The Egg and the Nest: Obtaining information about the reproductive biology of *Apteryx spp.* (Family: Apterygidae), a cryptic avian taxon, through eggshells

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Cover painting: The Haywain Triptych by Hieronymus Bosch. Circa 1516

Museo del Prado, Madrid.

Back painting: The Concert in the Egg by a follower of Bosch. Circa 1561.

Palais des Beaux-Arts, Lille.

Engraving from Stultitia Laus by Hans Holbein, 1511

Abstract

The amniotic eggshell is a bioceramic that can endure the passing of time without major alteration to its physical structure. Not surprisingly eggshells have been found in numerous paleontological excavations around the world and have been used to identify the presence of major taxa and furthermore, to propose hypotheses on how extinct amniotes lived and nested. In the past decades the information that has been obtained solely through eggshells, ranges from inferences of a specie's habitat, to diet, and nesting behaviours. This information can be obtained using techniques such as microscopy, stable isotopes, ancient DNA, allometry, physics and biochemistry. The possibility of making inferences about an organism's biology using its eggshells make them an invaluable asset in biological research, as we can obtain information about species that are difficult to observe otherwise, that are endangered, or that are not even alive anymore. It may also allow a comparison of the conditions that affected a species or a group during evolutionary time. In addition, these studies can be done with eggshells obtained after hatching, therefore without compromising the welfare of the study animals.

The genus *Apteryx* (commonly known as Kiwi) is an endemic taxon to the three main islands of New Zealand/Aotearoa. This genus presents five distinct species and several taxa (possible subspecies). *Apteryx* is a unique clade because of its many unusual characteristics, including that all its species are ground-dwelling nocturnal insectivores, are winter breeders and nest in ground burrows or hollowed trees. *Apteryx* also presents a unique eggshell that has been part of scientific debates since the 1960's. The physical features of *Apteryx* eggshells such as eggshell porosity, thickness and overall gas exchange, do not fit in most allometric models. Hypotheses to address this phenomenon have explored answers in the *Apteryx*'s extremely long incubation period (74 days or more) and low basal metabolic rate, because these require lesser oxygen. *Apteryx* are also the only ratite that nest in ground burrows or hollowed trees; all other ratite species lay in scrape type nests.

Another oddity of *Apteryx* is that this genus presents diverse incubation behaviours, with some species having single male incubation, and others male and female incubation, as well as co-operative and group incubation. The mating system of *Apteryx* has been suggested to be monogamous for some of the species; however, *Apteryx* females are bigger than males, and at least in two of the *Apteryx* species, the female does not contribute to incubation whatsoever, suggesting some degree of sex role reversal. This could mean that the mating system of *Apteryx* revolves around polyandry, or as it is the case of most ratites, promiscuity (or polygynandry). The importance of studying *Apteryx* lays in its rarity; it is a group of species that defies allometric predictions and presents a wide variety of unusual adaptations. In many respects, it could be said that *Apteryx* presents adaptations to a niche that in other ecological contexts has been filled by mammals. Terrestrial mammals, except for chiropterans, are naturally absent in New Zealand ecosystems giving the opportunity to birds, which are plentiful in New Zealand, to exploit these niches and adapt accordingly. The series of oddities in *Apteryx* makes it a "must study" species to explore evolutionary pathways and the extremes in evolution.

All *Apteryx* species are under some category of endangerment and are within the top priorities of restoration and conservation programs in New Zealand, which makes non-invasive methods

of study the only way to approach biological and evolutionary questions about the group. This makes the use of eggshells an ideal method to approach biological and reproductive questions in *Apteryx*. The nests of *Apteryx* can be easily identified during breeding and non-breeding seasons, and eggshells are frequent remains amongst unoccupied nests; making them accessible with minimal disturbance to the birds.

In this thesis, I explored the gaps in knowledge regarding the allometric predictions for the *Apteryx* eggs and eggshells, re-testing many of these assumptions, considering that there are five species, and not three as was the common belief before 2003. I explored the eggshell using optical techniques (scanning electron microscopy and micro-computed tomography) and experimentally (by determining the water vapour conductance of the eggshell for three of the five species). I found that *Apteryx* eggshell thickness decreased from north to south, and so did the water vapour conductance, porosity was much higher than what was previously measured in all of the sampled species, and it was higher than the allometric predictions. I found that *Apteryx* eggs presented a mineral "cuticle" composed by triangular crystals only reported in the literature for the eggshells of a Theropod dinosaur from the early Cretaceous. Morphological characteristics of the eggshell have been used taxonomically by palaeontologists to identify species; I found that it is possible to distinguish between the currently accepted *Apteryx* species by comparing the eggshell thickness, porosity, cuticle thickness and mammillary area.

I next looked at the thermal properties of eggs and nests, using Newton's law of cooling; I determined the cooling rate of eggs and nests of Brown Kiwi (*Apteryx mantelli*) and compared it with those of other precocial bird species. I found that Brown Kiwi eggs do not have any adaptation towards heat retention. The nests, however, have good temperature buffering and heat retention capacity. The nest architecture allows the nest to remain cooler than environmental temperature during the day and warmer than environmental temperature at night, which is when the incubating parent leaves the nest to forage.

Finally, I examined *Apteryx mantelli*'s mating system by extracting DNA from the chorioallantoic membrane of hatched eggshells from Operation Nest Egg, a conservation strategy for kiwi. I determined the degree of parentage of the membranes found in several radio-tagged males over a period of five years using fragment analysis, and eight different microsatellite markers. I used very cost-effective techniques that make this study highly replicable and adaptable to other species. I found that Brown Kiwi is not a genetic monogamous species, having multiple contributor parents to a particular nest within and between years.

In conclusion, I found that this non-invasive methodology using eggshells of hatched or abandoned eggs is very valuable to study cryptic and endangered species. Therefore, I advocate for further studies of this type, as the information that can be gained about a wide range of species and species behaviours using eggshells, together with relatively cheap techniques is of immense value to science.

This thesis is dedicated to my Parents,

Claudia and Fernando.

To them I owe my love for science

and the desire to know the universe

Acknowledgements

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Chapter One
General Introduction

Qui Rosarum intrare conatur Philosophicum absque claves,

assimilatur homini ambulare volunti absque pedibus

(He that endeavours to enter into the Philosophers' Rosarye without a Key,

Is like him who wants to walk without feet)

Michael Maierus, 1619

The amniotic egg is a compartmentalised unit that evolved to carry out the complex biochemical reactions that would be necessary to sustain the development of a new life on land (Burley and Vadehra, 1989; Reisz, 1997; Maina, 2017). Many amniotes evolved a mineral shell that encloses the egg, in most species this shell is composed of calcium carbonate in a variety of its structural forms (calcite, vaterite or aragonite); however, a great variability exists between species in terms of the arrangement of the mineral units that constitute the eggshell. In other words, the architecture of the eggshell is specific for different taxa, and differences and similarities in the eggshell structure have been the basis for the development of a parallel taxonomy that has been extensively used to identify and classify eggshell remains of extinct taxa (Mikhailov, 1991). The type of characters used taxonomically include the way columns of calcium carbonate radiate from aggregation points in the shell membrane, how layers of different texture constitute the palisade and external zone, whether these layers present a smooth or a squamatic texture, and the shape and arrangement of pores (Fig.1.1).
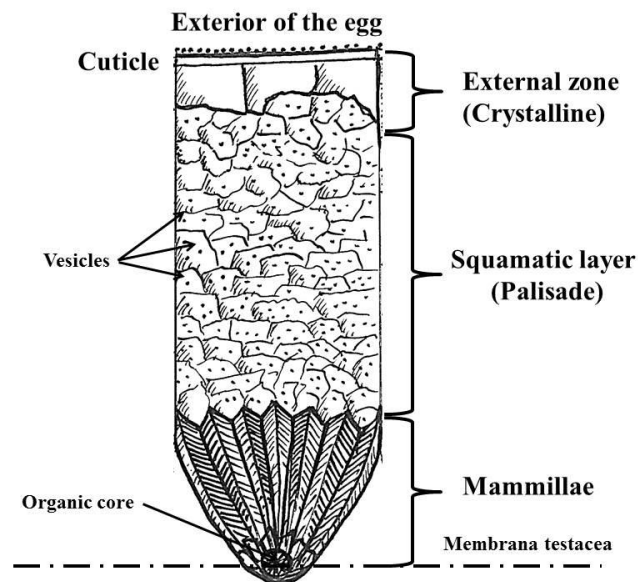
Figure 1. 1 Diagram of a single column belonging to the eggshell structure. (This example is characteristic of the Ornithoid morphotype). Adapted from Mikhailov (1996).

Beyond the possibility of identifying species using the eggshell's morphological characters, the eggshell structure contains within its composition, information about the environment the egg was laid. In addition, the functional characteristics of the eggshell can inform about the nesting environment and incubation requirements. The works of Ar, Paganelli and Rahn since the 1970's explored the gas exchange of the eggshell using the principles of Fick's law of diffusion and the relationship between mass, volume, and the development of avian embryos (Ar et al., 1974; Paganelli et al., 1974; Rahn et al., 1974; Paganelli, 1980; Rahn and Paganelli, 1990; Maina, 2017). Gas exchange in the egg is an interesting topic, as a balance between oxygen diffusion into the egg while allowing carbon dioxide and water to exit the egg are necessary to maintain appropriate respiration in the developing embryo. Water is particularly important, as it needs to be lost in a precise manner to prevent desiccation of the embryo. At the same time, the "apertures" or pores through which gases diffuse need to also prevent and minimise ingress of pathogens into the egg.

In general, Ar, Paganelli and Rahn's research focused on the relationships between the egg mass and volume and its effect on the eggshell gas conductance considering eggshell pore geometry and shell thickness. Their results have shown how allometric predictions can be made regarding the vapour conductance of eggs based on egg weight and eggshell thickness. Furthermore their findings imply that we can make interspecific comparisons between species with similar egg masses to examine the adaptations of the eggs to the nesting environment (Birchard and Kilgore, 1980; Seymour and Ackerman, 1980; Birchard et al., 1984). It has become widely accepted that some of the functional characteristics of the eggshell (shell thickness, pore density, pore area and distribution) can be used to accurately infer the nesting environment. By using these models, researchers have been able to propose hypotheses on the nesting behaviour and nest architecture of extinct taxa, based on comparisons of egg mass between seemingly different pore geometries and furthermore, gas exchange.

Beyond the eggshell itself there are a wide variety of behaviours that co-evolved with the physical structure of the eggshell to ensure hatching of chicks (Higham and Gosler, 2006). Incubation provides parental regulation of environmental conditions to sustain the life of the embryo inside the egg as all biochemical and enzymatic reactions require specific temperature ranges for optimal function (White and Kinney, 1974). Parental attentiveness evolved congruently in many species to reduce the temperature variability the egg might be exposed to during incubation (Carey, 1980). For these reasons, birds (and reptiles) have developed a great variety of incubation techniques that respond to the environmental challenges the egg of each species faces (Deeming, 2002). The diversity of physiological adaptations that allow different species to thrive in their environment, may have coevolved with different combinations of incubation length (Boersma, 1982), nesting, and incubating behaviours (Collias, 1964), and shaped some of the morphological attributes of specie's eggs. Therefore, to study the reproductive biology of birds, the relationship between the eggshell functional characteristics, nest environment, habitat of each species, and parental behaviour need to be considered. Nevertheless, the eggshell is an accurate reflection of the aforementioned factors, making the eggshell an excellent non-invasive source of information about species and their environments.

Functional characteristics of the eggshell like the cuticle and colouration, and their relation to the nest environment have been studied in several avian taxa (Westmoreland et al., 2007; Cassey et al., 2008; D'Alba et al., 2014, 2017) as well as their phylogenetic relations (Zelenitsky and Modesto, 2003; Grellet-Tinner, 2006; Winkler, 2006; Zelenitsky and Therrien, 2008). However, there are few species whose unique attributes do not fit the usual models and predictions. One of them is the genus *Apteryx*, a group of ratite birds endemic to New Zealand (Calder, 1979; Board and Scott, 1980; Tullett, 1984).

Apteryx (commonly known as Kiwi) was first described to science in 1813 by George Shaw (1751-1813). Since its discovery it has become a zoological curiosity due to its unusual traits of being flightless, a nocturnal insectivorous bird that in many aspects looks and behaves more like a mammal than a bird. There are five recognised species of *Apteryx* and around 11 taxa but this classification is still debated (Weir et al., 2016; White et al., 2018). In this genus the male can incubate the eggs; in the case of Brown Kiwi (*Apteryx mantelli*) and Pukupuku (*A. owenii*) the male is the sole care provider for the egg, while in the other species various systems have been

recognised, such as male and/or female incubation in Roroa (*A. haastii*) and Rowi (*A. rowi*) to co-operative breeding in Tokoeka (*A. australis*). Throughout this thesis I will use the term *Apteryx* to refer to the genus and will refer to the individual species by their Māori name.

Due to its atypical appearance compared to other birds, the anatomy of *Apteryx* was extensively studied during the 19th and early 20th centuries (Owen, 1839; Hyrtl, 1862; Parker, 1890). In the late 20th century its behaviour and ecology became of interest, and since, studies have been undertaken to study its physiology (Reid, 1971; Body and Reid, 1983; Prinzinger and Dietz, 2002), habitat use (Taborsky and Taborsky, 1992, 1995; Dixon, 2015), sociality (Cunningham, 1840; Cunningham and Castro, 2011; Ziesemann, 2011), diet (Colbourne and Kleinpaste, 1983; Dixon, 2015; Shapiro, 2005), and reproductive biology (McLennan, 1988,1991; Jolly, 1989 ; Potter, 1989; Colbourne, 1991, 2002; Jensen and Durrant, 2006; Ziesemann et al., 2011).

After European colonization, populations of *Apteryx* underwent rapid decline due to the introduction of mammalian predators (McLennan, 2006) and habitat loss (Holzapfel et al., 2008). Because of this decline most recent research efforts have focused on identifying threats and increasing *Apteryx* populations by different means; programmes have been established to create predator free areas, and a captive incubation program (Operation Nest Egg or ONE) was devised (Colbourne et al., 2005).

The Operation Nest Egg programme uses males tagged with Very High Frequency (VHF) radios that are followed to their nesting sites annually. When active nests are identified, the eggs are monitored, collected either before they hatch, or the chicks after hatching, then transferred to an incubating/caring facility, where the eggs are artificially incubated until hatch. Chicks and captive hatchlings are fed until they reach ≥ 1 kg, a body size sufficient to reduce the risk of predation by stoats, *Mustela erminea,* their main introduced mammalian predator (McLennan et al., 2004). These juveniles are then released back to the wild, in some cases to crèches in offshore islands, or in other cases translocated to mainland populations (for more information visit: https://www.kiwisforkiwi.org/what-we-do/how-were-saving-kiwi/learn-more-about-operation-nest-egg/how-it-works/). Operators at several ONE facilities have stored some of the eggshells from eggs they have incubated; these eggshells have been stored individually with code names that allow retrieval of information such as the date and area the egg was collected, as well as possible parentage (the male is usually identified but the mother is suspected based on field observations). In the framework of these conservation efforts and especially the ONE programme, I address several evolutionary questions in *Apteryx* making use of the mentioned eggshells.

The general aim of this thesis is to use eggshells and eggs to explore evolutionary hypotheses in *Apteryx*, and as a non-invasive method to study an endangered species. First, I review the literature in the fields of oology and palaeontology to propose studies that could improve the current knowledge in the study of ecology and phylogeny using avian eggshells, especially advocating for a greater use of extant species eggshells in relation to phylogenetic and ecological context, and a more active dialogue between biologists and palaeontologists (Chapter 2).

Second, I examined the functional characteristics of the eggshell in a phylogenetic, ecological and adaptive context relating them to latitudinal distribution of species and to the nest environment by looking at the eggshell thickness, cuticle and water vapour conductance (Chapter 3). The main question I ask in this chapter is: what are the functional characteristics of the very thin eggshell in relation to incubation period and gas exchange? Gas exchange has been measured in Brown Kiwi eggshells, but this was done before five species were recognised, therefore a comparative analysis of the different species does not exist. Because *Apteryx* are endemic to New Zealand and all the species are under some endangerment category, most previous studies had very small sample sizes or resorted to samples of eggs laid in captivity. Captive laid eggs are much smaller than those laid in the wild (Reid, 1981), thus changing the functional properties of the eggshell. Therefore, a closer look at gas exchange in *Apteryx* is needed with a bigger sample size using wild laid eggs. This chapter was written with the help of Nikki Minards and Michael Loh in the Scanning electron microscopy and Micro CT respectively, and written in collaboration with Isabel Castro, Patrick Morel and Wei-Hang Chua. I expect to find differences in the eggshell physical structure between the species, specifically differences in eggshell thickness and porosity; these differences will be related to latitudinal distribution and average body weight of each species, and altitudinal distribution (observed as a greater variation in species with wider altitudinal distributions and less variation in locally restricted species).

Third, I made a first approximation to the thermal properties of nests and eggs of Brown Kiwi to explore their adaptive value for a winter breeder and a single incubating parent, also with an additional purpose to promote similar studies for the other *Apteryx* species and eventually lead towards a comparative analysis (Chapter 4). Kiwi presents a unique nest architecture when compared with all the other ratites; this nest architecture is shared amongst all *Apteryx* species (as far as we know), therefore it is worth asking questions regarding the evolutionary pressures that might have resulted in such innovation. A description of the nest architecture exists for the Pukupuku (Jolly, 1989), and despite Brown Kiwi being the most studied species and its nest being been included in several studies (Potter, 1989; Colbourne, 2002; McLennan et al., 2004; Ziesemann et al., 2011) a formal description that includes detailed measurements has not been produced. I present a description of the Brown Kiwi nest in Chapter 4. The main hypothesis I tested in this chapter is: The evolution of nests is usually driven to reduce the effect of weather variability and/or predation, therefore, in the absence of known natural predators, the Brown Kiwi nest must have thermal buffering capacities. Furthermore, I explored the egg's thermal properties as well in case the egg itself had adaptations to withstand cold temperatures. I have done this using Newton's law of cooling. This chapter was revised and edited by Isabel Castro, Patrick Morel and Wei-Hang Chua.

Finally, I tested the alleged monogamous mating system of Brown Kiwi (Colbourne, 2002; Taborsky and Taborsky, 1999) by extracting DNA from eggshell membranes retrieved from ten tagged males in the population of Maungataniwha (Northern Hawke's Bay) during the breeding seasons of 2012 to 2017 (Chapter 5). In this chapter I proposed the hypothesis that Brown Kiwi is unlikely to be monogamous, since all of the other species of ratites that have been studied using molecular techniques exhibit mating systems that revolve around polygynandry (where a female mates either simultaneously or sequentially with more than one male and males mate

either simultaneously or sequentially with several females). I did this by examining the relatedness of the chicks that hatched from eggs retrieved from the same nest. Brown Kiwi present many morphological characters that are frequently associated with polyandry, such as incubation provided by the father, a bigger and more aggressive female (Corfield, Gillman, and Parsons, 2008), multiple clutches in one reproductive season, and little to no parental care. For at least one Brown Kiwi population (Ponui Island) non-monogamous associations have been observed, trios and quintets of roosting birds have been recorded as well as more than one male incubating in one nest (Ziesemann, 2011). Some of the other species of *Apteryx* (Roroa, Rowi and Tokoeka) exhibit male and female incubation, therefore a closer examination of the breeding system is needed, first to examine the actual mating system of Brown Kiwi and second, to understand how mating systems evolve from a ratite polygynandrous type to a more monogamous mating system if this is the case. This chapter was produced under the supervision of Kristene Gedye as she developed and optimised the extraction and amplification protocols used, the fragment analysis was carried at Massey Genomic Services under the management and supervision of Xiaoxiao Lin; the chapter was revised and edited by Isabel Castro, Patrick Morel and Wei-Hang Chua.

To conclude I discuss possible evolutionary explanations for the eggshell characteristics in *Apteryx*, and their relation to the nest architecture in Brown Kiwi. I suggest further observations that could be made to further our knowledge about the other *Apteryx* species. I discuss as well, the non-monogamous mating system of Brown Kiwi in relation to the costs of egg production.

Chapter Two
Avian eggshells: Witnesses in Avian Evolution

Seminate aurum vestrum in terram albam foliatam

(Sowe thy gold in the White Foliate Earth)

Michael Maierus, 1619

Abstract

Recent decades have seen the discovery of countless numbers of fossilised eggshells in numerous sites all over the world; a parataxonomic system has been designed to deal with the classification of eggshells that are unable to be immediately associated with skeletal remains where the eggshells were unearthed. Phylogenetic hypotheses have been proposed to help with the identification of eggshell remains and in order to explain plausible paths of evolution of eggshell morphology. Nesting ecology has been considered the main environmental factor affecting the morphology of eggshells and research has been conducted on eggshell porosity and water vapour conductance as means to theorize on the nesting behaviour of dinosaurs. In this review, I discuss the importance of studying the eggshell morphology of extant species in the light of the advances of palaeontology in species identification using eggshells. Palaeontologist have classified and identified species based solely on eggshell remains, demonstrating that eggshells are taxonomically informative. However, in only some cases a relationship can be established between eggshell remains and bone or embryonic remains confirming eggshell identification. The physical characteristics of eggshells of extinct organisms have been also used to formulate hypothesis of the environment and nesting ecology of these organisms. I advocate for further studies using extant organisms to validate the advances in palaeontology because the possibility of accurate identification of the laying species (either by observation or genetic identification), and because direct relationships can be established between physical traits and environment, and nesting ecology of extant species. Furthermore, there is an intrinsic problem with eggshell identification of extinct species, which is convergent evolution. Convergent evolution could produce very similar if not identical features in the eggshells of not-closely related species. This problem could be better understood and accounted for by observing how related species eggshells can be different in different environments and how non-related species could exhibit similar adaptations to similar environment. The inferences made using extinct organisms have oversimplified some ecological variables and omitted others, such as atmospheric gas composition and overall climate, even though eggshells themselves provide accurate information about climatologic conditions. I advocate for the use of extant species to study the relationship between environmental conditions, nest microclimate and eggshell functional characteristics, as well as the influence that both phylogeny and ecology have on shaping these characteristics.

Introduction

In the mid-19th century Wilhelm von Nathusius (1821-1899) produced one of the first systematic observations of the avian eggshell (Tyler, 1964) and since then there has been a steady effort to understand the evolution of the eggshell. The consensus is that the eggshells of all bird species

are composed of six distinct layers, the decalcified internal membranes (the inner and outer membranes), the mammillary zone, the squamatic zone (or palisade) the external zone, and the cuticle or accessory layer (Mikhailov, 1997) (Fig.1.1).

Two main factors can affect the morphology of an eggshell. First, phylogenetic factors, with changes in ancestral stages having been traced to produce eggshell based phylogenies (Mikhailov, 1992). This has been the basis for taxa identification in the geological strata, where a parataxonomic system based solely on eggshell remains has been developed, and eggshell structure used to establish relationships (Mikhailov, 1991). Second, are ecological factors, as eggshell by design need to respond to environmental pressures such as the nesting environment (Norell et.al, 1996; Hechenleitner, Grellet-Tinner and Fiorelli, 2015). Adaptations to the nest environment can be reflected on in differences in eggshell thickness, porosity, and cuticular composition (Winkler and Sánchez-Villagra, 2006; D'Alba et al., 2017). Also, eggshells' composition can record information regarding their environment, namely stable isotopes of carbon, oxygen and nitrogen can be used to inform on rainfall, vegetation and diet during their formation and incubation (Montanari, 2018). Climatic conditions have been assessed by examining the deposition of specific varieties of stable isotopes. For instance, carbon isotopes have been used to determine the type of vegetation present in the environment of herbivorous species (Angst et al., 2015), while oxygen (Ségalen and Lee-Thorp, 2009) and nitrogen (Johnson et al., 1997) have been used to estimate rainfall.

Interactions between phylogeny and ecology may obscure the level of phylogenetic information that could be obtained from eggshells in the cases were convergent evolution produces characteristics with difficult phylogenetic interpretations. In this review, I discuss the existing literature proposing phylogenetic hypotheses built using eggshells with the aim of proposing studies that could help clarify the information that can be obtained from eggshell phylogenies, and emphasising how such phylogenies can improve the understanding of the evolution of birds. To achieve this, I reviewed the published literature in the fields of palaeontology, cladistics and paleoecology focusing on the phylogenetic approaches used by numerous researchers and the inferences made about the organism's life history and behaviour.

Materials and methods

I used Google Scholar, Web of Science and Scopus to gather and compare publications on topics of taxonomy and cladistics using eggshells of birds and dinosaurs. Publications on reptiles such as turtles, geckos and crocodiles were marginally included due to their similarities in eggshell calcification and preservation and because these taxa provide relevant information about the evolution of eggshell structures in birds (Deeming, 2007). I searched for literature of ecological reconstruction using eggshells and focused mostly on research using stable isotopes. Finally, I searched for literature containing research that made inferences about the nesting and incubation ecology of extinct birds and dinosaurs using eggshells. This is, however, not a review about parataxonomy or a summary of paleontological discoveries. This review is not intended as a

thorough literature review in the field of palaeontological oology but rather using representative studies and methods from that field to demonstrate the need for performing these studies with extant species.

Key terms searched:

Avian eggshells, theropod, dinosaur nesting behaviour, dinosaur eggshells, eggshell parataxonomy. Eggshells AND fossils, Eggshell AND phylogeny, Avian eggshells AND genetics.

Results and Discussion

The ultrastructure of the amniotic egg has been used to assess phylogenetic relationships using morphological characters in a traditional taxonomic way and an autonomous parataxonomy was developed to deal with the classification of oolithes (fossilised eggs and eggshells) (Mikhailov,1991;1996). Eggshell remains have been found all over the world, giving testimony of the species that inhabited these areas. In many cases, the oolithes are found by themselves without the possibility to directly associate the eggshell remains to a particular organism (Hirsch, Kihmand and Zelenitsky, 1997); hence, necessitating a system of species classification using eggshell characteristics by themselves. Major differences in morphological characters at high taxonomic levels have been found, and several morphotypes of fossil and extant eggshells have been proposed (Mikhailov, 1991). Mikhailov's parataxonomy clearly distinguished between testuoid (turtles), geckoid (geckos), cocodriloid (crocodiles) dinosauroid (for dinosaurs distinguishing between spherulitic and prismatic types) and ornithoid eggshell structures (distinguishing between the ratite morphotypes seen in theropod dinosaurs and ratites; and a prismatic morphotype, which is present in most neoaves). These morphotypes have been revised from time to time, as new and unique eggshells have been discovered and characterised, making the diagnoses more complete (Vianey-Liaud et al., 1997; Vila et al., 2017).

In some cases, it has been possible to directly associate eggshell remains to skeletal remains of adults and embryos. The identity of eggshells of the small bird-like dinosaur *Troodon formosus,* for instance, was determined when a complete nest was found with an articulated tibia-fibula and caudal vertebrae, amongst other bones, in direct contact with the fossilized eggs (Varrichio et al., 1997). Hirsch and Quinn (1990) linked four different egg morphotypes to skeletal remains, mostly nests, complete clutches, and neonates in the Two Medicine formation in Montana. In this case, the presence of embryonic remains near eggs of *Orodromeus*, a late Cretaceous herbivorous parkosaurid dinosaur, allowed assignment of these types of eggshells to that genus. In a similar manner, complete nests with hatchlings of *Maiasaura*, an upper Cretaceous hadrosaurid dinosaur, found in the vicinity of the eggshell remains allowed assignment of these eggshells to a real taxon. In another unearthed fossil, embryonic bones were found inside the eggs of *Troodon formosus* (Jackson, Horner and Varricchio, 2010). Embryonic material has also been found and identified for the *Torvosaurus*, a late Jurassic theropod (Araujo et al., 2013), a megalosaurid theropod dinosaur, as well as Cretaceous avian taxa (Schweitzer et al., 2002), and

the tibia of a neonate specimen of a possible *Hypsilophodon* which is a late Cretaceous ornithischian dinosaur (Maxwell and Horner, 1994) to cite a few examples.

In 1996, Norell et al., reported a very unique specimen, a fossil of an *Oviraptor*, a theropod from the Cretaceous, sitting on top of what looked like a nest; it was suggested first that this was a predatory attempt (Osborn, 1924) but it seems that this is indeed a case of a nesting dinosaur. However, these types of discoveries are extremely rare; without direct association with skeletal remains the diagnoses can only allow inferences that relate broad taxonomic groups. The other specimens, even after being classified in oogenera and oospecies, will have to wait for the discovery of evidence that can further clarify their identities (Hirsch and Mikhailov, 1995). Two relatively complete reviews on the clades that have been associated with skeletal remains was published by Zelenitsky and Hirsch, (1997) and Grellet-Tinner et al., (2006).

An alternative that can be employed to identify the eggshell to lower taxonomic levels (i.e. genus or species) is to construct phylogenetic hypotheses that include the specimens that have been associated to skeletal remains. Tanaka et al., (2011) used the dataset published by Zelenitsky (2004) to construct a phylogeny to identify 145 eggshell fragments that varied in thickness and ornamentation found in the Luanchuan excavation (West Hunan province, China), comparing them to 28 different taxa and using 16 different characters. Among 36 equally parsimonious trees the eggshells were placed in the Elongatoolithidae oofamily (Norell et al., 1994; Fanti et al., 2012). There are five oogenera known for this oofamily in this region, thus further identification would be guesswork. Similar studies were conducted by Moreno-Azanza (2013) and Varricchio and Jackson (2004) by constructing phylogenies using eggshell morphological characters to try to identify new remains. Phylogenetic analyses were used in a similar way in the works of Vianey-Liaud et al., (1997 and 2003) but in these cases the authors found identical species distributed in Peru and France (1997), and France and India (2003). The implications for biogeography in these cases are very important, because it is possible that they were dealing with a very broadly distributed species, however, how different were the climates and habitats of these locations, and what evidence did they leave behind about the specific adaptations of this "cosmopolitan" species?

A strong criticism to phylogenies constructed with this purpose was posed by Mikhailov (2014). He stated that "*the choice and the given status of characters were inadequate to recent knowledge on eggshell structure and mineralisation, and the characters' coding was done disregarding the concept of homology*". This is a very important concern as synapomorphies indeed can obscure the interpretations made with eggshell phylogenies. For instance, the ratite morphotype that has been associated with theropods and paleognathes is also present in Cuculiformes and Piciformes, both Neoaves, which usually display the ornithoid prismatic morphotype. This is an example of homology rather than representing a close phylogeny (Hirsch et al., 1997). In a similar manner the mammillary wedges of the ratite morphotype are absent in *Apteryx* and Tinamous, but present in some of the neognaths (Vianey-Liaud and Zelenitsky, 2003).

Environmental weathering is another factor that can obscure the phylogenetic interpretation of fossil eggshells, as weathering can change the eggshell structure. However there have been good

attempts to associate specific natural forces with identifiable characteristics in these fossils, such as predation, dragging and dissolving, and a taphonomic narrative can be proposed for each scenario limited by the obtainable evidence (Hayward, Hirsch, and Robertson, 1991; Hayward et al., 2000).

Diagenesis (which is the mineral replacement over time, which in the case of eggshells can happen to calcium carbonate, which can be dissolved and replaced by secondary calcium carbonate) can also alter the eggshell morphology making it more challenging to interpret its features (Pickford, Senut, and Dauphin, 1998). However, this process has been studied and in some cases the diagenesis can be identified, and its effects accounted for. By using cathodoluminescence, it is possible to determine the diagenetic minerals from fossils (Cusack et al., 2008; Grellet-Tinner, Corsetti and Buscalioni, 2010), and in some other cases is a more simple process as seen in turtle eggshells where the aragonite is replaced by calcite (Hirsch, 1996).

It becomes apparent that a better understanding on how eggshells evolve in a phylogenetic context is necessary; in other words, constructing eggshell phylogenies with extant birds and reptiles is the way to determine the extent to which the eggshells are phylogenetically conserved, and the effect that the environment exercises on them. This is particularly relevant today because of the possibility of contrasting eggshell based phylogenies with molecular phylogenies. Therefore, there is a need to place the eggshell adaptations in an ecological context and allowing a closer examination of how different characteristics of the eggshells of different taxa adapt to similar environments, and how different environments drive the evolution of features of closely related taxa.

Some studies have already constructed eggshell phylogenies with extant taxa, yielding interesting results. Zelenitsky and Modesto, (2003) built the phylogeny of ratites using eggshell characters, however, the resulting trees did not agree with the most recent molecular phylogenies (Mitchell et al., 2014) that include extant and extinct taxa. Instead, Zelenitsky and Modesto's (2003) phylogeny uncovered a biogeographic pattern, or perhaps an ecological pattern. This phylogeny for instance associates *Apteryx* with Moa (Dinornithidae), both endemic to New Zealand, Emu (*Dromaius*) and Cassowary (*Casuarius*) both present in the Australia-Papua New Guinea area and finally Ostrich (*Struthio camelus*) (Africa) with Rhea (*Rhea* and *Pterocnemia*) (South America). It is possible then, that the similarity of these eggshells is based on their ecological adaptations to similar climates and environments, and not so much reflective of phylogenetic relationships (Figure 2.1).
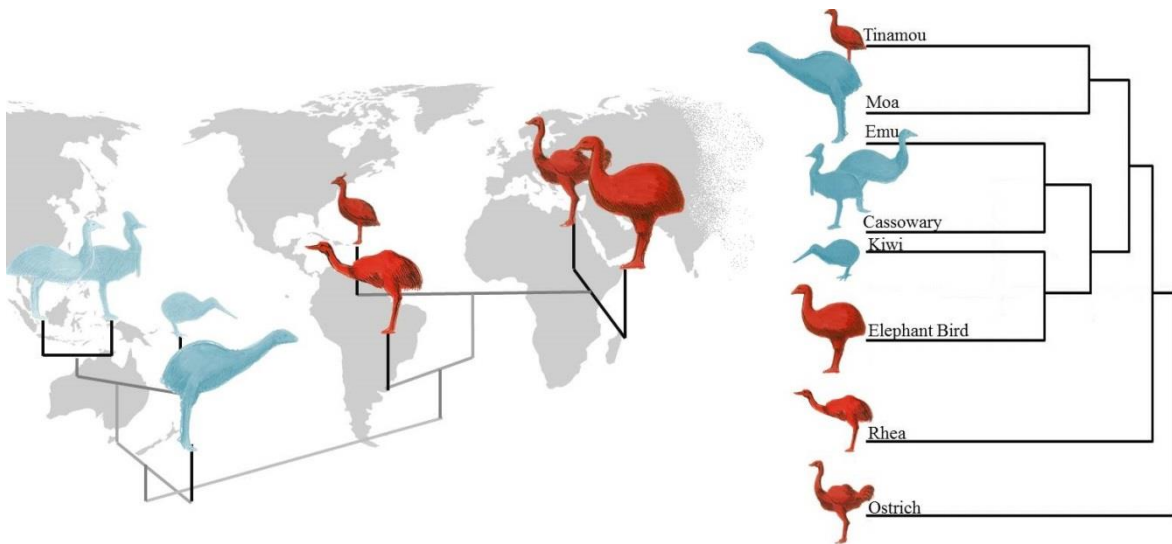
Figure 2.1. Two ratite phylogenies adapted from the literature. To the left, a phylogeny constructed using eggshell morphological characters presented by Grellet-Tinner (2006), in the background the maps indicate the places of origin of the species. On the right a molecular phylogeny presented by Mitchell et al., (2014). The colour of the birds indicates an Afro-American distribution (Red) or Australasian distribution (Blue).

A study by Grellet-Tinner (2006) that included more extinct taxa, in this case *Dinornis* (Moas), *Aepyornis* (Elephant Bird), *Struthiolithus* (an extinct species of Ostrich), *Genyornis* (Dromornithidae) and three other dinosaurian taxa as an outgroup, revealed a similar pattern to that published by Zelenitsky and Modesto. His phylogeny clearly distinguished between Australian-New-Zealand clade and an African-South American and Madagascan clade; and within the Australian-New Zealand clade again Dinornithidae appeared closely related to *Apteryx*. Again, seeing that these two independent phylogenetic approaches retrieve the same pattern begs the question: are these phylogenies actually revealing information about the adaptations of these species to their environments?

In 2016, Winkler used 13 eggshell morphological characters and tested which of these characters better supported well established clades. She found that three of the characters supported the molecular phylogeny; these characters were the size of the interunit spaces (or the ridge space between the mammillae and the columnar unit), development of the pore apertures, and the distribution of pore apertures (or the pore density). However, there was ambiguity with the remaining characters. She found that when examined in an ecological context this ambiguity could be explained by different species exhibiting similar traits in similar environments.

In another example the coloration and maculation of British passerine eggshells was contrasted with phylogenetic information and ecological information (nesting ecology and life history); it was revealed that while phylogeny was strongly correlated with the colour and appearance of the egg, ecology was not (Brulez et al., 2016). Interestingly, and in contrast to the above study, Cassey et al., (2012) reported that in British non-passerine species egg colouration co-varies with nesting ecology and life history as well as to be phylogenetically influenced (Cassey et al., 2012). These two studies suggest that phylogeny and ecology can have different effects on egg coloration, despite ecology being the main driver in phylogenetic changes.

Among the ecological aspects that affect eggshell morphology, the nest environment and nesting behaviour have received the most attention. It is the general consensus that nesting ecology is one of the main drivers behind the functional characters of the egg, as water vapour conductance depends on the humidity of the environment where the egg is being incubated (Paganelli and Ar, 1974; Walsberg, 1980; Ar and Rahn, 1985). Deeming (2006) explored the water vapour conductance in 47 extinct taxa by collating information about pore density and distribution as well as pore area in fossil specimens. Using allometric equations he estimated the water vapour conductance for these species and inferred that the dinosaurs most likely buried their eggs in a similar way as crocodiles and megapodes do today. He reached this conclusion by comparing the water vapour conductance obtained for the dinosaurs with that of avian eggs of similar size. Water vapour conductance depends on the egg temperature and pressure difference across the eggshell, which in turns depends on humidity. Therefore, the water vapour conductance can be correlated with the environment of the nest. When considering egg mass, mound or burrow nesters for example tend to have higher water vapour conductance, or in other words higher daily water loss, than scrape or open cup nesters (Grellet-Tinner, Lindsay and Thompson, 2017).

Tanaka et al., (2015) stated that due to the lack of preservation of nesting materials, and the inaccuracy of methods used to estimate water vapour conductance, the inferences made using solely water vapour conductance to assess nesting behaviour could be equivocal; instead they explored the nesting behaviours in dinosaurs by focusing on the porosity of the eggshell. They first established the relationship between porosity and nest type for 127 extant bird species and then applied these relationships to the calculation of water vapour conductance of 120 extant archosaurians and 29 extinct taxa to allow comparisons between the extant and extinct species. They concluded that dinosaurs most likely nested in two different nest types: fully covered nests, where the eggs are buried in the nest substrate, and open nests, where the eggs are incubated and brooded by a parent. Fully covered nests had eggs with higher porosity than those eggs of open nests. Buried eggs are in a high humidity environment with low oxygen available, making it necessary to have more pores to allow water vapour to escape and enough oxygen to come in, while open nests might desiccate fast due to direct exposure with the atmosphere, making it necessary to have less pores. These authors went a step further and gathered information about nest temperatures of extant crocodiles and Megapodes and compared these results with the lithographic information of dinosaur nests, by this they inferred the possible nest type, materials and incubation heat source for several dinosaur species (Tanaka et al., 2018).

Climate is suggested to have an effect on the eggshell (Hayward et al., 2000), and some attempts have been made to determine the environment in which dinosaurs incubated by using features found in the eggshell. Welsh et al., (2008) suggested that the type of pores on eggshells of different species found in the Aguja formation (Texas, USA) could be related to the arid climate assessed for this region during the late Campanian period (late Cretaceous). Further studies relating pore type and climate are needed, especially in extant fauna. Paleognathous birds for instance, despite their known monophyly (Mitchell et al., 2014) present a variety of pore shapes, from funnel-like (*Apteryx* and Tinamous), to simple branches (Emu and Cassoward), and completely branched (Ostriches) (Board, 1982). It would be logical to test the relationship of these pore types with the nesting environment and the external environment, as all extant ratite

eggs (except *Apteryx*) are incubated in open nests and are exposed directly to the elements. The variety of pore arrangements seen in dinosaur eggshells could indicate adaptation to very distinct environments.

The problem with predictions of nesting behaviour based on water vapour conductance and other functional characteristics of the eggshells is that other climatic variables are not considered, for example, the levels of atmospheric oxygen shifts during the Mesozoic. It has been suggested that the levels of atmospheric oxygen reached a height of 29% during the Albian-Cenomanian (at the beginning of the late Cretaceous 121-112 Myr) compared to the modern day 21% (Glasspool and Scott, 2010). The eggshells of dinosaurs could have been adapted to this oxygen superabundance making broad comparisons with modern taxa potentially equivocal.

Finally, the effect of convergent evolution would hinder taxonomic classification of eggshells using morphological characters (Mikhailov, 2014). For sub-fossil eggshells, DNA can be obtained, facilitating the identification and recognition of analogous traits in fossil eggshells (Montanari, 2018). For example, DNA was obtained from the eggshells of Moa found in a 13th century dirt oven (Oskam et al., 2011). Using ancient DNA obtained from Elephant bird's eggshells, Grealy et al., (2017) constructed a ratite phylogeny that was congruent with that by Mitchell et al., (2014), the latter being also based on ancient DNA but in this case from the tibiotarsus and femur of two different species of elephant birds, *Mullerornis agilis* and *Apyornis hilderbrandti.* There are more than 150 studies using ancient DNA sourced from bone, eggshells and coprolites (Grealy, Rawlence, and Bunce, 2017). Overall, there is a need to establish in modern taxa and as much as possible on extinct taxa, whether physical characteristics are caused by convergent evolution or by direct ancestry.

