos are assumed to continue developing until temperature drops below 26°C (Drent 1975; Webb 1987; Ewert 1992). Below this temperature, embryonic development will slow down and eventually cease (Webb 1987), depending on the egg's cooling or heating rates and on the embryo's resistance to extreme temperatures (Webb 1987).

Outside the breeding season, LBP generally undertake one-day foraging trips (Weavers 1992; Collins *et al.* 1999), however, during incubation period these trips can extend for over 7 days, thus increasing the risk of nest desertion (Renner 1998, Numata *et al.* 2000). In this study, eggs were temporarily left unincubated for up to seven days in a row. Upon their return, parents would generally go back to the nest and incubate the eggs for a further 1-3 weeks, after which they would permanently abandon it. Most embryos died within the first 1-2 days following abandonment, however the parents continued to incubate them upon their return, often for the remaining incubation period needed for a chick to hatch. This suggests that parents may not be able to detect embryo death until they have reached the end of a normal incubation time. Three embryos survived the first abandonment but died when left for a second time. For two of these eggs, the first abandonment had been for three days after 20 and 22 days of incubation. Given the difficulty in precisely aging embryos, it could not be confirmed whether this embryo continued to develop normally in the absence of incubation or whether its development was slowed down or even paused until the return of the parent. In most

bird species, embryos shift from poikilotherm (body temperature varies with surrounding temperature) to homeotherm (body temperature maintained regardless of surrounding temperature) within the last 20% of incubation (Black and Burggren 2004). In LBP, the last 20% of incubation is reached after around 28 days. However, during those three days the two eggs always felt relatively warm, suggesting that perhaps these birds may be able to begin maintaining their body temperature earlier than other birds. Another contributor to the egg's warmth could have been the nest's exposure to the sun and the favorable weather conditions during the absence of the parents. Although optimum incubation temperatures were unlikely to be reached, Webb (1987) showed that penguin species tend to have both a lower optimum and a broader range of acceptable incubation temperatures, which may explain the slow embryonic development during the absence of the incubating parent. The third egg had been abandoned only two days after lay date. Given the development of the embryo at death, it seems that incubation had already begun prior to abandonment and embryo development either slowly continued in the absence of the parent or was paused and resumed upon the return of the parent. As the nest and the outside of the egg were relatively cold, it seems unlikely that temperature maintained this embryo alive. It is more likely that the antimicrobial proteins and perhaps the daily cleaning of this egg during the absence of the parent contributed to its survival.

This year, parental abandonment of both eggs and chicks was very high during the second six-week period. Although the fate of chicks was not the main focus of this study, their high abandonment rate and the very rapid death of the youngest chick in each clutch reinforced the theory of a general lack of food resources causing the penguins to sacrifice the energy already invested in the eggs and chicks to survive and attempt to rear at least one chick. Further studies investigating the foraging behaviour

and food availability in the Hauraki Gulf would thus be crucial to better understand the fluctuating rates of egg and chick abandonment.

4.5.8 Considerations and Limitations

While physical manipulation of chicks and adults was limited in this study, many eggs were handled daily. Originally, the objective of this study was to swab all first laid eggs at laying and on the day the second egg of the clutch was laid in order to get measures of shell infection during the whole period prior to incubation. However, as a result of two second-laid eggs cracking due to the agitation of the parents when the first-laid eggs were returned to the nest, it was decided that first laid eggs were to be taken out of the nest more than once only if they had to be cleaned and not when a second egg was present, thus limiting the risk of losing eggs due to physical manipulation. This resulted in no additional eggs cracking due to human manipulation. In addition, a comparison between labelled and unlabelled eggs revealed no significant difference in hatching success, or stage at which eggs failed, suggesting that proper handling does not decrease egg viability.

The other major limitation encountered was the late plating of samples due to the lack of boat transport on certain days. To ensure an equal treatment of all samples, they were all refrigerated for 3 days prior to plating, however, this resulted in a significant loss of live platable bacteria. Although the goal of this study was to compare rather than quantify microbial infection, a loss as substantial as this could have compromised the accuracy of these results.

4.5.9 Conclusions

It appears that the 2009 breeding season was particularly unsuccessful in comparison to previous studies on this population, as well as other populations in New Zealand and Australia. While the nesting environment and exposure to microbes prior to incubation did not seem to impact on egg viability, other factors such as the lay date significantly influenced the outcome of eggs. Overall, early laid eggs had a lower number of temporary abandonments and thus a better chance of survival than eggs laid later in the season. Geurts (2006) found that egg laying on Tiritiri Matangi begins at a relatively low SST, which is usually a sign of high food resources. In 2009 however, egg laying began a month before the lowest SST and suddenly dropped as SST decreased, suggesting that low SST might not always be a good indicator of breeding initiation and high food availability. Future research should therefore include an ongoing monitoring of this population in addition to studies focusing on the correlation between food abundance, foraging behaviour and breeding success, in order to better assess the state of this population and, if needed, implement a management programme to increase its breeding success.