Studies focusing on the different effects of phylogeny and ecology are needed to understand how the eggshell characteristics vary in time among taxa. A thorough phylogenetic analysis that includes molecular, morphological, eggshell and ecological information could help to place patterns of avian evolution in context. An effort needs to be made to try to understand how climatic variables affect the eggshell structure, as this will provide a very valuable groundwork to aid palaeontologists working on paleoecological reconstruction as well to understand the effect current climate change could have on modern biodiversity.

Studies that look at the effect of climate on eggshell morphology in species such as Black Birds (*Turdus merula*) and the Pigeon (*Columba livia*) are needed. These species have been introduced from Eurasia into many other regions of the world and adapted and naturalised to other continents. By looking at the differences and similarities in their eggshells, thickness, porosity, and pigmentation it could be possible to observe how much the eggshell has adapted to its new environment and the effect of human activities in such environments, also to understand the effect of bottle-necks and founder effects on eggshell structure in avian populations. Similarly, the nesting behaviour could be observed, and differences in nest construction or architecture could reveal how these species adapt so successfully to new environments. Finally, by looking at their genetics and the historic records it would be possible to establish a "phylogeny" of the introduction of these species to their new habitats, looking at the degree of similarity between populations inhabiting similar climatic regimes.

In conclusion, many phylogenetic studies could benefit greatly if eggshell and nest phylogenies are constructed alongside the usual morphological and molecular phylogenies, as this could help place the different adaptations seen both in the eggshells and in the organisms themselves in an ecological context.

Chapter Three

The eggshell structure in *Apteryx*; form, function and adaptation

Accipe ovum et igneo percute gladio

(Take an egg and smite it with a fiery sword)

Michael Maierus, 1619.

Abstract

The eggshell structure of *Apteryx* has generated much debate over the decades because it does not fit well with most allometric predictions. *Apteryx* eggshells are unusually thin and have been reported to be 65% less porous than expected. It has been suggested that the thickness and porosity are compensations for a very long incubation period. Most studies so far have been carried out in what has been reported as *Apteryx australis* from the North Island of New Zealand, and using infertile eggs or eggs laid in captivity. However, *A. australis* comprised all birds with brown plumage, now separated into three distinct species (*A. mantelli, A. rowi* and *A. australis*) using different habitats and living at different latitudes and altitudes. In addition, captive eggs are much smaller than wild laid eggs. These confounding factors may help explain the problems found in earlier studies of eggshells. In this study, I analysed the physical characteristics of the *Apteryx* eggshells making a comparison between three different species (*A. mantelli, A. rowi* and *A. australis*) and for some of the analysis I included some specimens of *A. haastii*, to determine if there are recognizable differences between species, and to determine the actual water vapour conductance of the eggshell in the context of a burrow nester. I used scanning electron microscopy to observe and measure eggshell thickness, and mammillary area, micro computed tomography (Micro CT) to look at the pore structure, and a methodology reported by Portugal et al., (2010) to measure the water vapour conductance of eggshell fragments. I found that there are significant morphological differences between the eggshells of *Apteryx* species. I also found that the porosity of *Apteryx* eggshells was higher than allometric predictions, as opposed to previous reports, but that it was consistent with what is expected for a burrowing bird. Regarding eggshell thickness I found that indeed *Apteryx* eggshells are about 60% thinner than allometrically expected. I described several new features that have not been reported in the literature so far, such as a cuticle composed of triangular particles only described for a Cretaceous theropod. The particles in the cuticle could be related to reduce the possibility of bacterial penetration but could also be a mechanism to increase conductance as the egg develops and these particles wear off. Finally, in this chapter I discuss how other variables besides egg mass should be looked at when comparing eggshell morphological character in different species.

Introduction

The eggshell is a protective structure that has to be adapted to respond to the ecological demands of a species while reflecting the species phylogenetic relationships (Winkler, 2006). The eggshell, like any other structure, evolves from an ancestral state and suffers modification of some characters depending on ecological pressures. Indeed, amniotic eggs of different species share many characters, and the phylogenetic relations between related species can be traced up to some extent using the eggs and eggshells as another phenotypical character (Mikhailov, 1991). Avian eggshells have comparable features, such as the inner structural layers of the calcified portion of the eggshell (cuticle, crystalline, palisade and mammillae; Fig.1.1) that can be used to

point to differences and similarities between the habitats the animals exploit and the phylogenetic relations among them (Chapter 2).

Eggshells of extant animals have been rarely used to examine current taxonomic relationships, but some particular characters seem to reflect phylogenetic patterns (Winkler, 2006). Indeed, at broader time scales, eggshells have been useful to elucidate the relationships between extant and extinct species (Grellet-Tinner, 2006; Igic et al., 2010; Zelenitsky and Modesto, 2003). To my knowledge, no studies have so far been done to determine if the geographical and temporal variability of eggshell characteristics within one species are lesser or greater than the variability of those characteristics between closely related species, therefore allowing the actual recognition of species using the eggshell characters.

Eggs of different species present adaptations to their characteristic nesting environment and climate, therefore, variation in egg size, shape, eggshell thickness, pigmentation, and gas exchange properties could help in the identification of a species based on their eggs. The different layers of the avian eggshell can reveal information about the biology of the species; by themselves, both the cuticle and the mammillary layer are very informative about the ecology, life history and development of birds respectively.

The cuticle, which is the outermost layer, has been proposed to have antimicrobial properties (Board and Fuller, 1973), UV wavelength modulation properties, (Cooper et al., 2011), and water resistant properties. These characteristics suggest that the cuticle maybe under selection by the nesting environment (D'Alba et al., 2014; D'Alba et al., 2017). However, indicated that there is a strong phylogenetic influence on the composition of the cuticle thus obscuring somehow the inferences that could be made about the nesting ecology using this character. Nevertheless, the cuticle in conjunction with other characteristics should reflect the nesting ecology of species in phylogenetic context, as the nesting ecology evolves as any other trait by selection of pre-existing traits under specific ecological conditions.

Specialised cuticle traits can be found in some species; Portugal et al., (2018) showed how a coating of vaterite present in the cuticle of a communal nester, the Greater Ani (*Crotophaga major*) could act as a shock absorber protecting the egg from potential breakage. By having a coat that can be lost as the egg experiences friction, this cuticle would break instead of the eggshell itself in a busy nest.

Eggshell thickness as a functional character is associated with structural support for the egg, reduction of bacterial infection as a solid barrier (Board and Fuller, 1973) and more importantly, gas exchange as the eggshell possesses pores. The number and shape of pores are related to the incubation period (Zimmermann, Hipfner and Burger, 2007), altitude where the birds live (Rahn et al., 1977), and the nest microclimate (Birchard and Kilgore, 1980). It could be argued that a contributor to eggshell thickness is the bioavailability of calcium and the mechanisms different species use to obtain calcium around the breeding season. It has been observed in Great Tits (*Parus major*) that they travel long distances before laying eggs to obtain the necessary calcium to produce eggs, and in turn that birds that are not able to obtain enough calcium lay smaller eggs or smaller clutches (Wilkin et al., 2009).

Eggshell porosity is often defined as Ap/L, where Ap is the total pore functional area (mean area of individual pores multiplied by the total number of pores) and L is the thickness of the eggshell. These two characteristics of the eggshell define the gas conductance of the eggshell. Gas conductance is a measurement of the gas transfer through a medium (Paganelli, 1980). Gas exchange provides the embryo with the necessary oxygen for its development and allows carbon dioxide and water to escape (Mueller, Burggren and Tazawa, 2014; Maina, 2017). This poses a series of constraints for different species incubating in different environmental conditions (Carey, 1980). Birds in hot environments need to retain more water to avoid desiccation (Grant, 1982) while species in cold or wet environments need to increase the water vapour conductance to lose enough water for the embryo to develop (Deeming, 2011; Maina, 2017). Further, some authors suggest that altitude is an important factor in regulating water vapour conductance due to the differences in barometric pressure that affect the rate of gas diffusion (Rahn et al., 1977; Rahn et al., 1982).

Water vapour conductance is a function of eggshell thickness and pore diameter (Ar et al., 1974; Rahn and Paganelli, 1990), therefore it is expected that different species will present particular adaptations to regulate the water loss in different habitats and using different incubation techniques and nesting behaviours (Birchard and Kilgore, 1980; Portugal et al., 2010; Whittow et al., 1987). In support of this prediction, different eggshell characteristics have been observed in closely related species with different nesting behaviours (Birchard and Kilgore, 1980).

Water vapour conductance is particularly important for burrowing species, as these species' eggs are exposed to environments with high humidity, low concentrations of O_2, and high concentrations of CO_2. Therefore, it is expected that these species have adaptations regarding pore size and eggshell thickness to deal with these environments. In turn, burrow nesting may improve egg survivorship by providing better protection from predators or a more insulated environment for embryo development (Boccs, Kilgore and Birchard, 1984; Boggs and Kilgore, 1983; Whittow et al., 1987).

As mentioned above, calcium availability determines eggshell thickness; *Apteryx* eggshells are composed of 40% calcium, which is not different from that observed in other ratites (Tyler and Simkiss, 1958). The available calcium for chick development is concentrated in the mammillary zone, the innermost calcified layer of the eggshell. It is in this layer where, during egg production, nuclear keratan-sulphate rich crystals attach to the non-calcified membranes and start the crystal nucleation of calcium carbonate which will result in the formation of the mineral matrix of the eggshell (Fernandez, Araya and Arias, 1997). The embryo uses the mammillary tips to obtain calcium for development of brain, skeleton and muscle (Blom and Lilja, 2004). There is a difference in the way altricial and precocial birds use these mammillary tips (Rogulska, 1962; Bond et al.,1988; Starck, 1994;1996). Karlsson and Lilja (2008) have shown how the use of the calcium from the mammillary tips varies between precocial, semi precocial and altricial birds, "the faster the growth the less ossified the skeleton". This means that slow developing species (precocial) will require more access to calcium during development, which could result in bigger eggs with higher inner surface area as opposed to fast developing birds (altricial) where lesser amounts of calcium are required. Therefore, the mammillae become informative regarding the life history of species, as the eggs of precocial species need to have more available calcium, not

necessary a higher amount of calcium but calcium in a form that could be easily accessible to the chick.

The different layers of the eggshell can be informative regarding the adaptations of species to their environments. Therefore, I will apply these approaches to a species that belongs the Apterygidae family. This family is endemic to New Zealand and exhibits unique traits that seem difficult to explain in an ecological and evolutionary context. Among these traits are the egg's size, which is large in relation to bird size (Calder, 1979; Taborsky and Taborsky, 1999), comprising between 14-23% of the female's body weight (Dyke and Kaiser, 2010); the very thin egg shell compared to the size of the egg, which is 60% thinner than allometrically expected (Calder, 1979), and the use of a burrow nest (Chapter 4).

Apteryx nest in globular cavities dug in the ground or existing cavities in dead trees or tree roots (Ziesemann, 2011) lined with nesting materials (Chapter 4). This constitutes an evolutionary innovation for this clade as *Apteryx* nests are more complex in contrast to other ratite "nests". For example, Rhea (*Rhea Americana*) nests consist solely of a few twigs arranged in a circle (Barri et al., 2009), while Cassowaries (*Casuarius sp.*) (Hindwood, 1960), Ostriches (*Struthio camelus*) (Magige et al., 2009) and Emus (*Dromaius novaehollandiae*) (Patodkar et al., 2009) have nests that consist of a few branches and twigs on the surface of the soil. *Apteryx* is a unusual ratite as it has evolved to be mostly entirely nocturnal and mostly insectivorous (Cunningham and Castro, 2011; Le Duc et al., 2015).

The genus *Apteryx* contains five well defined species distributed in the three main Islands of New Zealand (Burbidge et al., 2003; Weir et al., 2016). *Apteryx* species present a very localized distribution due to the decline of their natural populations as a result of predation by introduced mammals and loss and fragmentation of habitat by deforestation (Holzapfel et al., 2018). Brown Kiwi (*Apteryx mantelli*) is widely distributed on the New Zealand's North Island and some offshore islands. Pukupuku (*A. owenii*) is currently restricted to offshore islands and one mainland sanctuary in the city of Wellington, as part of their translocation conservation program; in the past, Pukupuku was distributed along the North Island being sympatric with Brown Kiwi and the South Island probably being sympatric with several other Kiwi species. Roroa (*A. haastii*) is distributed in the northern part of the South Island and is a montane species. Rowi (*A. rowi*) is distributed only in the Okarito Forest in the middle of the west coast of the South Island, but in the past, it was distributed along the northwest part of the South Island and the Southeast part of the North Island. Finally, Tokoeka (*A. australis*) is distributed in Haast, Fiordland and on Rakiura Island but their range used to overlap with that of the Rowi, extending towards the north of the South Island (Weir et al., 2016).

Kiwi species are distributed over 1600 kms, the narrow and long shape of New Zealand and therefore, the climate encountered by *Apteryx* species varies from sub-tropical in the north to sub-Antarctic in the south (Fig.3.1) Therefore, adaptive variation is expected between the different species in terms of the nesting behaviour, egg size and possibly incubation period; however, some traits are expected to remain the same between the species reflecting their phylogenetic relationships.
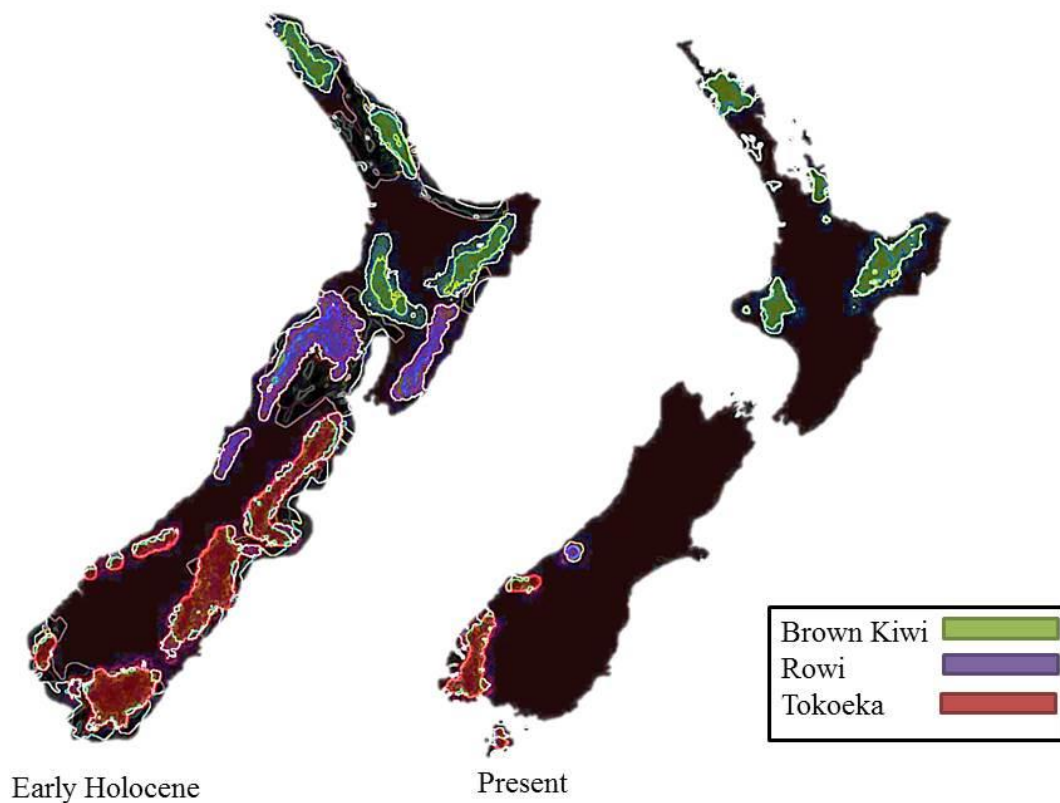
Figure 3.1. Map of ancient (left) and present (right) distribution of the three main species studied in this chapter. The ancient map was adapted from Weir et al.,2016 showing the land bridges during the Holocene, the left map (present) is adapted from Colbourne 2002.

Each *Apteryx* species presents a different incubating strategy and possibly mating system. The reason for this variability has not been explained yet. The incubating strategy of Brown Kiwi and Pukupuku is especially perplexing as the incubating father needs to abandon the egg for long periods of time during the night to forage; this puts the egg at risk of overcooling.

In other bird species, long incubation periods seem to be an adaptation to resist over-cooling and egg neglect (Boersma, 1982; Boersma and Wheelwright, 1979). However, the other *Apteryx* species show an equally long incubation period while having female egg attendance or possible helpers (Colbourne, 2002). It has been observed that species with long incubation periods have a reduced eggshell porosity (Zimmermann et al., 2007) likely to reduce the daily water loss and thus avoiding embryo desiccation.

The development of a nest inside a burrow requires further modification of the egg functional characteristics. For example, increased water vapour conductance, a resistant shell to endure the incubating adult's weight (and helpers in the case of Tokoeka), and a powerful line of defence against micro-organisms. This last modification is required because kiwi eggs are incubated for

about two and a half months in a humid, organic matter rich environment that is warmed periodically, which makes it ideal for the growth of micro-organisms (Hiscox, 2014). A reduced porosity would be beneficial for *Apteryx* as it would reduce the risk of bacterial penetration however, in a damped burrow environment not losing enough water could equally compromise the embryo; hence, an increased water vapour conductance (WVC) could be expected. Differences in WVC can be achieved by means of increasing pore area or number, or by decreasing eggshell thickness. There are other adaptations that could compensate for the need of increased gas exchange concurrently with a need to prevent microbial contamination, and a reduction in pore size through mechanical means such as cuticular particles or opercula partially or fully plugging the pores (Board and Perrott, 1979).

Some hypotheses about the function of the characteristics of the Apterygian egg and the eggshell to respond to certain ecological demands have been proposed. Reid (1971) proposed that the large *Apteryx* egg is a response to low temperatures during incubation that would select for an egg with higher volume-area ratio. Calder (1979) suggested that the increased amount of ovoinhibitors and lysozymes in the albumen of *Apteryx* eggs arose to reduce the risk of microbial infection during the very long incubation period. Maloney (2008) and Prinzinger and Dietz (2002) suggested that the slow metabolic and developmental rate allows the egg to withstand long periods of abandonment. However, interspecific variation in eggshell thickness and water vapour conductance are still not fully understood in *Apteryx*. *Apteryx* has been included in some studies on water vapour conductance variability in different species as an example of extreme adaptations (Calder, 1979; Tullett, 1984). However, this data has mostly been based on morphometric equations rather than direct measurements or using very few eggs from what was considered *Apteryx australis*, a species now known to comprise three species and up to nine different taxa (Weir et al., 2016). Especially outside New Zealand, samples are usually obtained from captive bred birds, which are known to produce eggs much smaller than those laid in the wild (Jensen and Durrant, 2006). Some authors have tried to address these questions but unfortunately with very few eggs and eggshells (Silyn-Roberts, 1983), leaving this matter to be researched in more depth.

The presence of the unusual traits in *Apteryx* has made this genus a zoological curiosity and efforts have been made to try to understand these species in their evolutionary and ecological context. The selective pressures that shaped this species are still matter of speculation. New Zealand ecosystems suffered drastic changes in the past 800 years, resulting in loss of the context of *Apteryx*, for example, its potential predators and competitors, the components of its diet, and the climate during its evolution.

It is the aim of this chapter to explore: 1. the physical characteristics of the eggshell in terms of the water vapour conductance of a burrowing precocial species; 2. the adaptations in the cuticle to incubation in the nest environment, and 3. To corroborate the assumption of calcium intake in precocial birds proposed by Karlsson and Lilja, (2008).

The framework of operation nest egg (See Chapter One: General Introduction, page 6) is an exceptional opportunity to study the functional characteristics of the Kiwi eggshell. These eggs are wild laid and have developed mostly in their natural environment; and for the first time we

can study kiwi eggshells using sample sizes bigger than previously. I achieve the aims of this chapter by first studying if there are any observable differences in the structure of the eggshell of different species of *Apteryx,* secondly testing the assumptions made regarding the water vapour conductance of these species in the context of their burrow nesting, and finally contrasting this information with their life history traits.

Materials and methods

Hypotheses

The different *Apteryx* species have different distributions, from sub-tropical to sub-Antarctic climates, they also present different incubating arrangements, ranging from a single parent in Brown Kiwi to group incubation in Tokoeka; all species nest in burrows. I therefore hypothesise that there are differences in the eggshell structure of the different species in terms of thickness of the different constituent layers.

Based on their distribution it is expected that the different species have differences in their water vapour conductance. This should be observable as differences in pore density and functional area and/or eggshell thickness. Thickness is expected to be less in species with higher altitude distributions, it would also be expected that thickness scales with body mass. Porosity is expected to vary according to climate, with higher porosities in colder climates to account for a reduced water loss due to lower overall temperatures. I also expect to find eggshell adaptations to burrow nesting that are comparable to those of other burrow nesting birds, such as modified pores and modified cuticle or accessory layer. In this context I will compare the water vapour conductance of the *Apteryx* species with other burrowing species reported in the literature.

It is expected that *Apteryx* present high calcium intake from the mammillae as it is a precocial species, this being observed by strong reduction or erosion of the mammillae during incubation as the embryo develops.

Sample sourcing

Eggshell measurements were made on samples obtained from Operation Nest Egg (ONE). ONE is a program for the captive rearing of wild *Apteryx* eggs. It was developed in 1994 and its purpose is to assist in increasing wild population numbers by hatching wild-laid eggs in captivity and rearing the chicks until they gain enough weight to survive predation. Then they are released into the wild (Colbourne et al., 2005).

The eggs in this programme are generally collected after day 20 of incubation to increase the hatching success through artificial incubation. The eggs used in this experiment were candled and determined infertile or early embryo death if there was no gross visual evidence of a developing embryo.

Eggshells from infertile/early embryo death eggs and hatched eggs were used as conservation policies of these endangered species are very strict and would not allow risking or ending the life of any bird, and we acted according to the permits obtained from the Department of Conservation of New Zealand (Authorisation number: 56537-DOA). Eggshells from 30 Brown Kiwi (*A. mantelli*), four Roroa (*A. haastii*), 25 Rowi (*A. rowi*), and 20 Tokoeka (*A. australis*)

from the 2013-2017 breeding seasons were used (n=79) (Table.3.1). Three additional Brown Kiwi eggshells from hatched eggs were used to compare the calcium depletion in the mammillae of the hatched eggs with the intact mammillae of infertile eggs.

The eggshells were obtained from seven different locations in the two islands of New Zealand (coordinates in Table.3.1, Fig.3.2). All of the eggshells used for thickness and porosity measurements came from eggs that hatched to ensure that the ratio of calcium intake from the mammillae during ontogenesis (Deeming, 2002a) was approximately the same between all the samples and did not interfere with thickness measurements. Three eggshells of infertile eggs were used to determine the calcium intake during development comparing them with eggshells from hatched eggs from the same parents (collected in the same nest). The eggshells were relatively intact except for the section broken by the chick during hatching. Different comparisons use different number of eggshells depending on the initial state of the eggshells and the purpose of the comparison. For example, for measurements of different eggshell regions, I only used eggshells from eggs that presented the three regions intact. Rahn and Ar (1980) suggested that pore distribution varies according to the latitude of the egg; therefore, sub-samples were taken manually from the equatorial region of each eggshell (Fig.3.3) to ensure accurate comparisons. This area of the egg was chosen for two reasons; because of hatching, the blunt end of most egg samples was destroyed, this being the usual exit point for the chick when hatching. In addition, for some of the samples the eggshells were somewhat crushed, and the equatorial region was the only identifiable part of the eggshell. For the study of the water vapour conductance I used fragments from the three regions that were intact enough and were identifiable (Fig.3.3).

Table 3.1 **Sites of origin of the eggshell samples, indicating co-ordinates and their respective species. The temperature data is an average of the past 10 years, and was obtained from world weather online (www.worldweatheronline.com/lang/en-nz). L= Location (O=Ohope, M=Maungataniwha, T=Tongariro, C=Coromandel, P=Paparoa National Park, Ok=Okarito Forest and H=Haast sanctuary). DAI=Number of days in artificial incubation, EA=Estimated age at the start of incubation. *Average value for all Brown Kiwi samples.** *Masl: meters above sea level.*

Sp.	L	Co-ordinates		No.	DAI	EA	Elevation	Mean
		Latitude	Longitude	Eggs	(Mean±SD)	(Mean±SD)	(masl)	T (C°)
A. mantelli	O	-37.980	177.028	3	26 ±12*	48±12*	0	14.5-26
	M	-38.809	176.783	7			744-859	12-20
	T	-39.159	175.550	12			610-1500	0-25
	C	-37.054	175.666	8			0-539	12-31
A. haastii	P	-42.021	171.360	4	Not known	Not known	151-1200	5-17
A. rowi	Ok	-43.273	170.176	25	39±12	35±12	0-158	-6-17
A. australis	H	-44.025	168.214	20	36±17	38±17	650-1587	-1-23
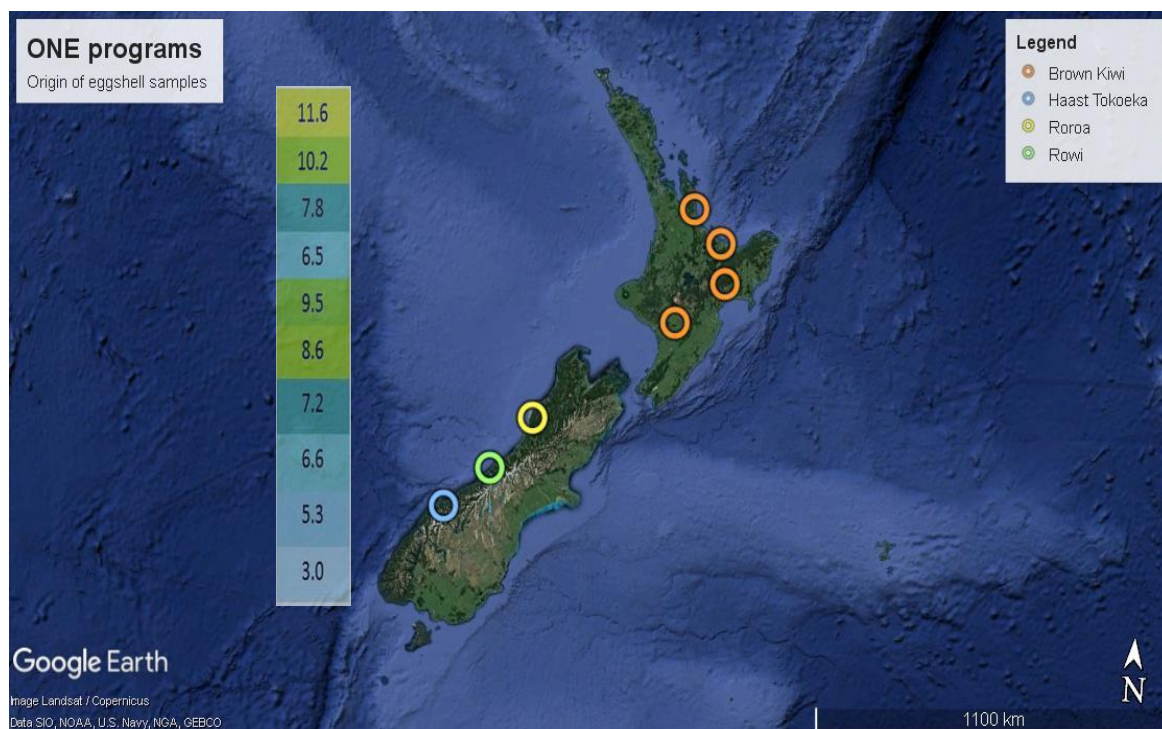
Figure 3.2. Map of New Zealand indicating the areas of origin of the samples used. To the left a bar showing the average temperatures during the winter months north to south; note how the temperatures are warmer up north and decrease towards the south but there is an increase in temperature in the centre of the North Island. The temperature information was obtained from NIWA.
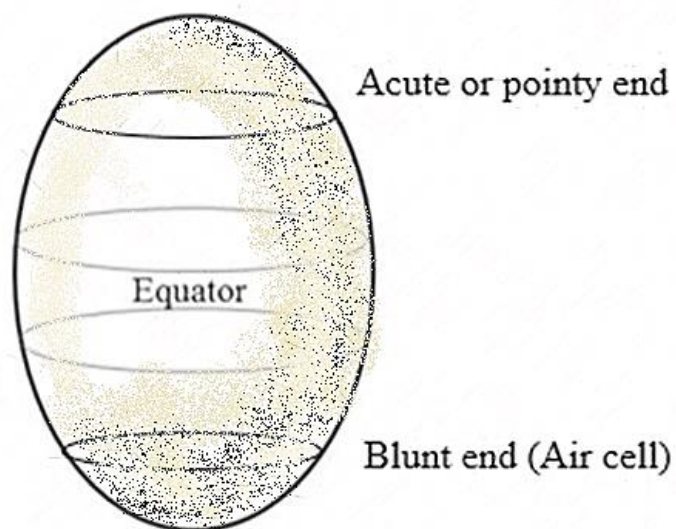
Figure 3.3 . Diagram of an egg showing the different regions of the egg where samples were taken. Drawing by the author.

Optical techniques

To determine the variation in porosity and thickness between species and the thickness of each structural layer, I used eggshell fragments from the equatorial region of eggs from each species. The fragments were manually broken and washed in deionized water to remove remaining dirt and egg fluids and allowed to air dry.

Micro-computed tomography Scan (MicroCT)

MicroCT (Zeiss) was used to determine the functional area of pores and their abundance. Twelve eggshell fragments (three eggs from each species) were used. The fragments were manually broken into three smaller fragments each, and a full scan of each fragment was used to count the number of pores and their area. Three measurements of the pore diameter were made around the funnel shaped external area, the middle of the pore, and the lower section of the pore. The eggshell fragments were scanned by Michael Loh at Fonterra imaging Lab. Images were obtained at 45Kj, 133 μA, 6 W; Source distance 20 mm, Detector at 8 mm, pixel size of 1.9266, Mag 10X, exposure time 11 seconds, and 1000 projection 180˚. The images were analysed using Xradia mxct software. Images from the cuticle of all individuals were taken to see its physical features.

The number of pores of each fragment was counted and their individual area measured in cm by averaging the three pore diameters, using the formula:

$$Pore\ area\ (cm^2) = \frac{\pi * r (\mu m)^2}{10,000^2} \qquad \text{Eq. (3.1)}$$

Where r is the average radius of each pore and $10,000^2$ is the conversion factor into centimetres.

The estimated total number of pores across the eggshell was calculated by counting the number of pores in each fragment and dividing the number of pores by the fragment area (pore density) then multiplying that number for the surface area of the eggshell.

The surface area of the eggshell was calculated using the formula recommended by Smart (1991):

$$Calculated\ Surface\ area\ (cm^2) = (4.393 + \frac{0.3941}{b})(0.51 lb^2) \qquad \text{Eq. (3.2)}$$

Where *b* is the maximum egg breadth (or the wider region of the egg) in cm and *l* is the egg length in cm.

Eggshell porosity was calculated following Ar et al., (1974) as:

$$Porosity = \frac{Ap}{L} \qquad \text{Eq. (3.3)}$$

Where *Ap* is the total functional pore area (mean pore area (cm^2) multiplied by the total number of estimated pores) and *L* is the eggshell thickness in cm.

I calculated the expected porosity based on the morphometric equations also provided by Ar et al., (1974) based on the egg weight (*W*):

$$\frac{Ap}{L} = 0.018\,W^{0.780} \qquad \text{Eq. (3.4)}$$

Scanning electron microscopy (SEM)

SEM (FEI Quanta 200 ESEM, Eindhoven, Netherlands) was used to observe the thickness of the eggshell, the thickness of each constituent layer, external features and mammillary area and density. The samples were rinsed with reverse osmosis water to further remove any particles and allowed to air dry; samples were then mounted on aluminium stubs and gold spluttered with approximately 100 nm of gold in a vacuum (Baltec SCD 050 sputter coater). The images were taken by the Manawatu Microscopy and Imaging Centre (MMIC), using an accelerating voltage of 20kV and a spot size of 3 to 4. For this technique 17 eggshells of Brown Kiwi, 21 of Rowi and 15 of Haast Tokoeka were used. Each eggshell fragment was further broken in two smaller fragments. The first eggshell fragment was used to produce images of the external features, pores and cuticle and then it was tilted at 90 degrees to measure the internal layers. The second eggshell fragment was coated with silver paint over the aluminium stub to increase conductivity and it was mounted exposing the internal side of the eggshell. This sample was used to measure the area of the mammillary tips and their density.

Images of the cuticle were resolved at 20 μm resolution, cross sectional images and internal side images were produced at 300 μm. The measurements were analysed using ImageJ free software (Rueden et al., 2017).

The average pore radius was measured using SEM images of the external layer. Only images that showed opened pores in which the diameter could be measured were used (N=87), these images belonged to 28 individual eggs. In this case, I used ImageJ to take three measurements of diameter per pore. These measurements were then divided by two to estimate the radius and the radii were averaged per individual pore. A species average was used for further calculations.

Dissecting microscope

Porosity

The pore density and porosity (Ap/L) of the three species eggshells was observed in a dissecting microscope (Olympus SZX12) to compare the measurements obtained with the micro CT and the predicted values by allometric equations.

Three eggshell fragments from the equatorial region of Brown Kiwi, Rowi, and Haast Tokoeka were washed in deionized water, let air dry and stained with an alcohol-based solution of Malachite green (1%). The eggshells were placed on paper towels and two drops of the dye was applied to the inner eggshell surface. The eggshells were let to set and to allow the dye to penetrate the pores (Fig.3.4).
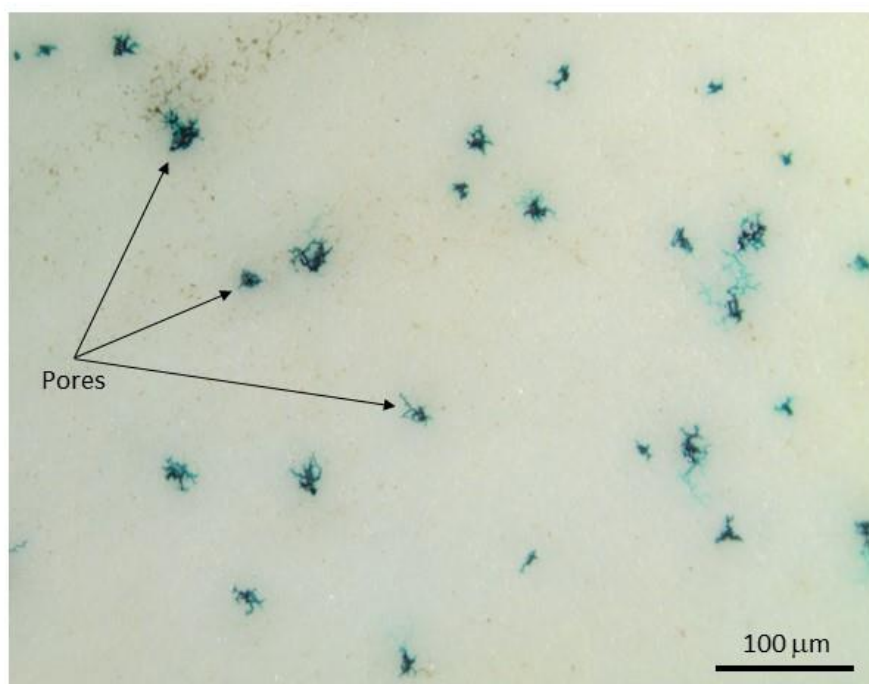
Figure 3.4. External (Cuticle) view of a Brown Kiwi eggshell obtained with a dissecting microscope. The malachite green has penetrated through the pores making them visible.

The eggshells were observed under stereo microscope (Olympus SZX12) and photographed using an attached camera (Olympus S30) and then the pores were individually counted using ImageJ. The eggshells from the same individuals were later used in the Water Vapour Conductance experiments.

Information collected by the ONE (Rainbow Springs, Kiwi Encounter, Rotorua) program about the size of the egg (breadth and width) was used to calculate the surface area of the egg. The conductance could be correlated to eggshell thickness and different porosities could be calculated and compared using equations 3 and 4. In this way, it was possible to determine the difference between the morphometric equations and an approximation to the experimental approach to water vapour conductance (below).

Mammillary erosion and calcium intake
The mammillary erosion was studied by comparing eggshells of infertile eggs with those of hatched chicks. Eggs coming from the same nests were selected to control for intra-specific variability. The eggs were also selected based on their location of origin to control for habitat variability (the eggs in this case came from the Project Kiwi Trust, located in the Coromandel peninsula). This comparison was done to assess the amount of calcium intake during embryo development.

Eggshell fragments from the egg equator were boiled in 5% NaOH solution for several minutes to detach the inner membranes and any remaining debris. The fragments were then rinsed in deionized water and dried at 70°C for a period of 12 h (Silyn-Roberts, 1983) and then dyed with

malachite green, as this pigment binds well with the calcium carbonate but it is repelled by the cuticle. Finally, the fragments were observed under a dissecting microscope Olympus SZX12. Photographs with scale bars where taken with a camera (Olympus S30) attached to the stereomicroscope to compare the hatched and infertile eggshells. The total area covered by mammillary tips was estimated using ImageJ.

Water vapour conductance

I used an alternative method for the estimation of the water vapour conductance by using the eggshells following the Portugal, Maurer, and Cassey (2010) methodology, instead of using a whole infertile egg (Rahn, 1985). I glued an eggshell fragment (inside down) to a PCR tube (SSI, 0.5 ml, Cat. No. 1110-02) filled with 200 µL of distilled water (Fig.3.5). The tubes were placed in PCR trays for easy handling and the trays placed in a desiccator containing 550 g of colour indicating silica gel and the desiccator in a controlled temperature room at 25°C. Fragments from the blunt end (B), the acute end (A) and the equator (E) were taken from each eggshell. Water loss was measured every 24 hours for a period of three days. Eggshells from commercially produced chicken eggs where included in this experiment as control and only equatorial fragments were used, first, because no significant difference were observed between eggshell regions (Conrado et al., 2017; Steven J Portugal et al., 2010). Secondly, the purpose of using chicken eggshells was solely to determine if the values obtained by my experiment were congruent with those reported in the literature.

I determined the daily water loss (ΔM_{H_2O}) by weight loss and calculated the water vapor conductance as:

$$\Delta G_{H_2O} = \Delta M_{H_2O}/\Delta P_{H_2O} \qquad\qquad \text{Eq. (3.5)}$$

Where ΔG_{H_2O} is the water vapor conductance, and ΔP_{H_2O} is the pressure difference at standard conditions (1 atmosphere and 25°C). The air cell pressure and nest environment pressure difference has been calculated for most avian species, including burrow nesters (23.77 mg.d^{-1}.torr^{-1}). Therefore, I used this value as the ΔP_{H_2O}. The water vapour conductance values obtained in this experiment are independent of surface area (Portugal et al., 2010), therefore by multiplying these values by the egg surface area (calculated as indicated above) it is possible to estimate the total water vapour conductance. ,
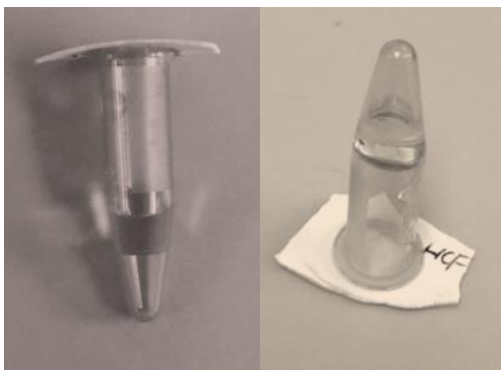
Figure 3.5. Eggshells glued to Eppendorf tubes filled with 200 µl of distilled water.

Finally using the data obtained in the SEM and MicroCT scan procedures I included the thickness and functional pore area in the model following Ar et al., (1974) as follows:

$$\Delta G_{H_2O} = 23.42 \; Ap/L \qquad\qquad \text{Eq. (3.6)}$$

This was done to compare water vapour conductance values predicted by Calder (1978,1979) and Tullett (1984) with those obtained in my experiment. A paired t test was performed to compare the theoretical values with those obtained in our experiment.

I calculated the porosity for all of these specimens based on Ar et al., (1974) formula using the mass of the egg as follows:

$$Ap = 9.2 * 10^{-5} * W^{1.236} \qquad\qquad \text{Eq. (3.7)}$$

Where W is the fresh weight of the egg, in this case I used the arrival weight of the eggs). The porosity data was not normally distributed therefore a Mann-Whitney test was performed to compare the experimental values (measured) with the predicted values.

Treatment comparison

Before the experiment, I compared two types of glue, PolyVinyl Acetate (PVA) and Cyanoacrylate (SG) to test which would allow for better sealing around the rim of the PCR tube. Both glues were tested under the same experimental conditions (T=25˚, RH=0%). Water conductance data was not normally distributed (Anderson Darling test =3.475, p=0.005) therefore a Mann Whitney was used to test if there were differences between the two glues.

Water vapour conductance of eggs during incubation

ONE incubates eggs at a constant temperature of 35.5°C and maintains humidity constant at about 70% (approximately). As part of their protocol eggs are weighted upon arrival and before hatching, to account for excessive water loss. Using this information, I calculated the water vapour conductance of the eggs under these circumstances, with the intention of obtaining a value of water vapour conductance of the whole egg in addition for the water vapour conductance of individual eggshell fragments. I used the relative humidity equation to correct for these conditions and calculate the partial pressure in the artificial incubator, thus calculating the pressure difference across the eggshells (considering that the pressure difference is 23.77 Torr in 0% humidity).

$$p_{H_2O} = \frac{RH * p^*_{H_2O}}{100\%} \qquad\qquad \text{Eq. (3.8)}$$

Here, p_{H_2O} is the partial pressure of water vapour in the incubator, RH is the relative humidity (70%), and $p^*_{H_2O}$ is the total pressure of water vapour at 35.5. Once the water vapour

conductance was calculated for these conditions, the water loss was calculated for the standard conditions (25°C and 0% humidity) using the formula developed by Ar et al., (1974):

$$\dot{M}_{H_2O} = c * D_{H_2O} * \frac{Ap}{L} * \Delta P_{H_2O}$$ Eq. (3.9)

Where $\dot{M}_{H_2O}$ is the daily water loss, c is a conversion constant ($155.52*10^7$/R*T, where R is the ideal gas constant:$6.24*10^4$cm^3*Torr/mol*°K, and T is absolute temperature in °K), D_{H_2O} is the diffusion coefficient of water in air at a given temperature (in °C), $\frac{Ap}{L}$ is porosity, and ΔP_{H_2O} the difference in pressure across the eggshell. From this using equation 3.5 water vapour conductance of whole eggs at 25 and 0% humidity was calculated and compared with the results reported by Calder (1978a) and Sylin-Roberts (1983) for Brown Kiwi, and with the values reported by Ar et al., (1974) for other avian species.

Statistical analysis

A discriminant analysis was used to determine the degree of association of each sample with the donor species to determine the degree of differentiation between the four species. A linear regression was performed to determine the relation between eggshell thickness and egg mass during incubation, prior to hatching, and with chick weight (Birchard and Deeming, 2009). This was done to determine if it was possible to identify species using the eggshell alone, and to see how the eggshell characteristics could correlate with environmental variables.

A one-way ANOVA was performed using the samples from all species to determine if there was a significant difference in terms of eggshell thickness and the proportional thickness of the constituent layers; Roroa was not included in this analysis, as the sample size of eggs was too small.

A paired t-test was conducted to assess the degree of mammillary erosion in the hatched eggs using values from unfertilised eggs and eggs that hatched.

An ANOVA was used to test if there were differences in water vapour conductance between the species and a nested linear model was used to test the different eggshell regions. In this case the fragments (E, A and B) were nested within species. All the ANOVA's performed in this chapter were followed by a Tukey *post hoc* test to assert which species differ from each other. A repeated measures ANOVA was used to determine the difference in water vapour conductance of three different regions of the eggshell of the three species of *Apteryx,* and to determine the extent of the interaction of the species and the region of the eggshell. All the statistical analysis in this chapter were made using MiniTab^{TM} 18 Statistical Software.

A paired T-test was performed to compare the water vapour conductance of the whole eggs being incubated by ONE and the water vapour conductance calculated from Eq. 3.6. using the mass of the egg.

Results

The different layers of the Apteryx eggshell

Apteryx eggshells showed a clear demarcation between the four constituent layers (Fig.3.6). With exception of the mammillary layer (ANOVA, F=1.85, *p*=0.150; n=59), the constituent layers of the eggshell showed variation between species. The proportion of cuticle from the total eggshell thickness (ANOVA, F=8.85, *p*<0.001; n=59) and the proportional thickness of the crystalline and palisade layers (F=6.76, p = 0.001; n=59) varied significantly between the four *Apteryx* species.

Interspecific comparison

Discriminant analysis

Using simultaneously the thickness of the eggshell, the mammillary density and area, and the thickness of the different constituent layers it was possible to associate a particular eggshell to its right species in 78 % of the cases (Table .3.2).

Table 3.2 Discriminant analysis showing the number of individuals that were correctly assigned to their species using eggshell thickness, mammillary area and density, and thickness of the constituent layers.

Put into group	True Group				
	Brown Kiwi	*Roroa*	*Rowi*	*Tokoeka*	**Total**
Brown Kiwi	13	0	4	1	**13**
Roroa	1	3	0	2	**3**
Rowi	3	0	15	0	**15**
Tokoeka	0	1	0	12	**12**
Total N	**17**	**4**	**19**	**15**	**55**
Percentage Correct	**76.5**	**75.0**	**78.9**	**80.0**	**78.2**

Eggshell thickness

Eggshell thickness varied with species and seemed to follow a latitudinal pattern with thinner eggshells in the northernmost species and thicker eggshells in the southernmost species (Table 3.3). Roroa was not included in the ANOVA because of the small sample size, but it roughly followed the same trend as the other species.

The cuticle (or external layer)

All *Apteryx* spp. presented very thin cuticles, barely discernible in a cross section of the eggshell. However, its roughed texture in contrast to the smooth laminar texture of the crystalline layer made it easy to identify.
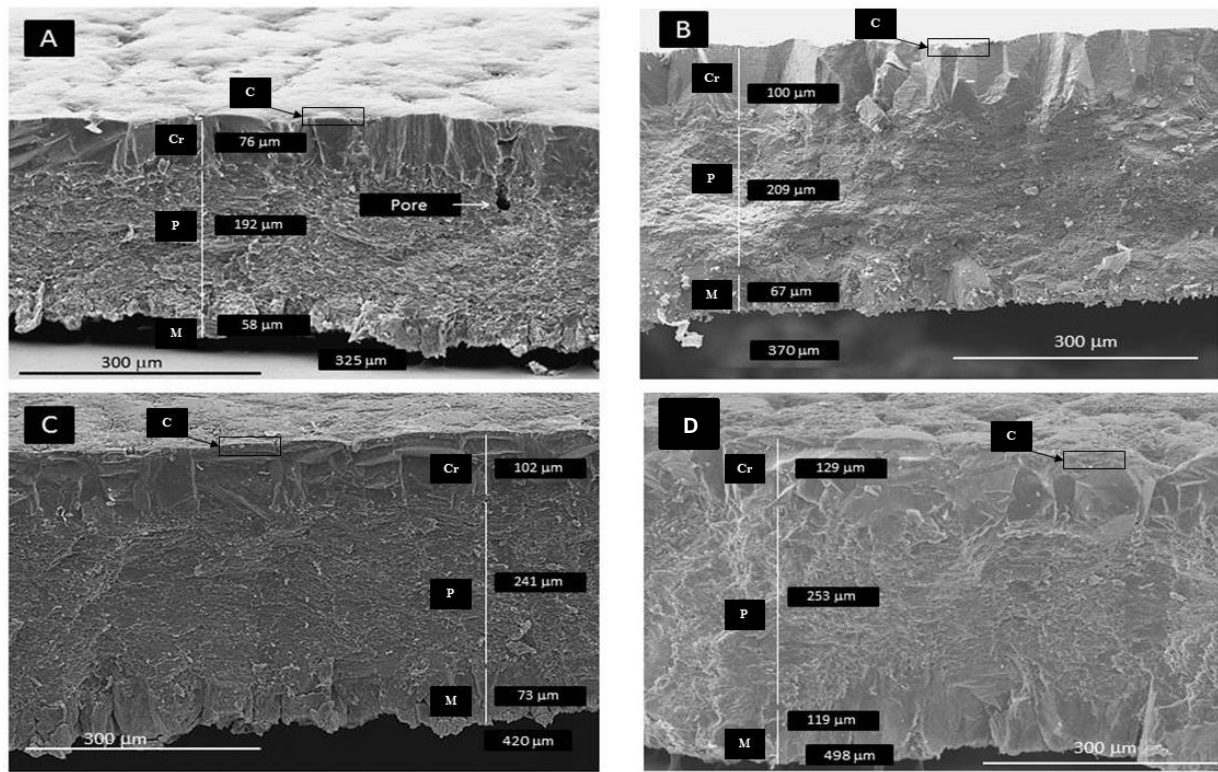
Figure 3.6. Cross section of A) Brown Kiwi B) Roroa, C) Rowi and D) Haast Tokoeka eggshells obtained using SEM. Here the constituent layers are indicated with the vertical bar and their measurements. C=cuticle, Cr=crystalline, P=Palisade, and M=mammillae. Total thickness is indicated at the bottom of each picture.

Table 3.3. Summary of the eggshell thickness and eggshell cuticle thickness obtained by SEM imaging of the eggshell cross-section (N=61), the superscript next to the species name represent the Tukey post hoc test indicating which are the significantly different species.

Species	N	Total Thickness (μm)	SE mean	Cuticle Thickness (μm)	SE mean
Brown Kiwi[a]	23	303.25	8.79	3.996	1.26
Rowi[b]	21	367.0	12.3	3.009	0.86
Tokoeka[b]	16	386.25	11.2	3.045	1.09
		F value	*p value*	**F value**	*p value*
		16.54	<0.001	5.63	0.006

The cuticle thickness decreased significantly with latitude, being thicker in Brown Kiwi and thinnest in Rowi and Tokoeka, the cuticle was significantly different between Brown Kiwi and Rowi (Table.3.3). In all species, to the naked eye and under SEM, the cuticle had a waxy appearance. Under the micro CT scan, the crystalline structure of the cuticle became apparent, and it was possible to discern the individual crystals that formed it (Fig.3.7).
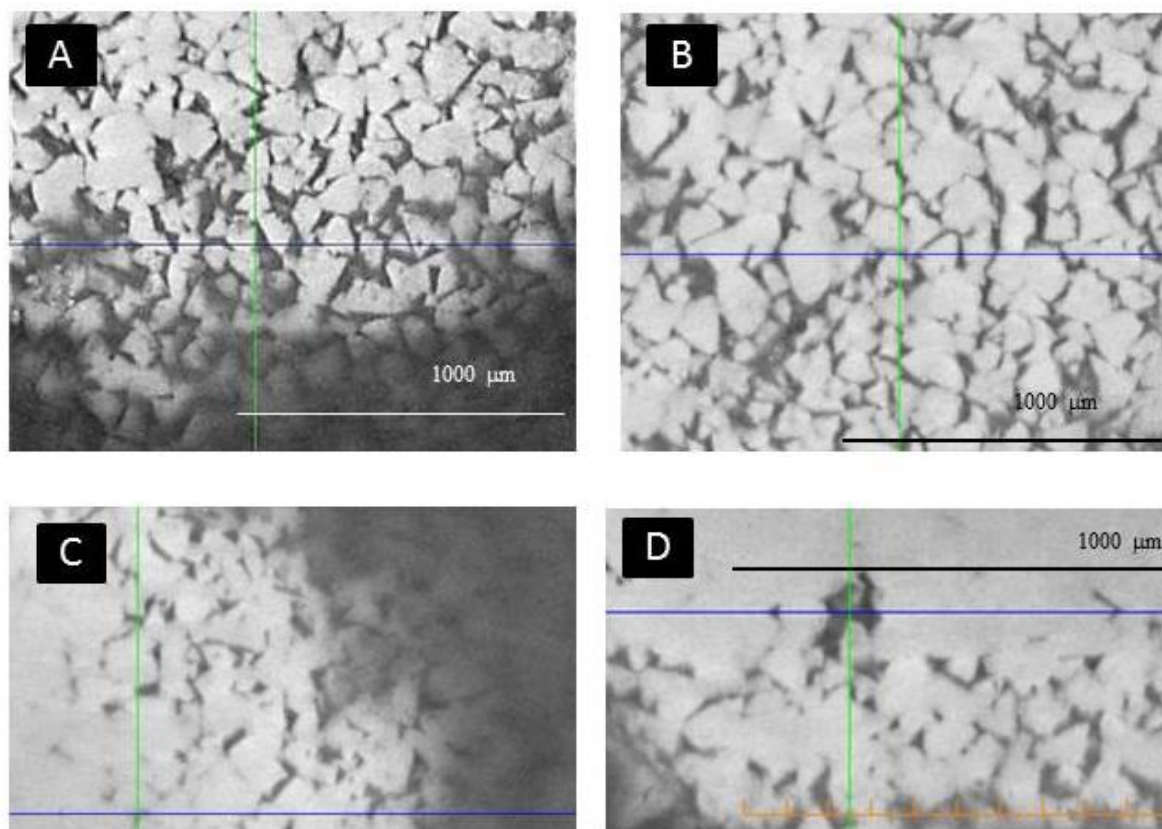
Figure 3.7. Comparison of the view of the Cuticle of A) Brown Kiwi, B) Roroa, C) Rowi, D) Haast Tokoeka. Using Micro CT it is possible to see the individual triangular crystals that form the cuticle and that are present in all Apteryx species

The Pores

Some pores were hard to identify in the external images of the eggshells because a "cap" made from the same mineral as the cuticle (Fig. 3.8) covered them. *Apteryx* eggshells had cylindrical (or funnel shaped) pores that usually crossed the thickness of the eggshell slightly on an angle. In each fragment, very few pores reached the cuticle surface of the eggshell, with most reaching only half or less of the total thickness of the eggshell and many smaller pores that barely went beyond the mammillary layer (Occluded pores, Fig. 3.8C).

The observations made using the micro CT were confirmed by Scanning Electron Microscopy images of the exterior of the eggshells, where in some cases caps are seen covering the pores and in others the mineral occlusions are observable (Fig.3.8). These caps and occlusions were observed in all four species and in all individuals.

Accordingly, fewer pores were visible at the cuticle side of the egg and many more from the inside. Since the CT is an X-ray technique, it is possible to see at different "depths" through the eggshell. When observed near the cuticle few pores were discernible but when looking at midway between the cuticle and the mammillae more pores become visible, confirming what was been seen in the cross sections (Fig.3.9).

36
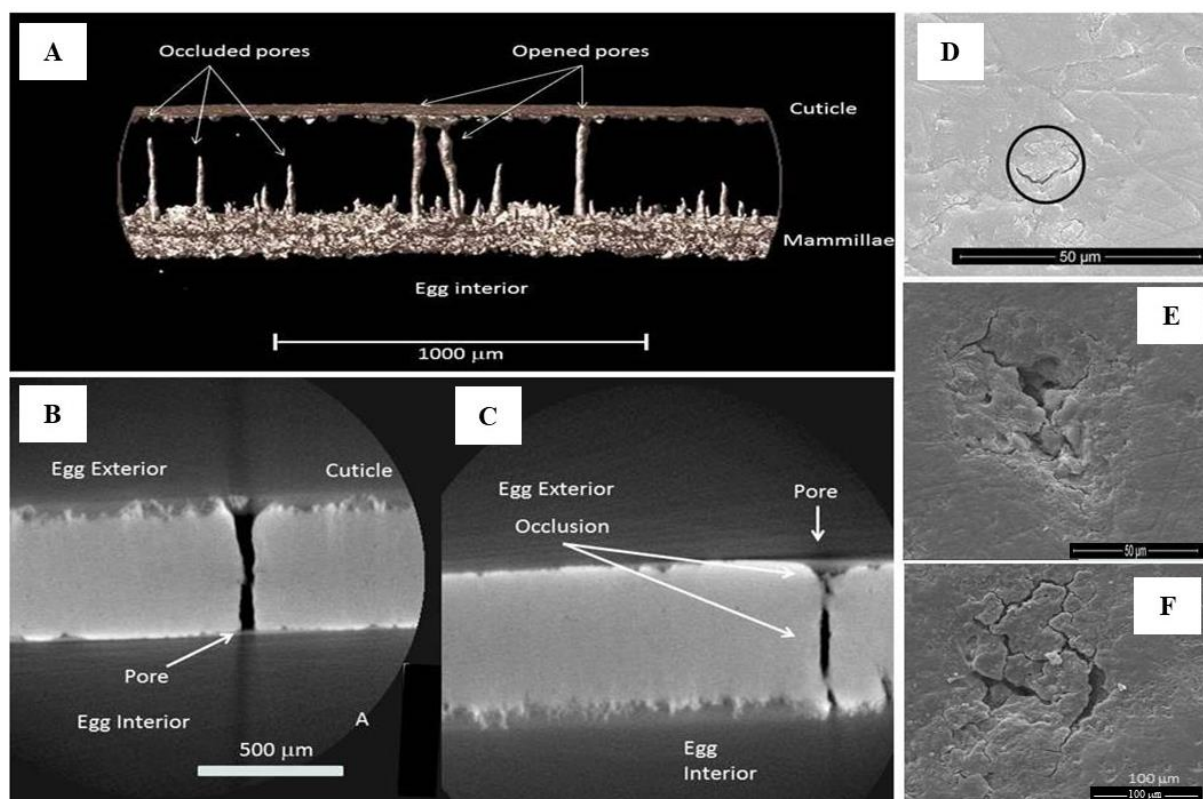
Figure 3.8. Pores of Apteryx. A) transversal view of a Brown Kiwi eggshell, the spaces have been inverted and the pores are shown as solid columns. B) Brown Kiwi eggshell showing an open pore, C) The same eggshell showing another pore, in this case occluded with mineral material. External views of capped pores on D) Brown Kiwi E) Rowi and F) Tokoeka. Pictures A to C were obtained using micro CT, pictures D to F were obtained using scanning electron microscopy.

Porosity

Pore size measured as pore radius was different between the southern species and Brown Kiwi, the latter having smaller pores; pore density in contrast was higher in Brown Kiwi and lowest in Tokoeka, the functional pore area (which considers the size of the egg) was significantly different between Brown Kiwi and the Southern species (Table.3.4).

Table 3.4. Summary of the pore density, average pore radius and porosity for three species of *Apteryx*, Ap (functional pore area) was calculated as the total area of pores (pore density*Egg Surface area*average pore area). Porosity was calculated from the later (Ap). Superscripts indicate the statistical differences found using Tukey's test.

Species	Pore radius (μm)		N	Pore density (Pores/cm^2)		Ap (cm^2)		Porosity (Ap/L)	
	Mean	**SE**		**Mean**	**SE**	**Mean**	**SE**	**Mean**	**SE**
Kiwi	21.8[b]	1.92	13	51.3[a]	3.40	0.20[b]	0.013	6.87[b]	0.49
Rowi	33.4[a]	1.78	12	45.7[ab]	3.63	0.40[a]	0.034	11.02[a]	1.21
Tokoeka	32.2[a]	2.33	10	36.1[b]	2.40	0.32[a]	0.023	8.67[ab]	0.78
F value	11.47			5.19		18.25		6.24	
P value	<0.001			0.011		<0.001		0.005	
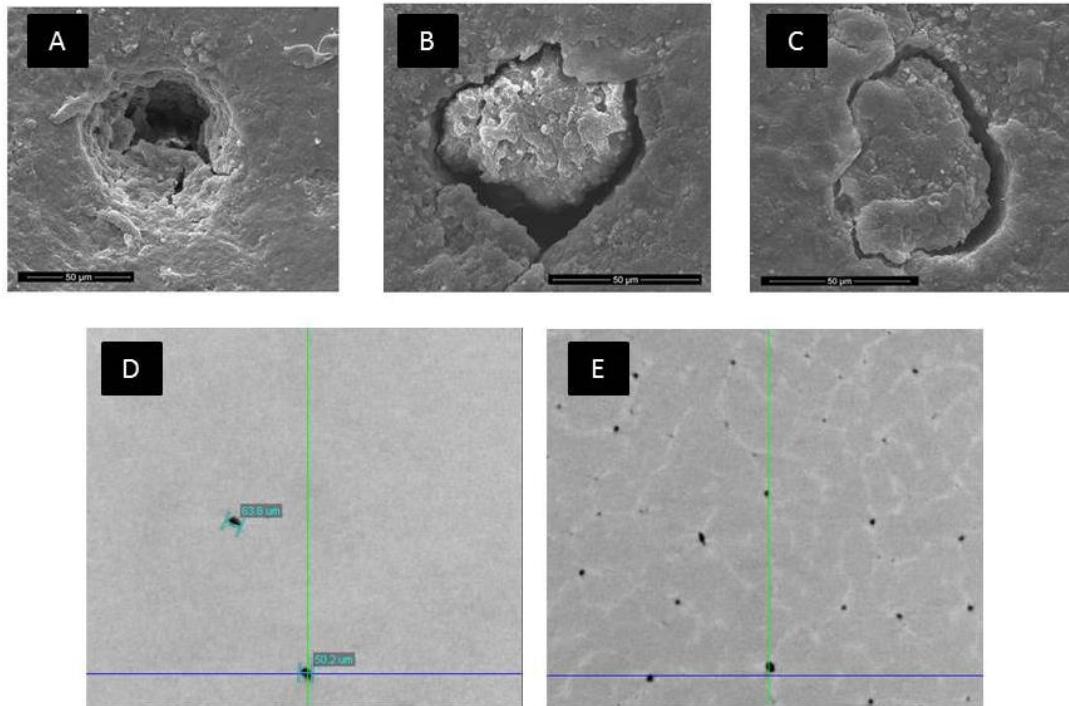
Figure 3.9 Closer look at the plugs inside the Apteryx eggshell. A) an open pore B) and C) different types of plugs. D) View from the exterior towards the inside of two pores near the surface of a Brown Kiwi eggshell E) same fragment, this time half way through the eggshell showing more pores and the outline of the calcium units as white lines.

There was a significant difference between measured and predicted porosity (Mann-Whitney, W=1682.0, $p<0.001$, N=35). The measured porosity was higher than the predicted porosity (Fig.3.10) and more variable.
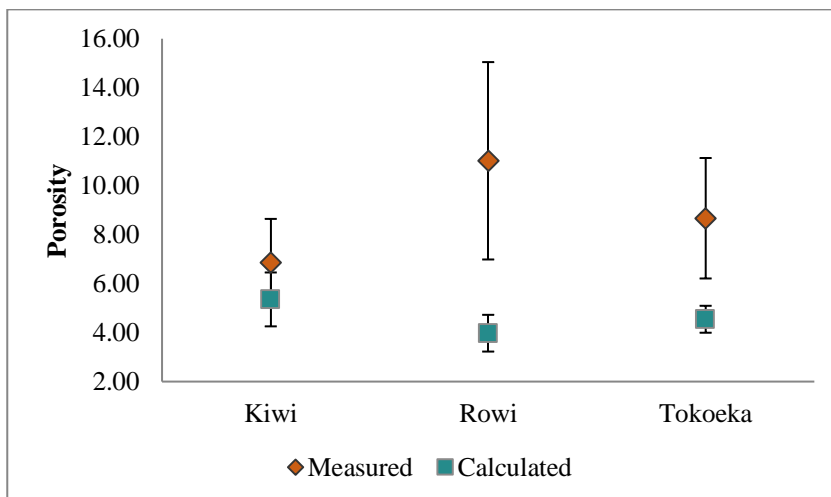
Figure 3.10 Comparison between the Measured porosity (Diamonds), and the predicted porosity, obtained by the formula found in Ar et al.,1974 (squares). Average± SD are presented. N=35 (Brown Kiwi n=13, Rowi n=12, Tokoeka n=10).

Water vapour conductance

Glue trial

The two glues did not seem to affect drastically the water vapour conductance (Mann-Whitney, W=196.0, p=0.51; N=27) (Table .3.5) as the results were very similar. However, the Super glue showed a greater standard deviation and less accurate results. I therefore chose to use the polyvinyl acetate (PVA) glue because of the lesser standard deviation and a more compact distribution of the data (Fig.3.11).

Table 3.5. Summary of the glue trial indicating the mean Water vapour conductance of Brown Kiwi eggshells.

Treatment	N	Mean±SD (mg/d.torr)	SE
PVA glue	13	0.172±0.066	0.02
Super Glue	14	0.344±0.112	0.11
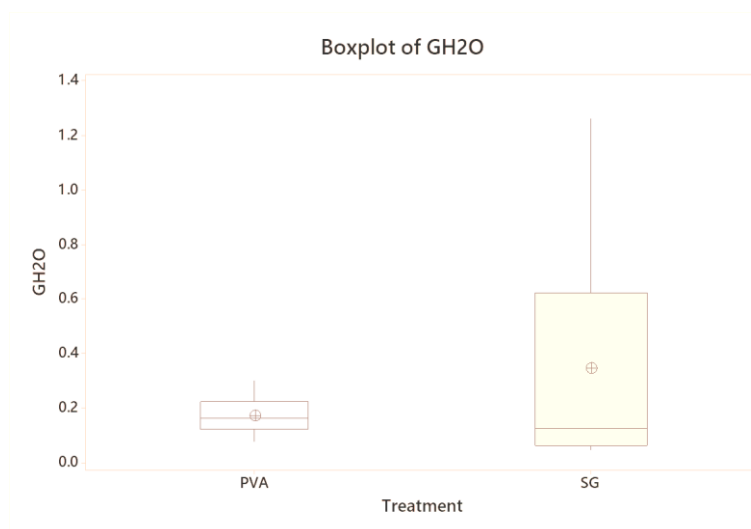
Figure 3.11. Boxplot of the measured water vapour conductance of Brown Kiwi eggshells (N=27) with two different types of glue (PVA and Super Glue).

Experimental Water vapour conductance

The three species of *Apteryx* differed significantly in the water vapour conductance of their eggshells (ANOVA F=6.36, p=0.003, N=36; Fig.3.12) with Brown Kiwi having a significantly higher water vapour conductance when compared to Tokoeka (F=3.87, p=0.001). Rowi did not differ significantly from Brown Kiwi (F=1.91, p= 0.062), and neither differ significantly to Tokoeka (F=2.06, p=0.05). These two last comparisons, however, are very close to being significant. The different regions of the eggshell confirmed existing differences in water vapour

conductance between species and showed that these differences are between the equatorial regions. The eggshell regions also showed significant within species differences, for Brown Kiwi and Rowi, the blunt end was different from the other regions. In contrast, Tokoeka showed no difference between the three regions (Table. 3.6).
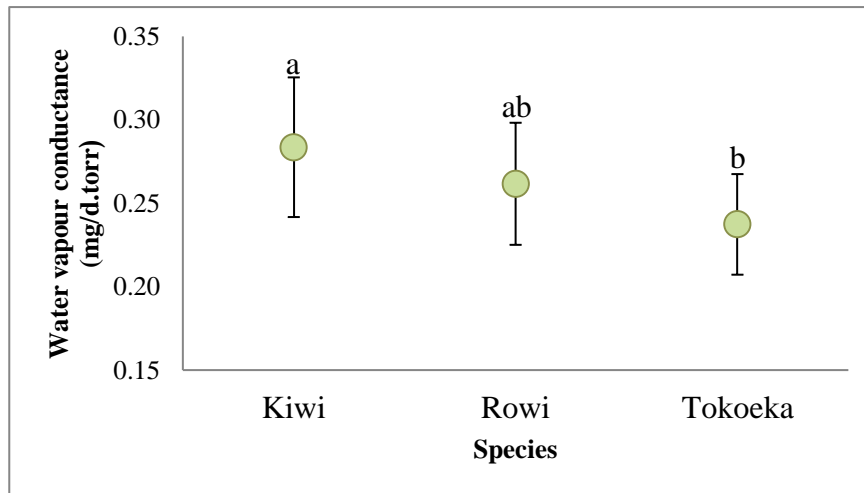
Figure 3.12. Mean values of water vapour conductance for three species of Apteryx. Kiwi N=27, Rowi N=21 and Tokoeka N=14. Means with different letters have significant statistical differences between them. The bars represent the Standard deviation.

Table 3.6. Repeated measures analysis of the three different eggshell regions. The super script in the first species column indicates the differences between the three species; the superscript in the fragment column indicates the differences between the fragments.

Species	N	Fragment	Water vapour conductance		Species compared to	*Species*Fragment*
			Mean	SE		*P value*
Kiwi[a]	7	A[a]	0.223	0.02		0.14
		E[ab]	0.221	0.01	Rowi	0.16
		B[b]	0.221	0.03		0.49
Rowi[b]	5	A[a]	0.255	0.03		0.65
		E[a]	0.206	0.02	Tokoeka	0.002
		B[b]	0.330	0.03		0.53
Tokoeka[ab]	8	A[a]	0.241	0.01		0.23
		E[a]	0.224	0.02	Kiwi	0.04
		B[a]	0.220	0.03		0.92
Species		Fragment			Species*Fragment interaction	
p value	0.049	*p value*	0.023		*p value*	0.11

A general pattern emerged where the eggshell thickness increased towards the south, while the water vapour conductance decreased, Porosity, however, was the lowest in Brown Kiwi, and the highest in Rowi (Table 3.7).

Table 3.7.Comparison of the eggshell Thickness, porosity (Ap), water vapour conductance (WVC) and related environmental variables between Brown Kiwi (BK), Rowi (R) and Tokoeka (T).

Sp	Eggshell thickness (μm)	Ap	WVC $\frac{mg}{d.torr}$	Cuticle thickness (μm)	Incubating strategy	Altitude masl	T range (C°)
BK	303.25	6.87	0.26	3.996	M	0-1500	0 to 31
R	367.0	11.02	0.25	3.009	MF	0-158	-6 to 17
T	386.25	8.67	0.23	3.045	MF-Co.	650-1587	-1 to 23

Mammillary density and area

The mammillary area was measured as the individual area of each mammillary knob (Fig.3.13) and the mammillary density was calculated as the number of mammillae per unit of area. The mammillary area showed an inverse correlation in a fit regression model (S=13.8272, R^2=86.45) with mammillary density (Fig.3.14).
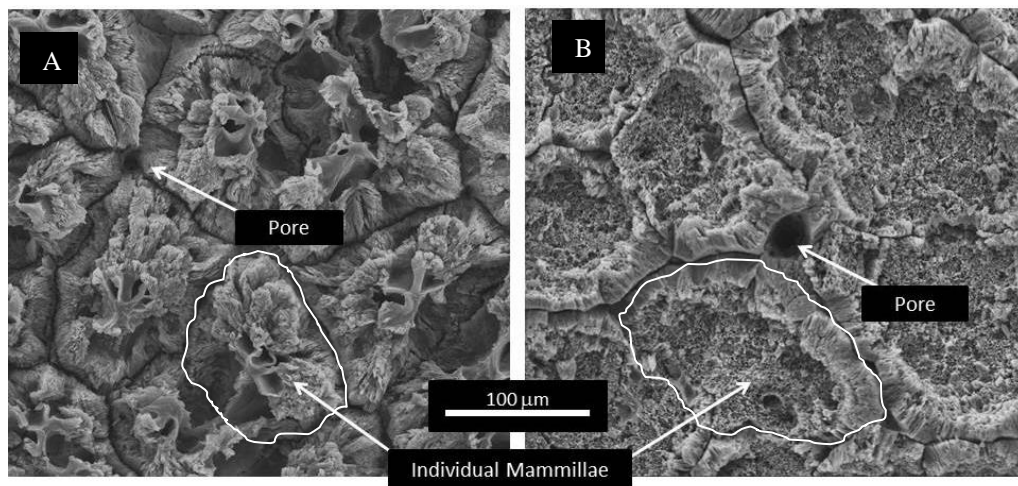
Figure 3.13 Mammillae, area and density. A) Brown Kiwi mammillae; B) Haast Tokoeka mammillae. Note how the mammillary density is greater in Brown Kiwi while the individual mammillae are smaller.
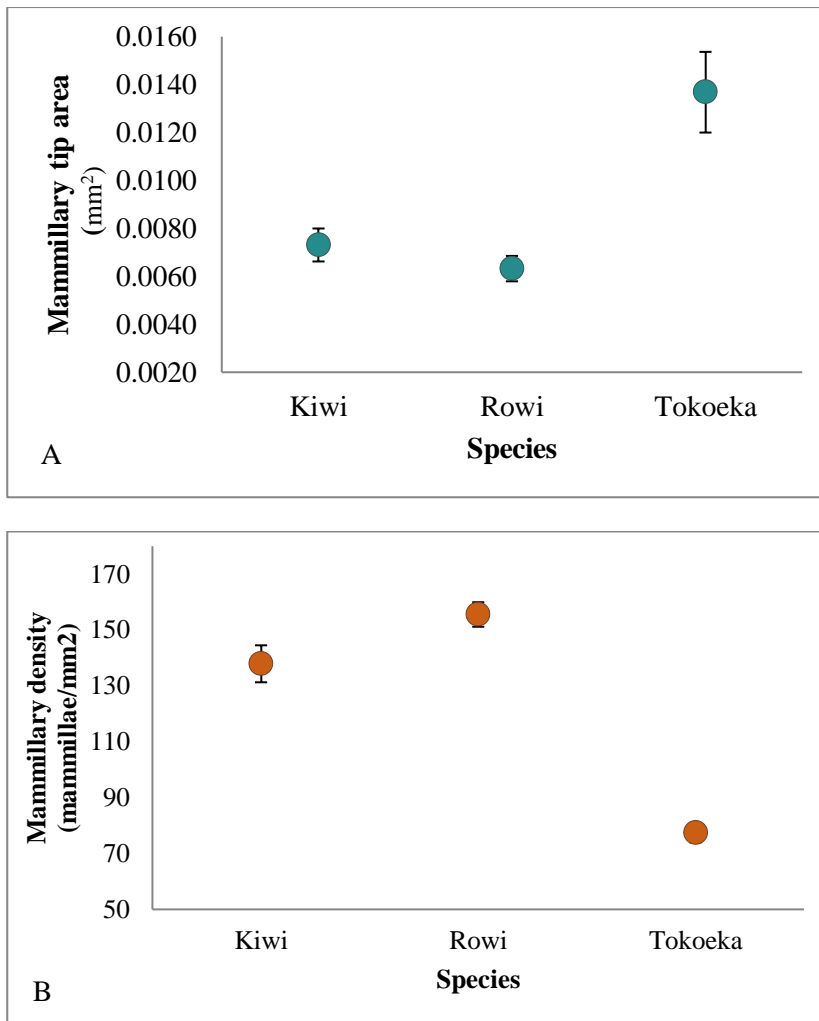
Figure 3.14. A) Average ± SE of mammillary area in three species of Apteryx. Kiwi N=17, Rowi N=21 and Tokoeka N=15. B). Average ± SE mammillary density defined as the number of mammillary tips per mm2

Mammillary erosion

When the mammillary layer of infertile eggshells was compared with that of hatched eggshells, it was evident that most of the once round (Fig.3.15, A) and well-defined mammillary tips had been eroded (Fig.3.15, B). Overall, in average 75% (SD=10.74) of the total surface area of infertile/early death embryo eggs is covered with the mammillary tips but after hatching only 5% on average (SD=1.05) remains (N=6). The erosion can also be observed in the remaining mammillary tips of the incubated eggshells as a reduction in surface area (Fig.3.16). When dyed with malachite green the core of the mammillary tips was coloured always in a much darker shade than the rest of the eggshell.
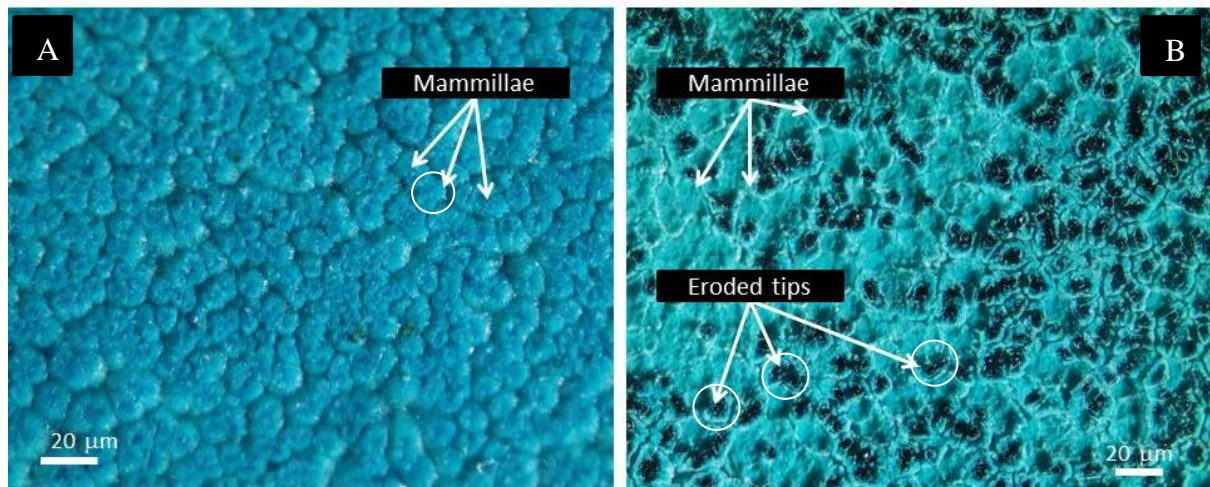
Figure 3.15. Dissecting microscope images of A) an infertile Brown Kiwi eggshell and B) a hatched Brown Kiwi eggshell dyed with malachite green. The round tips are clearly eroded in the hatched eggshell with only some cores left (darker spots).

Mammillary erosion cannot be observed in the same individual, but if we assume that infertile/early embryo death eggs represent normal not-incubated eggs, the mammillary tips would reduce in size from 95 μm^2 (SD=27 μm^2) to 38 μm^2 (SD=15 μm^2) during the embryonic development process.
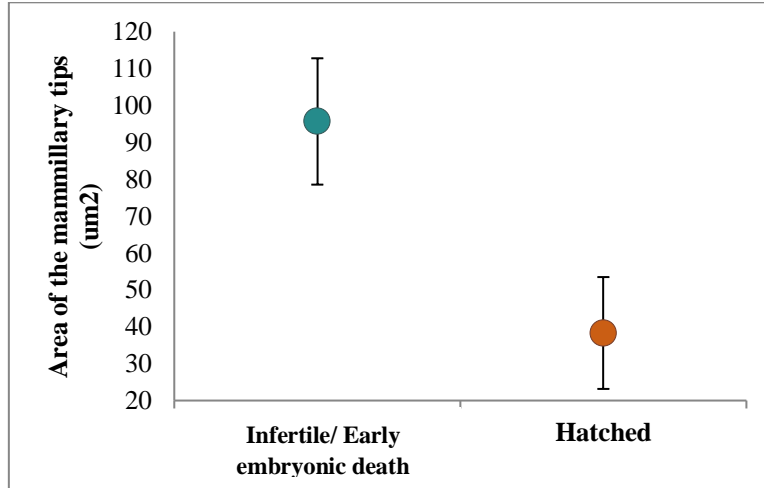
Figure 3.16. Mammillary area comparison between Infertile and hatched eggshells. Mean ±SD (N=6)

Water vapour conductance of eggs during incubation

The water vapour conductance of whole eggs was not significantly different to that calculated from allometric equations using the egg's mass (df=14, T=2.88, *p*=0.012). However, the water vapour conductance calculated from water loss was generally higher than the allometric calculation (Fig.3.17).
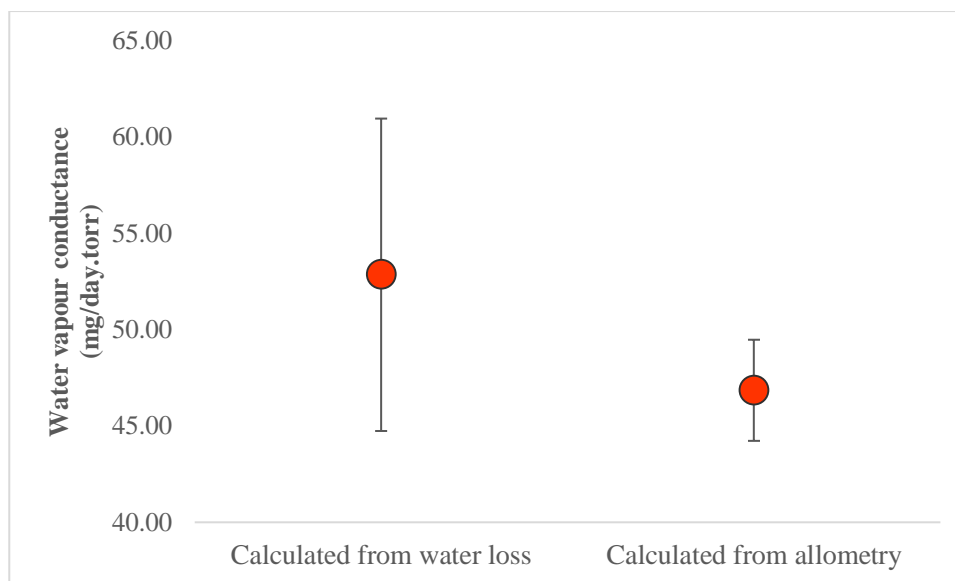
Figure 3.17. Comparison of the water vapour conductance calculated from water loss of incubated eggs and the water vapour conductance calculated using the allometric equation (eq.3.6) based on egg mass, Mean and SD are represented (N=15).

I compared the water vapour conductance calculated in this study for whole incubated eggs with that reported in the literature (Table 3.8). Calder (1978a) used infertile eggs and the methodology employed by Rahn et al., (1974); Sylin-Roberts (1983) devised a method for calculating the pore number and pore area and from three eggshell fragments calculated the water vapour conductance of the whole egg. Both these studies were done on *Apteryx australis* eggs and eggshells, but at this time, *Apteryx australis* included *A. mantelli*, *A. rowi,* and *A. australis*.