CHAPTER 5

General Summary and Recommendations for Future Research and Management

Plate 5.1. Little blue penguin chick on Tiriti Matangi Island. Photo by A.S Boyer

5.1 GENERAL SUMMARY

5.1.1 Causes and consequences of microbial infection

This study provides the first investigation of causes and consequences of microbial infection of avian eggs in New Zealand. The chicken egg study (Chapter 3), in association with previous studies (Cook *et al.* 2003; Beissinger *et al.* 2005; Cook *et al.* 2005a; Cook *et al.* 2005b; Godard *et al.* 2007; Shawkey *et al.* 2008) confirms the general idea that prolonged exposures to ambient conditions increase shell infection, however, this increase in shell infection does not always result in an increase in trans-shell infection or a decrease in hatching success. This could be the result of numerous single or combined factors, including the type of nest, the climatic conditions, the quantities and types of microbes present in the nesting environment, the quality of the shell's cuticle, or even perhaps the concentration of antimicrobial proteins inside the eggs. This study also reinforces the hypothesis that cleaning significantly reduces shell infection; however, its efficacy at reducing trans-shell infection is highly variable.

On Tiritiri Matangi, despite the nesting conditions that would seem to promote microbial infection, first laid little blue penguin (*Eudyptula minor*) eggs did not experience lower hatching success that second laid, whether they were cleaned or not. In addition, embryos did not cease to develop more often at very early stages than at later stages, revealing that microbial infection prior to full incubation was most likely not a major cause of LBP egg mortality.

5.1.2 Effect of other factors on LBP egg viability

Incubation length and egg length significantly varied between first and second-laid egg but this did not result in a difference in hatching success. Nest type did not affect hatching success either. Date on the other hand was an important factor affecting egg laying, with two distinct periods of laying. In the first period, over three quarter of the eggs were laid and these eggs had a significantly higher hatching success those laid later in the season. In contrast to the findings of Geurts (2006), initial lay date was not associated with low SST. Date also seemed to influence nest attendance, with an increasing number of temporary nest abandonments towards the end of the breeding season, perhaps associated with a decline in food availability. This study therefore recommends further research on the foraging range and parental effort of LBP from Tiritiri Matangi Island as well as the impact of humans and other marine species on the abundance of food in the Hauraki Gulf, as explained below.

5.2 FUTURE STUDIES AND MANAGEMENT

5.2.1 Little Blue Penguins

5.2.1.1. Foraging studies

In this study, temporary nest abandonment was found to be the major cause of LBP egg and chick failure. While the exact causes of such abandonments are unknown, it seems likely that the coordination, experience and foraging abilities of the parents, as well as the abundance of food within their foraging range would play a major role (Richdale 1957; Bull 2000a). The next logical step will be to investigate the foraging range of these birds using tracking devices, in association with prey and other predator

distribution surveys. This would enable the determination of the foraging effort of these birds in terms of duration, distance and depth. In a recent study, Chiaradia *et al.* (2007) showed that diving effort (duration) and bathymetry were very important factors affecting the fledging success of LBP in Australia and New Zealand. In Oamaru and Penguin Island for example, LBP were making shallower and shorter dives compared to Motuara and Philip Island and had an overall higher fledging success. For LBP on Tiritiri Matangi, bathymetry would not be the main issue as the maximum depth is only 40 m within 40 km of the island. Low prey abundances could however force LBP to swim greater distances and/or spend considerably more time at sea.

Second, this would allow a comparison with the foraging range of other marine birds or mammals preying on the same species and perhaps identify potential competition that these animals face. Based on diet analysis from several different regions, it was shown that LBP feed primarily on pilchard (*Sardinops sagax*) and slender sprat (*Sprattus anipodum*) but also on arrow squid (*Nototododarus sloanii*), anchovy (*Engralis australis*), yellow-eyed mullet (*Aldrichetta forsteri*) and red cod (*Pseudophycis bachus*), (Cullen *et al.* 1992; Van Heezik 1990; Montague and Cullen 1998; Williams 1995; Chiaradia *et al.* 2003; Geurts 2006), many of which are also preyed upon by sympatric seabird species such as southern black-backed gulls (*Larus dominicanus*) and australasian gannets (*Morus serrator*) (Wingham 1984; Robertson 1992), and marine mammals such as the common dolphin (*Delphius delphinus*) (Stockin 2008).