*Table 3.8. Water vapour conductance values reported in the literature. *The value reported by Sylin-Roberts is based on a calculation of the pores on three eggshell fragments.*

Water vapour conductance (mg/day.torr)	Source	N
26.00	Calder (1978a)	5
23.71*	Sylin-Roberts (1983)	1
52.84	This study	15

Finally, I plotted the resulting water vapour conductance alongside the data published by Ar et al.,1974, where they demonstrated how the water vapour conductance is proportional to the 0.78 power of the eggs mass (Fig. 3.18). Here I show how the water vapour conductance of Brown Kiwi fits perfectly with what is observed for other species.
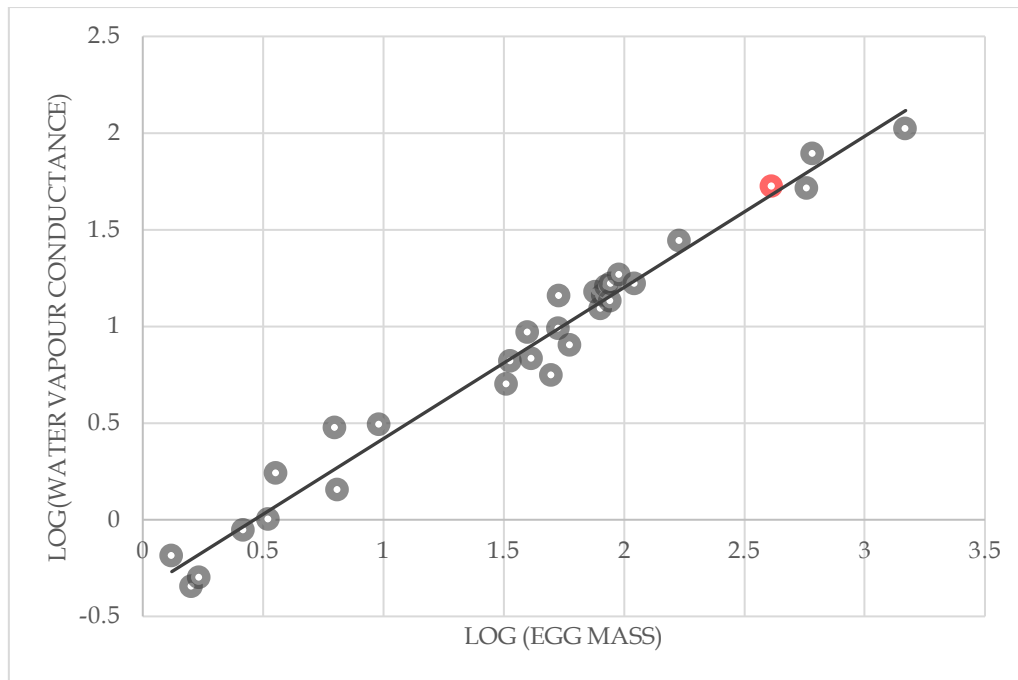
Figure 3. 18. Linear regression plot adapted from Ar et al.,1974 looking at the relationship between egg fresh mass and water vapour conductance. The original study included 29 species and in this study. I included Brown Kiwi (red dot) in the regression ($R^2=0.97$).

Discussion

In this study, I found that the eggshells of four of the five species of *Apteryx* are different enough to be assigned to their corresponding species using morphological characters. Eggshell thickness, mammillary area, and mammillary density were the most informative characters in this respect. The eggshells were thicker for the southernmost species, being the thickest for the most southern species, the Tokoeka. Cuticle thickness increased as we move north in distribution. The cuticle of *Apteryx* presented triangular particles that have not been reported in the literature so far; aggregations of these particles were observed to occlude the pores. In all the species of *Apteryx* capped and plugged pores were observed. Pore occlusions, caps and plugs have not been reported before in Apterygian eggshells either. This characteristic makes the identification of pores difficult and misleads the following inferences regarding water vapour conductance. Water vapour conductance of Apterygian eggshells is a much more complex phenomenon that previously assumed. Water vapour conductance was different between three species of *Apteryx,* and porosity appeared to be higher than predicted by weight, not lower as it has been assumed, and values for pore density and pore radius differed between the species. I will now discuss each of these findings on their own, linking them to their ecological significance.

Classification of species:

First, the identification of species using eggshell morphological characters has been used by palaeontologist for a very long time (Garcia, 2000) and has been proven effective in identifying taxa (Grellet-Tinner and Dyke, 2005; Harrison and Msuya, 2005). In this study, morphological

differences were expected as *Apteryx* species inhabit different habitats, and are distributed in reduced, non-sympatric areas, which makes the differences ecologically meaningful. In addition, the two larger islands of New Zealand exhibit a great deal of climatic variation depending on latitude, with sub-tropical climates in the north and sub-Antarctic in the south, which would require similar species to adapt to these regimes making eggshell differences likely to evolve.

Ecological influences on eggshell characteristics:

Eggshell thickness increased with latitude, with the southernmost species presenting a significantly thicker eggshell than the northernmost species (Table 3.7). There are several reasons that could account for this, including the different incubation strategy the species use. Tokoeka is known to incubate in groups, which would require a thicker eggshell to avoid breakage. In contrast Brown Kiwi incubates alone and it is lighter on average than the Tokoeka. Rowi, being the smallest of the tree species, presents an eggshell thickness similar to that of Tokoeka but in contrast to Brown Kiwi, both male and female have been observed incubating (Colbourne, 2002). It is interesting to see that porosity was the highest in Rowi, while the lowest in Brown Kiwi; an explanation for this could be that Brown Kiwi experiences overall warmer temperatures, which would cause gases to diffuse at a faster rate, hence a reduced porosity is needed, while in the colder climates of the South Island an increased porosity would be needed, the difference between Rowi and Tokoeka could be explained by the altitudinal difference between the species, with Tokoeka being exposed to lower barometric pressures which will increase the rate of diffusion, while Rowi being at sea level and experiencing higher barometric pressure would need to increase porosity to allow proper gas exchange. Cuticle followed an inverse pattern to that of overall eggshell thickness, in this case the warmer temperatures Brown Kiwi is adapted to are also propitious for micro-organisms, and hence a thicker cuticle would result possibly in more caps and reduction of the pore area. In contrast, Rowi and Tokoeka can have greater pores as the lower temperatures would reduce the risk of bacterial penetration.

Regarding the cuticle, the Apterygian cuticle consists of a very thin, waxy mineral layer that can occlude the pores by means of caps or plugs which had not been reported in the literature so far but have been reported in Tinamous (Perrot, 1979). It is important to note that all *Apteryx* species breed during the austral winter, which in New Zealand is characterised by frequent precipitation and heavy rain (Leathwick, Wilson, and Stephens, 2002) and it has been noticed that bacterial penetration is facilitated by water as a bacterial carrier (Berrang et al., 1999).

Apteryx does not present the accessory layer that has been described in Megapodidae, Phoenicopteridae and Podicipedidae (Board et al., 1984; D'Alba et al., 2014; Tullett et al., 1976) and that has been associated with decreasing bacterial penetration in high humidity environments. *Apteryx* cuticle was observed to have water repellent properties, these properties should be further studied in relation to bacterial penetration assisted by water at different stages of incubation. *Apteryx* exhibits triangular particles that have not been reported for any other bird. Subtriangular ornamentation was observed in the fossil eggshells of *Trigonoolithus amoae* a theropod from the lower cretaceous period found in La Cantalera, Spain (Moreno-Azanza, 2013). Since it has been suggested that many dinosaurs might have buried their eggs (Tanaka et al., 2015), and there is evidence of a nesting theropod (*Oviraptor philoceratos*) which might have

similar nesting behaviours to modern ratites (M. a. Norell et al., 1996), it could be possible to suggest that these triangular particles play some role related to the nest environment, however, it is difficult to assert which function could they have.

In this study, I report the presence of plugs and caps in *Apterygian* eggshells, which is a feature that has not been reported previously. Plugs and caps have been associated with nesting in humid environments (D'Alba et al., 2016) but these particles could also play a role in physically stopping bacteria and fungi to penetrate the eggshell. Antimicrobial mechanisms are certainly present in Apterygian eggs, particularly in the albumin (Prager, Wilson, and Arniheim, 1974) however, the cuticle is the first line of defence against microorganisms (D'Alba et al., 2017). In this case these plugs could serve both purposes of waterproofing the egg and reducing the possibility of infestation by microorganisms, as it has been proposed by Board (1981). In fact, water penetration and bacterial infection could go hand in hand, as water penetrating the pores can easily carry infectious bacteria (De Reu et al., 2006).

Abrasion can remove the cuticle with relative ease; cuticle erosion was very variable between individuals; however, the consequences of this were not explored in this study. All the eggs observed where fertile and hatched under artificial conditions making impossible to determine if there is an "expected" erosion of the cuticle for naturally hatched eggs. Cuticle erosion has been observed in *Crotophaga major*, and has been hypothesised that it functions as a shock absorber, eroding to absorb shock energy (Portugal et al., 2018). In *Numidia meleagris*, a bird belonging to the order Galliformes, a cuticle that erodes (or polishes) through the process of incubation has been observed. It is suggested that erosion serves the purpose of reducing the blockage of pores that can accumulate by contact with the nest, other eggs and\or dirt on the incubating parent (Board and Perrott, 2009). This process seems to remove both the plugs and debris, and it is noted that it would serve the purpose of increasing the oxygen diffusion towards the time it is needed the most, as the embryo develops. *Numidia meleagris* presents several similarities with *Apteryx* eggshells such as particles in the outer surface of the eggshells (spherical in *N. meleagris* but triangular in *Apteryx*) but in the case of *N. meleagris* the cuticle is completely polished through the incubation process. It is possible that this is also the case for *Apteryx* as it is known that the eggs are turned inside the nest (Colbourne, 2002). This would explain why we found some plugged pores and opened pores and why the cuticle erosion was different between individuals.

All *Apteryx* species presented capped or plugged pores. In the study of Silyn-Roberts (1983), this was not mentioned possibly due to the procedure to clean the eggshells by boiling them, as it is possible to remove the pore occlusions. Murphy et al., (2015) have shown how ultrasonic baths used to clean the eggshells can remove pore occlusions, which increases the number of observable pores. In our case as the eggshells were only rinsed with deionised water the occlusions remained. Unperforated pores have been reported in the eggshells of Ostriches (Willoughby et al., 2016; Maina, 2017). Capped pores have been reported in Tinamous, Jacanas, Emus and Rheas (Board and Perrot, 1979) and it has been suggested that in Jacanas the occluded pores are related to wet environments to provide water proofing (Board and Perrot, 1979); however this has not been tested. In the case of *Apteryx* both water and an increased physical barrier to prevent microbial penetration could be an explanation. Both the caps and plugs could

explain why when the pores where counted in the past, the porosity was under-estimated leading to exaggerated assumptions regarding the water vapour conductance of *Apteryx*

Gas exchange and water vapour conductance:

In this study I found that porosity is higher than was described by Calder (1978). He stated that Kiwi's porosity is 60% of the predicted value by Ar et al., (1974) equation. However, I found that the porosity is higher than the value predicted. This could be because the eggshells I used belonged to successfully hatched eggs, meaning that if there is any abrasion or pore opening, pores would be more visible at this stage than when an infertile egg is used. This still would need to be tested by comparing the porosity of freshly laid eggs versus the porosity of successfully hatched eggs. In this study, it was not possible to make such a comparison, as all the "infertile" eggshells were either infertile or early embryonic death, but they remained in the nest and were incubated for at least 25 days which would make any comparison in this regard equivocal.

Pore density decreased from north to south with the highest pore density observed in Brown Kiwi and the lowest in Haast Tokoeka, Pore radius, however, was the smallest in Brown Kiwi and the biggest in Rowi, but not showing significant differences with Tokoeka. Porosity differed between the three species being the highest in Rowi and the lowest in Brown Kiwi. Temperature and barometric pressure play a very important role in diffusivity of gases; higher temperatures and lower pressures allow the gases to diffuse at faster rates. In this case, Brown Kiwi experiences the highest temperatures while Rowi and Tokoeka experience the lowest; since Tokoeka eggs are bigger than those of Rowi and the average temperature is similar it would be expected that the eggshells presents a higher porosity. However, Haast Tokoeka inhabits higher altitudes, experiencing lower barometric pressures, which would be compensated by a reduced porosity. In summary, Brown Kiwi presents many small pores, while the southern species have fewer pores that are bigger. In addition to temperature and humidity this could also be associated with a reduced risk of bacterial penetration in colder climates for the southern species and as an adaptation to protect the egg from infection in warmer climates.

Water vapour conductance was in accordance to that measured for ground burrowing species reported by Portugal et al., (2014). Unfortunately at this point it cannot be said how this WVC relates to that of the whole egg, as our measurements relied on the fragments, however it could be suggested that the water vapour conductance would be higher than predicted by Calder (1979), due to the higher porosity measured in this study. It is also possible that the gas exchange changes during the incubation process as more pores could become opened as the egg is incubated and turned by the parent and the cuticle erodes. The different eggshell regions showed different water vapour conductance indicating a difference either in thickness or pore density. In some eggs, it was possible to observe a cluster of pores concentrated in the most apical extreme of the blunt end; however since the eggshells used in the study came from hatched individuals in most cases this particular region had fractures and a neighbouring fragment was used, possible underestimating the actual number of pores of that region. Significant differences were seen between the blunt end and the acute end for Brown Kiwi and Rowi in water vapour conductance, it is possible that this is also the case for Tokoeka but because of the difficulty of sampling this was not observed.

The general hypothesis was that the water vapour conductance of *Apteryx* was 65% lower than expected by allometric predictions because of low porosity in relation to egg mass, and as a compensation for a very long incubation period (Tullett, 1984). *Apteryx* does present a very slow metabolism in all life stages including the egg (Prinzinger and Dietz, 2002) and the nests do seem very poor in oxygen (Chapter IV), however there is a spike in oxygen consumption midway of the development making necessary for the egg to exchange gases efficiently in the nest environment (Prinzinger and Dietz, 2002).

The water vapour conductance of the whole egg was higher than previously reported, in this study I calculated the water vapour conductance at the conditions described by Ar et al., (1974) was 52.84 mg/day.torr as opposed to 26.00 and 23.71 mg/day.torr reported by Calder (1978a) and Sylin-Roberts (1983) respectively. And in contrast I found that is not significantly different to the water vapour conductance calculated from the egg's mass. Furthermore, when compared with the values reported by Ar et al.,1974 for a variety of avian species, *Apteryx mantelli* fitted perfectly into the expected relationship between egg mas and water vapour conductance, meaning that the egg loses water in the same proportion as any other avian egg.

If porosity is higher than predicted by allometry in all *Apteryx* species, then the water vapour conductance would be proportionally higher. However, for Brown Kiwi, it is found that the water vapour conductance is as expected by allometry. Previous studies suggested a lower water vapour conductance than expected, and the porosity measured in this study suggest that it should be higher. This phenomenon is possibly caused by the plugs and occlusions of the pores, which would reduce the rate of gas exchange by physically blocking the pore while maintaining numerous pores. It is difficult to imagine why more blocked pores are "better" than fewer opened pores, but it could have something to do with hatching. It is possible that a porous structure is more easily breakable for a hatching chick (Bond, Board, and Scott, 1988). In this study I found that many pores would not go all the way through the eggshell, the function of these types of pores is still not clear, however similar pores that do not transverse the eggshells have been observed in the eggs of Ostriches (Willoughby et al., 2016; Maina, 2017).

Gas exchange has been studied in closely related species of swallows that present burrowing and open cup nesting strategies, and it was found that burrowing species tend to have increased water vapour conductance. Birchard and Kilgore, (1980) explored the water vapour conductance of the bank swallow (*Riparia riparia*) a burrowing species and the barn swallow (*Hirundo rustica*) which is an open nester; they found an elevated water vapour conductance in the bank swallow eggs, supporting the hypothesis for a necessity of increased gas exchange in an enclosed environment. In this context, *Apteryx* is difficult to study as there are not many relatives that present a drastically different nesting behaviour but with similar sized egg. A comparison could be made between *Apteryx* and Tinamous as both taxa are ground-dwelling species with variable altitudinal and latitudinal distributions, but with very different nesting behaviours. The use of Micro CT has proven to be of great help to uncover the complexities of eggshell pore geometry and pore distribution (Willoughby et al., 2016; Maina, 2017) and I suggest when possible that other studies exploring porosity in avian eggshells make use of this technique.

Differences in porosity in different eggshell regions have been reported for ducks and gulls (Portugal et al., 2010), but the purpose of this trait has not been discussed. In comparison with Portugal's results *Apteryx* water vapour conductance showed similar results to those of chickens (approximately 0.20 mg.d^{-1}.torr^{-1}); unfortunately is difficult to assess this information in context as the species for which this experiment has been performed have very different egg sizes and masses. However, Portugal et al., (2014) reported water vapour conductance values using this same experimental approach I used for different nesting birds from Great Britain, and the values I obtained coincide with those reported for ground burrowing species.

Pores of Apterygian eggshells seem unique amongst ratites as most other ratites except Tinamous, have branched pores (Board and Scott, 1980; Szczerbińska and Wiercińska, 2014; Tullett, 1984). This could mean that funnel-like pores have evolved independently more than once, however, the funnel-like pores might be entirely different between *Apteryx* and Tinamous. All other ratite species show pores with branches of some sort, with ostrich having the most complex pores (Board, 1982). It could be then proposed that branched pores are the ancestral state of the Paleognathes, and *Apteryx* and Tinamous, present an evolutionary innovation by producing "unbranched" pores. According to Weir et al., (2016) *Apteryx* is a relatively recent species, as the taxon is believed to have originated approximately 8.6 Ma ago. However, when oological phylogenies are constructed (Zelenitsky and Modesto, 2003) *Apteryx* is placed as the most basal of the ratite species.

Eggshell Based phylogenies are interesting because Zelenitsky and Modesto (2003) used 67 discrete morphological characters to construct three possible phylogenies; in all three of them the Tinamous were placed at the base of the trees, followed by *Apteryx*. This could in theory suggest that *Apteryx* has maintained many ancestral characteristics even though its nesting strategy and overall physiology are so different to any other ratite. It is important not to discard this information in the light of more modern molecular phylogenies as it can be informative regarding the evolutionary steps that the ratites took to adapt to their individual nesting strategies and current climates. The possibility that the ancestor of the ratites buried its eggs, hence some of the physiological adaptations still be present, cannot be discarded.

Gas exchange appears to be a phenomenon more complex than previously described as there is a possibility that species develop mechanisms such as polishing the surface of the eggshell to change conductance over the incubation period. I believe this is the case for *Apteryx* as the possibility of open pores as the embryo develops would fit with a very long incubation period in a damp nest, as capped pores could help reduce the risk of microbial contamination in early stages of development where oxygen is barely required. It was noticed by Prinzinger and Dietz (1995) that precocial embryos would drastically increase the oxygen consumption later in the incubation period, by this point *Apteryx* could have "polished" the eggshell allowing an increased gas exchange, by that time the defences in the albumin would help protecting the embryo until hatching.

The eggshell characteristics found in this study suggest that *Apteryx* species are adapted to the environmental conditions where the eggs are incubated, and possibly the breeding strategies.

Each characteristic may be explained by more than one factor suggesting that it is the synergy between the factors that shaped the eggshells of the different species.

Chapter Four:

Thermal properties of Brown Kiwi eggs and nests

Fit pullus a nido volans, qui iterum cadit in nidum

(A chick attempts to fly out of its nest but falls into it again)

Michael Maierus, 1619

Abstract

Apteryx is the only ratite that nests in burrows or cavities, and in comparison, to other ratites, has a complex nesting ecology. I tested the hypothesis that the nest and the egg are adapted to a cold environment, because all *Apteryx* species start their breeding season at the beginning of the Austral winter. For this purpose, I measured the thermal properties of infertile Brown Kiwi eggs in an artificial incubator by heating them to incubation temperatures and calculating the time to thermal equilibrium in a controlled environment (10° C), then compared Brown Kiwi (*Apteryx mantelli*) to three other precocial species: Domestic Fowl (*Gallus gallus*), Greylag Goose (*Anser domesticus*) and Emu (*Dromaius navaehollandicus*). I also measured the thermal properties of nests in a Brown Kiwi population on Ponui Island using temperature dataloggers during the beginning of winter but before the breeding season, to determine the daily temperature variation with respect to the environment, I then conducted a heating experiment to determine the time it would take for a nest to attain thermal equilibrium with the environment once heated. In both cases I used Newton's law of cooling to determine the rates of heat loss. I found that kiwi eggs cool down as fast as the greylag goose eggs, which are a fraction of the mass of the Brown Kiwi eggs, suggesting that kiwi eggs do not have adaptations to deal with heat loss. In contrast, I found that nests could buffer environmental changes, maintaining the internal temperature above environmental temperature during the night, the period the incubating parent leaves to forage. These nests characteristics could be advantageous in a climate characterised by drastic weather changes.

Introduction

The nesting behaviour of birds is varied and complex; avian nests need to effectively respond to environmental pressures to ensure protection for the eggs against predation and the elements, and ultimately contribute to the successful hatching of eggs (Carey, 1980; Deeming, 2002b). The nest can be a complex architectural structure made from a wide variety of materials ranging from twigs to moss, leaves, feathers, dirt, mud, and even saliva, and can have many forms or architectural styles, such as burrows, mounds, open cups, and woven bags (Deeming, 2002a; Healy, Walsh, and Hansell, 2008). Nests have been used as extended morphological characters in phylogenies (Kusmierski, et al., 1993; Zyskowski and Prum, 1999), and there is evidence that nest architecture is a hereditary behaviour (Sheldon and Winkler, 1999). It has been suggested that nest architecture evolves in a similar way to species themselves, by modifications of an ancestral architecture (Winkler and Sheldon, 1993). These modifications are usually adaptations in terms of size, materials and quantity of accessories, however the architectural design seems to be much conserved (Kusmierski et al., 1993).

The materials used and the final appearance of the nest when combined make up what is termed the 'architectural strategy' (i.e. saliva woven cups or twig and grass woven bags). Each architectural strategy presents some benefits and some challenges in terms of protection against

predators, accessibility to resources, strength against the elements and good camouflage to avoid predation and parasitism; it could be thought that the architectural strategy is then under a strong selective pressure. However, an appropriate microclimate for embryo development and successful hatching is key to survival of all species and it is probably one of the most strongly selected attributes of nests (Lombardo, 1994). Birds are thought to select nest microclimates that can regulate drastic environmental conditions that could threaten the survival of the egg (Webb and King, 1983). For a nest to be successful three microclimatic requirements must be considered: temperature, humidity, and composition and concentration of respiratory gases (Walsberg, 1980; Walsberg and Schmidt, 1992).

Nest architecture has the advantage of allowing small adjustments and compromises depending on environmental conditions or predatory pressures (Forstmeier and Weiss, 2004; Warning and Benedict, 2015) and to be easily selected to maximize reproductive success; this could be done for example by changing the materials, the location, and/or the size of the nest. Nesting architecture is thus probably the driver behind nest evolution as small differences are constantly selected to increase fitness (Kusmierski et al., 1997). This means that nest architecture can differ greatly between closely related species depending on their ecological requirements (Greeney, 2008; Zyskowski and Prum, 1999). Therefore, it becomes very interesting when related species present drastically different nest architectures, as it begs the question: what are the ecological pressures that directed that change?

Burrow nesting can be an advantageous architectural strategy where predation and harsh weather conditions are frequent during the incubation period (Wheelwright and Boersma, 1979). Enclosed cavities have slower rates of temperature change which makes burrows a very efficient nest choice in rapidly changing environments (Howell and Bartholomew, 1961). Burrows of the Rainbow Bee-eater (*Merops ornatus*) for instance show very little variation with respect of ambient temperature (Lill and Fell, 2007). In most cases, the incubating parent can change temperatures in the nest simply by being present (warm) or not (cool) (White, Bartholomew, and Kinney, 1978). For example, deep burrows built in the sand retain the warmth left by Bonin petrel (*Pterodroma hypoleuca*) parents, maintaining incubation temperatures in the nest while the parents forage at night (Howell and Bartholomew, 1961). Crab plovers (*Dromas ardeola)* nest in burrows, and relie partially on solar radiation to help in the incubation process. In addition, being inside a burrow helps avoid direct solar radiation, and its negative effects, onto the eggs (De Marchi et al., 2008, 2015). The conditions in the burrow can be further manipulated by the parents, by means of the type and quantity of nest building materials (grass, moss or feathers as lining separating the soil from the egg), the burrow location/aspect (in relation to the wind and the sun), and the topology of the nest (having multiple chambers, openings, etc.) (Ramos et al., 1997; Heneberg, 2009). The Burrowing Owl (*Athene cunicularia*) seems to carefully control for the environmental characteristics of its nest location, and this "decision" is strongly associated with nest success (Green and Anthony, 1989). In general each burrowing species develops a balanced strategy where temperature control, humidity and ventilation, and even defence are carefully controlled in respect to environmental variables (Birchard et al., 1984; Boggs and Kilgore, 1983; Fisher, Poulin, Todd, and Brigham, 2004).

However, no matter how carefully a species chooses a nest site or builds its burrow, burrow nesting poses great difficulties for gas exchange and has an increased risk of hypercarbia (excess of carbon dioxide diffused into the blood stream in this case due to low availability of oxygen) (Birchard et al., 1984; Boggs and Kilgore, 1983). Water loss (Burton and Tullett, 1985) and an increased risk of microbial penetration are two further challenges for burrow nesting species as burrows often offer a dark, damped and warm environment suitable for many species of bacteria and fungi (D'Alba et al., 2014; White et al., 1978). Given the challenges imposed by burrow nesting, it would be expected that burrow nesting species develop engineering solutions in nest architecture to improve aeration while keeping appropriate temperature and humidity regulation (Ar and Piontkewitz, 1992; Lill and Fell, 2007; Poulin et al., 2005). Further, it could also be expected to encounter that these species possess physiological adaptations in both the parent and the embryo to withstand high humidity and low oxygen conditions for extended periods of time (Seymour and Ackerman, 1980; Boccs et al., 1984; Burton and Tullett, 1985).

All five species in the genus *Apteryx* nest in soil burrows, hollowed trees, or cavities formed in the gaps between tree roots and the soil. The soil burrows are dug by the birds (McLennan, 1988; Ziesemann, Brunton and Castro, 2011) while tree nests are naturally occurring cavities that the birds select to nest in. *Apteryx* nests have been known for a long time, but a formal description of many of the Apterygian nests has not been published. The only well documented example is provided by Jolly (1989) for the Little Spotted Kiwi (*A. owenii*).

Brown Kiwi (*Apteryx mantelli*) is the most common and widely distributed of the five species. Its breeding season occurs approximately from June to February, and the females lay up to two clutches of two eggs each (Sales, 2005). The first clutch is laid during the austral winter and hatches around October, which coincides with the austral spring; the second clutch is laid almost immediately after the hatching of first clutch, and the chicks hatch in late January, coinciding with the beginning of the Austral summer. Brown Kiwi's incubation period takes from 74 to 84 days (Holzapfel et al., 2008). The flexibility of hatching time as well as its duration exceeds the expectations for a species this size (Calder, 1978). However, long incubation periods and a flexible hatching time are often found in species that breed in cold climates and were the egg is subjected to long periods of abandonment (Olson, Vleck, and Vleck, 2006; Vleck and Kenagy, 1980); both are true for Brown Kiwi.

Unusually long incubation periods have been reported for various species of petrels where the incubation can take over 70 days (Boersma, 1982). Typically in many species of petrels the eggs can be left unattended for several days while the parents are foraging at sea (Boersma and Wheelwright, 1979; Wheelwright and Boersma, 1979). An increased incubation period appears to allow the embryo to deal with chilling and abandonment by retarding the development process. This requirement of retarded development can be accompanied with a reduction in the overall metabolic rate (McNab, 1996).

Brown Kiwi is a nocturnal insectivore, and the incubating parent leaves the nest to forage every night, usually just after dark, for approximately six to eight hours and returns before sunrise. The duration of egg abandonment decreases as the embryo develops, until at the end of the incubation period, the last week or so, the parent barely leaves the nest until the egg hatches (Colbourne,

2002). There is not a clear idea of why Brown Kiwi has such a long incubation period, but it has been suggested that it is due to the need for a very developed chick (Calder, 1979). A proximal explanation is that Kiwi, like Bonin Petrels, leaves the nest unattended for several hours and the embryo enters a form of dormancy where the development is delayed until the parent resumes incubation.

The heat exchange of the nest and the egg need to be better understood as it is not known, for example, the rate at which the nest loses heat while the father is foraging, or if the egg itself has adaptations to further diminish the heat loss.

Hypothesis

Nest thermal properties and egg physiological adaptations to neglect
The basis of a successful incubation is the maintenance of an appropriate microclimate for embryo development and successful hatching. The microclimate seems to be specific for each specie's life history; this means that metabolic rate, and developmental time, precociality-altriciality, and parental investment would affect and be affected by the nest microclimate.

In this study, I explored two possibilities. First, I examined whether the nest has some insulating capacity to maintain heat after the incubating parent has left the nest and possibly, until it returns, to reduce the risk of overcooling and consequential embryo death. Second, since the eggs of three species of *Apteryx* have been noted to have high levels of fatty acids in the yolk (Body and Reid, 1983, 1987), it is possible that that the egg can endure overcooling by having a low cooling rate, being able to maintain heat for an extended period of time. Cooling is related to the volume of an object, with larger volumes cooling at a slower rate due to the surface area to volume ratio. Volume in turn, is associated with mass in bird's eggs (Turner, 1985). A slower cooling rate could help explain the size of the kiwi egg and its composition. These two factors could work in conjunction to allow the egg to resist prolonged periods of abandonment in cold winter nights. This also requires the male to be aware of the cooling rate of the nest and the egg, so it can adjust the absence periods to those needs. However, in this study I will focus only in the physical thermal properties of the egg rather than the chemical ones.

The aim of this chapter is therefore to explore the evolutionary adaptations that could be present in *Apteryx* eggs and their nests in a cold environment context. First, I calculated the cooling rate of infertile Brown Kiwi eggs and the specific heat of the egg contents and compared them to three other precocial species: Domestic fowl (*Gallus gallus*), Greylag goose (*Anser domesticus*) and Emu (*Dromaius novaehollandicus*). Then, I produced a formal description of the Brown Kiwi nest architecture, and I explored the nest's temperature in relation to environmental temperatures. This was done to try to understand why *Apteryx* has such a different nesting behaviour to all the other ratites and suggest what could be the evolutionary pressures that have shaped this behaviour. I extracted the nesting materials from a single tree nest to classify the materials and perform a hydrophobicity test, with the purpose of determining if water proofing properties existed in these materials. As an additional step, I measured the pH of the nest, comparing tree nests and soil nests to explore the possibility that the respiratory gases in

conjunction with the products of rotting vegetation could increase the risk of the egg to bacterial penetration by weakening the shell.

Materials and Methods

Sourcing of the eggs

Eggs were obtained from the Operation Nest Egg program at Rainbow Springs Kiwi encounter in Rotorua and the West Coast Wildlife Centre in Franz Josef, New Zealand. The eggs in this programme are generally collected after day 20 of incubation to increase the hatching success through artificial incubation. The eggs used in this experiment were candled and determined infertile or early embryo death as there was no gross visual evidence of a developing embryo.

Only infertile/early embryo death eggs were used as conservation policies of these endangered species are very strict and would not allow risking or ending the life of any bird, and we acted according to the permits obtained from the Department of Conservation of New Zealand, Authorisation number: 56537-DOA. The eggs used in this experiment were laid in the breeding season of 2017-2018 and transported to Massey University facilities where they were kept in a chilling room at 4˚C until the beginning of the experiments. The chicken eggs were purchased from the local farmers market, the greylag goose eggs were obtained from an abandoned nest at Ponui Island (36˚50'S, 175˚10'E); finally, the Emu eggs were purchased from an Emu farm (Northland Ostrich and Emu Limited, Wiharara, Northland, New Zealand).

Egg thermal capacity and cooling rate

Cooling rate of egg contents

Infertile (or early embryo death) eggs from four precocial species, Brown Kiwi, Domestic Fowl, Greylag Goose, and Emu, were artificially "incubated" in an enclosed chamber at constant temperature and humidity, the incubation temperature was set at approximately 35°C. The purpose was to allow the egg to increase its temperature to 35°C and measure the rate at which heat was lost once the egg was exposed to a colder temperature (10°C). Eggs were weighed on arrival to Massey University and weighed again prior to the experiment to account for water loss (Calder and Parr, 1977). Each egg was placed in a metal mesh above the heating source and a probe data-logger (Tinytag TGP-4000, Australia), was inserted through a small opening in the eggshell (fig.4.1). The probe was fixed using the metal mesh for support and placed in the centre of the egg through the small orifice. The orifice was then covered with a thin plug of tissue paper and glue (Papier-mâché) to avoid the contents from leaking and maintaining the integrity of the egg.

Two thermometers were placed at the top and bottom of the incubator. The thermometers were able to be read without opening the incubator; this was done to monitor the temperature in the incubating chamber. Once both thermometers showed a temperature of 35°C the incubator was opened, and the egg transferred to a controlled temperature room at 10˚C. The data logger recorded temperature every 5 seconds.
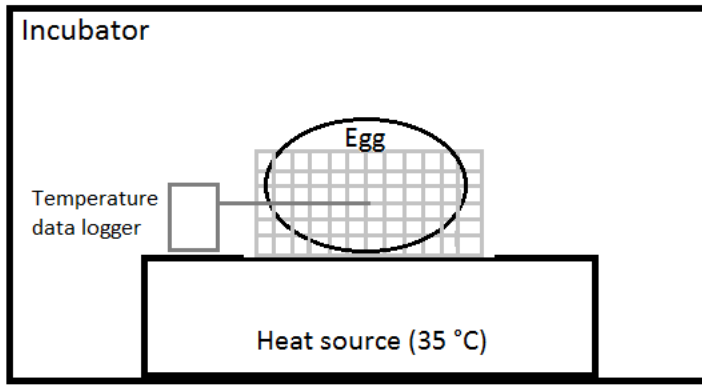
Figure 4.1 Diagram showing a transect section through the artificial incubator used in this study. The egg at room temperature was placed in the metal mesh above the heating source, at a height of 5 cm, the data logger was inserted to the centre of the egg.

Eggs follow Newton's law of cooling (Turner, 1985), therefore an integrated form of Newton's law was used at three different time intervals to determine the cooling constant (k) and the cooling average rate. Newton's law of cooling states that the rate of heat loss of a body is directly proportional to the difference in the temperatures between the body and its surroundings, assuming that the temperature difference is small and that the nature of radiating surface remains the same (Lienhard IV and Lienhard V, 2017). The cooling constants and heat loss rates were calculated and used to compare the insulation capacity of the individual eggs.

Newton's law of cooling states:

$$\frac{dT}{dt} = -K(T - T_a)$$

Eq. (4.1)

Where dT/dt is the change in the object's temperature (T) over the change in time (t), $-K$ is a constant, T is the object's temperature and T_a is the ambient temperature.

Developing this equation gives:

$$\frac{1}{(T - T_a)} dT = -Kdt$$

Eq. (4.2)

$$\int \frac{1}{(T - T_a)} dT = \int -Kdt$$

Eq. (4.3)

$$ln|T - T_a| = -kt + C_1$$

Eq. (4.4)

$$|T - T_a| = e^{-kt}e^{C_1} , e^{C_1} = C$$

Eq. (4.5)

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For a cooling object $T \geq T_a$ therefore $|T - T_a| = T - T_a$ thus:

$$T_{(t)} = Ce^{-kt} + T_a$$ Eq. (4.6)

By measuring a maximum temperature at t_0 it is possible to calculate the constant C which later will be used at t_i to calculate k. With these constants an average heat lose rate was calculated as follows:

$$\frac{f_{(t_1+h)} - f_{(t_1)}}{h}$$ Eq. (4.7)

Here t is the time and h the distance between two points in time in a Cartesian plane; in this case, two temperature points over time.

Specific heat of egg contents

Specific heat of eggs was measured to determine if the kiwi egg would require a higher amount of energy to elevate its temperature as compared to other species eggs, meaning it will be more costly to incubate, yet it will lose that temperature slower thus allowing the parent lo leave and forage for a longer period. Romanoff and Romanoff (1949) determined the specific heat of chicken egg contents and since then it has been assumed that this value is the same for most bird species. The composition of the Kiwi egg in terms of lipids seems very different to the chicken egg (Body and Reid, 1987); therefore, I determined the specific heat of the Brown Kiwi egg and compared it to that of chicken egg, Greylag goose, and Emu. Specific heat (defined as the heat required changing the temperature of a given substance by one degree Celsius) was measured by the calorimetric method. This was done by mixing a heated mass of a known substance whose specific heat is known (deionized water) with a mass of homogenised albumen/yolk whose temperature is known at the time of the experiment. The sample was obtained by pouring the whole egg contents in a beaker and mixing it thoroughly until no lumps or clusters were observable, then a subsample was taken for the experiment. This sample was poured into an adiabatic container with a digital probe thermometer (Traceable thermometer,4244CC). Then, the deionised water was poured and stirred, and the container sealed. The mixture temperature was then measured until equilibrium, and then the specific heat was calculated as follows:

$$Q_{lost\ by\ water} = Q_{gained\ by\ yolk\ or\ albumin}$$ Eq. (4.8)

Given that:

$Q = m. C_p(\Delta T)$ therefore:

$$m_{water} * C_{pwater} * (\Delta T)_{water} = m_{egg\ contents} * C_{pegg\ contents} * (\Delta T)_{egg\ contents}$$ Eq. (4.9)

thus:

$$C_{p\,egg\,contents} = \frac{m_{water} * C_{pwater} * (\Delta T)_{water}}{m_{egg\,contents} * (\Delta T)_{egg\,contents}}$$

Here Q is the heat transferred (the amount of energy), m the mass of the substance, C_p the specific heat, and ΔT the difference in temperature.

Once the specific heat was calculated the formula $Q = m.C_p(\Delta T)$ was used to estimate the energy required for raising the temperature of the egg from 25°C to incubation temperature (Table. 4.1). The incubation temperatures were obtained from the literature, and for this calculation, the total mass of the egg was used.

Table 4.1. Incubation temperatures of the species compared in this study.

Species	Incubation T (°C)	Source of incubation temperature
Domestic fowl	38	Tazawa, et al., (2001)
Greylag Goose	37	Webb (1978)
Brown Kiwi	35.5	Holzapfel et al., (2008)
Emu	34	Bassett (1996)

Nest Thermal properties

Study area

The fieldwork was carried out on Ponui Island, located in the Hauraki Gulf of New Zealand. Ponui is an island of 1770 ha of which two thirds are grazed pasture and the rest is a mixed broadleaf forest dominated by Kanuka (*Kunzea ericoides*) and Kauri (*Agathis australis*) (Miles and Castro, 2000). A Brown Kiwi population was established in the 1960s and has thrived reaching approximately 2000 individuals (Ziesemann et al., 2011). The study took place during the winters of 2016 and 2017 and the final stages during the summer of 2018. The Brown Kiwi population has been under study since 2004 and between 35 and 50 birds (males and females) have been tagged with radio transmitters annually and followed at least monthly. These transmitters allow us to locate the birds and since 2012, they log information about the activity of the wearers.

Nest description

The nests were visited during the non-breeding season to avoid the risk of birds deserting the nests. In this population, Brown Kiwi uses some but not all burrows for both roosting and nesting (Jamieson et al., 2016), thus some characteristics may be shared between roosting sites and nests. I focused only on those burrows that are known to have been used as nests in previous years.

Nest dimensions were measured using a STABILA laser meter (Stabila Messgeratäte D-76855, Hungary). Three measurements of depth, width and height were taken per nest, and averaged. The depth was measured from the entrance of the nest to the far-most wall; width was measured by placing the laser meter in one of the lateral walls of the burrow and pointing the laser to the

other wall; and height was measured by placing the laser meter on the bottom of the burrow and pointing the laser towards the ceiling.

Nest Microclimate

Nest Insulating capacity
The nest insulation properties were assessed in two ways: Temperature buffering capacity of the nest and heat retention capacity.

Temperature buffering capacity
The nest temperature in relation to environmental temperature flux was determined by placing paired temperature data loggers (Tinytag TGP-4000, Australia), one inside the nest, where the bird would usually sit and another one outside the nest (Ten data-loggers were used in total). The basal state of the nest was measured for 45 days, from 29 April to 13 June 2017. In addition, 15 nests (7 soil nests and 8 tree nests) were measured in the same way for a period of 24 hours in March 2018. These two experiments were done with the purpose of establishing the overall variance in temperature in the nest over a long period of time, allowing me to estimate the proportion of time the nests are above or below ambient temperature. I then estimated the relationship between nest inner temperature and ambient temperature daily and especially its changes throughout the day, allowing us to determine at what time the nest was warmer or cooler than ambient temperature.

The frequency the nest inner temperature was warmer and colder than the surroundings was estimated as follows:

$$f_{t_{above}} = \frac{\sum \Delta T > 0}{N}, f_{t_{below}} = \frac{\sum \Delta T < 0}{N} \qquad \text{Eq. (4.11)}$$

Where f_t represents the frequency of the nest being either above or below ambient temperature and $\Delta T = (T_n - T_a)$ the difference between the inner temperature of the nest (T_n) and the ambient temperature (T_a). N is the total number of measurements.

Heat retention capacity
To determine the insulating capacity of kiwi nests, ten recently used nests were selected after the breeding season of 2017-2018; the nests were visited in February to avoid nesting activity and risking nest abandonment. Temperature data-loggers (HOBO Pendant Data Logger 64K-UA-002-64) were placed inside the nests with a paired data logger outside monitoring environmental conditions for 24 hours. After this period a heat source (Zippo 12-hour hand warmers SKU:40334) was introduced in every nest along with the data-loggers. The heat source was set to warm the nest for a period of 12 hours. The temperature was monitored during this process and for the next 24 hours to determine the moment when the nest reached maximum temperature and started losing heat, as well as the point when it reached equilibrium with the environment. This method allows to establish the time the nest remains warm once a heat source is removed (or ceases to produce heat) indicating the time frame an incubating parent could forage without the risk of egg over- cooling.

Newton's law of cooling was used in the same way for this experiment; however, in this case, as the environmental temperature fluctuates and the equation requires a constant environmental temperature, the environmental temperature was averaged for the duration of the experiment. The resulting constants were compared using ANOVA.

Nest contents

Since little is known about the functional properties of Brown Kiwi nests, I extracted the entire contents of a single tree nest to describe the composition and characteristics of materials in kiwi nests, I only used one nest as it is not known if the removal of material would have a detrimental effect on birds that might reuse the nest or how important is the presence of such old materials in nests, and therefore the destruction of nests could not be justified.

The cavity was located in a living Puriri tree (*Vitex lucens*), a tree species often used by kiwi for roosting and nesting as it tends to hollow at the bottom of the trunk. This nest was known to have been used several times. Nesting material was 50 cm deep. The contents were described, and then different particle sizes were separated by using a gradation of sieves from >16 mm to <250 μm in size.

The hydrophobicity test was performed on the sieved materials of particle sizes equal or smaller than 500 μm, only these particle sizes were chosen, as the bigger material was too big to effectively measure hydrophobicity. Hydrophobicity was measured based on the methods of Shakesby et al., (1993), where a droplet of deionised water was placed on a smoothed dried soil and the time it takes for the water droplet to penetrate recorded. The nest contents were air dried over two months before the test, this is suggested to prevent interfering with the soil's natural hydrophobicity. The test was also performed in the argylosfaira (clumps of soil believed to be the product of nest digging) which was crushed in a mortar and pestle then sieved through a 1 mm sieve and then through a 250 μm sieve. Both soils were used, the less than 250 μm particles and the 1 to 250 μm particles.

Nest pH

The pH of 10 soil nests and 10 tree nests was measured using a direct soil pH meter with probe (HANNA HI99121) by inserting the probe at three points inside the nest in the area where the egg would normally be placed for incubation. The pH was compared with 10 measurements of the forest floor taken around the nests. Most of the nests had been unused since the previous season; however, I had the opportunity to measure the soil pH of two nests that where being used at the time. In these nests, the eggs were being candled for another study and while the crew was candling the egg, I took the three pH measurements.

Statistical analysis

The thermal properties and cooling times of eggs were not analysed statistically due to the very small sample sizes, and therefore only actual measurements are reported. The cooling time was compared using all species with a Spearman's rho correlation contrasting egg mass and cooling time.

The nest dimensions were compared using an ANOVA and a Tukey *post hoc* test in the same way as the nest substrate pH. The thermal properties of the nests were compared first using a χ^2 test to contrast the frequency of nests being either above or below environmental temperature. The cooling time and rate of tree nests was contrasted with that of soil nests using a one-way ANOVA.

Results

Egg thermal capacity and cooling rate

Cooling rate, resistance and capacitance of the egg contents
The Brown Kiwi egg took approximately 5:47 h (SD=0.19h) to attain thermal equilibrium with the environment (Fig.4.2) despite kiwi eggs being 2.8 times heavier than greylag goose eggs which took a similar amount of time (Table.4.2).
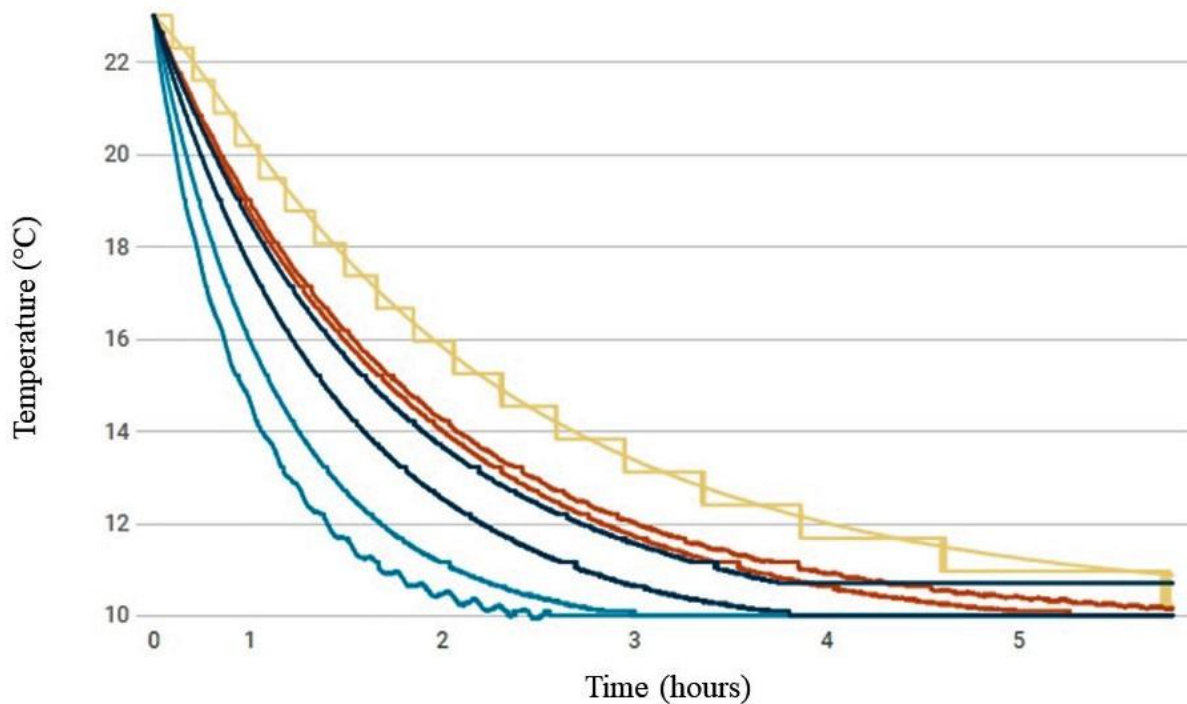
Figure 4.2 Egg cooling plot of four precocial bird species with different egg sizes. Chicken N=2 (light blue), Greylag Goose N=2 (Dark Blue), Brown Kiwi N=2 (Burnt orange), and Emu N=2 (Cream). The line for Emu1 is staggered because for this egg the data logger recorded temperature every ten seconds as opposed to five in the other eggs.

Table 4.2. Comparison of cooling rate and time between four species of precocial birds. All the values represent individual eggs.

Species	N	Cooling constant K (h^{-1})	Cooling rate (°C/h)	Actual cooling time (h)	Egg mass (g)
Chicken	1	$2.42*10^{-3}$	-5.41	2.48	69
Goose	2	$6.44*10^{-4}$	-3.73	3.11	100
		$5.87*10^{-4}$	-2.95	6.83	165
Brown Kiwi	2	$4.50*10^{-4}$	-3.62	4.44	341
		$3.08*10^{-4}$	-1.2	6.5	412
Emu	2	$2.93*10^{-4}$	-0.7	6.83	608
		$1.77*10^{-4}$	-1.98	11.3	658

I found that cooling time and cooling rate depended solely on egg's mass; with cooling time being positively correlated to egg mass (N=7, R^2=0.80) and the absolute value of cooling rate being negatively correlated with egg mass (N=7, R^2=0.91) (Fig.4.3).
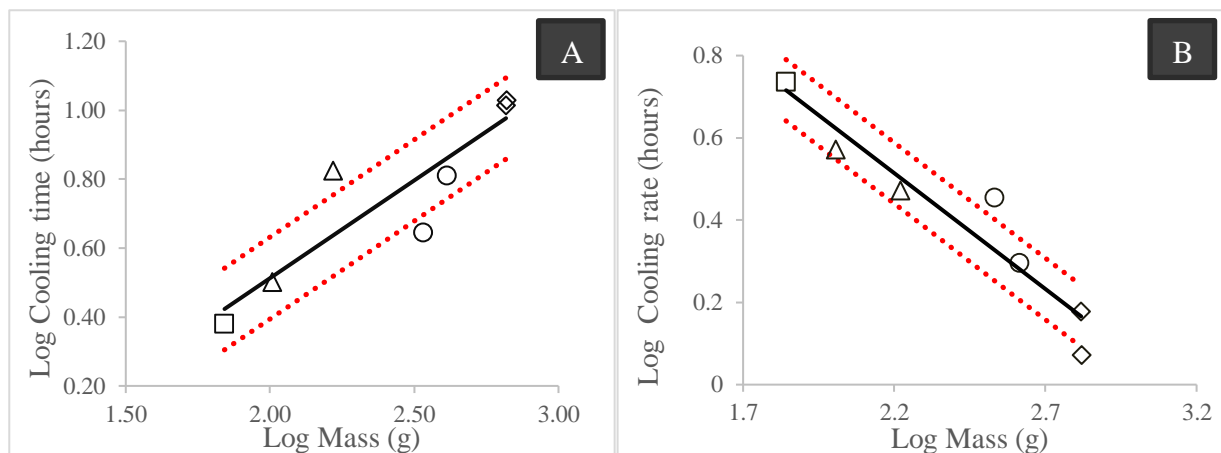
Figure 4.3. Linear regression with 95% confidence intervals of egg cooling and egg mass of four precocial species; Chicken (square), Greylag Goose (triangles), Brown Kiwi (circles), and Emu (diamonds). A) Log cooling time vs. Log gg weight (df= 5 R^2=0.80). B) Log cooling rate vs. Log egg weight (df=5 R^2=0.91).

Specific heat

When the specific heat of the four species was compared using the calorimetric method all species showed similar values as indicated by Romanoff and Romanoff (1949) The total energy to raise the egg from 25°C to incubation temperature was calculated based on the temperature difference from standard conditions (25°C) to incubation temperature (table 4.3).

Table 4.3. Summary of calculated specific heat and incubation energy requirement, this requirement is the energy need to raise the egg temperature from 25°C to the respective incubation temperature. Mean and SD are presented.

Species	N	Egg weight (g)	Specific heat (J/g.°C)	Incubation energy (J)
Domestic fowl	5	65.8±7.9	4.188±0.46	3,611.4±808.8
Greylag Goose	1	164.96	4.625	9,154.98
Brown Kiwi	1	412.0	3.663	11,249.77
Emu	1	608.0	4.128	24,446.02

Nest Thermal Properties

Nest description

Brown Kiwi nests consisted of either dug soil cavities on the slopes of gullies on the forest floor, or tree hollows, hollowed fallen logs, and spaces between tree roots and the soil. Soil nests could be quite shallow while some others could reach up to a meter deep. The shape of the nest depended on obstacles encountered in the soil: tree roots or rocks for instance, with the nests curving around them. Some nests presented a tunnel leading to the main chamber making them deeper. The main chamber was a bowl like space covered with vegetable lining. Since nests can be reused, the plant material can accumulate and rot, producing several layers of dust like material mixed with the soil. The soil nests were often found near the roots of Ponga (*Cyathea dealbata*) and Puriri (*Vitex lucens*), but sometimes they were built entirely on the soil or using the plant roots as support (Fig.4.5).
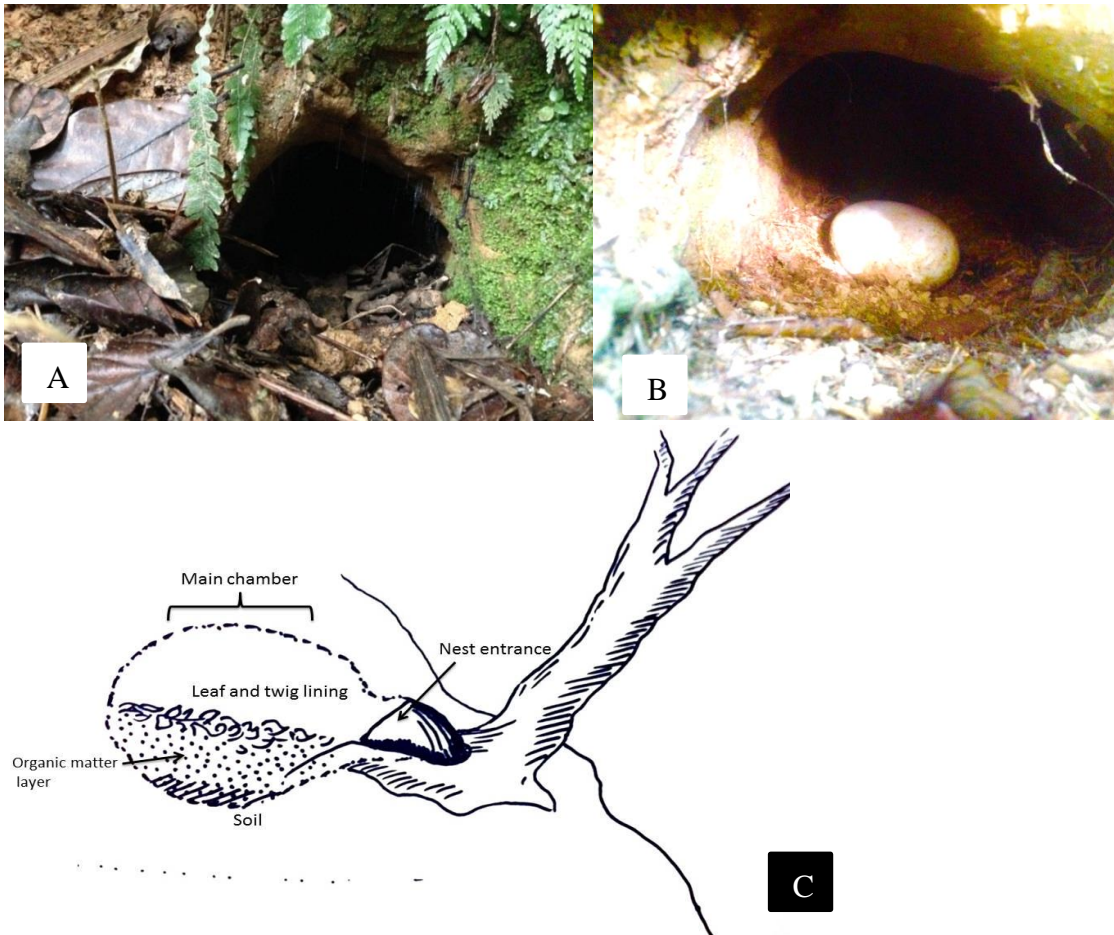
Figure 4.5. Typical Brown Kiwi soil nest. A) Entrance of a soil nest at Ponui Island. B) Nest with an abandoned egg pushed towards the entrance. C) Diagram showing the nest architecture, dotted lines show the underground cavity.

Brown Kiwi used fallen logs or hollowed trees as nests (Fig.4.6) choosing crevices or small chambers within as laying site. Tree nests were at times quite intricate, as the architecture depended on the tree structure and the available spaces within it. The dimensions of tree nests were difficult to grasp as the actual chamber tends to be difficult to reach and the size would depend on the tree itself. Brown Kiwi often covered the entrance of the nest with dried fern fronds and other litter (Fig.4.7).
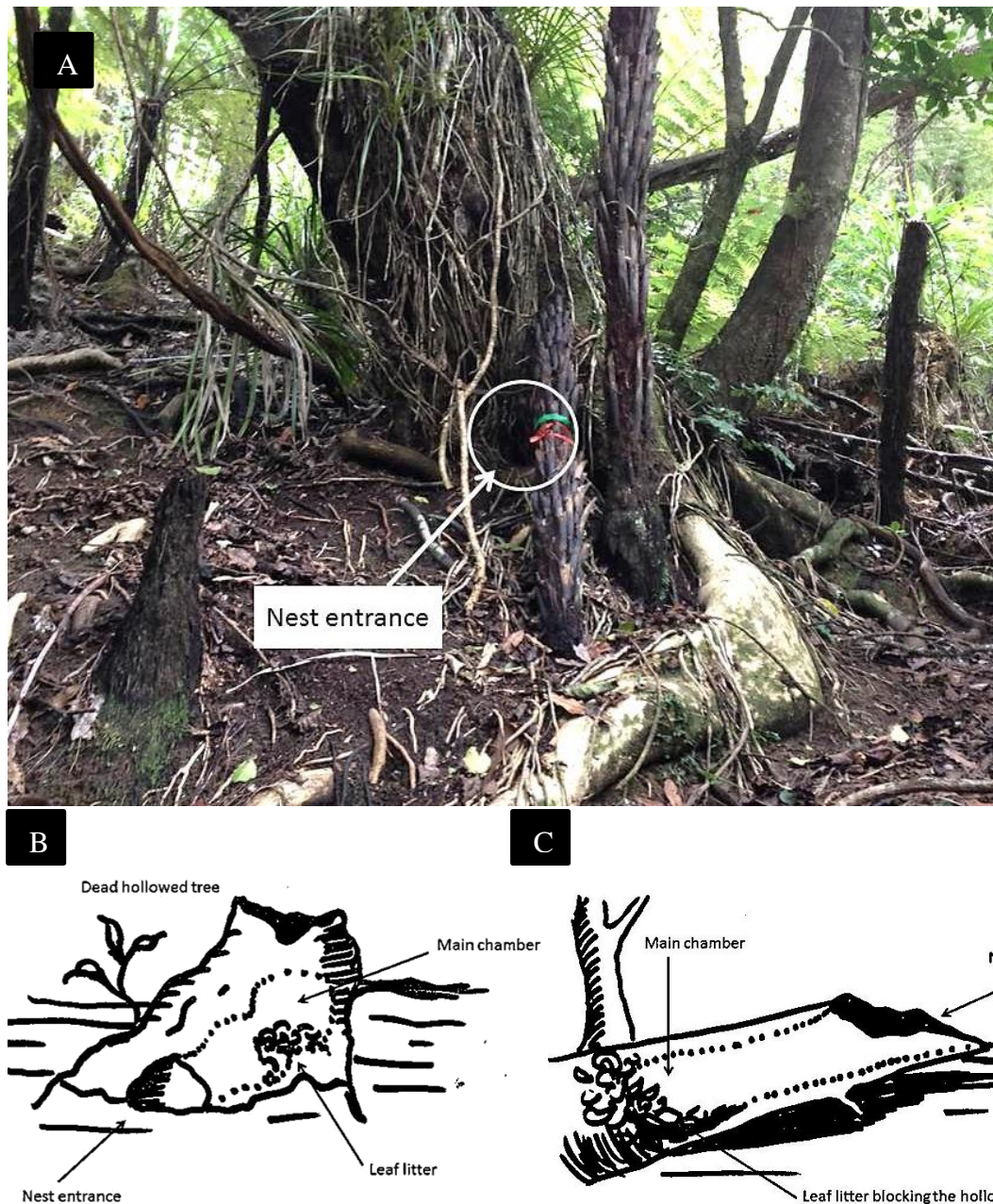
Figure 4.6. Examples of tree nests A) A Nest in a tree hollow (Puriri) covered by epiphytes. B) Diagram showing a nest cavity inside a dead tree. Note how the nest architecture depends on the tree structure. In this case the main chamber was up the dead tree in a very steep area. B) Nest on a fallen hollowed log. Here the Kiwi filled the log with plant material creating a plug. These are just some examples as tree nest can take any form as the Kiwi slightly modifies what it is available in the environment.

Figure 4.7. A) An example of a well concealed soil nest. The nest entrance, highlighted in white, is entirely covered by Ponga fronds. B) A soil nest seen from outside. Green tape and highlight was used to delimit the reach of the nest. These nests were all measured outside the breeding season and were therefore inactive.

Nest dimensions

The soil nests showed great variation in width and depth; however, height was very constant. Tree nests were more difficult to measure due to the tree structure, as often curves and crevices were out of reach; also, the measurements of tree nests could be less informative, as the dimensions depended on the tree and not the bird. However, some tree nests (n=4) were measured (Table 4.4).

Table 4.4. Measurements of soil nests and tree nests using a laser meter. Mean ± SD are presented.

Type	N	Width (cm)	Height (cm)	Depth (cm)
Soil	18	34.86±16.66	24.77±7.63	63.19±16.30
Tree	4	33.90±11.96	36.50±17.07	62.67±16.03

Nest microclimate

The burrow nest microclimate was slightly more stable than the external climate, as it was expected (Figure.4.8). The nest temperature lagged behind the changes in environmental temperature along the 45 days of measurements.
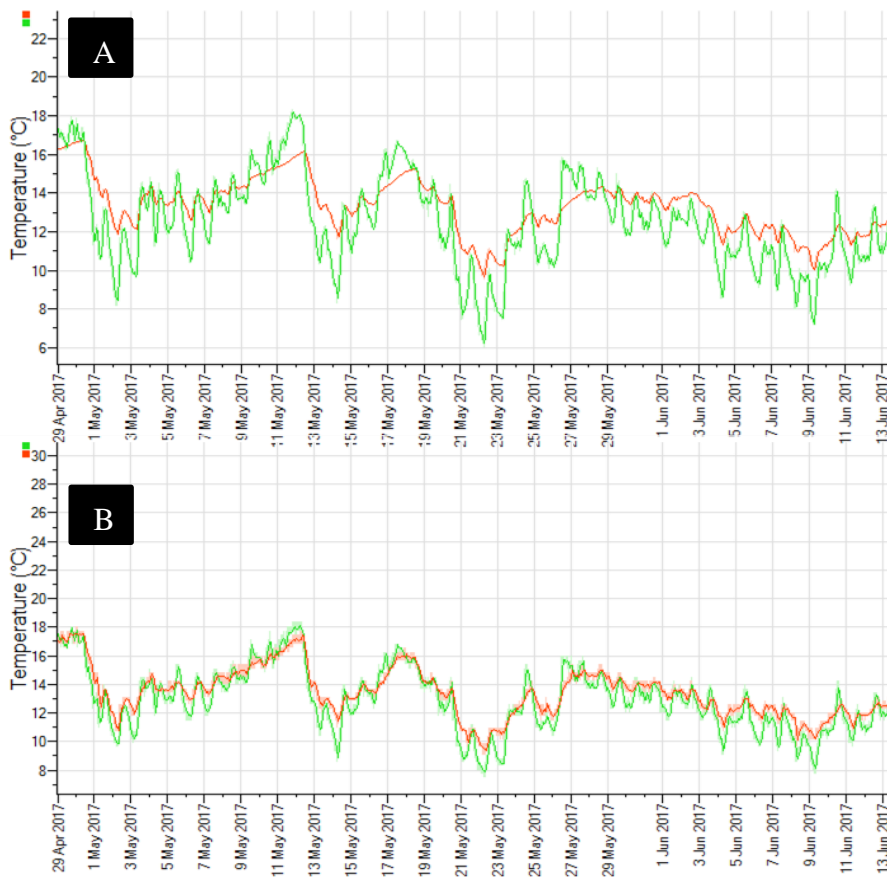
Figure 4.8 Comparison of temperature changes between the inside of a nest (Red) and the external temperature (Green). A) Soil nest, B) Tree nest in a period of 45 days.

The basal state of five nests was measured (three tree nests and two soil nests) and compared with the external temperature to determine the difference at each point and the frequency the nest is above or below the external temperature. The frequency of being above or below environmental temperature was also compared between tree and soil nests. (Table. 4.5).

Table 4.5. Frequency of nests being either above or below Environmental Temperature (ET) and the p values of the χ2 test applied.

Nest type	Soil	Tree	df	*p value*
Above ET	0.81	0.66		0.001
Bellow ET	0.19	0.34	4	0.001
	Comparison Soil vs. Tree, χ^2			0.002

Nest Temperature Buffering capacity

All the sampled nests (n=15) showed the same pattern of temperature fluctuation in respect to ambient temperature in an hourly basis, with very little temperature change (Fig.4.9). When the difference between the internal temperature and the ambient temperature are plotted together, it

becomes apparent that the nests remain cooler than ambient temperature during the day and remain a few degrees above ambient temperature at night (Fig.4.10).
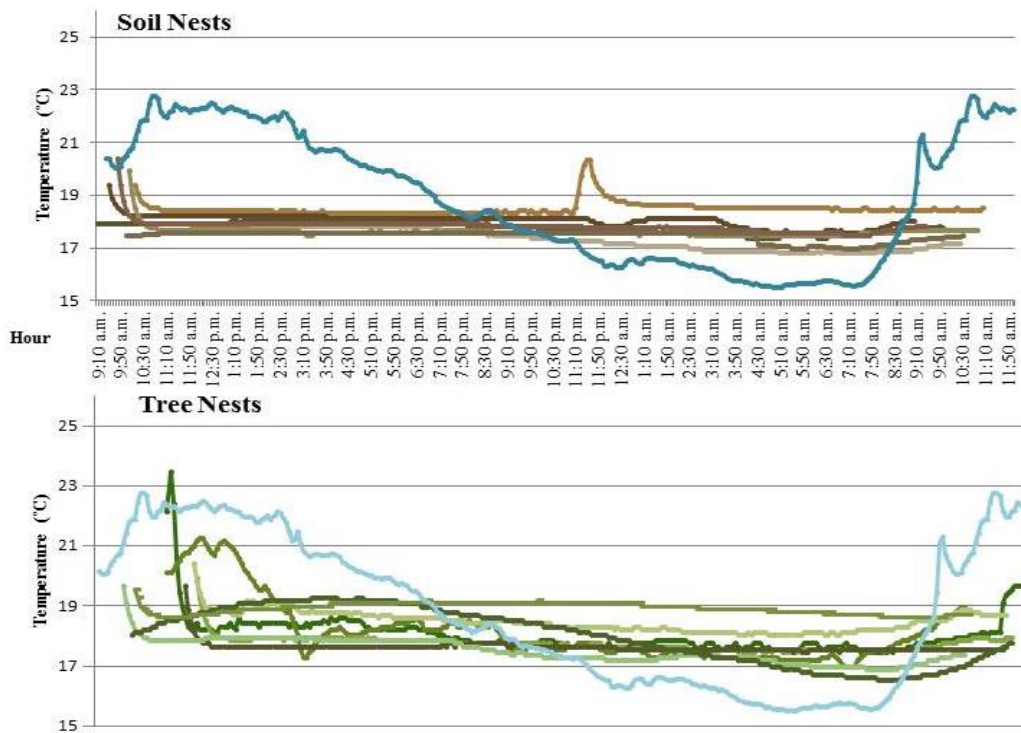
Figure 4.9 Two graphs showing the hourly temperature fluctuation inside soil nests (n= 7) and tree (n=8) nests with respect of ambient temperature (Blue lines).
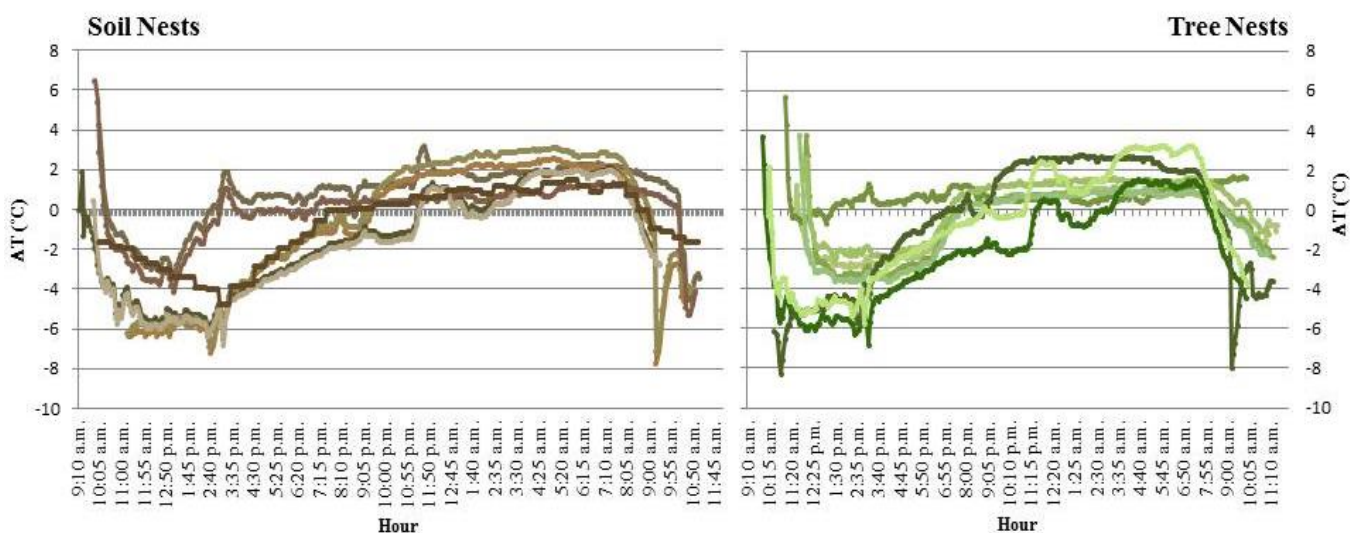
Figure 4.10 Difference between nest internal temperature and the ambient temperature (Tn-Ta). The zero line represents equilibrium, while anything below this line is cooler than ambient temperature and everything above is warmer than ambient temperature.

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Nest insulation capacity

The cooling rate of nests was very variable but on average, soil nests tended to keep warmth longer than tree nests (Table 4.6). Since not all nests reached temperatures near incubation during the experiment, I chose a similar initial temperature for all nests (average 19.85°C for soil nests and 19.76 for tree nests) at the initial time, from which the cooling constant and rate were calculated. There was no significant difference between the soil nests and tree nests.

Table 4.6 Summary of cooling time and rate of Soil and Tree nests from Ponui Island. Mean cooling time as an absolute measurement of time and the cooling rate is the temperature loss per hour.

Type	N	Mean cooling time (h)	SE mean	Cooling rate (°C/h)	SE mean
Soil	5	8.87	1.35	-0.42	0.143
Tree	5	6.66	1.00	-0.34	0.087
		F Value	1.73	F Value	0.19
		p value	0.224	*p value*	0.672

Nest contents

The nest was lined with dried leaves of the surrounding vegetation, soil clumps, argylosfaira, small twigs and dirt (Fig.4.11; table 4.7). The soil removal during nest construction produces dirt clumps (referred here as argylosfaira), that were often found outside of newly dug cavities and at the bottom of old nests (Fig. 4.11 A).

Table 4.7. Particle size and their weight percentage to the total lining contents of one Brown Kiwi nest. The capitalised letter in parenthesis after the type of soil matter relates to the photographs in Fig.11.

Contents	Particle size	Weight (g)	Percentage
Argylosfaira (A)	See figure	350	28.44
Soil clumps and big twigs (B)	>16 mm	59.4	4.83
Soil clumps and small twigs (C)	>4mm	586	47.62
Plant debris and dirt (D)	>2mm	50.5	4.10
Plant debris and dirt (E)	>1mm	57.9	4.70
Fine dust (F)	>500 μm	76.2	6.19
Fine dust (G)	<500 μm	41.7	3.39
Fine dust (H)	<250 μm	9	0.73

Figure 4.11 Particle composition of Brown Kiwi soil nests. A) Argylosfaira B) Twigs and rocks (Particle size >16 mm) C) Smaller twigs (Particle size > 4mm) D) Plant debris (Particle size > 2 mm) E) Plant debris (Particle size >1mm) F) Fine dust mostly organic matter (Particle size >500 μm) G) Fine dust, mostly organic matter (Particle size <500 μm) and H) Fine dust (<250 μm).

Hydrophobicity

The nest small particle material was very hydrophobic, as it was composed mostly of plant debris and chipped wood from twigs, the water droplet never penetrated this material. The clocks were stopped at 5 minutes, but the water droplet stayed on top of this material for a much longer period. On the contrary, the external soil material, which was mostly clay like soil and constituted the argylosfaira, was not hydrophobic, the water penetrated it almost immediately (Table 4.8).

Nest pH

All nest soils were acidic, with tree nests having the lowest pH (Table.4.9). There was a significant difference between the acidity of the nests and the external soil. This was due to tree nests being significantly more acidic than the surrounding soil, while soil nests did not significantly different from the external soil.

Table 4.8 Hydrophobicity (time taken for water particles to penetrate the nest soil and plant debris) of one Brown Kiwi nest. The Argylosfaira was crushed to produce particles of two sizes.

Particle size	Time to complete water penetration
>500 μm	>5 min
<500 μm	>5 min
<250 μm	>5 min
Argylosfaira	
>250 μm	16 sec
<250 μm	4 sec

Table 4.9. Summary of nest substrate pH. The superscript indicates the differences found using a Tukey post hoc test.

Nest type	N	Mean pH	SE mean	F-value	*P value*
Tree nest[b]	10	4.14	0.201	7.16	0.003
Soil nest[ab]	10	5.02	0.170		
External soil[a]	10	5.50	0.356		

Discussion

In this study, I found that Brown Kiwi eggs do not have any adaptation to prevent heat loss. I confirmed that egg cooling rate depends on egg mass. I also confirmed the assumption that the egg contents of different species have the same specific heat.

The Brown Kiwi nest can buffer the changes of environmental temperature maintaining a very stable microclimate for the egg development. In an hourly basis, the temperature inside the nest barely changed; at longer periods, the nest temperature lagged environmental temperature. The nests were above environmental temperature during the night when the parent is out foraging, and below environmental temperature in the morning, when the parent has returned to resume incubation. The nests took on average 8 hours to attain equilibrium with the environment once heated; the cooling rate for both tree and soil nests is less than 1°C per hour, showing the insulating capacity of the nest. There was not a significant difference between tree nests and soil nests in terms of cooling rate.

Egg cooling rates:
Overall, egg cooling depends on egg mass, as indicated by Turner (1985). However, it is possible that some aspects of the eggshell will affect cooling; this for example may explain why the Greylag Goose egg cooled at a similar rate as the much larger kiwi egg. This is perplexing as it

shows that there are other factors affecting egg cooling beyond mass. It would be expected that the cooling rate of the Brown Kiwi egg to be similar to that of the Emu and much higher than that of the Goose. It could be expected that egg composition , especially different species of fatty acid, would play a role on how slow or fast the egg will cool, as well as the thickness of the eggshell; the eggshell would have its own resistance to heat transfer and it should be proportional to the thickness. Tyler, (1962) indicated that the thickness for Greylag Goose was around 555 µm, the Kiwi egg is closer in size to that of the Emu, but the emu egg has a very thick eggshell, of approximately 1078 µm (Tyler and Simkiss, 1958). In comparison, the average Brown Kiwi eggshell is about 300 ± 47.9 µm (Chapter 3). Thicker eggshells would have the disadvantage of making the egg harder to warm up, therefore, the thin eggshell in the Brown Kiwi could allow for a faster rate of heat gain while is being incubated, and the nest will compensate by reducing the rate of heat loss.

Generally, for this type of study infertile eggs are used but it is possible that if the egg is fertile, the thermal properties of the egg change as the embryo develops and the egg loses water. In late stages of development the embryo passes from being poikilothermic to being homoeothermic and produces its own metabolic heat as a response to gradual cooling (Tazawa, et al., 1988). It has been noticed that ratites are efficient homeotherms and present all the same thermoregulatory adaptations present in other avian orders (Maloney, 2008). Unfortunately, since Brown Kiwi is an endangered species studies looking at the effect of gradual cooling on egg temperature at different stages are not possible, however, with the advances on ballistocardiography it would be possible to measure heart rate of Brown Kiwi in a non-invasive way while being artificially incubated. It would be expected that in agreement with a study by Ono, Hou, and Tazawa (1993), the Brown Kiwi embryo would respond to overcooling by retarding its development when at early stages, but after the 40th day of incubation (when metabolism increases drastically) the embryo would respond by producing metabolic heat.

An important aspect seen in all eggs is that the cooling is gradual. It took almost six hours for the Brown Kiwi egg to reach equilibrium with the environment; this feature can protect the embryo by slowly reducing metabolism and giving the incubating parent time to return and re-start the incubation by increasing the egg's temperature. Tazawa et al., (1988) showed how gradual cooling in chicken eggs is related to embryonic heat production at different stages of development. They found that the eggs respond to gradual cooling by either producing heat or slowly stopping development. However, this metabolic heat was insufficient to stop the egg from tending towards thermal equilibrium with the environment, and more importantly, it was insufficient to continue development (Whittow and Tazawa, 1991; Tazawa et al., 2001). Depending on the developmental stage of the embryo it seems that stopping temporarily the developmental process is a common strategy amongst birds and reptiles to withstand periods of low temperature. For American Kestrels (Sockman and Schwabl, 1998) and Japanese quails (Wentworth, Cigan, and Schaaf, 2009) at least, there is good evidence that long periods of egg cooling have no effect on embryo development.

Chicken embryos for instance can withstand temperatures as low as 8°C for a period of up to 20h and resume development once the egg is reheated at 38°C (Ono, Hou, and Tazawa, 1993).

Hypothermia seems to be less dangerous than hyperthermia in birds (Webb, 1978). Actually, many species experience very low incubation temperatures with no apparent consequence to the embryos (Webb, 1978). A physiological zero was suggested by White and Kinney (1974), as a low temperature (usually bellow 24℃) where the embryo development would stop and face imminent death, however embryos can actually tolerate cold temperatures by entering diapause, and depending on the species, this could happen for several hours to some days (Du and Shine, 2015).

Brown Kiwi eggs did not present any particular characteristic to reduce heat loss when compared with other precocial species. As a matter a fact the egg lost heat quite rapidly. However, the mechanisms the embryo might possess regarding slowing development or even entering diapause are unknown. Some species present very long incubation periods, and this has been hypothesised to be a response to long periods of egg neglect (Boersma and Wheelwright, 1979; Wheelwright and Boersma, 1979). Egg neglect refers to the time the parent spends foraging and not incubating the egg. Brown Kiwi has an incubation period of 74 days, which is 165% longer than expected for an egg its size (Calder, 1978, 1979; Tullett, 1984) . In birds, this requirement of retarded development is accompanied with a reduction in the overall metabolic rate (McNab, 1996) which is also the case for Kiwi (Prinzinger and Dietz, 2002). These are also adaptations that are suited to burrow nesting (Boccs et al., 1984). It is possible that the nightly egg abandonment in Brown Kiwi egg has shaped its incubation period, with the overall incubation period extending past what is expected from two reasons: the need of the embryo to slow down the developmental process during the coldest hors of the night, plus the production of a fully developed chick, both leading to a longer incubation period.

Nest cooling rate
The nest is a step-in egg protection, as it provides an environment that would buffer any sudden changes in temperature. It won't maintain temperatures of incubation but will reduce the rate at which the egg loses heat. An appropriate mathematical model for this interaction is needed, but in essence, as the father leaves to forage the nest would start losing heat but in an environment that would lose heat itself at a lower rate.

The nests were found above environmental temperature very frequently (75% of the time) but more importantly they seem to maintain cooler than ambient temperature during the day and above ambient temperature at night, precisely when the males go out and forage. The time it takes the egg for cooling is then reduced by the more stable environment of the nest. There is no evidence for a system of heat production inside the nests as it is the case of Megapodes (Dekker, 1988) or using heat radiation like in the case of Crab plovers (De Marchi et al., 2008).

The nest burrow lining material is laid in the bottom of the nest chamber and the hydrophobicity test suggests that the main purpose of the vegetable material is to keep the egg from dampening and being flooded. The weather during the Brown Kiwi breeding season is very wet, therefore having an insulating hydrophobic material between the egg and the nest soil could reduce the risk of dampness and help maintain temperature (i.e. a damped floor can extract heat much faster than a dried one; Oke,1988). Brown Kiwi are known to bury their eggs into this nest material (I. Castro, pers. comm.) supporting a moisture reducing and warmth maintenance role to this

material. In contrast, the soil (and main component of the argylosfaira) surrounding the nesting burrow is a very hydrophilic, indicating that this soil can be damped and remain wet for a long-time. The nest relative humidity has been measured in Ponui Island Kiwi burrows to be approximately 70%, remaining almost invariable during several months (Bansal et al., unpub data); the relative humidity reported for Brown Kiwi is not excessively high for a ground burrow, and it is possible that hydrophobic nesting material could help reduce the relative humidity of the nest by allowing condensed moisture to seep through the nest into the more hydrophilic ground (see argylosfaira hydrophobicity in table 4.8).

The fact that the nests are at about 70% Relative Humidity is very interesting, because this mean that the air in the nest is not completely saturated with water, thus allowing for a better gas exchange (Chapter 3), and it is not very different from the conditions outside the nest. Indeed, some New Zealand ecosystems could have much higher RH outside cavities. In previous models it would be difficult to understand how the reported low porosity of Brown Kiwi eggshells (Chapter 3) would be effective in alleged high humidity environments, as the water pressure from the saturated nest environment wouldn't allow gas exchange through small pores. However, it becomes apparent that the humidity in the nests is not as high and neither is the porosity.

There is evidence that Brown Kiwi selects vegetable material during the nest construction, as both males and females have been observed carrying plant matter from the immediate vicinity into the nest (I. Castro, pers. comm). It has also been mentioned that the birds clean the nests before the laying period, as often residues of eggshells, lining, and failed eggs are found near the entrance of the nests (I. Castro pers. comm.). This plant material in addition to water-proofing the egg would serve to insulate the egg from direct contact with the soil, preventing faster heat loss. In the southernmost *Apteryx* species, the Rakiura Island Tokoeka, this lining contains a great number of feathers (I. Castro, pers. comm), which could serve the same purpose plus reducing the heat loss of the egg. This feather use behaviour has been observed in numerous species including Tree Swallows (*Tachycineta bicolor*) and Barn Swallows (*Hirundo rustica*) (Møller, 1991; Lombardo et al., 1995; Peralta-Sanchez et al., 2010).

The gas composition within the nest has not been measured and it would be beneficial that the gas composition is looked in *Apteryx* nests as this is a fundamental piece to understand the relationship between eggshell porosity and thickness with nesting behaviour and architecture.

To ensure a functional egg, Kiwi needs to balance a thin eggshell with a big mass, a particular geometry to withstand the weight of the adult. In this respect there is evidence that Kiwi has an adaptation to seat on such a big egg, in the form of enlarged ribs (Abourachid et al., 2019), an adaptation seen also in the Hoatzin (*Opisthocomus hoatzin*) (Livesey & Zusi, 2007). Besides the structural support that combines egg resistance with anatomical adaptations in the incubating parent the Kiwi eggshell needs to allow proper gas exchange and a composition that would allow an extremely precocial chick to survive on its own. They also require an egg that can withstand abandonment, cooling and the high risk of bacterial and fungal infections. These traits need to be paired with an effective mating system and refined incubation behaviours to ensure reproductive success. The Brown Kiwi egg is adapted to a low oxygen environment as can be seen by its porosity, like other burrowing birds (Chapter 3) and a thicker cuticle that would allow

for a reduced risk of bacterial penetration in the nest environment. This is apparent that the nest evolved congruently with the egg, as the egg became bigger and the eggshell thinner, an environment that could allow for a controlled microclimate and a buffered temperature changes was necessary, especially in a rapidly weather changing environment as insular ecosystems tend to be. In contrast to the other ratites, whose nests are in an open scrap and the eggs directly exposed to the elements.