Tracking the foraging patterns of LBP would help assess the potential impact of commercial and recreational fishing. With its world-class snapper (Chrysophrys auratus) fishery the Hauraki Gulf is an increasingly popular fishing area in the Auckland region (Morisson *et al.* 2007; Morisson *et al.* 2008). However, this increase has both

direct and indirect effects on the Gulf's marine life. The main direct effect is the reduction in fish abundance, which is not only detrimental to the fish species but also to their predators. Indirectly, an increase in boat numbers also causes changes to underwater habitats due to damages to the seafloor, which also affects the abundance and distribution of fish (Morisson *et al.* 2008). Finally, given that a number of marine reserves exist within the Hauraki Gulf, LBP tracking will enable researchers to determine whether LBP from Tiritiri Matangi use these resource rich areas.

5.2.1.2 Egg removal

Along with the death of embryos, another issue associated with temporary abandonments of nests with eggs is the loss of time and effort incubating the eggs that have died. In some cases, eggs were abandoned several times during incubation suggesting that either the parents were inexperienced and poorly coordinated, or in too poor condition to successfully incubate and raise a clutch with limited food resources. However, in other cases, eggs were left only once and then continuously incubated for the remaining duration of a normal incubation period suggesting that initially parents might have needed a longer foraging trip to prepare for the long incubation stint but were therafter capable of coordinating incubation bouts. Checking on the eggs during the absence of the parents and regularly after their return would allow to look for abnormalities (e.g. cracks) and, with appropriate candling techniques, quantify developmental changes. If the eggs are no longer developing, early removal will directly benefit parents from unnecessary energy costs, and could encourage parents in a suitable condition to undertake additional breeding attempts.

5.2.1.3 Eggshell structure and chemical defences

In terms of microbial infection, an interesting next step would be to investigate the structure of the shell in terms of porosity, conductance and cuticle quality as well as the chemical defences present within the LBP egg. As mentioned previously, LBP were found to have low levels of lysozyme, an important antibacterial protein, thus raising the question of the presence and quantity of other antimicrobial proteins.

5.2.1.4 Incubation behaviour

It still remains unclear precisely how much incubation, if any, first-laid eggs receive prior to the laying of the second egg. This could be investigated by placing a temperature probe on the bare skin of the brood patch of the adults as well as by inserting a thermistor probe through the shell of a limited number of eggs, as done previously with other penguin species (Burger and Williams 1979). The thermistor probe is inserted to a depth of about 5 mm into the air sac through a 2mm diameter hole and sealed into position without puncturing the inner membrane. Although this could be seen as fairly invasive, using an artificial egg would not be suitable for this species for several reasons. First of all, if artificial eggs were used they would have to be tested simultaneously with real eggs to ensure an identical reaction to the incubation heat. But most importantly, if using an artificial egg it would need to be placed in the nest when the first egg is laid. This however, would result in two eggs in the nest and could modify the behaviour of the incubating parents by either discouraging them to lay another egg or randomly push one out. Another solution could have been to record temperature fluctuations in the nest. However, again this would not be suitable for this species as although it might not be actively incubating the first egg, the female generally remains

in the nest in between the laying of both eggs. The presence of the penguin would thus result in high temperatures not necessarily significative of incubation.

5.2.2 Microbial infection in other avian species

Although microbial infection prior to incubation was not shown to be an important cause of egg mortality in the LBP, previous studies have shown that it is an issue in other avian species (Cook *et al.* 2003; Beissinger *et al.* 2005; Cook *et al.* 2005a; Cook *et al.* 2005b; Godard *et al.* 2007). Management programs of other synchronously incubating species should thus include analyses of microbial risks. In New Zealand, this includes species such as the yellow-eyed penguin (*Megadyptes antipodes*), the weka (*Gallirallus australis*) and the shore plover (*Thinornis novaeseelandiae*). Understanding the role of egg microbes in hatchability remains an important issue and studies need to be conducted on a range of populations of species that incorporate different environmental conditions and nest types. In addition to investigating the microbial infection of eggs, these studies could examine the structure of the eggshell and the egg's chemical defences, as explained above. This would allow the establishment of a comparison of strategies evolved by various synchronously incubating species around New Zealand.

Microbial infection of eggs could also be an issue for all other species with clutch sizes greater than one. As described by Saino *et al.* (2002) first-laid eggs have an increased vulnerability as they undergo more cycles of warming and cooling as the mother lays the other eggs, cycles which promote microbial penetration. For species that lay eggs in very humid and soiled nests, eggs might be at an even greater risk. For example, this could be the case for the critically endangered kakapo (*Strigops*

habroptilus) which lays its eggs in burrows, on moist, muddy surfaces (Brunton and Beissinger, *pers comm.)*, and leaves its eggs unattended for up to several hours at night (Cockrem 2002). While microbial infection of the egg contents can only be examined by opening the eggs, the alternative for such endangered species would be to examine the microbial infection on the surface of the eggshells of a small number of eggs to at least obtain an indication of the quantity and type of microbes present. If pathogenic microbes are present in potentially harmful quantities, then management actions such as regular cleaning when eggs are unattended could be implemented, and, if successful, applied to more individuals.

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