The kiwi nest is unique amongst ratites and the evolutionary pressures that require for such a "jump" are long gone, but it could be suggested that during the glaciation period it became important for *Apteryx* to protect the egg from the elements and potential predators. It has been suggested that New Zealand avifauna presents more tree cavity nesters than the same families in other regions (Rhodes, O'Donnell and Jamieson, 2009). The aforementioned work examined only terrestrial tree cavity nesters, some of the ecological pressures experienced might be the same for *Apteryx*. The authors suggested that one of the main reasons for this behaviour to become widespread in New Zealand avifauna is likely explanation to be buffering of changing environmental conditions, but also potentially escaping predators.

To further understand the adaptations of Brown Kiwi to incubation it would be necessary to see how the egg loses and gains heat in the nest and the mechanisms the incubating parent uses to regulate temperature. A first approximation has been done by Rowe (1978), McLennan and McCann (1991), and Colbourne (2002), who have used dummy eggs to follow the temperature of the eggs while being incubated, mainly with the purpose of establishing the incubation temperature of Brown Kiwi eggs. However, a model exploring the temperature fluctuations of an actual egg in the nest would better clarify the incubation strategy of Brown Kiwi.

Chapter Five

A Genetic study of the Mating System of North Island Brown Kiwi in the Maungataniwha Forest

Coniunge fratrem cum sorore et propina illis poculum amoris

(Join the Brother and the Sister and drink to'em the Bowl of Love)

Michael Maierus, 1619

Abstract

Polygynandry is a mating system where females mate with several males and males with several females; this mating system seems to be the most common mating system amongst ratites. Molecular studies have demonstrated that most ratites have a high degree of mixed paternity and maternity. The genus *Apteryx* has been the most noticeable exception as it is usually described as monogamous. Brown Kiwi (*Apteryx mantelli*) present all the typical morphological traits of sex role reversal and most importantly, the female is not involved in incubation or parental care, leaving her with the opportunity to re-mate as soon as a male's clutch has been laid. In sex role reversal, usually the male incubates alone or provides most of the parental care, and females tend to be bigger and more aggressive than males. Most species that present this mating system lay eggs that are numerous and small, have precocial chicks that require little to no parental care after hatching. These characteristics are usually associated with polyandry, where a female mate either sequentially or successively with several males, while in polyandrous systems males tend to be monogamous. I tested the assumed monogamy of Brown Kiwi from a molecular perspective by assessing the relatedness of chicks obtained from the same male's nest. In this study I tested the hypothesis that the mating system of Brown Kiwi is either polygynandrous, like other ratites, or polyandrous, due to its sex role reversal. For this purpose, I developed a simple technique to extract DNA from eggshell membranes, meaning this study was entirely non-intrusive. I looked at the genetic mating system of Brown Kiwi in the Maungataniwha Forest, and I found that the eggs laid in a radio-tagged male's nest have different parentage. This occurs within clutches, between clutches during the same breeding seasons, and throughout the years. Interestingly, some chicks are related to other chicks hatched in different years, suggesting that Brown Kiwi probably re-mate with the same partners throughout the years. This suggest that either Brown Kiwi has a promiscuous mating system or at least a high degree of extra pair copulations.

Introduction

In recent years molecular studies have revealed the complexity of avian mating systems, suggesting that many of the former classifications (i.e. monogamy, polyandry, polygyny) based on observable mating and parental behaviour are not as well delimited as once thought. For example, extrapair paternity has been found to be extremely common, occurring in 90% of all avian species, including those classified as socially monogamous (Griffith, Owens and Thuman, 2002). A fascinating clade to study this diversity of mating systems is the ratites. The Ratites are an ancestral group that present interesting mating systems and associated parental strategies. It could be said that the mating system of ratites revolves around polygynandry, with major males and females controlling nests, but with multiple contributors to the parentage of those nests (Handford and Mares, 1985). Their incubating behaviours range from those where males are the main contributors to

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incubation like Ostriches (*Struthio camelus*), or not at all, as it is the case for Emus (*Dromaius novaehollandicus*) and Rheas (*Rhea americana*). The few studied species of Tinamous seem to also have male incubating and caring for young, this has been indicated for the genus *Eudromia* (Hanford and Mares,1985) and the Great Tinamou (*Tinamus major*). Only male care has also been reported in the Kiwi Pukupuku (*Apteryx owenii*) and Brown Kiwi (*Apteryx mantelli*). Another important aspect of ratites in general is that this clade also produces very precocial young.

In Ostriches, Emus and Rheas, males tend to hold harem like groups where several females lay eggs in different male's nests, but the males still perform courtship displays and defend territories (Patodkar et al., 2009; Sauer, 1972). For example, the male Ostrich defends a territory and prepares several scrape nests one of which is later chosen by a female that is regarded as "the major female" (the first female to lay eggs in a nest), this is followed by several other females that lay eggs in the same nest (known as minor females). The male is the one that initiates the mating displays (Magige et al., 2009). Using microsatellite DNA extracted from egg chorio-allantoic membranes and embryonic tissue, it has been determined that the major female contributed more to the central clutch but in the periphery mixed paternity and maternity occurred (Kimwele and Graves, 2003). In their study all males fertilized eggs in nests of neighbouring males and every major female was the minor female of some other nest.

Oring (1990) reported the mating behaviours of Rheas. He observed that groups of females form harems and lay eggs for a single male who takes care of the incubation. They found that females lay the eggs beside the nest and the male rolls them into the nest. Once the females have laid eggs for the male, they move onto a second male who defends territories as well. The male displays a great vigilance of the eggs leaving only for a few minutes every day. The male remains associated with the chicks until the next breeding season. Males defend the nest aggressively (Codenotti and Alvarez, 2006; Fernandez and Reboreda, 2007).

In a similar way to Rheas, Emu males undertake incubation alone and devote a considerable amount of energy to the incubation, reducing food intake and entering a torpor like state (Taylor et al., 2000). Despite the great investment made by the male, emu nests present a mixed parentage with approximately 50% of the chicks not sired by the sitting male, 11% not mothered by the "major female", and about 8% resulting from brood parasitism. Emus were thought to be monogamous; however extra-pair copulations have been reported, with 73% occurring towards the end of the breeding season (Blache, Barrett, and Martin, 2000).

In Cassowary (*Casuarius sp.*), Crome (1976) reported that the male provides all the parental care and that females might mate with more than one male in a year, however very little is known about the behaviours that accompany the mating system of cassowaries.

In the species studied (*Tinamus major*), male tinamous provide all the incubation effort and it has been observed that they have a "promiscuous" system where a male can mate sequentially with several females, gathering a nest of up to eight eggs, while females move

between males without forming a pair bond with any male (Brennan, 2012). Monogamy was first suggested for the Ornate Tinamou (*Nothoprocta ornata*) but recent studies using genetic analysis of the eggs laid in males' nests, present evidence of mixed paternity concordant with observations of females mating sequentially with males, and males mating simultaneously and caring for the eggs of several females (Giraldo-Deck et al., 2017). The mating system of many species of Tinamous is still not known.

Brown Kiwi has been classified as a monogamous species by some authors (Handford and Mares, 1985; Taborsky and Taborsky, 1999; Colbourne, 2002). More recent research with marked individuals in a high-density population on Ponui Island have reported kiwi breeding in groups in addition to pairs, trios formed by two males and a female as well as a male and two females and even quintets; pairs have been observed to re-mate in successive breeding seasons but "divorces" are also common (Ziesemann, 2011; I Castro pers. comm.). Brown Kiwi present sex role reversal as the male incubates alone and the female does not generally engage in any incubation behaviour or parental care post hatching. The female is larger than the male and lays two eggs, each weighing from 250 to 400 g in each clutch, with usually two clutches laid in the same breeding season (Colbourne, 2002). The male alone incubates the egg, spending between 74-84 days incubating the egg(s) (Calder, 1978). Females are known to take 35 days to produce an egg and with both ovaries functional, to develop two eggs simultaneously with one laid about two weeks ahead of the second (Colbourne et al., 2005). Under these circumstances, the female could re-mate and produce eggs with another partner while the male is incubating. The male does not seem to have many opportunities of re-mating, unless breeding and sharing incubation with others in a group. Nothing is known about sperm storage in kiwi, but it could be possible that the female copulates with multiple males and stores sperm for fertilising her eggs, as this has been observed in all other avian species in which sperm storage has been studied (Briskie and Montgomery, 1992; Birkhead, 1998).

Since the arrival of Europeans to New Zealand, Brown Kiwi populations have been in decline due to introduced mammalian predators (Holzapfel et al., 2008) and loss and fragmentation of habitat. One crucial aspect that influences the mating system of a species is the availability and density of nesting sites (Emlen and Oring, 1977; Goymann, Safari, Muck, and Schwabl, 2016). In an area with low, scarce or spaced nesting sites would make it difficult for the females and males, respectively, to control territories and thus making difficult the evolution of polyandry or polygynandry. For a polygynandrous species with ratite like systems, nest site limitation could make it difficult for sequential mating, as each individual would have difficulty finding another mate with an established nest. Population density will also play an important role in determining mating systems, as higher density populations will increase the probability of two or more birds coming in contact in a short amount of time.

It is therefore possible that monogamy in Brown Kiwi was assumed to be the case due to population densities being low enough that females would choose and mate with the same males twice every season, only because there were no other potential mates, or nesting sites in the area. Also, the cost of the egg has been traditionally thought to be too great for the

female to engage in other copulations (Calder and Parr, 1977), and this argument has been used as further support of the monogamous hypothesis, however, this could be debated as the energetic cost could be supplied by the insectivorous diet of Brown Kiwi, which would be rich in protein and lipids (Bell, 1990; Reynolds and Perrins, 2003). In addition, Dixon (2015) showed how Brown Kiwi can supplement this diet throughout the year, meaning that there is not a seasonal shortage of food. Calcium would be a limiting factor as many species need to accumulate the calcium required for reproduction prior to the breeding season (Ankney and Scott, 1980; Wilkin et al., 2009). However, many species present a mechanism to store calcium in the medullary bone in the form of citrate (Simkiss, 1961) and Brown Kiwi have considerable amounts of citrate in their bone (Dennison and Kooyman, 1991), which added to the fact that the eggshell is very thin (Chapters 3 and 4) would make it much less costly to have the necessary calcium to lay multiple eggs in one breeding season.

Brown Kiwi have all the traits that are classically associated with polyandry, and this was noted by Jenni (1974) and discussed by Ziesemann (2011) with evidence of long-term associations between females and two or more males. The observed plastic mating systems of other ratites and the fact that polyandry seems to be conserved in a select group of orders makes it necessary to revisit the monogamous status of Brown Kiwi, especially in the light of possible incubation contributions made by females in the Roroa, Rowi and Tokoeka, and the reported trios and quintets from the Ponui Island population (Ziesemann et al, 2011). It is not impossible that *Apteryx* evolved a monogamous system, but it becomes necessary that substantial evidence is produced to assert this mating system. It would be very interesting if it were the case, as new questions could be asked on how mating systems shift through time and what are the selective pressures behind them.

In polyandrous shorebird species, where the male is the sole contributor to incubation and parental care, there is a negative relationship with extra pair paternity, suggesting that the evolution of this strategy has required that males are "certain" that the chicks they are raising are theirs (Oring et al., 1992; Delehanty et al., 1998; Emlen et al., 1998). In most ratites, it has been demonstrated that high levels of extra pair paternity occur for both males and females, and the chicks are precocial, meaning that they require much less parental investment. In this scenario certainty of paternity may not be as necessary as the males increase their fitness by engaging in extra-pair copulations and ultimately the chicks will not require much caring.

In the last decade, studies employing molecular techniques have uncovered a great deal of mixed paternity. For species that have been described as socially monogamous, only 25% or less are genetically monogamous, meaning that they only incubate and raise their genetic offspring and no mixed paternity occurs (Griffith et al., 2002). Genetic studies are particularly relevant in this field as they accurately depict each parent's genetic input to future generations and serve as a testing ground of the traditional hypotheses proposed to explain the evolution of mating systems.

Paternity in birds has been frequently examined based on multilocus DNA fingerprinting with minisatellite probes (Westneat et al., 1995; Krokene et al., 1996); however, these methods require large amounts of good quality DNA. A more cost-effective and probably less invasive alternative is the use of microsatellite loci amplification, which is effective in providing reliable information about genetic variation (Taylor et al., 2000). Paternity of rare or endangered species is difficult to analyse because of the low probability of encountering the study species and the risk of causing nest abandonment, therefore non-invasive methods that could use tissues that are not obtained directly from the birds are necessary. This was done for the Ornate tinamou using embryonic and egg material from nests (Giraldo-Deck et al., 2017), however in this case it was necessary to destroy the eggs. In the current study, I use only eggshell material and the chorio-allantoic membrane that remains in the discarded eggshells after hatching.

In this chapter, I test the assumption of monogamy in Brown Kiwi from a genetic perspective, comparing the eggs laid in the same nest, in different clutches in the same nest and in the same nest over several years. In this way, I estimate the frequency in which chicks are parented by the same male and female in each clutch within a breeding season and throughout the years. This study was conducted using eggshells obtained from ONE, the eggs in turn were collected from the Maungataniwha Forest, Hawkes Bay.

Hypothesis

Based on what has been reported when molecular techniques have been previously applied to the mating systems of ratites, it is possible that Brown Kiwi present a similar mating system with females mating successively with different males, observational evidence for which has been reported in high density populations (Ziesemann et al., 2011). It is not impossible that Brown Kiwi present a genetically monogamous system, but it seems unlikely. Therefore, I expect to find extra pair paternity in the nests of males when the parentage of the hatched chicks is compared. I will compare the parentage between eggs belonging to the same clutch, between clutches in the same year and between years to determine the frequency of extra pair paternity if it exists.

Materials and Methods

Study area and Sample sourcing
Artificially incubated eggs from the Operation Nest Egg program (ONE) were used (see Chapter 3). The eggs were harvested from the Maungataniwha Forest (Lat -38.82 Long 176.90) in the Hawkes Bay, New Zealand (Fig. 5.1). The forest is bordered by the Te Urewera National Park on the north, and by the Whirinaki Conservation forest on the west. It comprises 6,120 hectares of native bush (Forest Lifeforce Restoration Trust, 2019). This population was chosen as it provides the highest number of eggs every year to ONE (E. Bean, pers. comm.) allowing for a greater sample size. Sixty-six eggshells were obtained; these belonged to eleven different males from the years 2012-2017 (Table 5.1).

Figure 5.1. Map of New Zealand showing the location of Maungataniwha Forest in the Hawke's bay. Map produced with Google Earth.

Table 5.1 Summary of the males used in this study and the total number of eggs used. The number of eggs produced per year was noted, the dashes are placed where no eggs were either produced or retrieved from the nests.

Male's code name										
	Mt.dew	**Mt.ts**	**Mt.twa**	**Mt.stt**	**Mt.q**	**Mt.sm**	**Mt.sh**	**Mt.pr**	**Mt.dm**	**Mt.wu**
Total No. of Eggs/male	12	11	10	8	6	6	5	4	2	2
Eggs per year										
2010	1	-	-	-	-	-	-	-	-	-
2012	-	-	3	1	-	1	-	1	1	1
2013	-	-	-	-	-	1	-	-	-	-
2014	3	4	3	1	1	-	2	3	-	-
2015	2	2	1	1	1	1	-	-	-	1
2016	5	2	1	3	1	1	-	-	1	-
2017	1	3	2	2	3	2	3	-	-	-

DNA Extraction

A fragment of the chorioallantoic membrane was removed from the inner eggshell of each egg; fragments containing vascular tissue or dried blood were favoured to maximise DNA yield. Membranes were placed in Eppendorf tubes with 400 µl of rat-tail lysis buffer (100 mM Tris.Cl pH 8.0, 5 mM EDTA, 0.2% SDS, 200 mM NaCl), 10 µl of proteinase K (Sigma 3115887001) was added to the membranes and vortexed for 2-3 seconds (s). The tubes were placed in a foam float in a water bath at 56°C overnight. Supernatants (lysates) were

transferred to 1.5 ml tubes and 400 μl of chloroform was added to each tube and then vortexed for 20-30 s. The lysate-chloroform mix was then centrifuged at 14,000 g for 10 min. The aqueous supernatant (approximately 300 μl) was then transferred to a fresh 1.5 ml tube. 400 μl of 100% ethanol and 40 μl of 3M sodium acetate was added to the lysate and mixed. The mixture was then placed for one hour to overnight in a freezer at -20°C.

The lysate mix was then centrifuged at 14,000 g for 10 min. The supernatant was carefully poured off and discarded. 400 μl of 70% ethanol was added to the tube and mixed thoroughly. The tubes were then centrifuged at 14,000 g for 10 min, and the supernatant discarded again. This process was repeated one more time discarding the supernatant at the end. The remaining DNA pellet was left to air dry overnight. 50 μl of R40 (TE (10 mM Tris, 1 mM EDTA, pH 8.0) with 40 μg/ml of RNaseA) was added and left overnight at 4 °C to resuspend the DNA.

Microsatellite markers and amplification protocols have been previously developed for *Apteryx* spp. by Shepherd and Lambert (2006) and Ramstad et al., (2010). I chose eight microsatellites with the highest degree of heterozygosity and amplified all of them (Table 5.2). Eight micro-satellites have been proven to be enough in similar studies, Brennan (2012) used eight microsatellites to assess Great Tinamou paternity, and Taylor et al., (2000) used only four, however in this case, DNA from incubating males was also extracted.

The amplifications were set at 50°C, 56°C, and 60°C. Amplification was carried using 5Prime HotMaster Taq DNA Polymerase (Dnature); individual PCR mixes were composed of 4.9 μl of nuclease free water, 2 μl of Taq, 0.3 μl of each of the forward and reverse primers, and 2.5 μl of DNA template. The PCR products were diluted 1:10 (10 μl of PCR products and 90 μl of nuclease free water) and from this dilution 30 μl were used for fragment analysis. The fragment analysis was performed using the HT DNA High Sensitivity LabChip Kit.

Table 5.2 Microsatellite DNA loci from North Island Brown Kiwi used for fragment analysis. Ta indicates the annealing temperature and HE the expected heterozygosity. This information was obtained from Shepherd and Lambert (2006) and Ramstad et al., 2010.

Locus	GeneBank Accession code	Sequence	T_a (C°)	H_E
Apt29	DQ157367	F: AGTAGCTACATGCGTACGTGTC R: TGGCCCACCTGGAGATGTGCA	56	0.820
Apt35	DQ157368	F: CAGCTTGTCTCAGGGAGCATTTGT R: CTATCTCAAGCGGCATCACAAAG	58	0.801
Apt59	DQ157362	F: TCTGTGCCTTGGAAGCAGTC R: GGAAGCTTGGGATCACTGGG	56	0.737

Apt68	DQ157365	F: GGACCAGTGTGTTTATATATTCTGC	56	0.558
		R: TGCAGATTCAGCCAGTAACG		
Aptowe8	HM064032	F: AAGTCCATCAGCTCAGCAATC	56	0.917
		R: AACAGCAAACTTAGGGACACG		
Aptowe15	HM064033	F: CCAAAAGTACTGCAGGGTTATTC	50	0.933
		R: ACATGGCTTAACTGCCACTG		
Aptowe31	HM064037	F: GTTAAAATCAATCAGTATCCATTCC	56	0.783
		R: GATCCCAGTAAAAGCCAACTT		
Aptowe39	HM064041	F: AAAATAGAACCTTCCAAGCTGAAC	56	0.665
		R: ATTCAGTGGGAAAAGCTTGC		

Analysis

Kinship coefficient

The relatedness of the chicks was calculated using a kinship coefficient. The kinship coefficient estimates the probability that alleles in a particular locus, chosen at random from two individuals are identical by descent (Lange, 2003). Identical by descent in this case means that the two identical alleles of two individuals arise from the same allele in an earlier generation.

The model is as follows:

$$Kf = \sum k \sum a \ (f_{ai} * f_{aj})/D \qquad \text{(Eq.5.1)}$$

Where $\sum k \sum a \ (f_{ai} * f_{aj})/D$ is the sum of all loci and all alleles; f_{ai} being the frequency of the allele a in a population i, and f_{aj} is the frequency of the allele a in the population j, D corresponds to the number of loci (Cavalli-Sforza and Bodmer, 1971). In this model individuals can be treated as populations. A kinship matrix was produced for all the eggs laid in the same nest; this indicated the relatedness of the chicks in a pair-wise relationship.

Since only one allele is being sampled at a time, the interpretation of the kinship coefficients is based on the probability of obtaining the same allele for each pair-wise comparisons, which is 50%; the calculation of the kinship coefficient takes into consideration this probability as an independent event (Oliehoek, Windig, Van Arendonk, and Bijma, 2006).

I interpreted then the kinship coefficient between two full siblings to be between 0.5 and 0.25, between half siblings to be >0.25 and 0.125 and finally any unrelated individuals would have a kinship coefficient of 0. This was calculated for all the nests.

Results

Kinship

The degree of Kinship was variable in all sampled nests except in two nests, which only had two eggs each, in both cases each egg laid in a different year. In the first nest (Mt.dm) the two eggs were unrelated and in the second nest (Mt.wu) the two eggs were full siblings. All nests presented full siblings, half siblings and completely unrelated individuals throughout the years (Table 5.3).

Table 5.3 Percentage of possible relationships per nest. The number of possible relationships is the number of possible pairwise combinations that could be achieved with the number of eggs. Each relatedness percentage (i.e. full siblings) is the percentage of associations found in each nest.

Male	No. Eggs	No. possible relationships	Full siblings (%)	Half Siblings (%)	Nonrelated (%)
Mt.dew	12	66	29	24	47
Mt.ts	11	55	24	42	35
Mt.twa	10	45	29	27	44
Mt.stt	8	28	18	39	43
Mt.q	6	15	20	13	67
Mt.sm	6	15	13	47	40
Mt.sh	5	10	30	50	20
Mt.pr	4	6	33	67	0
Average			24.5	38.5	37

The relationships between eggs of different years were difficult to assess because each individual was either related or not, to many other individuals in the nest, thus creating a very dense web of relatedness (Appendix II). To simplify, I looked at all the eggs laid per breeding season to clarify their relationships. In this part of the analysis, breeding seasons represented by a single egg had to be excluded (Table 5.4).

Table 5.4 Number of siblings, half sibling and unrelated individuals per breeding season. The numbers within parentheses () represent full siblings, the groups within braces {} indicate half siblings and the numbers outside any bracket represent the unrelated individuals; For example, in 2014, Mt.Dew had a pair of full siblings and an unrelated individual, while Mt.ts that same year had a pair of full sibling which had in turn two half siblings. A graphic representation of the relationships between the eggs of a nest can be found in Appendix II.

Male	Mt.dew	Mt.ts	Mt.twa	Mt.stt	Mt.q	Mt.sm	Mt.sh	Mt.pr
Breeding season								
2010	1	-	-	-	-	-	-	-
2012	-	-	{(2)1}	1	-	1	-	1
2013	-	-	-	-	-	1	-	-
2014	(2)1	{(2)2}	(3)	1	1	-	2	{(2)1}
2015	2	{2}	1	1	1	1	-	-
2016	{(3)2}	2	1	{2}1	1	1	-	-

| 2017 | 1 | {3} | (2) | 2 | (2)1 | {2} | {3} | - |

Discussion

I found that Brown Kiwi are not a genetically monogamous species, as 90% of the nests showed variable kinship coefficients, from full siblings to half-siblings and unrelated individuals. It was interesting to find that full siblings occurred between different years, suggesting that Brown Kiwi pairs re-mate in different years, or have stable groupings which could explain why the species was classified as monogamous before. Also, different contributors are found in the same clutch and in between the two clutches in a breeding season. This is the first time that the mating system of Brown Kiwi has been studied using eggshell membranes and it proved to be an effective method to assess relatedness between individuals.

The relatedness between individuals from the same breeding season was varied, less than 30% of the eggs in a breading season were full siblings, and almost 40% were unrelated individuals. Nevertheless, the relationships across years are complex. To illustrate, four eggs were obtained from Mt.pr's nest, three eggs laid in the same breeding season and one laid in a previous season; two of the eggs laid in the same breeding season were full siblings while being half siblings with the third egg. The third egg was a full sibling of the egg laid in the previous season, which in turn was half sibling to the other two, as it would be expected. This could mean that the male mated with a female which laid two eggs, but in the same breeding season he mated with another female who laid one egg, this male mated with that same female in a previous season.

In general my findings suggest that male Brown Kiwi mate with several females throughout the years, however, since I didn't obtain the parental DNA it is also possible that the female mated with several males, laying all the eggs in one male's nest, despite the male not being the sire of all the eggs in the clutch. This would also explain half siblings as the shared parent could be the mother and not the father.

The relatedness between the chicks was not always axiomatic; axiomatic in this case refers to the logical possibility of the described relationships. For example, there cannot be a half sibling to only one in a pair of full siblings, it necessarily must be half sibling to both. The presence of non-axiomatic relationships indicates the possibility of sampling errors, as in some cases a particular primer would not amplify for a particular individual thus losing information. A correction can be made with the available information to re-construct a more accurate web of relatedness between all the chicks associated to a male; to achieve such correction a series of logical axioms need to be defined to determine were the information might be lost and clarify further the relatedness. Also, obtaining the mother's DNA from the eggshell would not only help clarifying the relatedness between chicks but also determining in which cases the father is the shared parent or not.

Regardless of the fine detail of individual relationships my results demonstrate that Brown Kiwi is not genetically monogamous as unrelated individuals and half siblings are present in the same breeding seasons and across years. This is not surprising as most molecular studies performed on ratites have found similar mating systems, and furthermore, recent studies have shown that most avian species, regardless of the apparent mating system engage in extra-pair copulation (Akçay and Roughgarden, 2007). These results show in general that avian mating systems are not as rigid as the classical classification suggests. In the case of Brown Kiwi, such a system is not difficult to understand as kiwi chicks are extremely precocial, meaning that there is no parental investment after hatching, and more importantly, that only one parent is needed to ensure the hatching of the eggs and fledgling of the chicks.

From an ecological perspective several authors have suggested different scenarios where mating systems with a single incubating parent could arise. For example Andersson (2005) suggested that living in habitats were food is abundant would facilitate chick survival in the case that a single parent needs to take care of the clutch, but also would allow the female to store enough resources to produce multiple eggs and clutches; this scenario would give the female alternative reproductive opportunities. An important point is that the clutch size is determinate; therefore, the annual reproductive effort could only be increased by multiple clutching instead of laying more eggs in the same nest. As the clutch size increases it becomes more difficult for a single incubating parent to take care of all the eggs (Cody, 1966) thus making it more effective for the female to stay and help. Brown Kiwi have continuous sources of food throughout the year (Dixon, 2015) with the male continuing to forage throughout the incubation period making the incubation effort less energetically costly. In contrast, the male emu does not eat, drink or pass waste for the duration of the incubation (Patodkar et al., 2009). Also, as I have mentioned previously in this thesis the Brown Kiwi egg is big, therefore incubating more than two or three eggs at the same time would be very difficult for a single parent.

Maynard-Smith (1977) presents an alternative hypothesis for male parental care and female desertion, in his explanation male parental care would be favoured if the male has a low probability of finding another mate, the fecundity of the female increases with desertion, and parental care provided by a single parent would greatly increase chick survival but the aid of a second parent won't increase chick survival further. Female desertion would only contribute to individual fitness if she re-mates. If she does not re-nest, aiding in incubation would be favoured, even if a second parent does not drastically increase the probability of clutch survival. In this scenario if the female stays, she would have the opportunity to monopolise the male. In contrast by deserting, the female gives a chance to other females to lay eggs in her mate's nest.

It has been suggested that the Brown Kiwi egg is very expensive to produce in terms of energy (Calder, 1979), this has been used as an argument to support the hypothesis of a monogamous system in Brown Kiwi, under the notion that the female would be too exhausted after laying two eggs in one male's nest to try to find other mating opportunities. It could be argued that the constraint towards laying more eggs would be related to the size

of the egg, the proportional space that it occupies inside the female, and the possibility for males to incubate that many eggs at the same time, and not necessary the energetic and metabolic cost of production. The fatty acids needed for yolk production in Brown Kiwi are sourced from an insectivorous diet obtained prior and/or during the breeding season (Dixon, 2015). The remaining contents of the egg are protein that again would be abundant in an insectivorous diet (Bell, 1990). Nevertheless, any egg represents a significant cost for the female, but if the cost was so high it would be surprising that the female wouldn't make sure that the eggs laid in a nest successfully hatches, by being involved with or associated with their incubation; the only benefit to this would be increasing her own fitness by re-mating.

From the male's perspective, precocial chicks are inexpensive to raise as the only energetic cost for the male is incubation, and the male can replenish his resources by leaving the nest to forage during incubation. The nest provides an extra layer of care, protecting the egg from abrupt temperature changes (Chapter 4) and from potential predators, which in turn make incubation less costly. In this scenario, with relatively abundant resources it makes it possible for the Brown Kiwi male to "care" for chicks that might not be his with little cost.

Apteryx presents a unique opportunity to understand the evolution of mating systems, as this genus presents male incubation, male and female incubation and co-operative breeding (Chapter 3) and in Brown Kiwi a series of morphological and physiological adaptations towards these types of incubating systems (Chapter 4). It would be of great value to science to study the other *Apteryx* species in the same way as in this study, and the evolution of mating systems and incubation strategies in the speciation promoted by glaciations and habitat loss (Weir et al., 2016).

Chapter Six
Conclusion and General Discussion

Da ignem igni, Mercurium Mercurio, et sufficit tibi

(Give Fire to fire, Mercury to Mercury, and you have enough)

Michael Maierus, 1619

My main aim in this thesis was to answer questions about the reproductive biology and evolution of the genus *Apteryx* using eggshells, a non-invasive way. Non-invasive methods are needed to study endangered species, since direct experimentation or disturbance can be restricted. It is fundamental for the recovery of any species that its biology is well understood as this is the basis for successful recovery programs, as David Attenborough has said: "No one will protect what they don't care about; and no one will care about what they have never experienced".

Eggshells were used as a by-product of conservation efforts and the ONE program (Chapter One). I found that avian eggshells can provide much information about the life history traits of species, their environments and that hypotheses can be prosed on the evolution of species based on their eggshell adaptations to the nest environment and the incubating behaviours of the parents. Eggshells are fundamental to understand avian evolution as successful reproduction is what ultimately has the greatest impact on individual fitness; therefore, reproduction will be under the greatest selective pressure (Darwin, 1859).

In this thesis I have discussed and tested how eggshells can be used to identify the species that produced them. This statement holds true for extant and recently extinct species, as it is possible to extract intact DNA from the mineral matrix of the eggshell (Egloff et al., 2009; Oskam et al., 2010, 2011). However, the morphological characteristics of the eggshell are also informative regarding the species identity, nesting environment, diet and general environment. Mikhailov (1991;1996), and Zelenitsky and Hirsch (1997) have shown how the morphological features of eggshells reflect differences between major taxonomic groups (i.e. ratite birds, neognathous birds, testudines (turtles), geckos, etc.), but the possibility of identifying lower taxonomic levels using eggshells is still debated (Vianey-Liaud, Ashu and Garcia, 2003). I have found that differentiating species within the genus *Apteryx* was possible using characteristics of the eggshell structure. Body size was not included in my analyses because the individual body sizes of the females that laid the eggs was not known and using an average female weight for the analyses would have overlooked its actual effect. However, if body size was the determining factor for the differences in eggshell thickness and porosity, it would be expected that the pattern was different as Rowi and Brown Kiwi females have roughly the same weight and these species have significant differences in eggshell thickness. Juang et al, (2017) note that *Apteryx* presents a challenge to contact incubation due to the thickness of the eggshell, there is still much to be studied regarding the incubation behaviour of this species to understand how all the physical characteristics of the eggshell have evolved in this clade.

When species are compared or when we try to identify species using the morphological characteristics of eggshells alone, the possibility of convergent evolution presents issues. A strong critique came from the proponent of the eggshell parataxonomy, Mikhailov (2014), regarding the use of the oospecimen morphological characters as descriptors in phylogenetic analysis, as convergent evolution would hinder some of the identifying characteristics of oospecimens and the placement of such in a phylogeny would be necessarily equivocal. This objection is reasonable, as we do not know if eggshells of unrelated species would present similar adaptations to similar environments at lower

taxonomic levels. This does not mean that disentangling convergences in the eggshell morphology are not possible, is just that it has not been undertaken yet, and this is a task that must be carried with extant species in order to have certainty about the species that laid the eggs and its environment. This leads us to the question: how do the physical characteristics of eggshells evolve at genus or species levels?

Eggs, and therefore the eggshell needs to be adapted to the nest microclimate, as I discussed in Chapters 3 and 4. The eggshell needs to be able to respond to changes in gas composition and humidity of the nest microclimate by permitting adequate gas exchange while also preventing desiccation of the egg contents or ingress of excess water, such as that which might be experienced during rain or flooding of the nest. The eggshell of many species has features that minimise the risk of microbial infestation present in different types of nests. The nest itself is an adaptation to guard the egg from the elements, predation and parasitism, but is deliberate in its design and architecture to meet the needs of egg(s) in terms of gas exchange (Portugal et al., 2014) and microbial infestation (in the form of nesting materials) (Mennerat et al., 2009). At the same time parental behaviour is a response to (a) mitigate the risks of overcooling and overheating (Conway and Martin, 2000), (b) maintain optimal gas exchange and ventilation of the nest (Ar and Piontkewitz, 1992), and (c) predators in the form of vigilance, alerts, and nest modification (Lima, 2009). This means that physical characteristics of the nest and eggshell, and incubating behaviours cannot be understood separately as they influence each other, creating different possible evolutionary pathways to respond to the immediate threats in the environment.

More studies are needed to understand how eggshells adapt to environmental pressures in a phylogenetic context (Chapter 2), to see how basic eggshell structures, typical for a taxon (i.e. testudines, ratites, neognathous birds), might be affected by different environmental pressures. The variability in eggshell characteristics needs to be related to the nest architecture and incubating behaviours of adults. It could be thought that all of these variables evolve from an ancestral state. I reinforce the idea that any comparison should or must consider phylogeny, otherwise, comparisons might not be accurate or could become trivial, for comparing adaptations that might look similar but have different evolutionary origins. Considering phylogeny would also allow for comparisons made between "unrelated" taxa based on their similarities or differences, as a response to a particular set of environmental pressures.

In the context of climate change, studies on eggshells can become more relevant and interesting, because we can observe how species adapt to new environments as vegetation and weather patterns change, as species colonise new environments (Stein and Badyaev, 2011), and the borders between urbanised areas and forest become closer. For example, looking at the eggshell adaptations to different altitudinal, latitudinal and climatic regimes of cosmopolitan species (such as pigeons, house sparrows and blackbirds) in relation to (a) nest architecture and behaviour (this includes breeding season, number of clutches, etc.) and (b) the colonisation time of these species, could tell how plastic these attributes are in different taxa. In chapter 2, I discussed further studies using cosmopolitan species. In contrast, looking at closely related species with different spatial distributions and

behaviours can be used to examine the environmental pressures have driven the morphological and behavioural characteristics of related species apart.

Gas exchange

Gas exchange in the avian egg is a phenomenon more complex than has been previously described. In this thesis I found that *Apteryx* eggshells are more porous than previously thought, however the eggshell presents a mineral cuticle composed of triangular particles that plugs the pores, plus extensions in this cuticle create caps that reduce the functional area of the pores. This cuticle can be removed by friction and it is possible that the parent rotates the egg to reduce the cuticle by abrasion with the rough nest materials; this will allow more pores to have direct contact with the "nest atmosphere" increasing the gas exchange as the embryo develops. Contrasting freshly laid eggs with incubated ones would be the way to determine if there is cuticular wear in the eggshell, however this is not possible as access to freshly laid *Apteryx* eggs is and will continue to be restricted.

Previous studies on eggshell gas exchange have based comparisons only on the egg's fresh mass (Paganelli, 1980) and in some cases on nest microclimate (Tanaka et al., 2018). However, I believe that mass used this way is an imprecise characteristic to establish accurate comparisons; it cannot be denied that allometry plays an important role in determining gas exchange to a certain extent, as physical constraints are an important determinant in this process. I believe that phylogeny and incubation behaviour also need be considered. For incubation behaviour it is important to note the time the sitting parent spends on the egg, because when an incubating parent is sitting on top of the egg, one side of the egg will be covered by the brood patch and the other by the nest bottom, in which case airflow through the nest materials and the plumage of the sitting parent will affect the rate at which gases diffuse into and out of the egg. This possible reduction on the rate of gas diffusion may correlate with sitting time and egg or nest temperature; therefore, particular incubation time and bout lengths should be preferred by the sitting parent. For example, White-crowned Sparrows (*Zonotrichia leucophrys*) have been observed to modify the duration of on egg incubation, nest placement for solar radiation cap, and nesting materials at two different localities which experienced differences in weather and ambient temperature (Webb and King, 1983). These differences will influence the gas exchange rate of the eggs, as direct solar radiation and higher temperatures will increase the rate of water loss from eggs while shade and cooler temperatures will reduce the water loss. Therefore, it could be expected that some of the nest architectural adjustments and the parent's behaviour, to respond to the gas exchanges needs of the eggs as well as the eggs need to also be best adapted to a given nest environment.

Nest architecture

From this study I can conclude that one of the most important functions of the nest architecture is the buffering of environmental changes, more so than maintaining incubation temperatures. Brown Kiwi nests are efficient in maintaining above environmental temperatures at night while cooler temperatures during the day. However, in contrast to my hypothesis of the egg possessing adaptations to withstand overcooling, I believe that it is the developing embryo that can counteract the cooling of the egg by

metabolic heat production. Tazawa et al., (1988) have shown how avian species can be resistant to overcooling by either slowing embryonic development or in later stages, by compensating slow rate temperature changes with metabolic heat production. It is clear that the heat produced by the embryo is not enough to sustain incubation without exothermic input, but it can at least minimise the risks of significant temperature loss. Therefore, the nest will probably slow down the egg's heat loss by buffering the external temperature. In this scenario, features of the architecture and design of the Brown Kiwi nest, as a completely enclosed nest, slow down the rate of heat loss of the egg, and depending of the developmental stage the embryo, the possibility it has to produce heat to reduce the rate at which heat is lost. In the case of *Apteryx,* it is necessary to further explore the thermal capability of the embryo. As a ratite that inhabits cold climates, it is possible that the metabolic processes of heat production are either developed early in the Kiwi embryo, or the embryo might have a great resistance to overcooling. Any possible mechanism is at this time undescribed and not understood.

Much has been hypothesised about the ancestral nesting behaviour of early birds and dinosaurs; it becomes interesting then to look at the different forms nest architecture can take in a phylogenetic context. In the case of ratites it seems possible that *Apteryx* have a similar nesting strategy to that of other ratites (a scrap nest), but under selective pressure in a cold environment (New Zealand during the Pleistocene (Hellstrom, McCulloch, and Stone, 1998)) *Apteryx* exploited the available crevices and hollowed trees present in the environment to reduce the effect of weather on incubation. It could be suggested that at this point it would probably have been a single incubating parent, most likely the male; therefore, an adaptation was needed to survive the rapidly changing temperatures of New Zealand. The development of a dug cavity could follow from the reduction on usable spaces as *Apteryx* populations grew in numbers, forcing the fabrication of a similar structure to pre-existing hollows.

In this thesis the gas composition of the *Apteryx* nest was not studied, but considering the low metabolic rate of *Apteryx* embryos (Prinzinger and Dietz, 2002) it would be interesting to measure the composition of the nest gases as incubation progresses, to contrast with male incubating behaviour in terms of time spent sitting on the egg and the ventilatory strategies adopted by the male. It is possible that the low metabolic rate of Brown Kiwi (lower than most ratites (Maloney, 2008)) has arisen as an adaptation to survive in a very low oxygen environment (the nest cavity). Brown Kiwi embryos increase their oxygen demands around day 40, and it increases rapidly from there until hatching; at this time the incubating male spends more and more time in the nest, therefore it would be interesting to see if the male presents behavioural responses to increase the aeration in the nest or the embryo's access to oxygen.

Climate change is having an effect on the nesting behaviours of many species, and there is good evidence that animals are responding to rapid changes in the environment by adjusting the nesting behaviour (Mainwaring et al., 2017). However, in the short term this rapid changes in climate can have a detrimental effect on endangered species populations, therefore understanding the properties of the nest of endangered species is of immense

value for conservation plans, such as for Brown Kiwi, as appropriate nesting grounds need to be sought for translocations or to determine the location of further protected areas.

For the conservation of *Apteryx,* it is necessary that we understand the requirements of this species in terms of suitable nesting areas, also understanding the mating system of each species could inform about the territory requirements for each bird to breed normally. More importantly, *Apteryx* is a window into the incredible adaptation potential of living beings and the delicate relationship that exists between the nest architecture, the physical properties of the eggs and incubation behaviours; these three variables give birds the enormous potential of colonising new environments or adapting to changing ones.

It would be of immense value to understand how incubating behaviours and nesting ecology evolve in Aves, there is plentiful information about thousands of extant species that could be analysed further, by adding information of the nest architecture and incubation behaviours on top of molecular phylogenies. This effort will be very informative in "mapping" the history of birds, and they ways they have used to succeed in becoming such as diverse and cosmopolitan clades.

Mating system

In this thesis I confirmed the hypothesis that Brown Kiwi is not a monogamous species, by finding half siblings and unrelated individuals in a single male's nest throughout the years. It was very interesting, however, to find full siblings in different years, meaning that Brown Kiwi does mate with the same individual in different breeding seasons, which could explain the assumed monogamous system. It was not possible to determine if different females lay eggs from different fathers in the same nests, or if the male remains the same and mates successively with different females, which lay eggs in his nest. This could be achieved by extracting DNA from the eggshell to determine the identity of the females that laid eggs in a particular nest. However, it is very likely that both scenarios are occurring, as both the male and the female have opportunities to re-mate.

The variation in social groups and mating behaviours in the genus *Apteryx* is interesting; it would be of great benefit if this study were replicated using the Rakiura Island Tokoeka (*Apteryx australis australis*) as this sub-species is known for communal roosting and a co-operative incubation strategy. Looking at the other *Apteryx* species could reveal how mating systems evolve and I believe the findings will suggest that mating systems are inherently plastic, because depending on ecological variables such as food availability, territory, climate and nesting sites availability, as well as population density and viscosity, mating systems would oscillate to provide the highest fitness to individuals.

This variety should be addressed in terms of ecological and climatic constraints as it has been suggested by Jetz and Rubenstein (2011). These authors suggested that climatic variability between years is a good predictor of cooperation in birds when the phylogeny is taken into consideration. Their paper addresses the issue from the perspective of passerines, which are characterised for being extremely altricial, and the prediction might not hold as well for precocial species. For *Apteryx* several factors could be considered, such as an

ancestral plastic polygynandrous mating system, reduction in nesting sites due to climate change (since the Holocene), added to the limitations in dispersal as a ground dwelling species increasing population viscosity (Hatchwell, 2009). The extremes in the mating spectrum for *Apteryx* vary between the Brown Kiwi, which does well with a single incubating parent in a set of more benign and less changing weather pattern in the north island of New Zealand, and the Rakiura Island Tokoeka. The Tokoeka could be the perfect example of the ecological constraints cited above, because as Rakiura Island became separated from the mainland (Chapter 3) the nesting sites would become also reduced (note that for a species that digs a burrow, nest penetrability would affect the availability of nesting sites), as well as the dispersal of the species itself, by being confined to the island, making the neighbours more likely to be related, allowing for kin selection and promoting the evolution of a cooperative system. Looking at the intermediate species, such as Roroa and Rowi will also shed light as why two parents became necessary to ensure chick survival.

The next step in understanding the evolution and plasticity of mating systems in ratites is to determine the mating system of the other *Apteryx* species. The methods outlined in this thesis provide a good approach to examine the other specie's mating systems. Eggshells of Rowi and Haast Tokoeka are being kept by the rearing facility at Franz Joseph providing a great opportunity to compare Brown Kiwi with the southernmost species, also in this study I only used the eggshells from Maungataniwha forest, but there are several other locations were Brown Kiwi eggs are being obtained from (Chapter 3).

Egg production costs

The cost of the egg has been mentioned throughout this thesis, but I have come to the conclusion that the egg itself is not as expensive as previously thought. Calcium has been mentioned to be a limiting factor in reproduction (Reynolds and Perrins, 2003). There is evidence that *Apteryx* stores medullary bone which would allow for reserves of calcium for egg laying (Dennison and Kooyman, 1991) and shows anatomical adaptations towards carrying a big egg, such as the fused pygostyle that bends inwards allowing for better support for the egg while the bird walks (Abourachid, Castro and Provini, 2019). All the components of the egg can be obtained from an insectivorous diet, being mainly the protein and fat needed for yolk production (Bell, 1990). Finally, the thin eggshell might be an adaptation to reduce the risk of breakage while laying the egg, I noticed while handling the eggshells that the inner membranes are extremely thick, and when moist they are very resistant to breakage. This provides the egg with a structural support while conferring it a less rigid structure, which would be advantageous while the egg is being laid, furthermore it makes easier for the chick to hatch, as it lacks the pipping tooth (Calder, 1979). The need for a big egg must be related to the production of a very well-developed chick, taking a longer incubation time than birds of similar size, but possibly, in conjunction with the nest, allowing for a slower heat loss rate (on the basis of surface area-volume ratio). The father then can leave the nest to forage, which means that incubating is not going to deplete his own resources; the nest furthermore reduces the risk of egg failing by buffering the abrupt change of environmental conditions and keeps the eggs hidden from potential predators.

The egg might be costly in comparison to other eggs (Walsberg, 1983), however when the resources are considered I believe it is not as costly as previously believed. However, if female *Apteryx* are capable of laying several clutches in one breeding season is still to be discovered.

It has been suggested that for a generalist species (*Larus argentatus*) variation in the type of resources caused changes in egg size and shape, and eggshell maculation (O'Hanlon et al., 2019). It would be interesting to look at the effect of seasonal changes in arthropod diet on egg and eggshell characteristics in Brown Kiwi (Shapiro, 2005; Dixon, 2015), as it has been suggested that during the summer, Brown Kiwi that are found near pasture and graze lands supplement their diets with crickets, in contrast to Kiwi dwelling inside dense bush. Also, it would be fundamental to explore the seasonality of diet of the southernmost *Apteryx* species as harsher climates and often snowfall could have a severe effect on the availability of prey.

Studies using eggshells have the benefit of being non-intrusive, and much can be learnt about the biology of a species from this structure, if in addition to observational field work, eggshells were retrieved and analysed. With the appropriate tools, eggshells can be analysed to glean information about diet, weather, and migration. Breeding success and the input of individuals to the population in relation to their physical appearance, and behaviour could also be studied. This type of information can help answer questions about sexual selection, where ascertaining parentage would be impossible without significant intrusion. Finally, the morphological characteristics of eggshells will always be informative regarding the nesting environment of a species if the phylogenetic relationships of the species are explored beforehand with other methods, and when behavioural and ecological factors are controlled for and understood.

There is a need for a better understanding of the genus *Apteryx*, especially the southernmost species of which diet, habitat selection, nesting behaviour and mating system are not yet fully known, and as I have found in this study this taxon has the potential of revealing fundamental information for the understanding of evolution in birds.

References

Abourachid, A., Castro, I. and Provini, P. (2019). How to Walk with a Huge Egg? Trade-offs between locomotion and reproduction explain the special pelvis and leg anatomy in kiwi (Aves; Apteryx spp.). *Journal of Anatomy, 253(6),*1045-1056. https://doi.org/10.1111/joa.13072.

Ar, A., and Rahn, H. (1985). Pores in avian eggshells: Gas conductance, gas exchange and embryonic growth rate. *Respiration Physiology*, 61(1), 1–20. https://doi.org/10.1016/0034-5687(85)90024-6

Akçay, E., and Roughgarden, J. (2007). Extra-pair reproductive activity in birds: review of the genetic benefits. *Evolutionary Ecology Research*, 9(5), 855–868.

Andersson, M. (2005). Evolution of classical Polyandry: Three steps for female emancipation. *Ethology*, 111(23), 5–26.

Angst, D., Amiot, R., Buffetaut, E., Fourel, F., Martineau, F., Lazzerini, N., and Lécuyer, C. (2015). Diet and climatic context of giant birds inferred from δ13Cc and δ18Oc values of Late Palaeocene and Early Eocene eggshells from southern France. *Palaeogeography, Palaeoclimatology, Palaeoecology*, 435, 210–221. https://doi.org/10.1016/j.palaeo.2015.06.011

Ankney, C. D., and Scott, D. M. (1980). Changes in nutrient reserves and diet of breeding brown-headed cowbirds. *The Auk*, 97(4), 684–696. https://doi.org/10.2307/4085740

Ar, A., Paganelli, C. V, Reeves, R. B., Greene, D. G., and Rahn, H. (1974). The avian egg: water vapor conductance, shell thickness, and functional pore area. *The Condor*, 76(2), 153–158. https://doi.org/10.2307/1366725

Ar, Amos, and Piontkewitz, Y. (1992). Nest ventilation explains gas composition in the nest-chamber of the European bee-eater. *Respiration Physiology*, 87(3), 407–418. https://doi.org/10.1016/0034-5687(92)90021-N

Barri, F. R., Martella, M. B., and Navarro, J. L. (2009). Nest-site habitat selection by Lesser Rheas (*Rhea pennata pennata*) in north-western Patagonia, Argentina. *Journal of Ornithology*, 150(2), 511–514. https://doi.org/10.1007/s10336-009-0374-6

Bassett, S. M. (1996). Production and Incubation (*Dromaius novaehollandiae*). Thesis.

Bell, G. P. (1990). Birds and mammals on an insect diet: a primer on diet composition analysis in relation to ecological energetics. Studies in Avian Biology, 13, 416–422. 2

Berrang, M. E., Cox, N. A., Frank, J. F., and Buhr, R. J. (1999). Bacterial penetration of the eggshell and shell membranes of the chicken hatching egg: A review. Journal of Applied Poultry Research, 8(4), 499–504. https://doi.org/10.1093/japr/8.4.499

Birchard, G. F. and Kilgore, D. (1980). Conductance of water vapor in eggs of burrowing and nonburrowing birds: implications for embryonic gas exchange. *Physiological and Biochemical Zoology*: PBZ, 53(3), 284–292. https://doi.org/10.1086/664584

Birchard, G. F., and Deeming, D. C. (2009). Avian eggshell thickness: Scaling and maximum body mass in birds. Journal of Zoology, 279(1), 95–101. https://doi.org/10.1111/j.1469-7998.2009.00596.x

Birchard, G. F., Kilgore Jr., D. L., and Boggs, D. F. (1984). Respiratory gas concentrations and temperatures within the burrows of three species of burrow-nesting birds. *Wilson Bulletin*, 96(3), 451–456.

Birkhead, T. R. (1988). Behavioral aspects of sperm competition in birds. *In Advances in the Study of Behavior* 18, 35-72. Academic Press.

Blache, D., Barrett, C. D., and Martin, G. B. (2000). Social Mating System and Sexual Behaviour in Captive Emus *Dromaius novaehollandiae*. *Emu*, 100(1995), 161. https://doi.org/10.1071/MU9902

Blom, J., and Lilja, C. (2004). A comparative study of growth, skeletal development and eggshell composition in some species of birds. Journal of Zoology, 262(4), 361–369. https://doi.org/10.1017/S0952836903004746

Board, R.G., and Fuller, R. (1973). Non-specific antimicrobial defences of the avian egg, embryo and neonate. *Biological Review*, 49(1),15–49.

Board, R. G., and Scott, V. D. (1980). Porosity of the avian eggshell. *Integrative and Comparative Biology*, 20(2), 339–349. https://doi.org/10.1093/icb/20.2.339

Board, R. G. (1981). The microstructure of avian eggshells, adaptive significance and practical implications in aviculture. *Wildfowl*, 32, 132–136.

Board, R. G. (1982). Properties of avian eggshell and their adaptative value. *Biological Reviews of the Cambridge Philosophical Society*, 57, 1–28.

Board, R. G., Perrott, H. R., Love, G., and Scott, V. D. (1984). The phosphate-rich cover on the eggshells of grebes (Aves: Podicipediformes). *Journal of Zoology*, 203(3), 329–343. https://doi.org/10.1111/j.1469-7998.1984.tb02336.x

Board, R. G., and Perrott, H. R. (2009). The fine structure of the outer surface of the incubated eggshells of the Helmeted guinea fowl (*Numidia meleagris*). *Journal of Zoology*, 196(4), 445–451. https://doi.org/10.1111/j.1469-7998.1982.tb03516.x

Boccs, F., Kilgore, D. L., and Birchard, G. F. (1984). Respiratory Physiology of Burrowing Mammals and Birds. *Comparative Biochemistry and Physiology*, 77A(1), 1–7.

Body, D. R., and Reid, B. (1983). The lipid and amino acid composition of the kiwi (Apteryx australis mantelli) ratite eggs. *Journal of the Science of Food and Agriculture*, 34(6), 587–592. https://doi.org/10.1002/jsfa.2740340609

Body, D. R., and Reid, B. (1987). The lipid, fatty acid and amino acid composition of ratite eggs from three different species of kiwis Apteryx australis mantelli, *A. haastii* and *A. owenii* bred in captivity on the same diet. *Biochemical Systematics and Ecology*, 15(5), 625–628. https://doi.org/10.1016/0305-1978(87)90115-3

Boersma, P. D. (1982). Why Some Birds Take so Long to Hatch. *The American Naturalist*, 120(6), 733–750.

Boersma, P. D., and Wheelwright, N. T. (1979). Egg neglect in the Procellariiformes: reproductive adaptations in the fork-tailed storm-petrel. *The Condor*, 81(2), 157–165.

Boggs, D. F., and Kilgore, D. L. J. (1983). Ventilatory responses of the burrowing owl and bobwhite to hypercarbia and hypoxia. *Journal of Comparative Physiology*, 149, 527–533.

Bond, G. M., Board, R. G., and Scott, V. D. (1988). An account of the hatching strategies of birds. *Biological Reviews - Cambridge Philosophical Society*, 63(3), 395–415. https://doi.org/10.1111/j.1469-185X.1988.tb00723.x

Brennan, P. L. R. (2012). Mixed paternity despite high male parental care in great tinamous and other Palaeognaths. *Animal Behaviour*, 84(3), 693–699. https://doi.org/10.1016/j.anbehav.2012.06.026

Briskie, J. V., & Montgomerie, R. (1992). Sperm size and sperm competition in birds. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, 247(1319), 89-95.

Brulez K., Mikšík, I., Cooney, Christopher R.M., Hauber, E. Lovell, P.G., Maurer, G., Portugal S.J., Russell, D., Reynolds, S.J., Cassey P. (2016). Eggshell pigment composition covaries with phylogeny but not with life history or with nesting ecology traits of British passerines. *Ecology and Evolution*, 6(6), 1637–1645. https://doi.org/10.1002/ece3.1960

Burbidge, M. L., Colbourne, R. M., Robertson, H. A., and Baker, A. J. (2003). Molecular and other biological evidence supports the recognition of at least three species of brown kiwi. *Conservation Genetics*, 4(2), 167–177. https://doi.org/10.1023/A:1023386506067

Burton, F. G., and Tullett, S. G. (1985). Respiration of avian embryos. *Comparative Biochemistry and Physiology*, 82(4), 735–744. https://doi.org/10.1016/0300-9629(85)90476-1

Calder III, W. A. (1978). The Kiwi: A Case of Compensating Divergences from Allometric Predictions. J. Piiper (ed.), *Respiratory Function in Birds, Adult and Embryonic*. Springer-Verlag Berlin Heidelberg, 1978.

Calder, W. A. (1979). The kiwi and egg design: evolution as a package deal. *BioScience*, 29(8), 461–467.

Calder, W. A., and Parr, C. R. (1977). Energy content of eggs of the brown *Apteryx australis*; an extreme in avian evolution. *Comparative Biochemistry and Physiology*, 60A.

Carey, C. (1980). The Ecology of Avian Incubation. *BioScience*, 30, 819–824. https://doi.org/10.2307/1308374

Cassey, P., Honza, M., Grim, T., and Hauber, M. E. (2008). The modelling of avian visual perception predicts behavioural rejection responses to foreign egg colours. *Biology Letters*, 4(5), 515–517. https://doi.org/10.1098/rsbl.2008.0279

Cassey, P., G. H. Thomas, S. J. Portugal, G. Maurer, M. E. Hauber, T. Grim, P. G. Lovell and I. Mikšík (2012). Why are birds' eggs colourful? Eggshell pigments co-vary with life-history and nesting ecology among British breeding non-passerine birds. *Biological Journal of the Linnean Society*, 106(3), 657–672. https://doi.org/10.1111/j.1095-8312.2012.01877.x

Cavalli-Sforza, L. L., and Bodmer, W. (1971). Human population genetics. San Francisco, CA: Free-Man.

Codenotti, T. L., and Alvarez, F. (2006). Mating Behavior of the Male Greater Rhea. The Wilson Bulletin, 113(1), 85–89. https://doi.org/10.1676/0043-5643(2001)113[0085:mbotmg]2.0.co;2

Cody, M. L. (1966). a General Theory of Clutch Size. *Evolution*, 20(2), 174–184. https://doi.org/10.1111/j.1558-5646.1966.tb03353.x

Colbourne, R. (1991). Further evidence of Female incubation and Family grouping in Brown Kiwis. *Notornis*, 38, 248–249.

Colbourne, R. (2002). Incubation behaviour and egg physiology of kiwi (Apteryx spp.) in natural habitats. *New Zealand Journal of Ecology,* 26(2), 129–138.

Colbourne, R., Bassett, S., Billing, T., McCormick, H., McLennan, J., Nelson, A., and Robertson, H. (2005). The development of Operation Nest Egg as a tool in the conservation management of kiwi. *Science for Conservation,* (259), 1–24.

Colbourne, R. and Kleinpaste, R. (1983). A study of kiwis in an exotic forest. *New Zealand Forest Service*.

Collias, N. E. (1964). The evolution of nests and nest-building in birds. *Integrative and Comparative Biology*, 4(2), 175–190. https://doi.org/10.1093/icb/4.2.175

Conrado, I., Araújo, S. De, Susana, N., Leandro, M., Mesquita, M. A., Café, M. B., … Gonzales, E. (2017). Water vapor conductance: a technique using eggshell fragments and relations with other parameters of eggshell. *Brazilian Journal of Animal Science*.

Conway, C. J. (2000). Effects of ambient temperature on avian incubation behavior. Behavioral Ecology, 11(2), 178–188. https://doi.org/10.1093/beheco/11.2.178

Cooper, C. B., Voss, M. A., Ardia, D. R., Austin, S. H., and Robinson, W. D. (2011). Light increases the rate of embryonic development: Implications for latitudinal trends in incubation period. Functional Ecology, 25(4), 769–776. https://doi.org/10.1111/j.1365-2435.2011.01847.x

Corfield, J., Gillman, L., and Parsons, S. (2008). Vocalizations of the North Island Brown Kiwi (*Apteryx Mantelli*). *The Auk*, 125(2), 326–335. https://doi.org/10.1525/auk.2008.06234

Crome, F. H. J. (1976). Some observations on the biology of the cassowary in northern Queensland. *Emu*, 76(1), 8–14.

Cunningham, A. (1840). XXXVI.—On the habits of the *Apteryx australis*, a bird of New Zealand, closely allied to the Struthionidæ, and named by the native inhabitants Kiwi. *Annals and Magazine of Natural History*, 4(25), 312–314. https://doi.org/10.1080/00222934009496714

Cunningham, S., and Castro, I. (2011). The secret life of wild brown Kiwi: studying behaviour of a cryptic species by direct observation. *New Zealand Journal of Ecology*, 35.

Cusack, M., England, J., Dalbeck, P., Tudhope, A., Fallick, A., and Allison, N. (2008). Electron backscatter diffraction (EBSD) as a tool for detection of coral diagenesis. *Coral Reefs*, 27, 905–911. https://doi.org/10.1007/s00338-008-0414-3

D'Alba, L., Jones, D. N., Badawy, H. T., Eliason, C. M., and Shawkey, M. D. (2014). Antimicrobial properties of a nanostructured eggshell from a compost-nesting bird. *Journal of Experimental Biology*, 217(7), 1116–1121. https://doi.org/10.1242/jeb.098343

D'Alba, L., Torres, R., Waterhouse, G. I. N., Eliason, C., Hauber, M. E., and Shawkey, M. D. (2017). What Does the Eggshell Cuticle Do? A Functional Comparison of Avian Eggshell Cuticles. *Physiological and Biochemical Zoology*, 90(5), 588–599. https://doi.org/10.1086/693434

D'Alba, L., Maia, R., Hauber, M. E., and Shawkey, M. D. (2016). The evolution of eggshell cuticle in relation to nesting ecology. *Proceedings of the Royal Society B: Biological Sciences*, 283(1836), 20160687. https://doi.org/10.1098/rspb.2016.0687

Darwin, C. (1859). On the origin of species. Routledge.

De Marchi, G., Chiozzi, G., Dell'Omo, G., and Fasola, M. (2015). Low incubation investment in the burrow-nesting Crab Plover *Dromas ardeola* permits extended foraging on a tidal food resource. *Ibis*, 157(1), 31–43. https://doi.org/10.1111/ibi.12208

De Marchi, G., Chiozzi, G., and Fasola, M. (2008). Solar incubation cuts down parental care in a burrow nesting tropical shorebird, the crab plover *Dromas ardeola*. *Journal of Avian Biology*, 39(5), 484–486. https://doi.org/10.1111/j.0908-8857.2008.04523.x

De Reu, K., Grijspeerdt, K., Heyndrickx, M., Messens, W., Uyttendaele, M., Debevere, J., and Herman, L. (2006). Influence of Eggshell Condensation on Eggshell Penetration and Whole Egg Contamination with Salmonella enterica Serovar Enteritidis. *Journal of Food Protection*, 69(7), 1539–1545. https://doi.org/10.4315/0362-028X-69.7.1539

Deeming, D C. (2006). Ultrastructural and functional morphology of eggshells supports the idea that Dinosaur eggs were incubated buried in a substrate. *Palaeontology*, 49, 171–185. https://doi.org/10.1111/j.1475-4983.2005.00536.x

Deeming, D Charles. (2002a). Avian incubation: behaviour, environment and evolution. Oxford: Oxford University Press, 2002. Deeming, D Charles. (2002b). Avian incubation. Behaviour, environment, and evolution. *Oxford Ornithology Series*, 119(4), 421. https://doi.org/10.1126/science.186.4159.107

Deeming, D Charles. (2011). Importance of nest type on the regulation of humidity in bird nests. *Avian Biology Research*, 4(1), 23–31. https://doi.org/10.3184/175815511X13013963263739

Dekker, R. W. R. J. (1988). Notes on Ground Temperatures at Nesting Sites of the *Maleo Macrocephalon maleo* (Megapodiidae). *Emu*, 88(1900), 124–127.

Delehanty, D. J., Fleischer, R. C., Colwell, M. A., and Oring, L. W. (1998). Female benefits and the absence of extra-pair fertilization in Wilson's phalaropes. *Animal Behaviour*, 55, 995–1002. https://doi.org/10.2105/AJPH.2011.300600

Dennison, K. J., and Kooyman, B. P. (1991). The potential of citrate estimation in determining the sex of moa and kiwi. *Journal of Archaeological Science*, 18(4), 417–427. https://doi.org/10.1016/0305-4403(91)90035-N

Desmond Maxwell, W., and Horner, J. R. (1994). Neonate dinosaurian remains and dinosaurian eggshell from the Cloverly Formation, Montana. *Journal of Vertebrate Paleontology*, 14(1), 143–146. https://doi.org/10.1080/02724634.1994.10011547

Dixon, T. (2015). What they do in the shadows: habitat utilisation and diet of brown kiwi (*Apteryx mantelli*) adults within a high-density island population: a thesis submitted in partial fulfilment of the requirements for the degree of Master of Science in Ecology, Massey.

Du, W. G., and Shine, R. (2015). The behavioural and physiological strategies of bird and reptile embryos in response to unpredictable variation in nest temperature. *Biological Reviews*, 90(1), 19–30. https://doi.org/10.1111/brv.12089

Dyke, G. J., and Kaiser, G. W. (2010). Cracking a developmental constraint: egg size and bird evolution. In Proceedings of the VII International Meeting of the Society of Avian Paleontology and Evolution, ed. W.E. Boles and T.H. Worthy. *Records of the Australian Museum*, 62(1), 207–216. https://doi.org/10.3853/j.0067-1975.62.2010.1547

Egloff, C., Labrosse, A., Hebert, C., and Crump, D. (2009). A non-destructive method for obtaining maternal DNA from avian eggshells and its application to embryonic viability determination in herring gulls (*Larus argentatus*). *Molecular Ecology Resources*, 9(1), 19–27. https://doi.org/10.1111/j.1755-0998.2008.02214.x

Emlen, S. T., and Oring, L. W. (1977). Ecology, sexual selection, and the evolution of mating systems. Science, 197(4300), 215–223.

Emlen, S. T., Wrege, P. H., and Webster, M. S. (1998). Cuckoldry as a cost of polyandry in the sex-role-reversed wattled jacana, *Jacana jacana*. Proceedings of the Royal Society B: Biological Sciences, 265(1413), 2359–2364. https://doi.org/10.1098/rspb.1998.0584

Fairfield Osborn, H. (1924). American Museum Novitates Three New Theropoda, Protoceratops Zone, Central Mongolia'. Number 144 THz American Museum of Natural History, 7(7), 51.

Fanti, F., Currie, P. J., and Badamgarav, D. (2012). New specimens of nemegtomaia from the Baruungoyot and Nemegt formations (late cretaceous) of Mongolia. PLoS ONE, 7(2), 20–23. https://doi.org/10.1371/journal.pone.0031330

Fernandez, G. J., and Reboreda, J. C. (2007). Adjacent Nesting and Egg Stealing between Males of the Greater Rhea *Rhea americana*. Journal of Avian Biology, 26(4), 321. https://doi.org/10.2307/3677047

Fernandez, M. S., Araya, M., and Arias, J. L. (1997). Eggshells are shaped by a precise spatio-temporal arrangement of sequentially deposited macromolecules. Matrix Biology, 16(1), 13–20. https://doi.org/10.1016/S0945-053X(97)90112-8

Fisher, R. J., Poulin, R. G., Todd, L. D., and Brigham, R. M. (2004). Nest stage, wind speed, and air temperature affect the nest defence behaviours of burrowing owls. *Canadian Journal of Zoology*, 82(5), 707–713. https://doi.org/10.1139/z04-035

Forstmeier, W., and Weiss, I. (2004). Adaptive plasticity in nest-site selection in response to changing predation risk. *Oikos, 104(3),* 487-499. https://doi.org/10.1111/j.0030-1299.1999.12698.

Garcia, G. (2000). Diversité des coquilles "minces" d'oeufs fossiles du Crétacé supérieur du Sud de la France. *Geobios*, 33(1), 113–126. https://doi.org/10.1016/S0016-6995(00)80155-2

Giraldo-Deck, L. M., Habel, J. C., Meimberg, H., and Garitano-Zavala, A. (2017). Genetic evidence for promiscuity in the Ornate Tinamou *Nothoprocta ornata* (Aves: Tinamiformes). *Biological Journal of the Linnean Society*, 120(3), 604–611. https://doi.org/10.1111/bij.12911

Glasspool, I. J., and Scott, A. C. (2010). Phanerozoic concentrations of atmospheric oxygen reconstructed from sedimentary charcoal. *Nature Geoscience*, 3(9), 627–630. https://doi.org/10.1038/ngeo923

Goymann, W., Safari, I., Muck, C., and Schwabl, I. (2016). Sex roles, parental care and offspring growth in two contrasting coucal species. *Royal Society Open Science*, 3, 1335–1353. https://doi.org/10.1111/jeb.12657

Grant, G. (1982). Avian Incubation: Egg Temperature, Nest Humidity, and Behavioral Thermoregulation in a hot environment. *The American Ornithologist's Union*, (30), 1–75.

Grealy, A., Phillips, M., Miller, G., Gilbert, M. T. P., Rouillard, J. M., Lambert, D., Bunce, M., Haile, J. (2017). Eggshell palaeogenomics: Palaeognath evolutionary history revealed through ancient nuclear and mitochondrial DNA from Madagascan elephant bird (*Aepyornis* sp.) eggshell. *Molecular Phylogenetics and Evolution*, 109, 151–163. https://doi.org/10.1016/j.ympev.2017.01.005

Grealy, A., Rawlence, N. J., and Bunce, M. (2017). Time to spread your wings: A review of the avian ancient DNA field. Genes, 8(7). https://doi.org/10.3390/genes8070184

Green, G. A., and Anthony, R. G. (1989). Nesting Success and Habitat Relationships of Burrowing Owls in the Columbia Basin, Oregon. *The Condor, 91(2).*

Greeney, H. F. (2008). Nest construction behavior and variability in nest architecture and nest placement of the Spotted Barbtail (*Premnoplex brunnescens*). *Boletín de La Sociedad Antioqueña de Ornitologia*, 18(1), 26–37.

Grellet-Tinner, G., Corsetti, F., and Buscalioni, A. D. (2010). The importance of microscopic examinations of eggshells: Discrimination of bioalteration and diagenetic overprints from biological features. *Journal of Iberian Geology*, 36(2), 181–192. https://doi.org/10.5209/rev_JIGE.2010.v36.n2.6

Grellet-Tinner, G., Lindsay, S., and Thompson, M. B. (2017). The biomechanical, chemical and physiological adaptations of the eggs of two Australian megapodes to their

nesting strategies and their implications for extinct titanosaur dinosaurs. *Journal of Microscopy*, 267(2), 237–249. https://doi.org/10.1111/jmi.12572

Grellet-Tinner, G. (2006). Phylogenetic interpretation of eggs and eggshells: implications for phylogeny of Palaeognathae. *Alcheringa, 30*. https://doi.org/10.1017/CBO9781107415324.004

Grellet-Tinner, G., Chiappe, L., Norell, M., and Bottjer, D. (2006). Dinosaur eggs and nesting behaviors: A paleobiological investigation. *Palaeogeography, Palaeoclimatology, Palaeoecology*, 232(2–4), 294–321. https://doi.org/10.1016/j.palaeo.2005.10.029

Grellet-Tinner, G., and Dyke, G. J. (2005). The eggshell of the Eocene bird Lithornis. Acta Palaeontologica Polonica, 50, 831–835. https://doi.org/10.1016/j.femsle.2005.01.054

Griffith, S. C., Owens, I. P. F., and Thuman, K. A. (2002). Extra pair paternity in birds: a review of interspecific. *Molecular Ecology*, 11, 2195–2212.

Handford, P., and Mares, M. A. (1985). The mating system of ratites and tinamous: an evolutionary approach. *Biological Journal of the Linnean Society*, 25, 77–104.

Harrison, T., and Msuya, C. P. (2005). Fossil struthionid eggshells from Laetoli, Tanzania: Taxonomic and biostratigraphic significance. *Journal of African Earth Sciences*, 41(4), 303–315. https://doi.org/10.1016/j.jafrearsci.2005.07.001

Hatchwell, B. J. (2009). The evolution of cooperative breeding in birds: Kinship, dispersal and life history. *Philosophical Transactions of the Royal Society*: *Biological Sciences*, 364(1533), 3217–3227. https://doi.org/10.1098/rstb.2009.0109

Hayward, J. L., Hirsch, K. F., and Robertson, T. C. (1991). Rapid dissolution of avian eggshells buried by Mount St. Helens ash. *Palaios*, 6(2), 174–178. https://doi.org/10.2307/3514881

Hayward, J. L., Zelenitsky, D. K., Smith, D. L., Zaft, D. M., and Clayburn, J. K. (2000). Eggshell taphonomy at Modern Gull Colonies and a dinosaur clutch site. *Palaios*, 15(4), 343–355. <0343:ETAMGC>2.0.CO;2

Healy, S., Walsh, P., and Hansell, M. (2008). Quick guides Nest building by birds. *Current Biology*, 18(7), 271–273. https://doi.org/10.1016/j.cub.2008.01.020

Hechenleitner, E. M., Grellet-Tinner, G., and Fiorelli, L. E. (2015). What do giant titanosaur dinosaurs and modern Australasian megapodes have in common? *PeerJ*, 3:e ,1341. https://doi.org/10.7717/peerj.1341

Hellstrom, J., McCulloch, M., and Stone, J. (1998). A Detailed 31,000-Year Record of Climate and Vegetation Change, from the Isotope Geochemistry of Two New

Zealand Speleothems. *Quaternary Research*, 50(2), 167–178. https://doi.org/DOI:
10.1006/qres.1998.1991

Heneberg, P. (2009). Soil penetrability as a key factor affecting the nesting of burrowing
birds. *Ecological Research,* 24(2), 453–459. https://doi.org/10.1007/s11284-008-
0520-2

Higham, J. P., and Gosler, A. G. (2006). Speckled eggs: Water-loss and incubation
behaviour in the great tit *Parus major*. *Oecologia*, 149(4), 561–570.
https://doi.org/10.1007/s00442-006-0484-2

Hindwood. (1961). Nesting of the Cassowary. *Emu* 61(4) 283 – 284.
https://doi.org/10.1071/MU961283

Hirsch, K. F. (1996). Parataxonomic classification of fossil chelonian and gecko eggs.
Journal of Vertebrate Paleontology, 16(4), 752–762.
https://doi.org/10.1080/02724634.1996.10011363

Hirsch, K. F., Kihm, A. J., and Zelenitsky, D. K. (1997). New eggshell of ratite morphotype
with predation marks from the eocene of colorado. *Journal of Vertebrate
Paleontology*, 17(2), 360–369. https://doi.org/10.1080/02724634.1997.10010980

Holzapfel, S., Robertson, H. A., McLennan, J. A., Sporle, W., Hackwell, K., and Impey, M.
(2008). Kiwi (*Apteryx* spp.) recovery plan 2008-2018. Threatened Species
Recovery Plan 60. Department of Conservation, 1–72

Howell, T. R., and Bartholomew, G. A. (1961). Temperature regulation in nesting bonin
island petrels, wedge-tailed shearwaters, and Christmas Island shearwaters. *Auk*,
78(3), 343–354.

Hyrtl. (1862). On some points in the anatomy of the *Apteryx australis*. Annals and Magazine
of Natural History, 9(50), 187. https://doi.org/10.1080/00222936208681204

Igic, B., Greenwood, D. R., Palmer, D. J., Cassey, P., Gill, B. J., Grim, T., Brennan, P.L.R.,
Bassett S.M., Battley, P.F., and Hauber, M. E. (2010). Detecting pigments from
colourful eggshells of extinct birds. *Chemoecology*, 20(1), 43–48.
https://doi.org/10.1007/s00049-009-0038-2

Jackson, F. D., Horner, J. R., and Varricchio, D. J. (2010). A study of a Troodon egg
containing embryonic remains using epifluorescence microscopy and other
techniques. *Cretaceous Research*, 31(2), 255–262.
https://doi.org/10.1016/j.cretres.2009.11.006

Jamieson, S. E., Castro, I., Jensen, T., Morrison, K. W., and Durrant, B. (2016). Roosting
Preferences of North Island Brown Kiwis (*Apteryx mantelli*). *The Wilson Journal of
Ornithology*, 128(4), 857–866. https://doi.org/10.1676/15-064.1

Jensen, T., and Durrant, B. (2006). Assessment of reproductive status and ovulation in female brown kiwi (*Apteryx mantelli*) using fecal steroids and ovarian follicle size. *Zoo Biology*. https://doi.org/10.1002/zoo

Jetz, W., and Rubenstein, D. R. (2011). Environmental uncertainty and the global biogeography of cooperative breeding in birds. *Current Biology*, 21(1), 72–78. https://doi.org/10.1016/j.cub.2010.11.075

Johnson, B. J., Miller, G. H., Fogel, M. L., and Beaumont, P. B. (1997). The determination of late quaternary paleoenvironments at Equus Cave, South Africa, using stable isotopes and amino acid racemization in ostrich eggshell. *Palaeogeography, Palaeoclimatology, Palaeoecology*, 136(1–4), 121–137. https://doi.org/10.1016/S0031-0182(97)00043-6

Jolly, J. N. (1989). A field study of the breeding biology of the little spotted kiwi (*Apteryx owenii*) with emphasis on the causes of nest failures. *Journal of the Royal Society of New Zealand*, 19(4), 433–448. https://doi.org/10.1080/03036758.1989.10421846

Juang, J. Y., Chen, P. Y., Yang, D. C., Wu, S. P., Yen, A., and Hsieh, H. I. (2017). The avian egg exhibits general allometric invariances in mechanical design. *Scientific reports*, 7(1), 14205.

Karlsson, O., and Lilja, C. (2008). Eggshell structure, mode of development and growth rate in birds. *Zoology,* 111(6), 494–502. https://doi.org/10.1016/j.zool.2007.11.005

Kimwele, C. N., and Graves, J. A. (2003). A molecular genetic analysis of the communal nesting of the ostrich (*Struthio camelus*). *Molecular Ecology*, 12(1), 229–236. https://doi.org/10.1046/j.1365-294X.2003.01727.x

Krokene, C., Anthonisen, K., Lifjeld, J. T., and Amundsen, T. (1996). Paternity and paternity assurance behaviour in the bluethroat, *Luscinia svecica svecica*. *Animal Behaviour*, 52(2), 405–417. https://doi.org/10.1006/anbe.1996.0184

Kusmierski, R., Borgia, G., Crozier, R. H., and Chan, B. H. Y. (1993). Molecular information on bowerbird phylogeny and the evolution of exaggerated male characteristics. *Journal of Evolutionary Biology*, 6(5), 737–752. https://doi.org/10.1046/j.1420-9101.1993.6050737.x

Kusmierski, R., Borgia, G., Uy, A., and Crozier, R. H. (1997). Labile evolution of display traits in bowerbirds indicates reduced effects of phylogenetic constraint. *Proceedings of the Royal Society B: Biological Sciences,* 264(1380), 307–313. https://doi.org/10.1098/rspb.1997.0044

Lange, K. (2003). Mathematical and statistical methods for genetic analysis. Springer Science and Business Media.

Le Duc, D., G. Renaud, A. Krishnan, M. S. Almén, L. Huynen, S. J. Prohaska, M. Ongyerth, B. D. Bitarello, H. B. Schiöth, M. Hofreiter, P. F. Stadler, K. Prüfer, D. Lambert, J.

Kelso, J. and Schöneberg, T. (2015). Kiwi genome provides insights into evolution of a nocturnal lifestyle. *Genome Biology*, 16(1), 147. https://doi.org/10.1186/s13059-015-0711-4

Leathwick, J. R., Wilson, G., and Stephens, R. T. T. (2002). Climate Surfaces for New Zealand. Lincoln New Zealand, 21. Lill, A., and Fell, P. J. (2007). Microclimate of nesting burrows of the Rainbow Bee-eater. Emu, 107(2), 108–114. https://doi.org/10.1071/MU06046

Lima, S. L. (2009). Predators and the breeding bird: Behavioral and reproductive flexibility under the risk of predation. *Biological Reviews*, 84(3), 485–513. https://doi.org/10.1111/j.1469-185X.2009.00085.x

Livesey, B. C., & Zusi, R. L. (2007). Higher-order phylogeny of modern birds (Theropoda, Aves: Neornithes) based on comparative anatomy. Zool J Linn Soc, 149, 1-95.

Lombardo, M. (1994). Nest architecture and reproductive performance in Tree Swallows (*Tachycineta bicolor*). *The Auk*, 111(4), 814–824.

Lombardo, M. P., Bosman, R. M., Faro, C. A., Houtteman, S. G., and Kluisza, T. S. (1995). Effect of Feathers as Nest Insulation on Incubation Behavior and Reproductive Performance of Tree Swallows (*Tachycineta Bicolor*). *The Auk: Ornithological Advances*, 112(4), 973–981. https://doi.org/10.2307/4089028

Magige, F. J., Stokke, B. G., Sortland, R., and Røskaft, E. (2009). Breeding biology of ostriches (*Struthio camelus*) in the Serengeti ecosystem, Tanzania. *African Journal of Ecology*, 47(3), 400–408. https://doi.org/10.1111/j.1365-2028.2008.01002.x

Maina, J. N. (2017). Structure and Function of the Shell and the Chorioallantoic Membrane of the Avian Egg: Embryonic Respiration. In the Biology of the Avian Respiratory System (219-247). Springer, Cham.

Mainwaring, M. C., Barber, I., Deeming, D. C., Pike, D. A., Roznik, E. A., and Hartley, I. R. (2017). Climate change and nesting behaviour in vertebrates: a review of the ecological threats and potential for adaptive responses. *Biological Reviews*, 92(4), 1991–2002. https://doi.org/10.1111/brv.12317

Maloney, S. K. (2008). Thermoregulation in ratites: A review. *Australian Journal of Experimental Agriculture* 48.1293–1301. https://doi.org/10.1071/EA08142

Maynard-Smith, J. (1977). Parental investment: A prospective analysis. *Animal Behaviour*, 25, 1–9. https://doi.org/10.1016/0003-3472(77)90062-8

McLennan, J. A. (2006). Strategies to Reduce Predation on Bird Populations. *Biological Invasions in New Zealand*, 186, 371–387. https://doi.org/10.1007/3-540-30023-6_24

McLennan, J. A. (1988). Breeding of north island brown kiwi, *Apteryx australis mantelli*, in Hawke's Bay, New Zealand. *New Zealand Journal of Ecology*, 11, 89–97.

McLennan, J. A., Dew, L., Miles, J., Gillingham, N., and Waiwai, R. (2004). Size matters: predation risk and juvenile growth in North Island brown kiwi (*Apteryx mantelli*). *New Zealand Journal of Ecology*, 28(2), 241–250.

McLennan, J. A., and McCann, A. J. (1991). Incubation temperatures of great spotted kiwi, *Apteryx haastii*. *New Zealand Journal of Ecology*, 15(2), 163–166.

McNab, B. (1996). Metabolism and temperature regulation of kiwis (Apterygidae). *The Auk*, 113(3), 687–692.

Mennerat, A., Mirleau, P., Blondel, J., Perret, P., Lambrechts, M. M., and Heeb, P. (2009). Aromatic plants in nests of the blue tit *Cyanistes caeruleus* protect chicks from bacteria. *Oecologia*, 161(4), 849–855. https://doi.org/10.1007/s00442-009-1418-6

Mikhailov, K. E., Bray, E. S., and Hirschl, T. F. (1996). Parataxonomy of fossil egg remains (veterovata): principles and applications. *Journal of Vertebrate Paleontology*, 16(4), 763–769.

Mikhailov, K. (1991). Classification of fossil eggshells of amniotic vertebrates. *Acta Palaeontologica Polonica*, 36(2), 193–238.

Mikhailov, K. (1992). The microstructure of avian and dinosaurian eggshell: Phylogenetic implications. *Natural History Museum of Los Angeles County Science Series*, (36), 361–373. https://doi.org/10.1136/annrheumdis-2015-209055

Mikhailov, K. (1997). Fossil and recent eggshell in amniotic vertebrates: fine structure, comparative morphology and classification. *The Palaeontogical Association*, 56.

Mikhailov, K. (2014). Eggshell structure, parataxonomy and phylogenetic analysis: some notes on articles published from 2002 to 2011, 2963. https://doi.org/10.1080/08912963.2013.829824

Miles, J., and Castro, I. (2000). Survey of northern brown kiwi (*Apteryx mantelli*) on Ponui Island, Hauraki Gulf, 1999. Unpublished Report. Department of Conservation, New Zealand.

Mitchell, K. J., Llamas, B., Soubrier, J., Rawlence, N. J., Worthy, T. H., Wood, J., Lee, M.S.Y. and Cooper, A. (2014). Ancient DNA reveals elephant birds and kiwi are sister taxa and clarifies ratite bird evolution. *Science*, 344(6186), 898–900. https://doi.org/10.1126/science.1251981

Møller, A. P. (1991). The Effect of Feather Nest Lining on Reproduction in the Swallow *Hirundo rustica*. *Ornis Scandinavica (Scandinavian Journal of Ornithology)*, 22(4), 396–400. https://doi.org/10.2307/3676514

Montanari, S. (2018). Cracking the egg: the use of modern and fossil eggs for ecological, environmental and biological interpretation. *R.Soc.Open.sci.* https://doi.org/10.1098/rsos.180006

Moreno-Azanza, M. (2013). Unusual theropod eggshells from the Early Cretaceous Blesa Formation of the Iberian Range, Spain. *Acta Palaeontologica Polonica*, 59(4), 843–854. https://doi.org/10.4202/app.2012.0069

Mueller, C. A., Burggren, W. W., and Tazawa, H. (2014). The Physiology of the Avian Embryo. Sturkie's Avian Physiology: Sixth Edition (Sixth Edit). Elsevier. https://doi.org/10.1016/B978-0-12-407160-5.00032-4

Murphy, J. P., Swanson, M. T., Jaeckle, W. B., and Harper, R. G. (2015). Corrosion casts: A novel application of a polyurethane resin (PU4ii) for visualizing eggshell pore morphology. *The Auk*, 132(1), 206–211. https://doi.org/10.1642/AUK-14-158.1

Norell, M. a., Clark, J. M., Chiappe, L. M., and Dashzeveg, D. (1996). A nesting dinosaur. *Nature*, 379(6561), 186–186. https://doi.org/10.1038/379186a0

Norell, M. A., Clark, J. M., Demberelyin, D., Rhinchen, B., Chiappe, L. M., Davidson, A. R., McKenna, M.C., Altangerel, P., Novacek, M.J. (1994). A Theropod Dinosaur Embryo and the Affinities of the Flaming Cliffs Dinosaur Eggs Science. 266 (5186), 779–782. DOI: 10.1126/science.266.5186.779.

Oliehoek, P. A., Windig, J. J., Van Arendonk, J. A. M., and Bijma, P. (2006). Estimating relatedness between individuals in general populations with a focus on their use in conservation programs. *Genetics*, 173(1), 483–496. https://doi.org/10.1534/genetics.105.049940

Olson, C. R., Vleck, C. M., and Vleck, D. (2006). Periodic Cooling of Bird Eggs Reduces Embryonic Growth Efficiency. *Division of Comparative Physiology and Biochemistry, Society for Integrative and Comparative Biology,* 79(5), 927–936. https://doi.org/10.1086/506003

Ono, H., Hou, P.-C. L., and Tazawa, H. (1993). Responses of developing chicken embryos to acute changes in ambient temperature: non-invasive study of heart rate. *Israel Journal of Zoology*, 37–41. https://doi.org/10.1080/00212210.1994.10688766

Oring, L.W., (1990). Avian Polyandry. Current Ornithology, 3, 309-351. Plenum Press, London and New York.

Oring, L. W., Fleischer, R. C., Reed, J. M., and Marsden, K. E. (1992). Cuckoldry Through Stored Sperm in the Sequentially Polyandrous spotted sandpiper. *Nature*, 631–633.

Oskam, C. L., Jacomb, C., Allentoft, M. E., Walter, R., Scofield, P., Haile, J., Holdaway, R.N., and Bunce, M. (2011). Molecular and morphological analyses of avian eggshell excavated from a late thirteenth century earth oven. *Journal of*

Archaeological Science, 38(10), 2589–2595.
https://doi.org/10.1016/j.jas.2011.05.006

Oskam, C. L., Haile, J., McLay, E., Rigby, P., Allentoft, M.E., Olsen, M.E., Bengtsson, C., Miller, G.H., Schwenninger, J-L., Jacomb, C., Walter, R., Baynes, A., Dortch, J., Parker-Pearson, M., Gilbert, M.T.P., Holdaway, R.N., E., Willerslev and Bunce, M. (2010). Fossil avian eggshell preserves ancient DNA. *Proceedings of the Royal Society B: Biological Sciences*, 277(1690), 1991–2000. https://doi.org/10.1098/rspb.2009.2019

Owen. (1839). On the Anatomy of the Southern Apteryx (*Apteryx australis*, Shaw). *The Transactions of the Zoological Society of London*, 2(4), 257–301. https://doi.org/10.1111/j.1469-7998.1839.tb00024.x

Paganelli, C. V., Olszowka, A., and Ar, A. (1974). The Avian Egg: Surface Area, Volume, and Density. The *Condor*, 76(3), 319–325. https://doi.org/10.2307/1366345

Paganelli, C. V. (1980). the Physics of Gas-Exchange Across the Avian Eggshell. *American Zoologist*, 20(2), 329–338.

Parker, T. J. (1890). Observations on the anatomy and development of *Apteryx*. *Philosophical Transactions of The Royal Society* B, 73 (182), 509–512. https://doi.org/10.1098/rstb.1891.0002

Patodkar, V. R., Rahane, S. D., Shejal, M. A., and Belhekar, D. R. (2009). Behaviour of Emu bird (breeding), *Veterinary World,* 2(11), 439–440.

Peralta-Sanchez, J. M., Møller, A. P., Martin-Platero, A. M., and Soler, J. J. (2010). Number and colour composition of nest lining feathers predict eggshell bacterial community in barn swallow nests: An experimental study. *Functional Ecology*, 24(2), 426–433. https://doi.org/10.1111/j.1365-2435.2009.01669.x

Perrot, R. G. and B. H. R. (1979). The plugged pores of tinamou (Tinamidae) and jaçana (Jacanidae) eggshells. *Ibis*, 121(4), 469–474. https://doi.org/10.1111/j.1474-919X.1979.tb06686.x

Pickford, M., Senut, B., and Dauphin, Y. (1998). Diagenetic changes in the mineral and organic phases of fossil avian eggshells from Namibia. *Applied Geochemistry*, 13(2), 243–256.

Portugal, S. J., Maurer, G., Thomas, G. H., Hauber, M. E., Grim, T., and Cassey, P. (2014). Nesting behaviour influences species-specific gas exchange across avian eggshells. *Journal of Experimental Biology*, 217(18), 3326–3332. https://doi.org/10.1242/jeb.103291

Portugal, S. J., Bowen, J., and Riehl, C. (2018). A rare mineral, vaterite, acts as a shock absorber in the eggshell of a communally nesting bird. *Ibis*, 160(1), 173–178. https://doi.org/10.1111/ibi.12527

Portugal, S, J., Maurer, G., and Cassey, P. (2010). Eggshell permeability: a standard technique for determining interspecific rates of water vapor conductance. *Physiological and Biochemical Zoology*, 83(6), 1023–1031. https://doi.org/10.1086/656287

Potter, M. A. (1989). Ecology and reproductive biology of the North Island brown kiwi (*Apteryx australis mantelli*) a thesis presented in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Zoology at Massey University.

Poulin, R. G., Todd, D. L., Dohms, K. M., Brigham, R. M., and Wellicome, T. I. (2005). Factors associated with nest- and roost-burrow selection by burrowing owls (*Athene cunicularia*) on the Canadian prairies. *Canadian Journal of Zoology*, 83, 1373–1380. https://doi.org/10.1139/Z05-134

Prager, E. M., Wilson, A. C., and Arniheim, N. (1974). Widespread Distribution of Lysozyme-G in Egg-White of Birds. *Journal of Biological Chemistry*, 249(22), 7295–7297.

Prinzinger, R., and Dietz, V. (1995). Qualitative course of embryonic O2 consumption in altricial and precocial birds. *Respiration Physiology*, 100(3), 289–294. https://doi.org/10.1016/0034-5687(94)00140-U

Prinzinger, R., and Dietz, V. (2002). Pre- and postnatal energetics of the North Island brown kiwi (*Apteryx mantelli*). *Comparative Biochemistry and Physiology. Part A, Molecular and Integrative Physiology*, 131(4), 725–732. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11897183

Rahn, H., Paganelli, C. V., and Ar, A. (1974). The avian egg: air-cell gas tension, metabolism and incubation time. *Respiration Physiology*, 22(3), 297–309. https://doi.org/10.1016/0034-5687(74)90079-6

Rahn, H., Carey, C., Balmas, K., Bhatia, B., and Paganelli, C. (1977). Reduction of pore area of the avian eggshell as an adaptation to altitude. *Proceedings of the National Academy of Sciences of the United States of America*, 74(7), 3095–3098. https://doi.org/10.1073/pnas.74.7.3095

Rahn, H., Ledoux, T., Paganelli, C. V, and Smith, A. H. (1982). Changes in eggshell conductance after transfer of hens from an altitude of 3,800 to 1,200 m. *Journal of Applied Physiology: Respiratory, Environmental and Exercise Physiology*, 53(6), 1429–1431.

Rahn, H., and Ar, A. (1980). Gas exchange of the avian egg time, structure, and function. *Integrative and Comparative Biology*, 20(2), 477–484. https://doi.org/10.1093/icb/20.2.477

Rahn, H., and Paganelli, C. V. (1990). Gas fluxes in avian eggs: Driving forces and the pathway for exchange. *Comparative Biochemistry and Physiology - Part A: Physiology*, 95(1), 1–15. https://doi.org/10.1016/0300-9629(90)90002-A

Ramos, J. A., Monteiro, L. R. L., Sola, E., Moniz, Z., and Encarnacion, S. (1997). Characteristics and competition for nest cavities in burrowing Procellariiformes. *The Condor*, 99(3), 634–641. https://doi.org/10.2307/1370475

Ramstad, K. M., Pfunder, M., Robertson, H. A., Colbourne, R. M., Allendorf, F. W., and Daugherty, C. H. (2010). Fourteen microsatellite loci cross-amplify in all five kiwi species (*Apteryx* spp.) and reveal extremely low genetic variation in little spotted kiwi (*A. owenii*). *Conservation Genetics Resources*, 2(SUPPL.1), 333–336. https://doi.org/10.1007/s12686-010-9233-2

Reid, B. (1981). Size discrepancy between eggs of wild and captive Brown Kiwi (*Apteryx australis mantelli*). *Notornis*, 28.

Reid, B. (1971). The weight of the kiwi and its egg. *Notornis*, 18(4), 245–249.

Reynolds, S. J., and Perrins, C. M. (2003). Dietary Calcium Availability and Reproduction in Birds. *Comparative Biochemistry and Physiology Part A: Physiology,* 80. https://doi.org/10.1016/0300-9629(85)90550-x

Romanoff, A. L., and Romanoff, A. J. (1949). The avian egg. *The Avian Egg. Wiley and Sons, New York.*

Rowe, B. (1978). Incubation temperatures of the North Island brown kiwi (*Apteryx australis mantelli*). *Notornis*, 25, 213–217.

Rueden, C. T., Schindelin, J., Hiner, M. C., DeZonia, B. E., Walter, A. E., Arena, E. T., and Eliceiri, K. W. (2017). ImageJ2: ImageJ for the next generation of scientific image data. *BMC Bioinformatics*, 18(1), 1–26. https://doi.org/10.1186/s12859-017-1934-z

Sales, J. (2005). The endangered kiwi: A review. *Folia Zoologica*, 54(1–2), 1–20.

Sauer, E. G. F. (1972). Aberrant Sexual Behavior in the South African Ostrich. *The Auk*, 89(4), 717–737. https://doi.org/10.2307/4084105

Schweitzer, M. H., Jackson, F. D., Chiappe, L. M., Schmitt, J. G., Calvo, J. O., and Rubilar, D. E. (2002). Late Cretaceous avian eggs with embryos from Argentina. *Journal of Vertebrate Paleontology*, 22(1), 191–195. https://doi.org/10.1671/0272-4634(2002)022[0191:LCAEWE]2.0.CO;2

Ségalen, L., and Lee-Thorp, J. A. (2009). Palaeoecology of late Early Miocene fauna in the Namib based on 13C/12C and 18O/16O ratios of tooth enamel and ratite eggshell carbonate. *Palaeogeography, Palaeoclimatology, Palaeoecology,* 277(3–4), 191–198. https://doi.org/10.1016/j.palaeo.2009.03.018

Seymour, R. S., and Ackerman, R. A. (1980). Adaptations to underground nesting in birds and reptiles. *Integrative and Comparative Biology*, 20(2), 437–447. https://doi.org/10.1093/icb/20.2.437

Shakesby, R. A., Coelho, C. D. O. A., Ferreira, A. D., Terry, J. P., and Walsh, R. P. D. (1993). Wildfire impacts on soil erosion and hydrology in wet Mediterranean forest, Portugal. *International Journal of Wildland Fire*, 3(2), 95–110. https://doi.org/10.1071/WF9930095

Shapiro, L. M. (2005). Diet overlap and potential competition between North Island brown kiwi chicks (*Apteryx mantelli*) and ship rats (*Rattus rattus*) for limited resources on Ponui Island, New Zealand: a thesis presented in partial fulfilment of the requirements for the degree of Doctor of Philosophy, Massey University.

Sheldon, F. H., and Winkler, D. W. (1999). Nest architecture and avian systematics. *The Auk*, 116(4), 875–877.

Shepherd, L. D., and Lambert, D. M. (2006). Nuclear microsatellite DNA markers for New Zealand kiwi (*Apteryx spp.*). *Molecular Ecology Notes*, 6(1), 227–229. https://doi.org/10.1111/j.1471-8286.2005.01201.x

Silyn-Roberts, H. (1983). Interior Openings of Functional Pores in the Avian Egg Shell: Identification with the Scanning Electron Microscope. *British Poultry Science*, 24(4), 497–499. https://doi.org/10.1080/00071668308416767

Silyn-Roberts, H. (1983). The pore geometry and structure of the eggshell of the North Island brown kiwi, *Apteryx australis mantelli. Journal of Microscopy*, 130(1), 23–36. https://doi.org/10.1111/j.1365-2818.1983.tb04195.x

Simkiss, K. (1961). Calcium metabolism and avian reproduction. *Biological Reviews*, 36(3), 321–359. https://doi.org/10.1111/j.1469-185X.1961.tb01292.x

Smart, I. H. M. (1991). Egg-shape in birds. Egg Incubation: Its Effects on Embryonic Development in Birds and Reptiles, 101–116. Cambridge University Press.

Sockman, K. W., and Schwabl, H. (1998). Hypothermic tolerance in an embryonic American kestrel (*Falco sparverius*). *Canadian Journal of Zoology*, 76, 1399–1402. https://doi.org/10.1139/cjz-76-7-1399

Stein, L. R., and Badyaev, A. V. (2011). Evolution of eggshell structure during rapid range expansion in a passerine bird. *Functional Ecology*, 25(6), 1215–1222. https://doi.org/10.1111/j.1365-2435.2011.01887.x

Szczerbińska, D., and Wiercińska, M. (2014). Ultrastructure of the eggshell of selected Palaeognathae species - A comparative analysis. *Annals of Animal Science*, 14(1), 167–178. https://doi.org/10.2478/aoas-2013-0079

Taborsky, B., and Taborsky, M. (1995). Habitat use and selectivity by the brown kiwi
(*Apteryx australis mantelli*) in a patchy environment. *The Auk*, 112(3), 680–689.

Taborsky, B., and Taborsky, M. (1999). The mating system and stability of pairs in kiwi
Apteryx spp. *Journal of Avian Biology*, 30(2), 143–151.

Taborsky, M., and Taborsky, B. (1992). Spatial organization of the North Island brown kiwi
Apteryx australis mantelli: sex, pairing status and territoriality. *Ibis*, 134(1), 1–10.
https://doi.org/10.1111/j.1474-919X.1992.tb07222.x

Tanaka, K., Junchang, L., Zelenitsky, D. K., and Kobayashi, Y. (2011). Description and
Phylogenetic position of Dinosaur Eggshells from the Luanchuan Area. *Acta
Geologica Sinica*, 85(1), 66–74.

Tanaka, K., Zelenitsky, D. K., and Therrien, F. (2015). Eggshell Porosity Provides Insight
on Evolution of Nesting in Dinosaurs. *PLoS ONE*, 10(11), 1–23.
https://doi.org/10.1371/journal.pone.0142829

Tanaka, K., Zelenitsky, D. K., Therrien, F., and Kobayashi, Y. (2018). Nest substrate reflects
incubation style in extant archosaurs with implications for dinosaur nesting habits.
Scientific Reports, 8(1), 1–10. https://doi.org/10.1038/s41598-018-21386-x

Taylor, E. L., Blache, D., Groth, D., Wetherall, J. D., and Martin, G. B. (2000). Genetic
evidence for mixed parentage in nests of the emu (*Dromaius novaehollandiae*).
Behavioral Ecology and Sociobiology, 47(5), 359–364.
https://doi.org/10.1007/s002650050677

Tazawa, H., Moriya, K., Tamura, A., Komoro, T., and Akiyama, R. (2001). Ontogenetic
study of thermoregulation in birds. *Journal of Thermal Biology*, 26(4–5), 281–286.
https://doi.org/10.1016/S0306-4565(01)00031-6

Tazawa, H., Wakayama, H., Turner, J. S., and Paganelli, C. V. (1988). Metabolic
compensation for gradual cooling in developing chick embryos. *Comparative
Biochemistry and Physiology -- Part A: Physiology*, 89(2), 125–129.
https://doi.org/10.1016/0300-9629(88)91068-7

Tullett, S. (1984). The porosity of avian eggshells. *Comparative Biochemistry and
Physiology Part A*, 78(1), 5–13.

Tullett, S. G., Board, R. G., Love, G., Perrott, H. R., and Scott, V. D. (1976). Vaterite
Deposition During Eggshell Formation in the Cormorant, Gannet and Shag, and in
'Shell-less' Eggs of the Domestic Fowl. *Acta Zoologica*, 57(2), 79–87.
https://doi.org/10.1111/j.1463-6395.1976.tb00213.x

Turner, J. S. (1985). Cooling rate and size of birds' eggs-A natural isomorphic body. *Journal
of Thermal Biology*, 10(2), 101–104. https://doi.org/10.1016/0306-4565(85)90033-6

Tyler, C. (1964). A study of the eggshells of the anatidae. *Proceedings of the Zoological Society of London*. 142:547-583.

Tyler, C., and Simkiss, K. (1958). A study of the eggshells of ratite birds. *Proceedings of the Zoological Society of London*, 133, 201–243. https://doi.org/10.1111/j.1469-7998.1965.tb01873.x

Varricchio, D. J., and Jackson, F. D. (2004). A phylogenetic assessment of prismatic dinosaur eggs from the Cretaceous Two Medicine Formation of Montana. Journal of Vertebrate Paleontology, 24(4), 931–937.

Varrichio, D. J., Jackson, F. D., Borkowski, J. J., and Horner, J. R. (1997). Nest and egg clutches of the dinosaur *Troodon formosus* and the evolution of avian reproductive traits. Nature, 385(16), 247–250.

Vianey-Liaud, M., Ashu, K., and Garcia, G. (2003). Relationships between European and Indian dinosaur eggs and eggshells of the oofamily Megaloolithidae. Journal of Vertebrate Paleontology, 23(3), 575–585. https://doi.org/10.1671/0272-4634(2003)023

Vianey-liaud, M., Hirsch, K., Sahni, A., and Sigi, B. (1997). Late cretaceous Peruvian eggshells and their Relationships with Laurasian and Eastern Gondwanian material. *Geobios,* 30(1), 75-90. https://doi.org/10.1016/S0016-6995(97)80259-8

Vianey-Liaud, M., and Zelenitsky, D. K. (2003). Historical and new perspectives on the parataxonomy of fossil eggs. *Palaeovertebrata*, 32, 189–195.

Vila, B., Sellés, A. G., and Beetschen, J. C. (2017). The controversial Les Labadous eggshells: A new and peculiar dromaeosaurid (Dinosauria: Theropoda) ootype from the Upper Cretaceous of Europe. *Cretaceous Research*, 72, 117–123. https://doi.org/10.1016/j.cretres.2016.12.010

Vleck, C. M., and Kenagy, G. J. (1980). Embryonic metabolism of the fork-tailed storm petrel: physiological patterns during prolonged and interrupted incubation. *Physiological Zoology*, 53(1), 32–42.

von Nathusius, W., and Tyler, C. (1964). Wilhelm Von Nathusius 1821-1899 on Avian Eggshells: A Translated and Edited Version of His Work. University of Reading.

Walsberg, G. E. (1980). The gaseous microclimate of the avian nest during incubation. Integrative and *Comparative Biology*, 20(2), 363–372. https://doi.org/10.1093/icb/20.2.363

Walsberg, G. E. (1983). Avian ecological energetics. *Avian Biology*, 7, 161–220.

Walsberg, G. E., and Schmidt, C. A. (1992). Effects of variable humidity on embryonic development and hatching success of mourning doves. *The Auk*, 109(2), 309–314.

Warning, N., and Benedict, L. (2015). Paving the way: Multifunctional nest architecture of the Rock Wren. *The Auk*, 132(1), 288–299. https://doi.org/10.1642/AUK-14-186.1

Webb, D. R., and King, J. R. (1983). An Analysis of the Heat Budgets of the Eggs and Nest of the White-Crowned Sparrow, *Zonotrichia leucophyrs*, in Relation to Parental Attentiveness. *Physiological Zoology*, 56(4), 493–505.

Webb D.R. (1978). Thermal Tolerance of Avian Embryos: A Review. *The Condor*, 89(4), 874–898.

Weir, J. T., Haddrath, O., Robertson, H. A., Colbourne, R. M., and Baker, A. J. (2016). Explosive ice age diversification of kiwi. *Proceedings of the National Academy of Sciences*, 201603795. https://doi.org/10.1073/pnas.1603795113

Welsh, E., Sankey, J. T., and Farlow, J. O. (2008). First dinosaur eggshells from Texas, USA: Aguja Formation (late Campanian), Big Bend National Park. *Vertebrate Microfossil Assemblages: Their Role in Paleoecology and Paleobiogeography*, 166–177.

Wentworth, B., Cigan, J., and Schaaf, T. (2009). Tolerance of Japanese quail embryos and young chicks to hypothermia. *Poultry Science*, 88(5), 1040–1043. https://doi.org/10.3382/ps.2008-00195

Westmoreland, D., Schmitz, M., and Burns, K. E. (2007). Egg color as an adaptation for thermoregulation. *Journal of Field Ornithology*, 78(2), 176–183. https://doi.org/10.1111/j.1557-9263.2007.00101.x

Westneat, D. F., Clark, A. B., Rambo, K. C., and Westneat, D. F. (1995). Within-brood patterns of paternity and paternal behavior in red-winged blackbirds. *Behavioral Ecology and Sociobiology*, 37(5), 349–356. https://doi.org/10.1007/BF00174140

Wheelwright, N. T., and Boersma, P. D. (1979). Egg chilling and the thermal environment of the fork-tailed storm petrel (*Oceanodroma furcata*) nest. *Physiological Zoology*, 52(2), 231–239.

White, D. J., Ramón-Laca, A., Amey, J., and Robertson, H. A. (2018). Novel genetic variation in an isolated population of the nationally critical Haast tokoeka (*Apteryx australis* 'Haast') reveals extreme short-range structure within this cryptic and flightless bird. *Conservation Genetics*, 19(6), 1401–1410. https://doi.org/10.1007/s10592-018-1109-0

White, F. N., and Kinney, J. L. (1974). Avian Incubation. *Science*, 186(4159), 107–115. https://doi.org/10.1126/science.186.4159.107

White, F. N., Bartholomew, G. A., and Kinney, J. L. (1978). Physiological and Ecological Correlates of Tunnel Nesting in the European Bee-Eater, *Merops apiaster*. Physiological Zoology, 51(2), 140–154. https://doi.org/10.1086/664584

Whittow, G. C., Pettit, T. N., Ackerman, R. A., and Paganelli, C. V. (1987). Temperature regulation in a burrow-nesting tropical seabird, the Wedge-tailed Shearwater (*Puffinus pacificus*). *Journal of Comparative Physiology B: Biochemical, Systemic and Environmental Physiology*, 157(5), 607–614. https://doi.org/10.1007/BF00700981

Whittow, G. G. C., and Tazawa, H. (1991). The early development of thermoregulation in birds. *Physiological Zoology,* 64(6), 1371–1390. https://doi.org/10.2307/30158220

Wilkin, T. A., Gosler, A. G., Garant, D., Reynolds, S. J., and Sheldon, B. C. (2009). Calcium effects on life-history traits in a wild population of the great tit (*Parus major*): Analysis of long-term data at several spatial scales. *Oecologia*, 159(2), 463–472. https://doi.org/10.1007/s00442-008-1222-8

Willoughby, B., Steyn, L., Bam, L., Olivier, A. J., Devey, R., and Maina, J. N. (2016). Micro-Focus X-Ray Tomography Study of the Microstructure and Morphometry of the Eggshell of Ostriches (*Struthio camelus*). The Anatomical Record, 299(8), 1015-1026.

Winkler, D. W., and Sheldon, F. H. (1993). Evolution of nest construction in swallows (Hirundinidae): a molecular phylogenetic perspective. *Proceedings of the National Academy of Sciences of the United States of America*, 90(12), 5705–5707. https://doi.org/10.1073/pnas.90.12.5705

Winkler, J. D. (2006). Testing phylogenetic implications of eggshell characters in side-necked turtles (Testudines: Pleurodira). *Zoology*, 109(2), 127–136. https://doi.org/10.1016/j.zool.2005.12.003

Winkler, J. D., and Sánchez-Villagra, M. R. (2006). A nesting site and egg morphology of a miocene turtle from Urumaco, Venezuela: Evidence of marine adaptations in pelomedusoides. *Palaeontology*, 49(3), 641–646. https://doi.org/10.1111/j.1475-4983.2006.00557.x

Zelenitsky, D. K., and Hirsch, K. F. (1997). Fossil eggs: identification and classification. In *Dinofest International: A Symposium Held at Arizona State University*, 279–286. Academy of Natural Sciences.

Zelenitsky, D. K., and Therrien, F. (2008). Phylogenetic analysis of reproductive traits of maniraptoran theropods and its implications for egg parataxonomy. Palaeontology, 51(4), 807–816. https://doi.org/10.1111/j.1475-4983.2008.00770.x

Zelenitsky, D. K., and Modesto, S. P. (2003). New information on the eggshell of ratites (Aves) and its phylogenetic implications. Canadian Journal of Zoology, 81(6), 962–970. https://doi.org/10.1139/z03-076

Ziesemann, B., Brunton, D., and Castro, I. (2011). Nesting success and breeding ecology in a high-density population of Brown Kiwi (*Apteryx mantelli*). *Emu*, 148–154.

Ziesemann, B. (2011). The social organisation and mating system of the brown kiwi (Apteryx mantelli). Massey University, Albany, New Zealand.

Zimmermann, K., Hipfner, J. M., and Burger, A. E. (2007). Egg size, eggshell porosity, and incubation period in the marine bird family Alcidae. *The Auk*, 124(1), 307–315. https://doi.org/10.1642/0004-8038(2007)124

Zyskowski, K., and Prum, R. O. (1999). Phylogenetic Analysis of the Nest Architecture of Neotropical Ovenbirds (Furnariidae). *The Auk*, 116(4), 891–911. Retrieved from http://www.jstor.org

Appendix I

Data from eggshell measurements using scanning electron microscopy. Measurements in μm, Species: 1-Brown Kiwi, 2-Roroa, 3-Rowi, 4-Haast Tokoeka.

Individual identification	Species	Year	Cuticle	Crystalline	Palisade	Mammillae	Total thickness
MT.FM1516.1	1	2016	2.967	61.782	150.751	44.859	261.511
MT.MPH1516.14	1	2016	1.987	52.717	166.333	45.379	283.35
MT.STT1516.18	1	2016	3.947	52.766	210.087	45.854	304.323
MT.TS1516.34	1	2016	2.055	77.206	101.477	25.368	264.708
O.JM1516.30	1	2016	3.927	43.85	198.186	62.495	295.627
O.RT1516.27	1	2016	3.623	60.925	128.431	43.23	253.526
OM.ROB1516.9	1	2016	4.559	42.944	87.588	36.076	165.75
T.DAN1516.6	1	2016	5.045	71.737	192.37	60.103	348.532
T.DAN1516.7	1	2016	4.78	60.896	162.179	49.286	297.38
T.LG1516.31	1	2016	5.168	60.229	215.971	66.824	348.182
T.MR1516.20	1	2016	1.594	58.695	232.61	81.528	391.315
KR.KKG	1	2016	2.835	65.527	161.187	41.785	284.115
PK.ATR4	1	2016	4.201	69.83	173.833	54.699	298.535
PK.WILL12	1	2016	4.033	69.518	169.25	63.857	311.398
PK.WILL14	1	2016	6.052	63.33	192.717	49.665	330.159
PK.WILL11	1	2016	6.296	61.649	181.049	68.959	320.762
MT.Q5	1	2016	5.699	66.063	205.017	44.63	339.842
MT.WU43	1	2016	2.743	67.599	207.526	38.395	329.292
MT.TEH3	1	2016	4.852	84.522	192.279	51.586	366.45
T.RK1516.14	1	2016	3.479	75.553	201.529	47.13	318.612
T.SP1516.8	1	2016	7.478	48.225	225.55	31.771	312.113
T.TAI1516.20	1	2016	2.492	69.777	156.151	48.759	292.259
G.J1617.1	2	2017	2.375	88.693	203.661	93.072	377.752
G.RR1617.1	2	2017	3.9	70.198	118.132	41.124	261.94
G.T1516.1	2	2016	2.206	87.132	205.174	71.767	370.59
R.BC1516.1	3	2016	3.832	78.265	242.633	72.304	423.491
R.BG1516.2	3	2016	1.205	96.279	152.304	105.762	375.927
R.BU1516.1	3	2016	3.623	71.164	209.805	44.578	361.961
R.BW1516.3	3	2016	4.217	77.782	187.275	75.291	339.317
R.CH1516.1	3	2016	2.428	52.759	196.814	82.447	334.095
R.CO1516.1	3	2016	4.278	108.79	207.717	75.824	407.762
R.CS1516.1	3	2016	3.191	76.087	154.703	38.499	289.763
R.CS1516.2	3	2016	2.921	67.671	206.206	54.556	359.072
R.DA1516.2	3	2016	4.089	65.729	226.736	50.365	367.77
R.DF1516.1	3	2016	4.314	80.172	250.867	52.593	368.138
R.DG1516.1	3	2016	2.782	81.029	220.932	50.718	390.952
R.DG1516.2	3	2016	1.601	73.734	249.429	71.163	349.722

R.DK1516.1	**3**	2016	4.161	94.096	280.128	66.908	423.061
R.DY1516.1	**3**	2016	4.152	70.675	240.687	38.691	381.708
R.DZ1516.2	**3**	2016	4.19	68.321	270.742	36.404	396.987
R.EB1516.1	**3**	2016	2.637	65.675	230.424	100.028	411.057
R.EL1516.4	**3**	2016	4.101	71.598	285.376	60.345	405.174
R.EM1516.1	**3**	2016	2.03	63.112	185.046	68.349	336.35
R.EN1516.2	**3**	2016	1.806	82.137	181.589	69.186	333.719
R.EU1516.2	**3**	2016	1.741	35.345	129.356	51.724	221.201
R.F1516.1	**3**	2016	2.785	74.158	253.454	97.418	430.215
H.AA1617	**4**	2017	4.043	63.865	242.872	58.495	362.61
H.BJ1516.2	**4**	2016	3.788	48.704	179.127	100.763	377.204
H.BR1415.2	**4**	2015	4.221	50.839	208.243	61.391	328.338
H.HF1516.1	**4**	2016	1.522	56.571	259.765	122.718	382.301
H.HI1415.1	**4**	2015	4.486	51.468	259.106	60.495	372.027
H.IT1516.1	**4**	2016	2.061	69.285	228.52	34.661	318.49
H.JC1617.1	**4**	2017	5.26	74.326	230.219	63.444	356.235
H.MG1516.1	**4**	2016	3.456	62.063	254.142	84.14	428.469
H.MT1516.1	**4**	2016	4.51	73.164	222.715	99.326	384.048
H.MT1516.2	**4**	2016	0.977	70.369	224.586	65.194	366.081
H.SL1516.1	**4**	2016	4.689	103.597	307.743	117.705	493.17
H.TI1516.1	**4**	2016	2.136	69.895	218.315	52.217	341.586
H.TR1516.1	**4**	2016	3.248	90.19	271.422	54.871	410.991

Appendix II

Kinship matrix

In the matrix the closest the number is to one the more closely related the individuals are. The expected values for full siblings is between 0.5 and 0.25 (green); half siblings from o.25 to 0.125 (Beige) and below this value the individuals were considered unrelated (pink).

	Mttwa11	Mttwa12	Mttwa13	Mttwa19	Mttwa20	Mttwa21	Mttwa23	Mttwa28	Mttwa30	Mttwa3
Mttwa11		0.14	0.14	0.14	0.00	0.00	0.00	0.00	0.00	0.0
Mttwa12	0.14		0.25	0.25	0.00	0.25	0.00	0.29	0.17	0.0
Mttwa13	0.14	0.25		0.25	0.25	0.25	0.13	0.00	0.17	0.1
Mttwa19	0.14	0.25	0.25		0.25	0.13	0.00	0.14	0.50	0.1
Mttwa20	0.00	0.00	0.25	0.25		0.25	0.38	0.00	0.00	0.0
Mttwa21	0.00	0.25	0.25	0.13	0.25		0.25	0.00	0.00	0.1
Mttwa23	0.00	0.00	0.13	0.00	0.38	0.25		0.00	0.00	0.0
Mttwa28	0.00	0.29	0.00	0.14	0.00	0.00	0.00		0.17	0.0
Mttwa30	0.00	0.17	0.17	0.50	0.00	0.00	0.00	0.17		0.5
Mttwa31	0.00	0.00	0.14	0.14	0.00	0.14	0.00	0.00	0.50	

	Mtts25	Mtts26	Mtts27	Mtts28	Mtts29	Mtts30	Mtts35	Mtts36	Mtts37	Mtts38	Mtts40
Mtts25	1.00	0.13	0.25	0.13	0.25	0.13	0.00	0.00	0.25	0.13	0.00
Mtts26	0.13	1.00	0.00	0.00	0.25	0.00	0.00	0.14	0.00	0.13	0.00
Mtts27	0.25	0.00	1.00	0.13	0.25	0.13	0.13	0.00	0.13	0.38	0.00
Mtts28	0.13	0.00	0.13	1.00	0.13	0.50	0.50	0.00	0.13	0.25	0.00
Mtts29	0.25	0.25	0.25	0.13	1.00	0.13	0.00	0.14	0.38	0.38	0.00
Mtts30	0.13	0.00	0.13	0.50	0.13	1.00	0.13	0.29	0.13	0.13	0.13
Mtts35	0.00	0.00	0.13	0.50	0.00	0.13	1.00	0.00	0.00	0.25	0.00
Mtts36	0.00	0.14	0.00	0.00	0.14	0.29	0.00	1.00	0.14	0.14	0.14
Mtts37	0.25	0.00	0.13	0.13	0.38	0.13	0.00	0.14	1.00	0.13	0.00
Mtts38	0.13	0.13	0.38	0.25	0.38	0.13	0.25	0.14	0.13	1.00	0.13
Mtts40	0.00	0.00	0.00	0.00	0.00	0.13	0.00	0.14	0.00	0.13	1.00

	Mttwa11	Mttwa12	Mttwa13	Mttwa19	Mttwa20	Mttwa21	Mttwa23	Mttwa28	Mttwa30	Mttwa31
Mttwa11		0.14	0.14	0.14	0.00	0.00	0.00	0.00	0.00	0.00
Mttwa12	0.14		0.25	0.25	0.00	0.25	0.00	0.29	0.17	0.00
Mttwa13	0.14	0.25		0.25	0.25	0.25	0.13	0.00	0.17	0.14
Mttwa19	0.14	0.25	0.25		0.25	0.13	0.00	0.14	0.50	0.14
Mttwa20	0.00	0.00	0.25	0.25		0.25	0.38	0.00	0.00	0.00
Mttwa21	0.00	0.25	0.25	0.13	0.25		0.25	0.00	0.00	0.14
Mttwa23	0.00	0.00	0.13	0.00	0.38	0.25		0.00	0.00	0.00
Mttwa28	0.00	0.29	0.00	0.14	0.00	0.00	0.00		0.17	0.00
Mttwa30	0.00	0.17	0.17	0.50	0.00	0.00	0.00	0.17		0.50
Mttwa31	0.00	0.00	0.14	0.14	0.00	0.14	0.00	0.00	0.50	

	Mtdew10	Mtdew12	Mtdew13	Mtdew14	Mtdew15	Mtdew19	Mtdew20	Mtdew21	Mtdew23	Mtdew24	Mtdew25	Mtdew04
Mtdew10		0.50	0.00	0.20	0.25	0.40	0.40	0.40	0.00	0.00	0.00	0.00
Mtdew12	0.50		0.00	0.33	0.00	0.17	0.20	0.17	0.00	0.17	0.00	0.00
Mtdew13	0.00	0.00		0.38	0.00	0.00	0.00	0.25	0.00	0.00	0.13	0.00
Mtdew14	0.20	0.33	0.38		0.00	0.25	0.14	0.13	0.00	0.00	0.00	0.00
Mtdew15	0.25	0.00	0.00	0.00		0.29	0.33	0.14	0.00	0.17	0.00	0.14
Mtdew19	0.40	0.17	0.00	0.25	0.29		0.43	0.25	0.00	0.00	0.00	0.00
Mtdew20	0.40	0.20	0.00	0.14	0.33	0.43		0.29	0.14	0.17	0.00	0.00
Mtdew21	0.40	0.17	0.25	0.13	0.14	0.25	0.29		0.14	0.00	0.13	0.00
Mtdew23	0.00	0.00	0.00	0.00	0.00	0.00	0.14	0.14		0.17	0.29	0.29
Mtdew24	0.00	0.17	0.00	0.00	0.17	0.00	0.17	0.00	0.17		0.14	0.14
Mtdew25	0.00	0.00	0.13	0.00	0.00	0.00	0.00	0.13	0.29	0.14		0.13
Mtdew04	0.00	0.00	0.00	0.00	0.14	0.00	0.00	0.00	0.29	0.14	0.13	

	Mtsm15	Mtsm17	Mtsm18	Mtsm22	Mtsm23	Mtsm09
Mtsm15		0.00	0.13	0.00	0.14	0.13
Mtsm17	0.00		0.25	0.00	0.14	0.00
Mtsm18	0.13	0.25		0.00	0.14	0.00
Mtsm22	0.00	0.00	0.00		0.14	0.13
Mtsm23	0.14	0.14	0.14	0.14		0.29
Mtsm09	0.13	0.00	0.00	0.13	0.29	

	Mtq10	Mtq11	Mtq12	Mtq02	Mtq03	Mtq08
Mtq10		0.00	0.25	0.00	0.00	0.00
Mtq11	0.00		0.00	0.14	0.00	0.29
Mtq12	0.25	0.00		0.13	0.00	0.00
Mtq02	0.00	0.14	0.13		0.00	0.25
Mtq03	0.00	0.00	0.00	0.00		0.00
Mtq08	0.00	0.29	0.00	0.25	0.00	

	Mtsh29	Mtsh35	Mtsh37	Mtsh38	Mtsh40
Mtsh29		0.00	0.14	0.00	0.29
Mtsh35	0.00		0.25	0.25	0.13
Mtsh37	0.14	0.25		0.13	0.13
Mtsh38	0.00	0.25	0.13		0.13
Mtsh40	0.29	0.13	0.13	0.13	

	Mtpr20	Mtpr27	Mtpr28	Mtpr30
Mtpr20		0.14	0.13	0.25
Mtpr27	0.14		0.29	0.14
Mtpr28	0.13	0.29		0.13
Mtpr30	0.25	0.14	0.13	

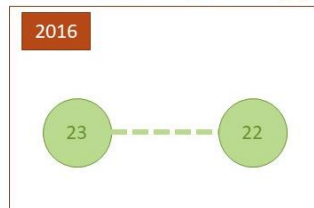|	Mtstt13	Mtstt16	Mtstt17	Mtstt19	Mtstt20	Mtstt22	Mtstt23	Mtstt05
Mtstt13		0.25	0.13	0.38	0.00	0.00	0.00	0.00
Mtstt16	0.25		0.13	0.13	0.13	0.25	0.00	0.00
Mtstt17	0.13	0.13		0.00	0.00	0.13	0.00	0.00
Mtstt19	0.38	0.13	0.00		0.13	0.13	0.13	0.13
Mtstt20	0.00	0.13	0.00	0.13		0.38	0.25	0.13
Mtstt22	0.00	0.25	0.13	0.13	0.38		0.00	0.00
Mtstt23	0.00	0.00	0.00	0.13	0.25	0.00		0.13
Mtstt05	0.00	0.00	0.00	0.13	0.13	0.00	0.13	

Relatedness amongst the chicks from the same breeding season and nThe ochre circles joined by a solid bar represent full siblings (K = 0.5 to 0.25), the green circles joined by a dashed line represent half siblings (K <0.25, K=12.5) and the blue circles represent unrelated individuals (K=0). The grey thin lines represent the reconstruction of relatedness made based on the existing relationships. Th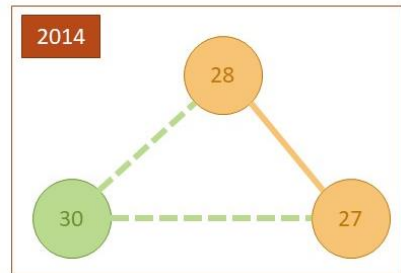e empty circles represent single eggs retrieved in a season.